# HEPARINIZED CROSSLINKED COLLAGEN STRUCTURES FOR THE EXPANSION AND DIFFERENTIATION OF HEMATOPOIETIC STEM CELLS

## PROEFSCHRIFT

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# List of Abbreviations

| | | |
|---|---|---|
| bFGF | - | basic Fibroblast Growth Factor |
| BFU | - | Burst Forming Unit |
| BM | - | Bone Marrow |
| BSA | - | Bovine Serum Albumin |
| CAFC | - | Cobblestone Area Forming Cell |
| CAM | - | Cell Adhesion Molecule |
| CB | - | Cord Blood |
| CD | - | Cluster of differentiation |
| CDS | - | Cell Dissociation Solution |
| CFU | - | Colony Forming Unit |
| CSF | - | Colony Stimulating Factor |
| d | - | day(s) |
| DSC | - | Differential Scanning Calorimetry |
| ECM | - | Extracellular Matrix |
| EDC | - | N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide |
| ELISA | - | Enzyme-Linked Immuno Sorbent Assay |
| EPO | - | Erythropoietin |
| Fn | - | Fibronectin |
| GAG | - | Glycosaminoglycan |
| h | - | hour(s) |
| HPC | - | Hematopoietic Progenitor Cell |
| HS | - | Heparan Sulfate |
| HSC | - | Hematopoietic Stem Cell |
| IL | - | Interleukin |
| LTC-IC | - | Long Term Culture Initiating Cell |
| MES | - | 2-Morpholinoethano sulfonic acid buffer |
| NHS | - | N-Hydroxysuccinimide |
| MNC | - | Mononuclear Cell |
| mPB | - | mobilized Peripheral Blood |
| NHS | - | N-hydroxysuccinimide |
| PBS | - | Phosphate Buffered Saline |
| PB | - | Peripheral Blood |
| PG | - | Proteoglycan |
| SCF | - | Stem Cell Factor |
| TCPS | - | Tissue Culture Polystyrene |
| TNBS | - | 2, 4, 6-trinitrobenzene sulphonic acid |
| TPO | - | Thrombopoietin |
| wk | - | weeks(s) |

# Chapter 1

# General Introduction

## 1. Background

In 1868 Neuman and Bizzozero proposed that the bone marrow (BM) is the major source of blood cells. The production of blood cells is called hematopoiesis, which is a continuous process because the life span of blood cells is limited. The daily production is about 2.5 billion red cells, 2.5 billion platelets and 1.0 billion granulocytes per kilogram of body weight [1].

The different blood cells are produced by a small pool of hematopoietic stem cells (HSCs) in the bone marrow. The idea of a common stem cell as source for the different blood cells was first coined in 1909 by Alexander Maximow [2]. The scientific search for HSCs really began after the first nuclear tests, when it was recognized that irradiation destroyed the hematopoietic system. It was found that radiation-induced hematopoietic failure in laboratory animals could be reversed by injection of unirradiated BM cells. It was later shown that these animals were restored in all blood cell types [3].

The ability to restore the hematopoietic system by injection of BM cells (or HSCs) is currently used after high dose chemotherapy or radiotherapy treatment. BM transplantation is necessary after these treatments as they do not only kill the cancer cells but also the sensitive HSCs. Since the first successful BM transplantation in the late 1960s, this procedure has become an established part of many therapies. Not only is it used for the treatment of leukemia, but also for other cancers and hematological malignancies [4]. Moreover, it could help in the treatment of Crohn's disease and probably other autoimmune diseases like rheumatoid arthritis and multiple sclerosis [5]. The number of patients that receive BM transplantation is estimated at about 35000 per year in the US [6].

In addition to BM, primitive hematopoietic cells can also be obtained in small amounts from peripheral blood (PB) and umbilical cord blood (CB). The most important difference in cell content between BM and PB or UC samples is that BM contains non-hematopoietic, supporting, stromal cells. Treatment with so-called

mobilization factors can increase the concentration of HSCs in 'mobilized peripheral blood' (mPB) [7]. mPB is rapidly replacing BM as source of HSCs, since the harvesting of BM is a painful procedure that needs to be performed in an operating room. CB is an alternative cell source that is just beginning to be explored. The clinical results obtained with these cells are encouraging [8].

Once the donor cells are infused into the patient, it takes the cells 8-30 days to home to their specific microenvironment in the BM, engraft and restore hematological cell counts to safe levels. This is called short-term engraftment. During this period close monitoring of the patient and administration of antibacterial and antifungal agents are required in order to avoid infections. The treatments during this period account for a substantial fraction of the expenses of the transplantation procedure. Long-term engraftment can be evaluated after approximately five months, when HSCs have started the lifelong production of blood cells [9].

*In vitro* expansion and controlled differentiation of HSCs or hematopoietic progenitor cells (HPCs) is of great interest for application in BM or HSC transplantation. This would allow the harvesting of smaller specimens and infusion of the expanded cells at multiple time points. This would then reduce the recovery period of the patients. When the cells can selectively be differentiated it would be possible to produce specific blood cells for transfusion. The expansion and delivery of HSCs could also be useful in gene therapy [10].

## 2. Approach

Mimicking the bone marrow environment in a bioreactor system is expected to improve the expansion of HSCs and provide a model to investigate the mechanisms of hematopoiesis and processes such as stem cell mobilization and homing. Here we will focus on the expansion of HPCs and their differentiation into erythroid cells.

The BM is thought to contain niches in which HSCs are localized in a specific microenvironment [11, 12]. In their niche the cells interact with stromal cells, extracellular matrix (ECM) components and cytokines. This environment can be mimicked using a bioreactor system in which a porous support structure provides the three-dimensional surroundings for the stem cells. This scaffold structure can be chemically modified in order to further mimic the ECM of the bone marrow. Mimicry of the bone marrow by using ECM or related molecules can help to modulate the function of growth factors, chemokines and cytokines.

## 3. Aim and structure of this thesis

The objective of the studies presented in this thesis was to develop a bioreactor system based on mimicry of the BM, for the expansion and differentiation of HSCs. Collagen type I and heparin were used to prepare films, beads and porous structures for HSC/HPC culture. The beads were used in a hematopoietic bioreactor as scaffold for the HPCs. Collagen type I is an important component of the bone marrow ECM. It can be processed into different morphologies to form a two- or three-dimensional structure suitable for cell culture. Additionally, chemical modification with heparin allows the presentation of heparin-binding factors. Heparin served as a model compound for the extracellular matrix component heparan sulfate.

An introduction on the bone marrow environment, hematopoiesis, erythropoiesis and different approaches to *in vitro* hematopoietic cell culture is presented in **chapter 2**. First, the chemical and cellular components of the bone marrow microenvironments are discussed. Secondly, an overview of the literature concerning hematopoietic cell culture and three-dimensional culture systems is presented. In the third part of the chapter both the biomimetic and bioreactor culture of HPCs are introduced.

**Chapter 3** describes the culture of PB $CD34^+$ cells on heparinized crosslinked collagen films and deals with the effects of immobilized heparin on cell adhesion, expansion and maintenance of progenitor cells.

In **chapter 4** the adsorption of the chemokine SDF-1α to the heparinized crosslinked collagen films as well as the effects on HPC culture are investigated. This chemokine serves as a model compound to study the interactions between surface material, hematopoietic factor and cells. Its ability to increase HPC adhesion could be used for retention of the cells in a three-dimensional structure when used in a bioreactor.

The binding and subsequent release of IL-3 by heparinized and non-heparinized crosslinked collagen films, as well as the use of these IL-3 pre-adsorbed films as substrate for culturing $CD34^+$ cells, are presented in **chapter 5**. The preliminary results obtained using bFGF pre-adsorbed heparinized and non-heparinized crosslinked collagen films as substrate for culturing $CD34^+$ cells are given in an **appendix** to chapter 5.

In **chapter 6** the HPC expansion when using heparinized crosslinked collagen films as culture substrate is compared with that in a packed bed of heparinized crosslinked collagen beads. Finally, **chapter 7** deals with a packed bed of these beads used in a bioreactor to expand the HPCs into erythroid progenitor cells. The use of this

hematopoietic bioreactor to differentiate HPCs into more committed erythroid cells like erythroblasts is described as well.

## 4. References


1. Beutler E, Yichtman AJ, Coller BS, Kipps TJ. Hematology. New York: Mc Graw-Hill, 1995.
2. Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. Biomed Pharmacother 2001;55:186-194.
3. Spangrude GJ, Heimfeld S, Weissman IL. Purification and Characterization of Mouse Hematopoietic Stem-Cells. Science 1988;241:58-62.
4. Mukhopadhyay A, Madhusudhan T, Kumar R. Hematopoietic Stel Cells: Clinical Requirements and Developments in Ex-Vivo Culture. Adv Biochem Eng Biotechnol 2004;86:215-253.
5. Craig RM. Autologous hematopoietic stem cell transplantation for Crohn's disease. Autoimmun Rev 2002;1:244-249.
6. Facts and Figures folder from the *National Marrow Donor Program* (last update June 2006, based on 2005 fiscal year numbers).
7. To LB, Haylock DN, Simmons PJ, Juttner CA. The Biology and Clinical Uses of Blood Stem Cells. Blood 1997;89:2233-2258.
8. Cairo MS, Wagner JE. Placental and/or umbilical cord blood: an alternative source of hematopoietic stem cells for transplantation. Blood 1997;90:4665-4678.
9. McAdams TA, Winter JN, Miller WM, Papoutsakis ET. Hematopoietic cell culture therapies – 2 – Clinical aspects and applications. Trends Biotechnol 1996;14:388-396.
10. Nordon RE, Schindhelm K. Ex vivo manipulation of cell subsets for cell therapies. Artif Organs 1996;20:396-402.
11. Yin T, Li LH. The stem cell niches in bone. J Clin Invest 2006;116:1195-1201.
12. Arai F, Hirao A, Suda T. Regulation of Hematopoietic Stem Cells by the Niche. Trends Cardiovasc Med 2005;15:75-79.

# Chapter 2

# *In vivo* and *in vitro* Hematopoiesis

## 1. The bone marrow and *in vivo* hematopoiesis

The bone marrow (BM) environment plays a key role in the regulation of hematopoietic stem cell (HSC) proliferation and differentiation. The BM is a complicated tissue; therefore it will be discussed in three parts. The development and macroscopic structure of the bone marrow will be discussed first. After an overview of the cellular components of the BM, their extra-cellular matrix (ECM) will be described in more detail.

### *1.1. Development and anatomy of the bone marrow*

Hematopoiesis in the embryo takes place in the yolk sac and in a region of the mesoderm surrounding the endothelium of the developing gut and blood vessels. After this embryonic phase, the HSCs colonize the developing liver and spleen. From the fifth month until birth the BM is seeded with cells from the liver and spleen and it becomes the exclusive site for the production of blood cells [1]. HSCs can also circulate in small numbers in peripheral blood (PB) and cord blood (CB), but only produce blood cells in the marrow. After 4 years the number of fat cells (adipocytes) in the BM of the long bones increases and after about 18 years, hematopoietic tissue only remains in the vertebrae, ribs, pelvis and in a part of the femora.

The adult BM has a complex structure. A simplified scheme is shown in figure 1.

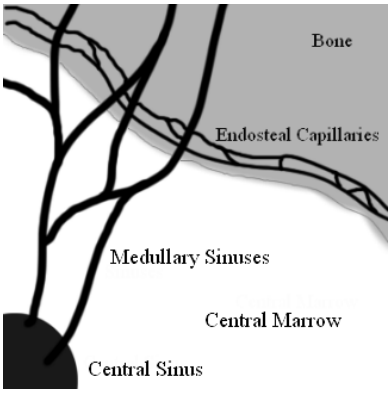


**Figure 1. The circulation of the bone marrow; adapted from Nilsson [2]**

The central sinus in the middle of the bone marrow is the central vein, which drains the highly branched network of medullary sinuses. The HSCs are located close to the endosteal region of the bone, while the more committed cells are distributed towards the central marrow [2]. The stem cells divide and develop through several stages and migrate through the endothelial cell layer of the sinuses when they are mature.

### *1.2. The cellular components of the bone marrow*

*Hematopoietic stem and progenitor cells*

HSCs are the source of all blood cells. The major distinction in blood cell types is the grouping into the myeloid and lymphoid lineage. The myeloid cells include erythrocytes (red blood cells), platelets (derived from noncirculating megakaryocytes), and white blood cells like monocytes and granulocytes (neutrophils, eosinophils, and basophils). The lymphoid white blood cells include T-lymphocytes and B-lymphocytes.

HSCs are defined as cells that can develop into every blood cell type and are capable of self-renewal. This means that the cell division of stem cells is asymmetrical: they divide into a cell that is similar to themselves and a cell that differentiates. They are also capable of symmetric division, creating two stem cells, thereby expanding the stem cell pool. There are many stages in differentiation between HSCs and the final blood cells. A scheme of the hematopoietic hierarchy is shown in figure 2.

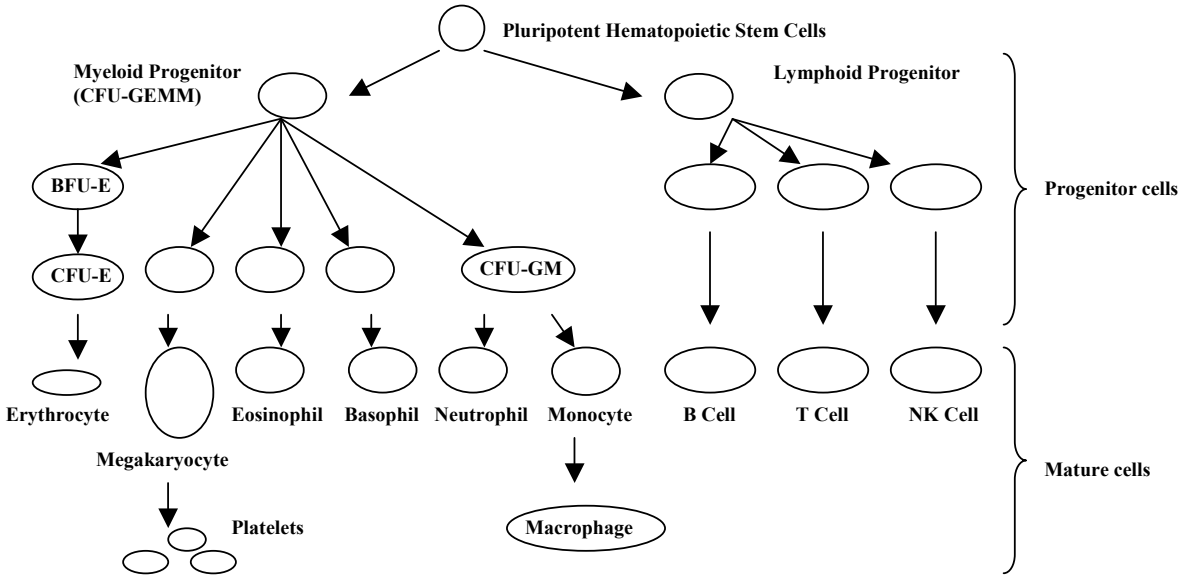


**Figure 2. Hematopoietic cell lineages ; adapted from Panoskaltsis [3]**

More primitive intermediate cells are referred to as hematopoietic progenitor cells (HPCs). Colony-forming units (CFUs) are progenitor cells that produce specific colonies in a semi-solid culture medium.

HPCs can be obtained using several separation steps. Mature erythrocytes and granulocytes are removed from BM or blood by centrifugation through a density gradient. The remaining fraction is referred to as mononuclear cells (MNCs). Further separation techniques have to be used to obtain fractions with a higher HSC content. Labeled antibodies can be used to identify distinct cell types based on the expression of certain molecules on the cell membrane. These labeled cells can be isolated by fluorescence activated cell sorting (FACS). Many of these surface molecules are referred to as clusters of differentiation (CD).

For the erythrocyte lineage the CD markers are illustrated in figure 3.

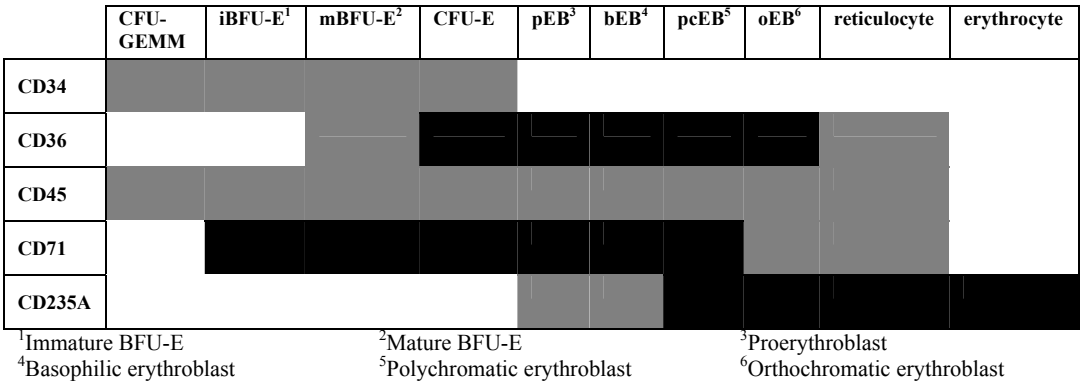


**Figure 3. Antigen expression in the erythroid lineage at the different stages of differentiation (grey: intermediate expression, black: high expression and white: no expression) [4,5]**

Despite HSCs being the most studied stem cells, the actual antigenic phenotype of 'the hematopoietic stem cell' is still unknown. Several purified cell fractions obtained using combinations of antibodies fulfill the criteria for primitive HPCs. Known combinations are $CD34^{+}38^{-}HLA^{-}Dr^{-}Thy^{+}$ or $CD34^{+}$ without any other differentiation markers (the $CD34^{+}Lin^{-}$ population) [1].

About 1-3% of BM-MNC express the CD34 antigen, the fraction in PB is 0.1-0.2% and in CB it is 0.8-1.2% [1]. The obtained cell fractions are enriched, but still heterogeneous with respect to HPC content. Additionally, the expression of the CD34

surface marker on HPCs decreases with aging* and its expression is influenced by cell cycle activity. This means that a small percentage of primitive cells can be $CD34^-$ [6]. Magnetic activated cell sorting (MACS) is usually used to obtain $CD34^+$ cells. MACS is based on immunolabeling the surface membrane of the targeted cells with very small paramagnetic beads and the subsequent isolation of the labeled cells in a high gradient magnetic field. Afterwards the beads are rapidly internalized by the cells and do not seem to interfere with their functioning [7].

Besides HSC identification with flowcytometry and colony forming assays, the gold standard remains the long-term *in vivo* reconstitution of hematopoiesis. For human cells immunodeficient (NOD/SCID mice) are used.

*Stromal cells*

In the BM the hematopoietic cells are surrounded by supporting cells called stromal cells. These cells produce an ECM, they present adhesion and signaling ligands to the hematopoietic cells and they produce cytokines, chemokines and other factors. The three-dimensional microenvironment of the HSCs, consisting of the stromal cells, the ECM and different factors (hormones, cytokines, chemokines, growth and colony-stimulating factors) is called the stem cell "niche" [8, 9].

The five most important stromal cell types are: endothelial cells, macrophages, adipocytes, osteogenic cells and fibroblast-reticular cells. The stromal system also contains stem cells. These are often referred to as mesenchymal stem cells (MSCs) or stromal stem cells. These MSCs can differentiate into many tissues, such as cartilage, bone, tendon, ligament, and marrow stroma [11].

Endothelial cells cover the inner surface of the medullary sinuses and the outside of these sinuses contains adventitial fibroblast-reticular cells. These cells form the barrier between the venous sinuses and the extravascular hematopoietic spaces between the sinuses. The reticular cells are extensively branched and produce collagen I and IV, fibronectin, laminin and decorin [12]. Fibroblast-reticular cells can trans-differentiate into adipocytes, which are most abundant in the inactive yellow (fatty) BM, but they are also present in the hematopoietic red BM.

* Aging of HSC is an important, but controversial issue. Aging does not only cause changes in surface marker expression but also a decrease in stem cell functioning. There is some evidence that the capacity of self-renewal declines with increasing age of the donor [10].

Macrophages are stromal-associated cells derived from blood monocytes and reside in the interstitial spaces of virtually all tissues and form the central component of many defense strategies. They also secrete regulatory factors and influence differentiation of HPCs [8,13].

Bone cells like osteoblasts play a central role in the maintenance of HSCs in their microenvironment [14, 15, 16]. The former cells also function as supporting stromal cells for *in vitro* culture of HPCs [17, 18]. The supporting osteoblast cells adjacent to primitive cells express collagen type I, laminin, fibronectin, decorin, hematopoietic factors (cytokines, chemokines and growth factors) and high levels of Jagged-1, which is an important ligand for cell-cell (*Notch*-pathway) signaling [12, 19, 20, 21]. A recent model of the HSC-niche with a central role for osteoblasts is represented in figure 4.

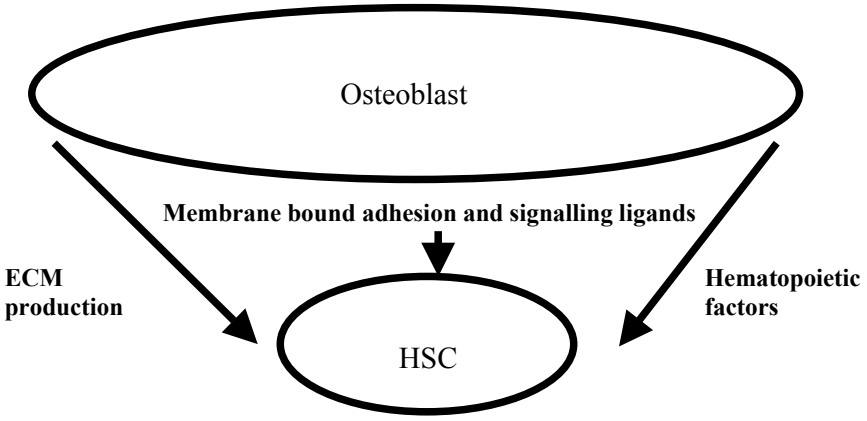


**Figure 4. Model of HSC-Osteoblast interactions, adapted from Taichman [9]**

### *1.3. The extracellular components of the bone marrow*

The ECM is a dynamic system in which different ECM components interact with each other to form a three-dimensional structure to which cells and signaling molecules can adhere. The main ECM constituents of the BM are proteoglycans, glycosaminoglycans (GAGs), collagens and non-collagenous glycoproteins.

Collagen in the BM ECM consists mainly of collagen types I, III, IV and V [13]. The general function of collagen in the body is mechanical. It provides the structural support of cells and it is the major component of connective tissues. Stromal cells and their precursors adhere to collagens through β1 integrins [22]. Additionally, several HPC cell-lines show some adherence to collagen type I [23]. The structure of fibrillar Type I collagen is shown in figure 5.

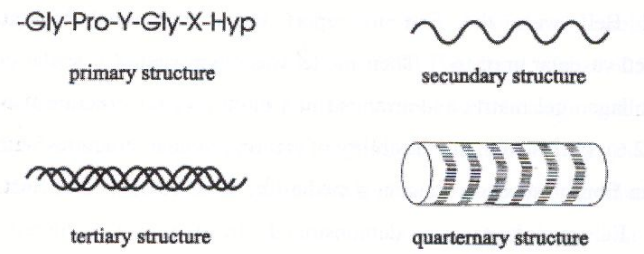


**Figure 5. Organization of collagen, showing the major polypeptide sequence (primary structure), the polypeptide chains (secondary structure), their triple helix structure (tertiary structure) and the subsequent organization into fibrils (quartenary structure).**

The collagen fibrils form the meshwork of reticular fibers throughout the marrow (type III), and form the connective layer in the sinusoids (type IV) and the bone matrix (type I) [24]. The collagens are also important in the structural organization of the ECM through their interactions with other components like proteoglycans.

Proteoglycans (PGs) are complex macromolecules with numerous GAGs linked to a protein core. PGs form aggregates linked onto a hyaluronic acid molecule. These GAGs are polymers of repeating amino sugar and uronic acid units that may be sulfonated. Common GAGs are chondroitin, heparan, dermatan, and keratan sulfates and hyaluronic acid [25]. The general structure of a PG is shown in figure 6.

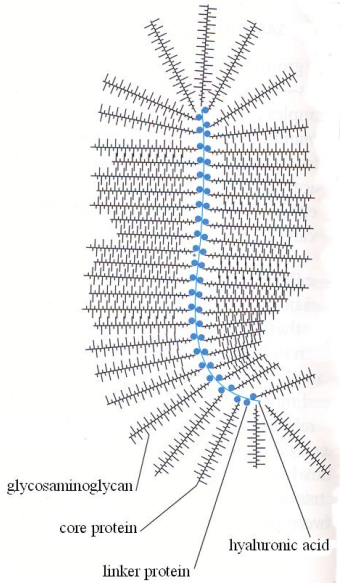


**Figure 6. The general proteoglycan (PG) structure. The figure is reprinted and adapted from Biochemistry 3rd by Mathews, Van Holde and Ahern [155].**

Especially HS-containing PGs are thought to play an important role in the presentation and activation of several molecules that influence hematopoiesis [26]. Hematopoietic factors such as cytokines and growth factors were identified and employed in culture media and they will therefore be discussed in the paragraphs concerning HPC culture. The presentation of hematopoietic factors by PGs and the other mechanisms by which these molecules can function are presented in figure 7.

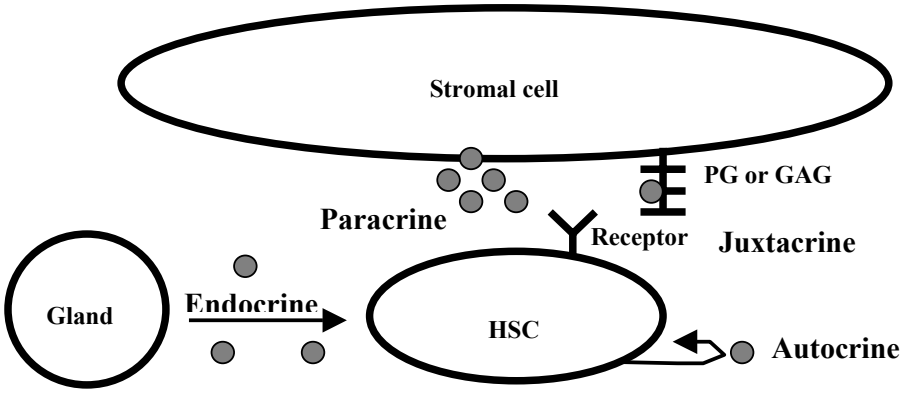


**Figure 7. The mechanisms by which hematopoietic factors can function, with hematopoietic factors represented as grey spheres. Endocrine: signaling by means of hormones transported to their target through the circulation. Paracrine: signaling by molecules secreted by cells close to the receiving cell. Autocrine: signaling to a cell by means of molecules secreted by the cell itself. Juxtacrine: signaling by molecules that are transmitted or presented by other cell membrane bound molecules.**

Binding of a hematopoietic factor to GAGs or PGs can influence its function in several ways. Binding can change the factor's conformation, thereby activating it [27, 28]. The association to heparinoids can also protect a factor from proteolytic degradation [29, 30]. Finally, the binding of a hematopoietic factor to GAGs or PGs can induce conformation-dependent association or polymerization of the factor and its receptors [31]. The binding of bFGF to heparin is the paradigm for all these processes. Similar mechanisms are expected to play a role in the functioning of other, less studied, heparin-binding molecules [26].

The addition of 5 μg/ml HS to HPC culture medium resulted in improved HPC maintenance [32-34], but at sustained high concentrations (160 μg/ml) heparin had toxic effects [35]. The sulfation of GAGs appears to be important for maintaining HPCs, since O-sulfated HS and heparin supported HPC maintenance, while desulfated and N-sulfated heparin did not [33]. Additionally, HPC adhesion to heparin-coated surfaces was investigated. It was found that $CD34^+$ cells adhered stronger to O-sulfated heparin than to N-sulfated of desulfated heparin [33]. The binding and

thereby co-localization of cells and cytokines like IL-3 or MIP-1α to O-sulfated heparin could explain the improved HPC maintenance [34, 36]. An overview of HS and heparin-binding hematopoietic factors is given in table 1.

**Table 1. Hematopoietically active factors with articles or reviews on binding to HS or heparin.**

| Abbreviation | Full name | Reference: |
|---|---|---|
| MIP-1α | Macrophage inflammatory protein 1α | 34, 36 |
| IL-3 | Interleukin-3 | 34, 37, 38 |
| GM-CSF* | Granulocyte/Macrophage-colony stimulating factor | 37, 38, 39 |
| SDF-1α | Stroma-derived factor 1α | 40, 41 |
| IGF | Insulin-like growth factor | 42 |

* does not bind at neutral pH [43]

Heparin-binding factors like bFGF [27-31] and TGF-β [44] have not been investigated for BM stroma or ECM binding.

Hematopoietic cells also adhere to GAGs like HS and hyaluronic acid. HS degradation on stromal cell lines reduces HPC binding to these substrates [47]. Additionally, isolated HS and HS proteoglycans can inhibit CFU binding to stromal cells [48, 49]. Hyaluronic acid and its receptor (CD44) are both present on the cell membrane of hematopoietic cells and are important for homing [45]. Hyaluronic acid chains from the ECM have been shown to bind to HPCs [46].

Non-collagenous glycoproteins like fibronectin, laminin, hemonectin and thrombospondin are the most important ECM ligands for cell binding. These adhesive proteins are concentrated in distinct regions of the BM and are also expected to play an important role in the formation of hematopoietic microenvironments [50]. An overview of Cell Adhesion Molecules (CAMs) is shown in table 2.

**Table 2. CAMs present on hematopoietic cell-lines and $CD34^+$ cells [51-54]**

| CAM | CD | Ligands |
|---|---|---|
| ICAM-1 | CD54 | LFA-1, MAC-1 |
| PECAM-1 | CD31 | PECAM-1, heparin, HS |
| LFA-3 | CD58 | LFA-2 |
| VLA-4 (α4β1) | CD49d/CD29 | Fn, VCAM-1 |
| VLA-5 (α5β1) | CD49e/CD29 | Fn |
| LFA-1 (α1β2) | CD11a/CD18 | ICAM-1, 2+3 |
| Vn receptor (αvβ2) | CD51/CD61 | Vitronectin |
| L-Selectin | CD62L | GlyCAM-1, MAdCAM-1 |
| HCAM | CD44 | Collagen, hyaluronic acid |
| Tsp receptor | CD36 | Collagen, thrombospondin |
| Mac-1 (αmβ2) | CD11b/CD18 | Heparin and HS |
| | CD45 | Heparin and HS |

Adhesion of HPCs to these adhesive proteins changes during differentiation [48]. The differences in adhesion are thought to be important in the formation of microenvironments for specific steps in the hematopoietic process [49]. The strength of adhesion and the expression of CAMs on HPCs also depend on the cell source [55], cell cycle phase [56] and treatments like cryopreservation, incubation with serum and the presence of cytokines and growth factors [57].

## 2. Approaches for *in vitro* hematopoiesis

The *in vitro* culture of HPCs is an extensive and often reviewed subject [58-66]. After a short historical introduction this overview will be focused on cytokines and other additives for serum-free cultures of human mPB $CD34^+$ cells. PB $CD34^+$ cells are clinically the most used HPCs and their application would be improved by a system for controlled expansion and differentiation [67]. Finally, current methods employing mimicry of the bone marrow and the use of bioreactors will be discussed. For these last subjects all HPC cell types will be discussed, as well as serum- and stroma-containing cultures.

### *2.1. Historical introduction: Dexter cultures*

In the mid-1970s, Dexter and co-workers developed a murine long-term BM culture (LTBMC) system in which murine hematopoiesis could be maintained for several months on a pre-established stromal layer. Primitive cells proliferated and accumulated below the stroma layer and mature cells migrated through the stroma [68].

The adaptation of Dexter's system for use with human cells was first reported by Gartner and Kaplan in 1980. In these cultures the use of fetal bovine serum and horse serum was found to be required, but still these never obtained the cell proliferation or longevity observed in murine culture systems. Recombinant hematopoietic factors are often used in combination with serum and greatly improve the productivity of these systems. Due to the clinical and regulatory problems associated with xenogenic serum, it becomes more common to use serum-free media, which are commercially available [69].

### *2.2. The current state: conventional HPC cultures*

The influence of hormones, growth factors, chemokines, colony-stimulating factors and cytokines on maintenance, expansion and differentiation of HPCs is part of a highly complex system involving endocrine, paracrine, juxtacrine and autocrine regulation. Glands, stromal cells, HPCs, and differentiating cells secrete these substances [70, 71].

The earliest discovered hematopoietic factors were called colony-stimulating factors (CSFs) as they led to the formation of specific cell colonies in culture. In addition to these, there is a growing list of other hematopoietic factors that influence HPC survival and expansion. A general overview of the most used factors can be found in table 3.

**Table 3. An overview of hematopoietic factors with relevant reviews or articles.**

| Abbreviation | Full name | Reference: |
|---|---|---|
| GM-CSF<br>M-CSF<br>G-CSF | Granulocyte/Macrophage colony-stimulating factor<br>Macrophage colony-stimulating factor<br>Granulocyte colony-stimulating factor | 83 |
| - | Dexamethasone and hydrocortisone | 72, 73 |
| SCF | Stem Cell Factor | 74, 75 |
| FL | Flk2/Flt3 ligand | 76, 77 |
| IL-3 | Interleukin-3 | 78 |
| IL-6 | Interleukin-6 | 79 |
| Tpo | Thrombopoietin | 80, 81 |
| Epo | Erythropoietin | 82 |
| SDF-1α | Stroma-derived factor 1α | 84, 85 |

For optimal culture conditions a synergistic interaction between multiple cytokines at the right concentration is needed. The optimal concentration depends on cell activity and differentiation stage of the HPCs [86].

*Expansion of HPCs*

FL and SCF receptors are expressed on primitive HPCs and both are involved in the regulation of the early stages of hematopoiesis. FL and SCF prevent apoptosis of early progenitor cells and work in synergy to stimulate proliferation [75]. SCF also synergizes with other factors (like Epo, IL-3, GM-CSF and G-CSF) to support colony growth of BFU-E, CFU-GM and CFU-GEMM. Moreover, SCF modulates the adhesive behaviour of HPCs [74].

The function of IL-3 in HPC expansion remains controversial, though in serum-free conditions it appears to improve the ability of FL, SCF and Tpo to stimulate

proliferation of primitive cells [78]. Tpo regulates the expansion and maturation of megakaryocytes, but also enhances the survival and proliferation of primitive hematopoietic cells, either directly or in synergy with other early-acting factors [81].

The highest expansion of human PB $CD34^+$ cells, using both stroma- and serum-free conditions, was obtained by Herrera *et al* [87]. Serum-free medium (X-VIVO 15, containing human serum albumine (HSA), insulin and transferrin) with SCF, FL, IL-3 and Tpo was supplemented with bovine serum albumin (BSA), L-glutamine, 2-mercaptoethanol, penicillin and streptomycin. In 6 days of cell culture this led to a $CD34^+$ expansion of 6 times. Total cell expansion was also 6-fold and CFUs expanded 2 times.

When IL-6 was also included in the serum-free medium, the $CD34^+$ expansion was 18 times, total cell expansion 20 times and CFU expansion 19 times. However, extensive $CD34^+$ amplification (more than 8-fold) resulted in loss of engraftment potential in NOD/SCID mice. These results are summarized in table 4.

**Table 4. Expansion of PB $CD34^+$ cells reported for two different cytokine combinations and their NOD/SCID engraftment capacity [87]**

| Cytokines | TVC expansion | $CD34^+$ expansion | CFU expansion | NOD/SCID engraftment |
|---|---|---|---|---|
| SCF, FL, IL3 and TPO | 6x | 6x | 2x | yes |
| SCF, FL, IL3 and TPO + IL6 | 20x | 18x | 19x | no |

TVC expansion: total viable cell expansion

SDF-1α plays an important role in the processes of HSC homing, mobilization and retention in the microenvironments [84]. SDF-1α has been reported to increase [89] and to have no influence on the expansion of CFU numbers [85]. Serum-free culture of CB $CD34^+$ cells using media with SDF-1α (or a small peptide analogue [88]) has also been reported to enhance engraftment [85].

*Differentiation of HPCs into the erythroid lineage*

There are several multi step approaches for *in vitro* erythropoiesis. A good serum-free cell expansion (34-fold with 4-fold $CD34^+$ expansion after 1 wk) combined with erythroid differentiation was achieved by first expanding $CD34^+$ cells into $CD36^+$ erythroid progenitors (65%, see table 2.5 first column for composition of the culture medium). The $CD36^+$ cells were subsequently isolated with a MACS system and cultured for 3 days in medium with 2 U/ml Epo, resulting in dramatic cell proliferation (152-fold expansion) with 98% of the cells $CD36^+$ and 40% also

Glycophorin-A positive ($GpA^+$). Additionally, after one week enucleation was observed [90].

Recently, even higher expansion factors were reported employing a three-step serum-free culture protocol [91, 92]. The composition of the media employed in the first step is listed in the second column of table 2.5. During the second and third step the cells were cultured on a stroma (or MSC) layer, with SCF, Epo and IGF-1 in the second step and Epo and IGF-1 in the third [91], or with only Epo in the second step and no factors added in the third [92]. The latter culture protocol resulted in a 2-fold expansion of erythroid CFUs and a 1000-fold total viable cell expansion during the first week of which >95% were erythroblasts. [92].

A single step serum-free 3 weeks culture protocol to produce enucleated erythrocytes has been reported, yielding ca 90-95% erythroid cells of which 10-40% were enucleated. However, this only resulted in enough cells for characterization and therefore expansion factors were not mentioned. Macrophages were essential for enucleation, since this occurred around erythrocyte-macrophage associations [93]. The medium used in this study is listed in the third column of table 5.

**Table 5. Cell culture serum-free media compositions for the expansion of erythroid progenitors**

| **Freyssinier et al [90]** | **Giarratana et al [92]** | **Malik et al [93]** |
|---|---|---|
| 5% BSA | 1% BSA | 1% BSA |
| 50 µg/ml insulin | 10 µg/ml insulin | 10-4 mol/l 2-mercaptoethanol |
| 1 mg/ml transferrin | 120 µg/ml transferrin | 10-6 mol/l hydrocortisone |
| 100 U/ml penicillin-streptomycin | 900 ng/ml ferrous sulfate | 100 U/ml penicillin-streptomycin |
| 2 mmol/l L-glutamine | 90 ng/ml ferric nitrate | 2 mmol/l L-glutamine |
| 10 ng/ml rh IL-3 | $10^{-6}$ mol/l hydrocortisone | 10 U/ml rh Epo |
| 10 ng/ml rh IL-6 | 100 ng/ml SCF | 0.001 ng/ml rh GM-CSF |
| 25 ng/ml rh SCF | 5 ng/ml IL-3 | 0.01 U/ml rh IL-3 |
| | 3 U/ml Epo | |

Most of these protocols include cell isolation or medium changes in between the different steps of the culture procedure. In a bioreactor system the media could be exchanged without disturbing the cell in culture.

## 2.3. The current state: biomimetic HPC cultures

Mimicry of the BM environment is a promising strategy to improve the maintenance and expansion of HPCs. There are several reports that claim improved HPC expansion by using either biomimetic two-dimensional or three-dimensional static culture systems or a three-dimensional system in a bioreactor.

Many reviews have been written about BM mimicry and bioreactors for *in vitro* HPC culture [94-100]. All reports on static and bioreactor systems for BM mimicry will be discussed, but not all types of bioreactors. Omitted are airlift reactors and hollow fiber reactors, due to very poor results [101, 102] and rotating wall vessel (RWV) bioreactors due to negative effects on erythropoiesis [103, 104]. Although stirred suspension reactors are the most investigated systems for HPC culture [105-110], these are also not discussed since they do not allow mimicry of the BM. Bioreactor and biomimetic systems will be discussed according to the material employed in the study.

*Synthetic Polymers*

The earliest reports on biomimetic HPC culture concerned a perfused nylon mesh with a pre-established stroma layer for murine and rodent HPCs [111]. More recently polyesters were used after surface modification. These materials were NaOH hydrolyzed poly(ethylene terephthalate) (PET) non-wovens [112] and plasma-treated Fibra-cell non-wovens [113-117]. In both systems serum-containing media were used. The PET scaffold could maintain CFUs for 8-10 weeks without the use of additional cytokines or a pre-established stroma layer [112]. The Fibra-cell scaffold was used for the culture of murine BM cells and these showed a 5 times increase in CFU-GEMM cells, while on a similar two-dimensional system the amount of these CFUs decreased [115].

A flat-bed bioreactor developed by Koller *et al,* in which serum-containing medium flows slowly over the culture bed with a pre-grown stroma layer, was used for the culture of human BM MNCs and resulted in an LTC-IC expansion of 3-10 times [118-124]. Aastrom Biosciences Inc. owns a number of patents covering this approach. Several synthetic polymers were investigated as material for this flat-bed bioreactor. The materials that supported PB $CD34^+$ cell cultures near the level of polystyrene (PS) are listed in table 6. Polycarbonate gave similar results compared to PS, but when reused the performance decreased dramatically.

**Table 6. Cell survival on non-ECM materials, expressed as percentage relative to PS [125]**

| Material | Total cells (%) | CFU-GM (%) |
|---|---|---|
| Polystyrene (PS) | 100 | 100 |
| Polymethylpentene (PMP) | 93 | ~ 40 |
| Polyacetonitrile-methacrylate (Barex) | 72 | 70 |
| Cellulose acetate | 93 | ~ 65 |
| Teflon perfluoralkoxy (PFA) | 99 | 98 |
| Titanium | 83 | 88 |
| Tissue culture polystyrene (TCPS) | 106 | 117 |

Aastrom Biosciences also investigated the performance of commercial and custom-made cell culture materials. The best performance was found using glow of corona discharge surface modified PS (TCPS) [126].

*Collagen type I*

The ECM component collagen is often used as material in biomimetic HPC cultures employing various culture conditions. Collagen is often used since it can be applied as a coating or processed into structures with different morphologies.

It was used as coating for porous polyvinyl Formal (PVF) scaffolds in static HPC cultures. These scaffolds were used to investigate the effect of pore size on total cell expansion. For murine BM cells the optimal average pore size was found to be 130 μm. An average pore size of 60 μm gave the largest expansion but also difficulties in collecting the cultured cells [127]. These cells were cultured for up to 6 weeks after which mainly cells of the granulocyte and macrophage lineage were obtained [127, 128].

Porous collagen beads (Cellex) were used in a static serum-containing culture of CB $CD34^+$ cells [129]. The presence of these beads did not influence total cell or $CD34^+$ cell expansion. After 9 days the CFU content in the culture containing beads was slightly higher compared to the same culture without beads. This static culture system with porous beads produced cells with a higher capacity to engraft sub-lethally irradiated NOD/SCID mice [129]. Similar collagen beads have been added to serum-containing stirred suspension cultures of BM MNCs, but this did not influence cell (total, $CD34^+$ and CFU) expansion [130].

Collagen beads, with a pre-grown murine stromal cell-line, were used as a packed bed and in suspension. The fixed system was optimized for the culture of CB $CD34^+$ cells. This resulted in a MNC expansion of 100 times, a CFU-GM expansion of 114 times

and a primitive CFU expansion of 15 times [131, 132]. Similar collagen beads with stromal cells were used in a packed bed system as illustrated in figure 8 [133-135].

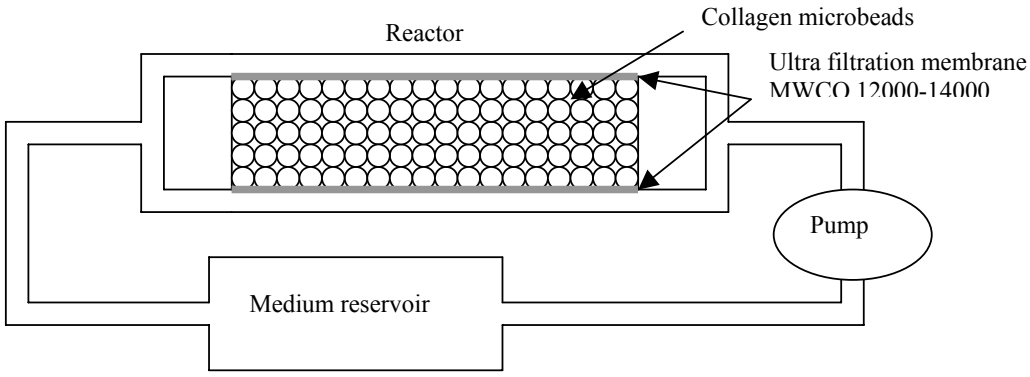


**Figure 8. Schematic drawing of the bioreactor used by Wang et al [133]**

This perfusion reactor was used for the culture of whole BM with serum-containing medium. The system supported the multi-lineage production of both murine [133, 134] and human blood cells [135] for at least one month.

Collagen sponges were also investigated as scaffold for BM culture systems. Glowacki *et al* investigated the influence of medium perfusion on stromal cell growth in collagen sponges. They found greater cellularity and ECM formation in perfused sponges and were able to grow cells from a hematopoietic cell line (CRX-1) on the stroma matrix. These cells did not survive without the stroma layer [136].

Coculture with stromal cells was also performed with gelatin-coated glass microcarriers in a perfused fixed-bed bioreactor. After 8 days of culture of human CB MNCs in serum-containing medium, the early progenitors CFU-GEMM and later progenitors CFU-GM and BFU-E expanded up to 4, 7 and 2 times, respectively [137].

Progenitor expansion without stromal cells was achieved with porous gelatin beads (Cultisphere-G). These supported a higher CFU content during the whole period of a 10 days static serum-free culture of CB MNCs [138]. Moreover, these beads were used in a stirred system after pre-seeding the beads with stromal cells. With this system a $CD34^+$ cell expansion of about 10 times and a CFU expansion of 23 times was achieved after 12 days. This was three times higher than with a comparable two-dimensional system [138].

The results of the reports discussed above are summarized in table 7. These cell expansions are difficult to compare to the results obtained with conventional systems as discussed in paragraph 2.2, since none of the biomimetic studies used PB cells and

most of the culture media contained serum. The use of stroma and serum usually leads to higher expansion ratios [139]. The use of CB instead of PB HPCs also contributes to higher expansion ratios, since CB HPCs expand easier [140, 141].

**Table 7. Overview of the conditions and results for the culture of human hematopoietic cells using porous collagen or gelatin beads as biomimetic environment.**

| Ref. | System | Cells | Stroma | Serum | Results |
|---|---|---|---|---|---|
| 129* | Static | CB $CD34^+$ | No | No | 12d: 8-11 x $CD34^+$ |
| 138# | Static | CB MNC | No | Yes | 8 d: 2x CFU |
| 138# | Stirred | CB $CD34^+$ | Yes | No | 7d: 13x $CD34^+$ & 22x CFU |
| 130* | Stirred | BM | Yes | Yes | 4 wk: 7x LTC-IC[i] & 22x CFU |
| 135 | Perfused | BM | Yes | Yes | >5 wk erythroid production |
| 132 | Perfused | CB $CD34^+$ | Yes | Yes | 10 d: 6x CAFC[ii] & 114x CFU-GM |
| 137 | Perfused | CB $CD34^+$ | Yes | Yes | 2 wk: 4x CFU-GEMM & 2x BFU-E |

* In these systems the expansion results of cultures without beads were the same.
# In these systems the expansion rates obtained using three-dimensional conditions were higher than for similar two-dimensional situations.
[i] Long-term-culture initiating-cells (LTC-ICs) are very primitive HPCs, which after differentiation on stroma can be characterized with a CFU assay.
[ii] Cobble-stone-area-forming cells (CAFCs) are primitive cells that form colonies below a stroma layer.

*Other proteins and glycoproteins*

The adhesion ligand Fibronectin (Fn) was coated on Cellfoam™ structures consisting of a tantalum-covered porous carbon matrix. These were seeded with human BM $CD34^+$ cells and cultured with serum-containing medium without additional cytokines. This scaffold allowed the culture of HPCs up to 6 weeks. After 1 week the number of $CD34^+$ cells increased 1.5 times using the scaffold, while it decreased on Fn-coated surfaces and was only maintained on a stroma surface [142]. The addition of cytokines to the three-dimensional system led to larger expansion of $CD34^+$ cells compared to the two-dimensional equivalents [143, 144]. Binding to Fn is not only important for homing and engraftment [145] but the activation of its CAM is also an important signal, that 'cross-talks' with that of cytokines [146-149], to support cell survival and proliferation [150, 151].

Besides adhesion ligands also signaling ligands like Jagged-1 can be immobilized on a surface to influence HPC behavior. The stimulation of the Notch receptor on murine BM cells with immobilized Jagged-1 increased their proliferation, while stimulation with Jagged-1 in solution decreased proliferation [152]. The chemokine SDF-1α coated onto a surface stimulated adhesion of human $CD34^+$ cells to co-immobilized adhesion proteins [153].

*Glycosaminoglycans*

As discussed in paragraph 2.1.3, proteoglycans and their GAG side chains are investigated for their role in binding and co-localization of hematopoietic factors with HPCs. HS and heparin are the most studied GAGs [154, 155]. Heparin is not present in the BM ECM, but is often used as model material for HS.

GAGs immobilized on chitosan membranes have been studied as substrate for CB $CD34^+$ cells. The use of chondroitin sulfate B and heparin-coated surfaces led to a 20 to 30-fold expansion of $CD34^+$ cells, while on the chitosan surface the HPCs only expanded 10 times [35].

Cytokines pre-adsorbed to immobilized GAGs have been used to study the effect of surface presentation and release. SDF-1α pre-adsorbed onto PGs on stromal cell lines improved the adhesion of KG-1a cells, which was reduced by GAG degrading ezymes [41]. Pre-adsorption of GM-CSF and IL-3 to HS-coated collagen [37] and HS-containing Matrigel [38] surfaces resulted in proliferation of cultured hematopoietic cell lines.

## 3. Concluding remarks

In adults the bone marrow is the principal site where the hematopoietic system produces blood cells. It is thought to contain niches in which HSCs are localized in a specific microenvironment. In their niche the cells interact with stromal cells, ECM components and hematopoietic factors like cytokines. ECM components can also interact with hematopoietic factors and present these to the HPCs.

In this chapter, the structure and components of the BM were introduced. Subsequently, serum- and stroma-free expansion of PB $CD34^+$ cells and *in vitro* erythropoiesis were reviewed. The overview of biomimetic and bioreactor HPC cultures showed that collagen is a suitable material for HPC culture in static and bioreactor systems. Collagen type I is an important component of the bone marrow ECM and can be processed into different morphologies to form a two- or three-dimensional structure suitable for cell culture. Additionally, chemical modification allows the immobilization of compounds present in the microenvironments of the bone marrow. This can be done with GAGs, since these play an important role in hematopoietic niches by binding and release of hematopoietic factors. A bioreactor containing these modified collagen structures could facilitate large scale HPC expansion.

## 4. References


1. Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. Biomed Pharmacother 2001;55:186-194.
2. Nilsson SK, Johnston HM, Coverdale JA. Spatial localization of transplanted hemopoietic stem cells: inferences for the localization of stem cell niches. Blood 2001;97:2293-2299.
3. Panoskaltsis N, Mantalaris A, Wu JHD. Engineering a Mimicry of Bone Marrow Tissue Ex Vivo. J Biosci Bioeng 2005;100:28-35.
4. Okumura N, Tsuji K, Nakahata T. Changes in Cell Surface Antigen Expression During Proliferation and Differentiation of Human Erythroid Progenitors. Blood 1992;80:642-650.
5. Nakahata T, Okumura N. Cell-Surface Antigen Expression in Human Erythroid Progenitors – Erythroid and Megakaryocytic Markers. Leuk Lymphoma 1994;13:401-409.
6. Ogawa M. Changing phenotypes of hematopoietic stem cells. Exp Hematol 2002;30:3-6.
7. Servida F, Soligo D, Caneva L, Bertolini F, De Harven E, Campiglio S, Corsini C, Deliliers GL. Functional and Morphological Characterization of Immunomagnetically Selected $CD34^{+}$ Hematopoietic Progenitor Cells. Stem Cells 1996;14:430-438.
8. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005;105:2631-2639.
9. Yin T, Li LH. The stem cell niches in bone. J Clin Invest 2006;116:1195-1201.
10. De Haan G. Hematopoietic stem cells: Self-renewing or aging? Cells Tissues Organs 2002;171:27-37.
11. Gregory CA, Prockop DJ, Spees JL. Non-hematopoietic bone marrow stem cells: Molecular control of expansion and differentiation. Exp Cell Res 2005;306:220-335.
12. Balduino A, Hurtado SP, Frazao P, Takiya CM, Alves LM, Nasciutti LE, El-Cheikh M, Borojevic R. Bone Marrow Subendosteal Microenvironment Harbours Functionally Distinct Haemosupportive Stromal Cell Populations. Cell Tissue Research 2005;319:255-266.
13. Clark BR, Keating A. Biology of Bone Marrow Stroma. Ann N Y Acad Sci 1995;770:70-78.
14. Calvi LM, Adams GB, Weibrecht KW, Weber JM, Olson DP, Knight MC, Martin RP, Schipani E, Divieti P, Bringhurst FR, Milner LA, Kronenberg HM, Scadden DT. Osteoblast cells regulate the haematopoietic stem cell niche. Nature 2003;425:841-846.
15. Zhang JW, Niu C, Ye L, Huang HY, He X, Tong WG, Ross J, Haug J. Johnson T, Feng JQ, Harris S, Wiedemann LW, Mishina Y, Li LH. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-841.
16. Zhu J, Emerson SG. A new bone to pick: osteoblasts and the haematopoietic stem-cell niche. BioEssays 2004;26:595-599.
17. El-Badri NS, Wang BY, Good RA. Osteoblasts promote engraftment of allogenic hematopoietic stem cells. Exp Hematol 1998;26:110-116.
18. Jung Y, Wang J, Havens A, Sun Y, Wang J, Jin T, Taichman RS. Cell-to-cell contact is critical for the survival of hematopoietic progenitor cells on osteoblasts. Cytokine 2005;32:155-162.
19. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: Stem Cells and Their Niches. Cell 2004;116:769-778.
20. Ohlstein B, Kai T, Decotto E, Spradling A. The Stem Cell Niche: Theme and Variation. Curr Opin Cell Biol 2004;16:693-699.

21. Kojika S, Griffin JD. Notch receptors and hematopoiesis. Exp Hematol 2001;29:1041-1052.
22. Gronthos S, Simmons PJ, Graves SE, Robey PG. Integrin-mediated interactions between human bone marrow stromal precursor cells and the extracellular matrix. Bone 2001;28:174-181.
23. Koeningsmann M, Griffin JD, Di Carlo J, Cannistra A.. Myeloid and Erythroid Progenitor Cells from Normal Bone Marrow Adhere to Collagen Type I. Blood 1992;79:657-665.
24. Fernandez M, Minguell JJ. The role of collagen in hemaopoiesis. Braz J Med Biol Res 1996;29:1201-1207.
25. Ruoslahti E. Proteoglycans in cell regulation. J Biol Chem 1989;264:13369-13372.
26. Tanaka Y, Kimata K, Adams DH, Eto S. Modulation of Cytokine Function by Heparan Sulfate Proteoglycans: Sophisticated Models for the Regulation of Cellular Responses to Cytokines. P Assoc Am Physician 1998;10:118-125.
27. Yayon A, Klagsbrun M, Esko JD, Leder P, Ornitz DM. Cell Surface, Heparin-like Molecules Are Required for Binding of Basic Fibroblast Growth Factor to Its High Affinity Receptor. Cell 1991;64:841-848.
28. Prestrelski SJ, Fox GM, Arakawa T. Binding of Heparin to Basic Fibroblast Growth Factor Induces a Conformational Change. Arch Biochem Biophys 1992;293:314-319.
29. Gospodarowicz D., Cheng J. Heparin Protects Basic and Acidic FGF From Inactivation. J Cell Physiol 1986;128:475-484.
30. Sommer A, Rifkin DB. Interaction of Heparin With Human Basic Fibroblast Growth Factor: Protection of the Angiogenic Protein From Proteolytic Degradation by a Glycosaminoglycan. J Cell Physiol 1989;138:215-220.
31. Pantoliano MW, Horlick RA, Springer BA, Van Dyk DE, Tobery T, Wetmore DR, Lear JD, Nahapetian AT, Bradley JD, Sisk WP. Multivalent Ligand-Receptor Binding Interactions in the Fibroblast Growth Factor System Produce a Cooperative Growth Factor and Heparin Mechanism for Receptor Dimerization. Biochemistry 1994;33:10229-10248.
32. Gupta P, McCarthy JB, Verfaillie CM. Stromal fibroblast heparan sulfate is required for cytokine- mediated ex vivo maintenance of human long-term culture-initiating cells. Blood 1996;87:3229-3236.
33. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche. Blood 1998;92:4641-4651.
34. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O- sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood 2000;95:147-155.
35. Madihally SV, Flake AW, Matthew HWT. Maintenance of CD34 Expression During Proliferation of $CD34^+$ Cord Blood Cells on Glycosaminoglycan Surfaces. Stem Cells 1999;17:295-305.
36. Stringer SE, Nelson MS, Gupta P. Identification of an MIP-1a binding heparan sulfate oligosaccharide that supports long-term in vitro maintenance of LTC-ICs. Blood 2003;101:2243-2245.
37. Alvarez-Silva M, Borojevic R. GM-CSF and IL-3 activities in schistosomal liver granulomas are controlled by stroma-associated heparan sulfate progeoglycans. J Leukoc Biol 1996;59:435-441.
38. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis. Nature 1988;332:376-378.

39. Gordon MY, Riley GP, Watt SM and Greaves MF. Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 1987;326:403-405.
40. Amara A, Lorthioir O, Valenzuela A, Magerus A, Thelen M, Montes M, Virelizier JL, Delepierre M, Baleux F, Lorat-Jacob H, Arenzana-Seisdedos F. Stromal Cell-derived Factor 1alpha Associates with Heparan Sulfates through the First Beta-strand of the Chemokine. J Biol Chem 1999;274:23916-23925.
41. Netelenbos T, Van den Born J, Kessler FL, Zweegman S, Merle PA, Van Oostveen JW, Zwaginga JJ, Huijgens PC, Drager AM. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. Leukemia 2003;17:175-184.
42. Andress DL. Heparin Modulates the Binding of Insulin-Like Growth-Factor (Igf) Binding Protein-5 to a Membrane-Protein in Osteoblastic Cells. J Biol Chem 1995;270:28289-28296.
43. Sebollela A, Cagliari TC, Limaverde GSCS, Chapeaurouge A, Sorgine MHF, Coelho-Sampaio, Ramos CHI, Ferreira ST. Heparin-binding Sites in Granulocyte-Macrophage Colony-stimulating Factor – Localization and regulation by histidine ionization. J Biol Chem 2005;280:31949-31956.
44. Mc Caffrey TA, Falcone DJ, Du BH. Transforming growth factor-beta-1 is a heparin binding protein – indentification of putative heparin-binding regions and isolation of heparins with varying affinity for TGF-beta-1. J Cell Physiol 1992;152:430-440.
45. Nilsson SK, Haylock DN, Johnston HM, Occhiodoro T, Brown TJ, Simmons PJ. Hyaluronan is synthesized by primitive hemopoietic cells, participates in their lodgment at the endosteum following transplantation, and is involved in the regulation of their proliferation and differentiation in vitro. Blood 2003;101:856-862.
46. Khaldoyanidi S, Moll J, Karakhanova S, Herrlich P, Ponta H. Hyaluronate-enhanced hematopoiesis: Two different receptors trigger the release of interleukin-1 beta and interlenkin-6 from bone marrow macrophages. Blood 1999;94:940-949.
47. Netelenbos T, Van der Born J, Kessler FL, Zweegman S, Huijgens PC, Drager AM. In vitro model for hematopoietic progenitor cell homing reveals endothelial heparan sulfate proteoglycans as direct adhesive ligands. J Leukoc Biol 2003;74:1035-1044.
48. Gordon MY, Riley GP, Clarke D. Heparan Sulfate is Necessary for Adhesive Interactions Between Human Early Hemopoietic Progenitor Cells and the Extracellular Matrix of the Bone Marrow Microenvironment. Leukemia 1988;2:804-809.
49. Siczkowski M, Clarke D, Gordon MY. Binding of Primitive Hematopoietic Progenitor Cells to Marrow Stoma Cell Involves Heparan Sulfate. Blood 1992;80:912-919.
50. Nilsson SK, Debatis ME, Dooner MS, Madri JA, Quesenberry PJ, Becker PS. Immunofluorescence characterization of key extracellular matrix proteins in murine bone marrow in situ. J Histochem Cytochem 1998;46:371-377.
51. Turner ML, Masek LC, Hardy CL, Parker AC, Sweetenham JW. Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. Br J Haematol 1998;100:112-122.
52. Holloway W, Martinez AR, Oh DJ, Francis K, Ramakrishna R, Palsson BO. Key adhesion molecules are present on long podia extended by hematopoietic cells. Cytometry 1999;37:171-177.
53. Coombe DR, Watt SM, Parish CR. Mac-1 (CD11b/CD18) and CD45 Mediate the Adhesion of Hematopoietic Progenitor Cells to Stromal Cell Elements Via Recognition of Stromal Heparan Sulfate. Blood 1994;84:739-752.

54. Peter K, Schwarz M, Conradt C, Nordt T, Moser M, Kubler W, Bode C. Heparin Inhibits Ligand Binding to the Leukocyte Integrin Mac-1 (CD11b/CD18). Circulation 1999;100:1533-1539.
55. Sovalat H, Racadot E, Ojeda M, Lewandowski H, Chaboute V, Henon P. $CD34^+$ cells and $CD34^+CD38^-$ subset from mobilized blood show different patterns of adhesion molecules compared to those from steady-state blood, bone marrow, and cord blood. J hematother stem cell res 2003;12:473-489.
56. Giet O, Van Bockstaele DR, Di Stefano I, Huygen S, Griemers R, Beguin Y, Gothot A. Increased binding and defective migration across fibronectin of cycling hematopoietic progenitor cells. Blood 2002;99:2023-2031.
57. Koenigsmann MP, Koenigsmann M, Notter M, Neuloh M, Mucke C, Thiel E, Berdel WE. Adhesion molecules on peripheral blood-derived $CD34^+$ cells: effects of cryopreservation and short-term ex vivo incubation with serum and cytokines. Bone Marrow Transplant 1998;22:1077-1085.
58. Mukhopadhyay A, Madhusudhan T, Kumar R. Hematopoietic Stem Cells: Clinical Requirements and Developments in Ex-Vivo Culture. Adv Biochem Engin / Biotechnol 2004;86:215-253.
59. McAdams TA, Miller WM, Papoutsakis ET. Hematopoietic cell culture therapies – 1 – Cell culture considerations. Trends Biotechnol 1996;14:341-349.
60. McAdams TA, Winter JN, Miller WM, Papoutsakis ET. Hematopoietic cell culture therapies – 2 – Clinical aspects and applications. Trends Biotechnol 1996;14:388-396.
61. Audet J, Zandstra PW, Eaves CJ, Piret JM. Advances in hematopoietic stem cell culture. Curr Opin Biotechnol 1998;9:146-151.
62. Devine SM, Lazarus HM, Emerson S. Clinical application of hematopoietic progenitor cell expansion: current status and future prospects. Bone Marrow Transplant 2003;31:241-252.
63. Yoshida T, Takagi M. Cell processing engineering for ex vivo expansion of hematopoietic cells: a review. Biochemical Engineering Journal 2004;20:99-106.
64. Attar EC, Scadden DT. Regulation of hematopoietic stem cell growth. Leukemia 2004;18:1760-1768.
65. Robinson S, Niu T, De Lima M, Ng J, Yang H, McMannis J, Karandish S, Sadeghi T, Fu P, Del Angel M, O'Connor S, Champlin R, Shpall E. Ex vivo expansion of umbilical cord blood. Cytotherapy 2005;7:243-250.
66. Sauvageau G, Iscove NN, Humphries RK. In vitro and in vivo expansion of hematopoietic stem cells. Oncogene 2004;23:7223-7232.
67. Jansen J, Hanks S, Thompson JM, Dugan MJ, Akard LP. Transplantation of hematopoietic stem cells from the peripheral blood. J Cell Mol Med 2005;9:37-50.
68. Dexter TM, Allen TD, Lajtha LG. Conditions Controlling the Proliferation of Haemopoietic Stem Cells In Vitro. J Cell Physiol 1977;91:335-344.
69. De Bruyn C, Delforge A, Bron D. Ex vivo myeloid differentiation of cord blood $CD34^+$ cells: comparison of four serum-free media containing bovine or human albumin. Cytotherapy 2003;5:153-160.
70. Janowska-Wieczorek A, Majka M, Ratajczak J, Ratajczak MZ. Autocrine/Paracrine Mechanisms in Human Hematopoiesis. Stem Cells 2001;19:99-107.
71. Majka M, Janowska-Wieczorek A, Ratajczak J, Ehrenman K, Pietrzkowski Z, Kowalska MA, Gewirtz AM, Emerson SG, Ratajczak MZ. Numerous growth factors, cytokines, and chemokines are secreted by human $CD34^+$ cells, myeloblasts, erythroblasts, and megakaryoblasts and regulate normal hematopoiesis in an autocrine/paracrine manner. Blood 2001;97:3075-3085.
72. Leberbauer C, Boulme F, Unfried G, Huber J, Beug H, Mullner EW. Different steroids co-regulate long-term expansion versus terminal differentiation in primary human erythroid progenitors. Blood 2005;105:85-94.

73. Von Lindern M, Zauner W, Mellitzer G, Steinlein P, Fritsch G, Huber K, Lowenberg B, Beug H. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. Blood 1999;94:550-559.
74. Broudy VC. Stem cell factor and hematopoiesis. Blood 1997;90:1345-1364.
75. Lyman SD, Jacobsen SEW. C-kit ligand and flt3 ligand: Stem/progenitor cell factors with overlapping yet distinct activities. Blood 1998;91:1101-1134.
76. Gilliland DG, Griffin JD. The roles of flt3 in hematopoiesis and leukemia. Blood 2002;100:1532-1542.
77. Namikawa R, Muench MO, Roncarolo MG. Regulatory roles of the ligand for Flk2/Flt3 Tyrosine Kinase receptor on human hematopoiesis. Stem Cells 1996;14:388-395.
78. Ivanovic Z. Interleukin-3 and ex vivo maintenance of hematopoietic stem cells: facts and controversies. Eur Cytokine Netw 2004;15:6-13.
79. Hauser SP, Kajkenova O, Lipschitz DA. The pivotal role of interleukin 6 in formation and function of hematopoietically active murine long-term bone marrow cultures. Stem Cells 1997;15:125-132.
80. Drachman JG. Role of trombopoietin in hematopoietic stem cell and progenitor regulation. Curr Opin Hematol 2000;**7:**183-190.
81. Verfaillie CM. Optimizing hematopoietic stem cell engraftment: a novel role for thrombopoietin. J Clin Invest 2002;110:303-304.
82. Testa U. Apoptotic mechanisms in the control of erythropoiesis. Leukemia 2004;18:1176-1199.
83. Barreda DR, Hanington PC, Belosevic M. Regulation of myeloid development and function by colony stimulating factors. Dev Comp Immunol 2004;28:509-554.
84. Kucia M, Jankowski K, Reca R, Wysoczynski M, Bandura L, Allendorf DJ, Zhang J, Ratajczak J, Ratajczak MZ. CXCR4-SDF-1 signaling, locomotion, chemotaxis and adhesion. J Mol Histol 2004;35:233-245.
85. Glimm H, Tang P, Clark-Lewis I, Von Kalle C, Eaves C. Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice. Blood 2002;99:3454-3457.
86. Zandstra PW, Petzer AL, Eaves CJ, Piret JM. Cellular determinants affecting the rate of cytokine depletion in cultures of human hematopoietic cells. Biotechnol Bioeng 1997;54:58-66.
87. Herrera C, Sanchez J, Torres A, Bellido C, Alvarez MA. Early-acting cytokine driven ex vivo expansion of mobilized peripheral blood $CD34^+$ cells generates post-mitotic offspring with preserved engraftment ability in non-obese diabetic / severe combined immunodeficient mice. Br J Haematol 2001;114:920-930.
88. Li K, Chuen CKY, Lee SM, Law P, Fok TF, Ng PC, Li CK, Wong D, Merzouk A, Salari H, Gu GJS, Yuen PMP. Small Peptide Analogue of SDF-1alpha Supports Survival of Cord Blood $CD34^+$ Cells in Synergy with Other Cytokines and Enhances Their Ex Vivo Expansion and Engraftment into Non-obese Diabetic / Severe Combined Immunodeficient Mice. Stem Cells 2006;24:55-64.
89. Broxmeyer HE, Kohli L, Kim CH, Lee Y, Mantel C, Cooper S, Hangoc G, Shaheen M, Li X, Clapp DW. Stromal cell-derived factor-1 / CXCL12 directly enhances survival/antiapoptosis of myeloid progenitor cells through CXCR4 and Galpha i proteins and enhances engraftment of competative, repopulating stem cells. J Leukoc Biol 2003;73:630-638.
90. Freyssinier JM, Lecoq-Lafon C, Picard F, Ducrocq R, Mayeux P. Purification, amplification and characterization of a population of human erythroid progenitors. Br J Haematol 1999;106:912-922.
91. Neildez-Nguyen TMA, Wajcman H, Marden MC, Bensidhoum M, Moncollin V, Giarratana MC, Kobari L, Thierry D, Douay L. Human erythroid cells produced ex vivo at large scale differentiate into red blood cells in vivo. Nat Biotechnol 2002;20:467-472.

92. Giarratana MC, Kobari L, Lapillonne H, Chalmers D, Kiger L, Cynober T, Marden MC, Wajcman H, Doay L. Ex Vivo generation of fully mature human red blood cells from hematopoietic cells. Nat biotechnol 2005;23:69-74.
93. Malik P, Fisher TC, Brasky LLW, Zeng L, Izadi P, Hiti A, Weinberg KI, Coates TD, Meiselman HJ, Kohn DB. An In Vitro Model of Human Red Blood Cell Production from Hematopoietic Progenitor Cells. Blood 1998;91:2664-2671.
94. Koller MR, Palsson BO. Tissue Engineering - Reconstitution of Human Hematopoiesis Ex- Vivo. Biotechnol Bioeng 1993;42:909-930.
95. Collins PC, Papoutsakis ET, Miller WM. Ex vivo culture systems for hematopoietic cells. Curr Opin Biotechnol 1996;7:223-230.
96. Nielsen LK. Bioreactors for hematopoietic cell culture. Annu Rev Biomed Eng 1999;1:129-152.
97. Cabral JMS. Ex vivo expansion of hematopoietic stem cells in bioreactors. Biotechnol Lett 2001;23:741-751.
98. Noll T, Jelinek N, Schmidt S, Biselli M, Wandrey C. Cultivation of Hematopoietic Stem and Progenitor Cells: Biochemical Engineering Aspects. Adv Biochem Eng Biotechnol 2002;74:111-128.
99. Cabrita GJM, Ferreira BS, Da Silva CL, Goncalves R, Almeida-Porada G, Cabral JMS. Hematopoietic stem cells: from the bone to the bioreactor. Trends in Biotechnol 2003;21:233-240.
100. Panoskaltsis N, Mantalaris A, Wu JHD. Engineering a Mimicry of Bone Marrow Tissue Ex Vivo. J Biosci Bioeng 2005;100:28-35.
101. Sardonini CA, Wu YJ. Expansion and Differentiation of Human Hematopoietic-Cells From Static Cultures Through Small-Scale Bioreactors. Biotechnol Prog 1993;9:131-137.
102. Highfill JG, Haley SD, Kompala DS. Large-scale production of murine bone marrow cells in an airlift packed bed bioreactor. Biotechnol Bioeng 1996;50:514-520.
103. Sytkowski AJ, Davis KL. Erythroid cell growth and differentiation in vitro in the simulated microgravity environment of the NASA rotating wall vessel bioreactor. In Vitro Cell Dev Biol Anim 2001;37:79-83.
104. Liu Y, Liu T, Fan X, Ma X, Cui Z. Ex vivo expansion of hematopoietic stem cells derived from umbilical cord blood in rotating wall vessel. J Biotechnol 2006;124:592-601.
105. De Leon A, Mayani H, Ramirez OT. Design, characterization and application of a minibioreactor for the culture of human hematopoietic cells under controlled conditions. Cytotechnology 1998;28:127-138.
106. Collins PC, Miller WM, Papoutsakis ET. Stirred culture of peripheral and cord blood hematopoietic cells offers advantages over traditional static systems for clinically relevant applications. Biotechnol Bioeng 1998;59:534-543.
107. Collins PC, Nielsen LK, Patel SD, Papoutsakis ET, Miller WM. Characterization of hematopoietic cell expansion, oxygen uptake, and glycolysis in a controlled, stirred-tank bioreactor system. Biotechnol Prog 1998;14:466-472.
108. Collins PC, Nielsen LK, Wong CK, Papoutsakis ET, Miller WM. Real-time method for determining the colony-forming cell content of human hematopoietic cell cultures. Biotechnol Bioeng 1997;55:693-700.
109. Kim BS. Production of human hematopoietic progenitors in a clinical- scale stirred suspension bioreactor. Biotechnol Lett 1998;20:595-601.
110. Kwon J, Kim BS, Kim MJ, Park HW. Suspension culture of hematopoietic stem cells in stirred bioreactors. Biotechnol Lett 2003;25:179-182.
111. Naughton BA, Tjota A, Sibanda B, Naughton GK. Hematopoiesis on suspended hylon screen-stromal cell microenvironments. J Biomech Eng 1991;113:171-177.
112. Li Y, Ma T, Kniss DA, Yang ST, Lasky LC. Human cord cell hematopoiesis in three-dimensional nonwoven fibrous matrices: In vitro simulation of the marrow microenvironment. J Hematother Stem Cell Res 2001;10:355-368.

113. Takagi M, Sasaki T, Yoshida T. Spatial development of the cultivation of a bone marrow stromal cell line in porous carriers. Cytotechnology 1999;31:225-231.
114. Tomimori Y, Takagi M, Yoshida T. The construction of an in vitro three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers. Cytotechnology 2000;34:121-130.
115. Sasaki T, Takagi M, Soma T, Yoshida T. 3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics. Cytotherapy 2002;4:285-291.
116. Takagi M, Iemoto N, Yoshida TU. Effect of concentrations of murine stromal and hematopoietic cells on the progenitors expansion in their three-dimensional coculture in nonwoven fabrics. J Biosci Bioeng 2002;94:365-367.
117. Sasaki T, Takagi M, Soma T, Yoshida T. Analysis of Hematopoietic Microenvironment Containing Spatial Development of Stromal Cells in Nonwoven Fabrics. J Biosci Bioeng 2003;96:76-78.
118. Koller MR, Bender JG, Miller WM, Papoutsakis ET. Expansion of Primitive Human Hematopoietic Progenitors in a Perfusion Bioreactor System With Il-3, Il-6, and Stem-Cell Factor. Bio/technology 1993;11:358-363.
119. Koller MR, Emerson SG, Palsson BO. Large-Scale Expansion of Human Stem and Progenitor Cells From Bone-Marrow Mononuclear-Cells in Continuous Perfusion Cultures. Blood 1993;82:378-384.
120. Palsson BO, Paek SH, Schwartz RM, Palsson M, Lee GM, Silver S, Emerson SG. Expansion of Human Bone-Marrow Progenitor Cells in a High Cell- Density Continuous Perfusion System. Bio/technology 1993;11:368-372.
121. Palsson BO, Oh DJ, Koller MR. Replating of bioreactor expanded bone marrow results in extended growth of primitive and mature cells. Cytotechnology 1995;18:125-131.
122. Sandstrom CE, Bender JG, Miller WM, Papoutsakis ET. Development of novel perfusion chamber to retain nonadherent cells and its use for comparison of human "mobilized" peripheral blood mononuclear cell cultures with and without irradiated bone marrow stroma. Biotechnol Bioeng 1996;50:493-504.
123. Koller MR, Manchel I, Maher RJ, Goltry KL, Armstrong RD, Smith AK. Clinical-scale human umbilical cord blood cell expansion in a novel automated perfusion culture system. Bone Marrow Transplant 1998;21:653-663.
124. Stiff P, Chen BH, Franklin W, Oldenberg D, Hsi E, Bayer R, Shpall E, Douville J, Mandalam R, Malhotra D, Muller T, Armstrong RD, Smith A. Autologous transplantation of ex vivo expanded bone marrow cells grown from small aliquots after high-dose chemotherapy for breast cancer. Blood 2000;95:2169-2174.
125. LaIuppa JA, McAdams TA, Papoutsakis ET, Miller WM. Culture materials affect ex vivo expansion of hematopoietic progenitor cells. J Biomed Mat Res 1997;36:347-359.
126. Koller MR, Palsson MA, Manchel I, Maher RJ, Palsson BO. Tissue culture surface characteristics influence the expansion of human bone marrow cells. Biomaterials 1998;19:1963-1972.
127. Tun T, Miyoshi H, Ema H, Nakauchi H, Ohshima N. New type of matrix support for bone marrow cell cultures: In vitro culture and in vivo transplantation experiments. Asaio Journal 2000;46:522-526.
128. Tun T, Miyoshi H, Aung T, Takahashi S, Shimizu R, Kuroha T, Yamamoto M, Ohshima N. Effect of growth factors on ex vivo bone marrow cell expansion using three-dimensional matrix support. Artif Organs 2002;26:333-339.
129. Kim HS, Lim JB, Min YH, Lee ST, Lyu CJ, Kim ES, Kim HO. Ex Vivo Expansion of Human Umbilical Cord Blood $CD34^+$ Cells in a Collagen Bead-Containing 3 Dimensional Culture System. Int J Hematol 2003;78:126-132.
130. Zandstra PW, Eaves CJ, Piret JM. Expansion of Hematopoietic Progenitor Cell Populations in Stirred Suspension Bioreactors of Normal Human Bone Marrow Cells. Bio/technology 1994;12:909-914.

131. Jelinek N, Schmidt S, Thoma S, Herfurth C, Biselli M. A novel minimized fixed-bed cultivation system for hematopoietic cells. Exp Hematol 2000;28:287.
132. Jelinek N, Schmidt S, Hilbert U, Thoma S, Biselli M, Wandrey C. Novel bioreactors for the ex vivo cultivation of hematopoietic cells. Chemical Engineering & Technology 2002;25:A15-A18.
133. Wang TY, Wu JHD. A Continuous Perfusion Bioreactor For Long-Term Bone-Marrow Culture. Ann N Y Acad Sci 1992;665:274-284.
134. Wang TY, Brennan JK, Wu JHD. Multilineal Hematopoiesis in a 3-Dimensional Murine Long-Term Bone-Marrow Culture. Exp Hematol 1995;23:26-32.
135. Mantalaris A, Keng P, Bourne P, Chang AYC, Wu JHD. Engineering a human bone marrow model: A case study on ex vivo erythropoiesis. Biotechnol Prog 1998;14:126-133.
136. Glowacki J, Mizuno S, Greenberger JS. Perfusion enhances functions of bone marrow stromal cells in three-dimensional culture. Cell Transplantat 1998;7:319-326.
137. Meissner P, Schroder B, Herfurth C, Biselli M. Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. Cytotechnology 1999;30:227-234.
138. Xiong F, Chen Z, Liu H, Xu Z, Liu X. Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system. Biotechnol Lett 2002;24:1421-1426.
139. Sandstrom CE, Miller WM, Papoutsakis T. Review: Serum-Free Media for Cultures of Primitive and Mature Hematopoietic Cells. Biotechnol Bioeng 1994;43:706-733.
140. Lewis ID, Verfaille CM. Multi-lineage expansion potential of primitive hematopoietic progenitors: Superiority of umbilical cord blood compared to mobilized peripheral blood. Exp Hematol 2000;28:1087-1095.
141. Tanavde VM, Malehorn MT, Lumkul R, Gao ZG, Wingard J, Garrett ES, Civin CI. Human stem-progenitor cells from neonatal cord blood have greater hematopoietic expansion capacity than those from mobilized adult blood. Exp Hematol 2002;30:816-823.
142. Bagley J, Rosenzweig M, Marks DF, Pykett MJ. Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device. Exp Hematol 1999;27:496-504.
143. Biber K, Ehring B, Popovsky M, Ballen K, Pigeon K, Plosky D, Pykett M, Rosenzweig M. Pre-clinical evaluation of a three-dimensional bioreactor for the maintenance and expansion of hematopoietic progenitors. Blood 2000;96:3347.
144. Banu N, Rosenzweig M, Kim H, Bagley J, Pykett M. Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine 2001;13:349-358.
145. Szilvassy SJ, Meyerrose TE, Ragland PL, Grimes B. Homing and engraftment defects in ex vivo expanded murine hematopoietic cells are associated with downregulation of beta 1 integrin. Exp Hematol 2001;29:1494-1502.
146. Levesque JP, Haylock DN, Simmons PJ. Cytokine Regulation of Proliferation and Cell Adhesion are Correlated Events in Human $CD34^+$ Hematopoietic Progenitors. Blood 1996;88:1168-1176.
147. Strobel ES, Mobest D, Von Kleist S, Dangel M, Ries S, Mertelsman R, Henschler R. Adhesion and Migration are Differentially Regulated in Hematopoietic Progenitor Cells by Cytokines and Extracellular Matrix. Blood 1997;90:3524-3532.
148. Levesque JP, Simmons PJ. Cytoskeleton and integrin-mediated adhesion signaling in human $CD34^+$ hemopoietic progenitor cells. Exp Hematol 1999;27:579-586.
149. Arai A, Nosaka Y, Kanda E, Yamamoto K, Miyasaka N, Miura O. Rap1 Is Activated by Erythropoietin or Interleukin-3 and Is Involved in Regulation of

beta 1 Integrin-mediated Hematopoietic Cell Adhesion. J Biol Chem 2001;276:10453-10462.
150. Dao MA, Hashino K, Kato I, Nolta JA. Adhesion to fibronectin maintains regenerative capacity during ex vivo culture and transduction of human hematopoietic stem and progenitor cells. Blood 1998;92:4612-4621.
151. Verfaillie CM. Adhesion Receptors as Regulators of the Hematopoietic Process. Blood 1998;92:2609-2612.
152. Vas V, Szilagyi L, Paloczi K, Uher F. Soluble Jagged-1 is able to inhibit the function of its multivalent form to induce hematopoietic stem cell self-renewal in a surrogate in vitro assay. J Leukoc Biol 2004;75:714-720.
153. Peled A, Grabovsky V, Habler L, Sandbank J, Arenzana-Seisdedos F, Petit I, Ben-Hur H, Lapidot T, Alon R. The chemokine SDF-1 stimulates integrin mediated arrest of $CD34^+$ cells on vascular endothelium under shear flow. J Clin Invest 1999;104:1199-1211.
154. Stringer SE, Gallagher JT. Heparan Sulphate. International Journal of Cell Biology 1997;29:709-714.
155. Mathews CK, Van Holde KE, Ahern KG. Biochemistry 3rd ed. San Francisco: Benjamin/Cummings, 2000.

# Chapter 3

**Heparinized crosslinked collagen films for hematopoietic progenitor cell culture**

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**Abstract**

A system to achieve proliferation and controlled differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great clinical interest and provides a model to investigate hematopoiesis.

Here we present a substrate for the culture of $CD34^+$ HPCs consisting of a crosslinked collagen film with covalently bound heparin. Type I collagen films were crosslinked and subsequently heparinized, both by using a water-soluble carbodiimide. Heparin was immobilized to an extent of $23 \pm 4$ μg per mg of collagen film.

$CD34^+$ HPCs were cultured on heparinized and non-heparinized crosslinked collagen films. Total cell numbers (the sum of adherent and non-adherent cells) only increased using heparinized films, on which a 5-fold expansion of the cell population was obtained after 2 wks. Heparinization of the films was also essential for expansion of colony-forming units (CFUs), both among cells adhering to the film and non-adhering cells. CFUs mainly expanded in the erythroid lineage.

Heparinized collagen can subsequently be processed into a three-dimensional microenvironment, loaded with hematopoietic factors and used in a bioreactor for hematopoietic cell culture.

## 1. Introduction

The *in vitro* expansion and controlled differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great interest for application in bone marrow

(BM) transplantation. Mimicking the BM environment in a bioreactor system is expected to improve HSC and HPC expansion and provide a model to investigate the mechanisms of hematopoiesis and processes such as stem cell mobilization and homing. There are several reports that claim good HPC expansion by using a bioreactor [1-8] or a three-dimensional culture system [9,10].

In adults the BM is the principal site where the hematopoietic system produces blood cells. It is accepted that the BM contains niches in which HSCs are localized in a specific microenvironment [11,12]. In their niche the cells interact with stromal cells, extracellular matrix (ECM) components and cytokines. This environment can be mimicked using a bioreactor system in which a porous structure, e.g. collagen, provides the three-dimensional surroundings for the stem cells. This substrate can be chemically modified in order to further mimic the ECM of the BM.

Collagen type I is an important component of the BM ECM. It can be processed into different structures suitable for cell culture. Additionally, collagen can be chemically modified with compounds present in the microenvironments of the BM. This can be done with glycosaminoglycans (GAGs), since these are important in keeping the ECM in a hydrated state and play an important role in chemokine function [13-18].

Here we investigated crosslinked type I collagen films, either heparinized or not, as culture substrates for HPC adhesion, expansion and differentiation. Heparin was used as a model compound for the GAGs present in the BM.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Collagen film preparation*

Collagen dispersions were prepared from type I insoluble collagen derived from bovine achilles tendon. The collagen (1 g) was swollen overnight in 0.05 M acetic acid solution (50 ml) at 4 ˚C. The mixture was dispersed with 50 g crushed ice for 4 min in a Philips blender and subsequently homogenized for 30 min at 4 ˚C using an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). The resulting slurry was

filtered through a 20 denier nylon filter (average pore size 30 μm) and a 25 μm filter (Cellector screen, Bellco, Feltham, UK) mounted in a disc filter holder. After degassing at 0.1 mbar the suspension was cast onto a poly(ethylene terephthalate) (PET) plate using a casting knife. The films were allowed to dry for 1 wk in a laminar flowhood.

Subsequently, these films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Fluka, Buchs, Switzerland) and N-hydroxysuccinimide (NHS) [19]. The scaffolds were first incubated in 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) for 30 min at room temperature. The samples were then transferred to the crosslinking solution, containing 2.3 g EDC and 0.56 g NHS in 215 ml MES-buffer per g of collagen (molar ratio of EDC/NHS = 2.5). After 2 h incubation under gentle shaking, the films were placed in a 0.1M $Na_2HPO_4$ (Merck, Darmstadt, Germany) solution for 2 h to stop the reaction and then rinsed with demineralized water for three times 30 min.

### *2.3. Heparin immobilization*

Crosslinked collagen films were subsequently heparinized. The films were first incubated in MES buffer (0.05 M, pH 5.6) for at least 30 min at room temperature. The carboxylic acid groups of heparin (from porcine mucosa, MW 6000-30000, activity>160 IU/mg) were pre-activated for 10 min. This was done by means of 0.43 g EDC and 0.16 g NHS in 188 ml of a 2 (wt)% heparin solution in MES-buffer [19].

The crosslinked collagen (1 g) was heparinized for 2 h in the EDC/NHS-activated heparin solution. Subsequently, the reaction was terminated by immersion of the films in 0.1 M sodium phosphate. The heparinized films were then washed in 2 M NaCl (24 h), 4 M NaCl (4 times 24 h) and MilliQ water (3 times 24 h).

### *2.4. Characterization of films*

The shrinkage temperature ($T_s$) of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC). This temperature, at which the collagen sample undergoes thermal denaturation, is used as a measure of the crosslink density [20]. Before measuring, samples of 5-10 mg were swollen overnight in 50 μl of phosphate buffered saline (PBS, B. Braun Medical, Oss, The Netherlands) in high-pressure capsules. The DSC thermogram was recorded on a DSC 7 (Perkin Elmer,

Wellesley, USA). Samples were heated from 20 ˚C to 90 ˚C at a heating rate of 10 ˚C/min. A sample containing 50 µl of PBS was used as a reference.

The residual number of free primary amino groups in (crosslinked) collagen was determined spectrophotometrically after reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fluka, Buchs, Switzerland) [21].

The heparin content of the films was determined by a colorimetric method based on the binding of toluidine blue [22]. A standard curve was prepared by mixing 0.5 ml of known heparin solutions and 0.5 ml of 0.15 mg/ml toluidine blue solution in water at room temperature. After adding 1 ml of cyclohexane and vortexing, the organic layer with the associated toluidine blue-heparin complex was removed by aspiration and the absorbance of the aqueous layer was measured at 630 nm. Samples (1 mg) of heparinized crosslinked collagen films were incubated overnight at room temperature in 1 ml of a 0.075 mg/ml toluidine blue solution. Subsequently, the absorbance of the solution was measured at 630 nm.

$^{3}$H-labelled heparin sodium salt (from porcine mucosa, MW 3000-30000, activity=195 IU/mg, Bufa Chemie, Uitgeest, The Netherlands) was also used to determine the heparin content of the structures. This heparin was tritiated using the method of Hatton *et al.* [23] and had an activity of 21 kBq/mg with a free label content of less than 3 % as determined by gel filtration using a PD10-Sepharose column (Pharmacia, Upsala, Sweden).

For quantification of the amount of immobilized heparin the samples were heparinized with the tritiated heparin as described for unlabelled heparin. The samples were dissolved in Luma Solve (2 ml, Lumac, Olen, Belgium) for 24 h at 60 ˚C. After addition of the scintillation cocktail (19 ml, Optiphase HiSafe 3, Wallac, Milton Keynes, UK) the radioactivity of the samples was measured using a 1410 Winspectral liquid scintillation counter (Wallac, Türku, Finland). The amount of immobilized heparin was calculated from the specific activity of the tritiated heparin.

To determine the amount of IL-3 adsorbed to the films from cell culture medium, circular crosslinked collagen films (d=15 mm) with and without immobilized heparin were fixed in the wells of a 24-well tissue culture polystyrene (TCPS) culture plate (Corning, NY, USA) with Viton O rings. These films were incubated overnight at 37ºC in a solution of penicillin (200 U/ml) and streptomycin (200 µg/ml; both from Gibco BRL, Paisley, UK). After rinsing 3 times with PBS, 0.5 ml of culture medium (see below) was added to the wells. After overnight incubation at 37ºC the

concentration of IL-3 in the medium was determined using an ELISA (Quantikine, R&D systems).

### *2.5. Hematopoietic cell culture*

Circular crosslinked collagen films (d=15 mm) with and without immobilized heparin were rinsed with 70 % ethanol. The films were fixed with Viton O rings in the wells of a 24-well tissue culture polystyrene culture plate and incubated overnight at 37 ˚C in a solution of 200 U/ml penicillin and 0.2 mg/ml streptomycin.

Human $CD34^+$ cells isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood were obtained from Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and cultured in a serum-free medium consisting of IMDM (with GlutaMAX I, Gibco), 1 (wt)% Bovine Serum Albumine (BSA), 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml recombinant human insulin, 200 μg/ml human partially iron-saturated holo-transferrin, 10 ng/ml TPO, 10 ng/ml IL-3, 100 ng/ml SCF, 50 ng/ml Flt3/Flk2-ligand. All four cytokines were from recombinant human origin (R&D systems, Minneapolis, USA). The cells were seeded by adding 0.5 ml of a cell suspension with a density of $1 \cdot 10^5$ cells/ml onto the heparinized and non-heparinized crosslinked collagen films. Wells with Viton O rings but no films were also seeded and used as control. The cells were cultured at 37 ˚C in a humidified 5 % $CO_2$ atmosphere. Half of the medium was exchanged twice a wk.

During cell culture 2 cell fractions were obtained. The cells that did not adhere to the films were removed by gentle pipetting. The adhering cells were obtained after rinsing the surfaces with PBS containing 1 (wt)% BSA and subsequent incubation in Cell Dissociation Solution (CDS) for 20 min at room temperature.

Flowcytometric phenotyping was performed using anti-CD45-FITC, anti-CD34-PE and 7AAD vitality dye (all from Immunotech, Marseille, France). Isotype-identical antibodies were used as control and for quantification Flow-Count beads were added to the samples. Cells were counted and analyzed using a Cytomics FC500 flow cytometry system (Beckman Coulter, Fullerton, USA). Moreover, differentiation was evaluated by Colony Forming Unit (CFU) assays. For CFU analysis, samples containing $10^3$ cells were taken from the HPC cultures, subsequently cultured in MethoCult (StemCell Technologies, Meylan, France) and scored after 2 wks. The

number of CFUs adhering to the heparinized and non-heparinized crosslinked collagen films as well as the number of non-adherent CFUs were determined. For quantification of the adherent CFUs, the cells were first removed from the films using CDS.

### *2.6. Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 3 experiments performed in duplicate. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values < 0.1.

## 3. Results

### *3.1. Heparinized crosslinked collagen film preparation*

Collagen films with a thickness of 50 μm were obtained. Crosslinking with EDC and NHS resulted in a decreased number of free primary amino groups while the shrinkage temperature increased. At the described conditions the number of free amino groups decreased from 27 to 16 per 1000 amino acid residues. The shrinkage temperature increased from 59 ˚C for uncrosslinked collagen to 76 ˚C for the crosslinked films. These results are in agreement with data previously reported by Wissink et al [19].

The toluidine blue assay indicated that 23 ± 4 μg heparin was immobilized per mg of collagen. This was confirmed by using radio-labelled heparin, of which 26 ± 3 μg was immobilized per mg of collagen. Toluidine blue was used for further measurements. Staining was observed throughout the thickness of the films, indicating that heparin was immobilized both on the surface and in the bulk of the film. These results also correspond to previously reported data [13].

Adsorption of IL-3 onto heparinized and non-heparinzed crosslinked collagen films during overnight incubation with 0.5 ml culture medium containing 10 ng/ml IL-3, decreased the IL-3 concentration in the culture medium in contact with these films with 4.1 ± 0.6 and 4.8 ± 0.6 ng/ml, respectively. Heparinized films subsequently released about 164 pg of the adsorbed IL-3 into cytokine-free culture medium during 1 d, while non-heparinized films released 8 times less IL-3 into cytokine-free culture medium (data not shown). This indicates that a part of the adsorbed IL-3 was reversibly bound to the heparinized film, while the remaining IL-3 was probably strongly adsorbed to the crosslinked collagen.

### *3.2. Adhering cells during cell seeding*

One h after seeding $CD34^+$ enriched (>90 %, on average 95 %) hematopoietic progenitor cells onto crosslinked and heparinized crosslinked collagen films, the viability of the non-adhering cells was not changed (on average 84 %). The viability of the adhering cells, however, was lower. On heparinized crosslinked collagen films the average viability of adhering cells 1 h after seeding was 77 % while on crosslinked collagen and TCPS this was even lower (60 and 63 %, respectively). The lower viability could partially be due to the procedure used to remove the cells from the surface.

The number of viable cells adhering to the heparinized crosslinked collagen surfaces and among these the number of $CD34^+$ progenitor cells were both significantly higher than the amount of these cells adhering to crosslinked collagen and TCPS (figure 1).

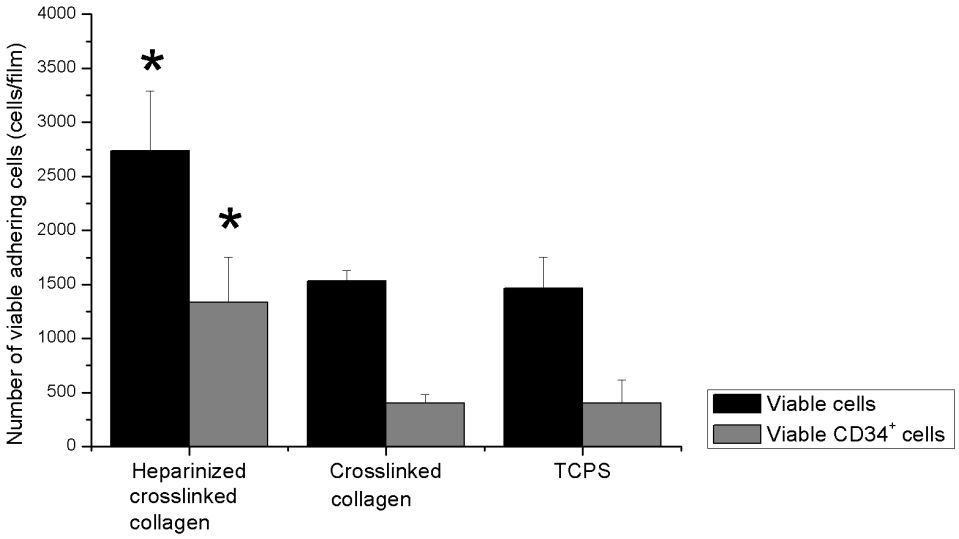


**Figure 1. Number of viable cells ($7AAD^-$ and $CD45^+$) and $CD34^+$ hematopoietic progenitor cells ($7AAD^-$, $CD45^+$ and $CD34^+$) adhering to the surfaces 1 h after seeding, determined from three independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to both crosslinked collagen films and TCPS surfaces.**

On heparinized crosslinked collagen 11 % of the seeded cells adhered to the surface and on crosslinked collagen and TCPS this value was 7 %. From the total $CD34^+$ cell population 9% adhered to the heparinized crosslinked collagen films, while 3-6 % adhered to TCPS and the crosslinked collagen films.

### *3.3. Adhering cells during cell proliferation*

The number of cells adhering to the surfaces increased during cell culture, except for the cells on crosslinked collagen (figure 2).

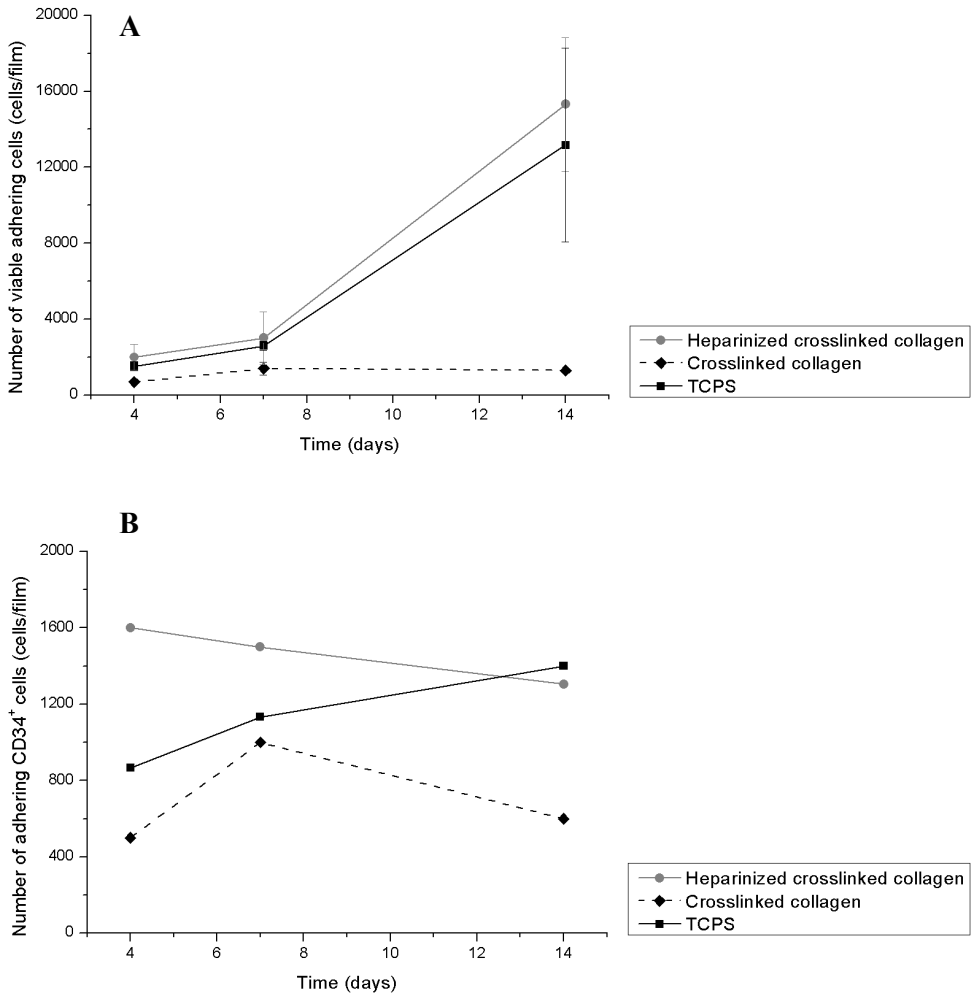


**Figure 2. Number of: A. viable cells (7AAD$^-$ and CD45$^+$) and B. viable CD34$^+$ cells (7AAD$^-$, CD45$^+$ and CD34$^+$) adhering to the surfaces during cell culture, determined from 3 independent experiments performed in duplicate (± S.E.M.).**

During 2 wks of cell culture the number of viable cells adhering to the surface as percentage of the total population remained constant at about 11 % for heparinized crosslinked collagen and 7 % for non-heparinized crosslinked collagen. On TCPS the number of viable cells adhering to the surface as percentage of the total population decreased to 3-5 %.

The number of CD34$^+$ cells adhering to the surfaces only increased during cell culture on TCPS (figure 2B). After 2 wks the initially lower number of CD34$^+$ cells adhering

to TCPS was similar to the numbers adhering to heparinized crosslinked collagen films. The number of $CD34^+$ cells adhering to the surfaces as percentage of the total $CD34^+$ cell population was about 3-4 % for heparinized crosslinked collagen and 10-15 % for non-heparinized crosslinked collagen. On TCPS the number of $CD34^+$ cells adhering to the surface as percentage of the total $CD34^+$ cell population increased from 9 to 16 %.

The average viability of adhering cells remained constant during 2 wks of cell culture, around 70 % for the cells adhering to heparinized crosslinked collagen and 60% for the cells adhering to TCPS and crosslinked collagen.

Films with 34 instead of 23 μg heparin per mg of collagen had 1.5 times more cells and even 2 times more $CD34^+$ cells adhering to the films during the 2 wks of cell culture (data not shown).

### *3.4. Hematopoietic cell proliferation during two weeks*

In the HPC cultures using heparinized crosslinked collagen films and TCPS the total (adherent and non-adherent) viable cell population expanded, while in cultures using non-heparinized films there was no proliferation (figure 3A).

During 2 wks the total number of $CD34^+$ cells remained the same on TCPS, but decreased on the heparinized and non-heparinized crosslinked collagen films (figure 3B). The total number of $CD34^+$ cells in cultures using heparinized films decreased slower compared to the numbers in cultures using crosslinked collagen films, indicating an improved environment for the maintenance of these cells.

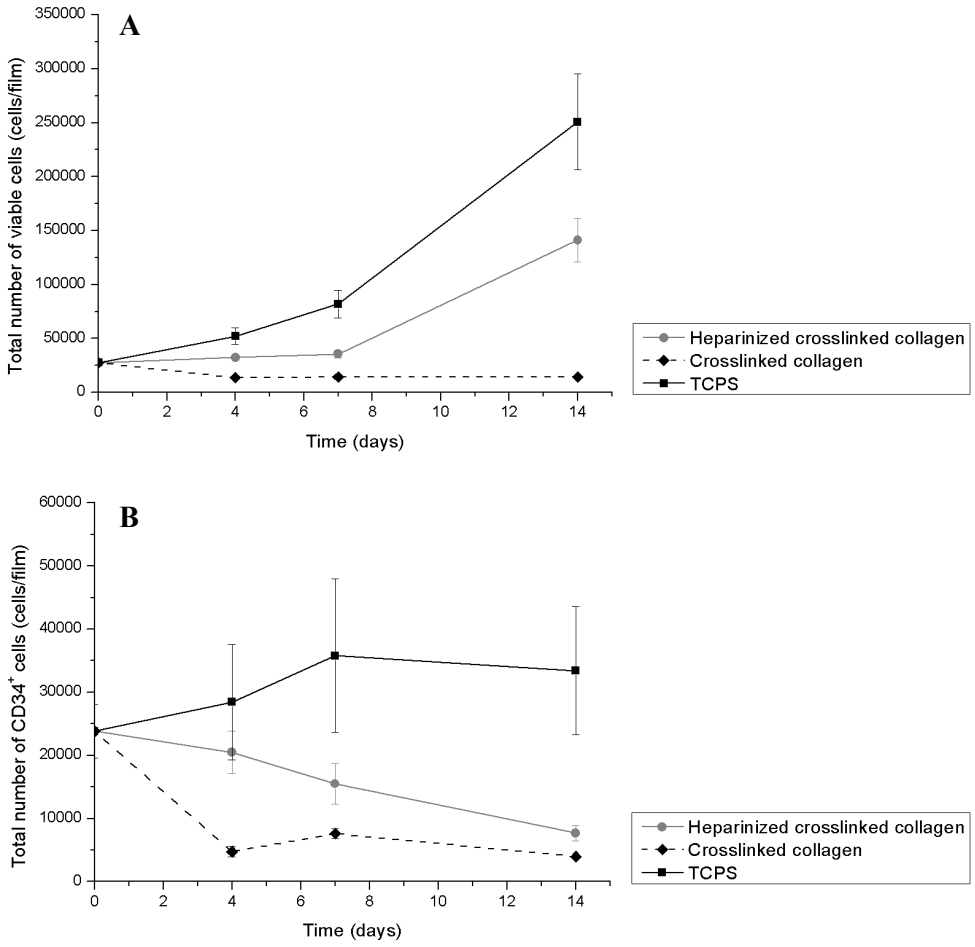


**Figure 3. Total number (adherent and non-adherent) of: A. viable cells ($7AAD^-$ and $CD45^+$) and B. viable $CD34^+$ cells ($7AAD^-$, $CD45^+$ and $CD34^+$), determined from 3 independent experiments performed in duplicate (± S.E.M.).**

The viability of non-adhering cells remained stable around 70 % during 2 wks of cell culture, except for the viability of cells on crosslinked collagen films, which decreased to almost 40 % during the first wk of culture and then recovered to 60 %. Using crosslinked collagen films with a higher heparin content (34 instead of 23 µg heparin per mg of collagen) did not influence the total viable cell or $CD34^+$ expansion (data not shown).

### *3.5. Colony forming cells during two weeks of culture*

The number of CFUs present among $10^3$ non-adhering cells increased 3-fold during the first days of cell culture using TCPS and heparinized crosslinked collagen (table 1). During cell culture using crosslinked collagen films without heparin, the amount of CFUs present among the non-adhering cells decreased to negligible numbers.

**Table 1. Colony-forming units (CFUs) per $10^3$ cells obtained at day 4 from non-adhering cells.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Initial cell batch* | 4 | 34 | 0 | 50 | 88 |
| TCPS | 1 | 59 | 9 | 27 | 95 |
| Heparinized collagen | 2 | 109 | 21 | 82 | 213 |
| Non-heparinized collagen | 0 | 3 | 1 | 1 | 6 |

* Cells from the same cell batch as used in the cell cultures on the different substrates but immediately seeded into MethoCult™.

For the cell cultures using TCPS, the amounts of CFUs among the non-adhering cells were 2 to 4 times lower compared to the numbers in cell cultures using heparinized crosslinked collagen (tables 2 and 3).

**Table 2. Colony-forming units (CFUs) per $10^3$ cells obtained from non-adhering cells when using TCPS.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 0* | 4 | 34 | 0 | 50 | 88 |
| Day 4 | 1 | 59 | 9 | 27 | 95 |
| Day 7 | 0 | 19 | 13 | 9 | 41 |
| Day 14 | 0 | 3 | 15 | 6 | 23 |

* Cells from the same cell batch as used in the cell cultures on the different substrates but immediately seeded into MethoCult™.

**Table 3. Colony-forming units (CFUs) per $10^3$ cells obtained from non-adhering cells when using heparinized crosslinked collagen.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 0* | 4 | 34 | 0 | 50 | 88 |
| Day 4 | 2 | 109 | 21 | 82 | 213 |
| Day 7 | 0 | 24 | 36 | 83 | 143 |
| Day 14 | 0 | 14 | 71 | 16 | 102 |

* Cells from the same cell batch as used in the cell cultures on the different substrates but immediately seeded into MethoCult™.

Differentiation mainly took place in the erythroid lineage and continued during the 2 wks of cell culture. This is shown by the continuous increase in CFU-E numbers during cell culture using heparinized crosslinked collagen films (table 3).

The amount of CFUs among the non-adhering cells during 2 wks of cell culture was comparable using crosslinked collagen films with a higher heparin content (34 instead of 23 μg heparin per mg of collagen).

The cell fractions adhering to TCPS and heparinized crosslinked collagen contained CFUs (tables 4 and 5). These were mainly of the erythroid lineage. At 2 wks about 10 % of the total amount of CFUs in the culture adhered to heparinized crosslinked collagen films. On crosslinked collagen no significant amount of CFUs adhered.

**Table 4. Colony-forming units (CFUs) per $10^3$ cells obtained from cells adhering to heparinized crosslinked collagen.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 4 | 0 | 3 | 0 | 4 | 7 |
| Day 7 | 0 | 2 | 2 | 2 | 6 |
| Day 14 | 0 | 2 | 47 | 20 | 69 |

**Table 5. Colony-forming units (CFUs) per $10^3$ cells obtained from cells adhering to TCPS.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 4 | 0 | 3 | 2 | 0 | 5 |
| Day 7 | 0 | 3 | 6 | 1 | 10 |
| Day 14 | 0 | 0 | 15 | 4 | 19 |

## 4. Discussion

Here we present a substrate for HPC culture, based on EDC and NHS crosslinked bovine type I collagen films. Crosslinked films were heparinized using the same EDC and NHS chemistry. Heparin was used as model material for GAGs like heparan sulfate, which are thought to play and important role in the BM. Heparinization of crosslinked collagen films resulted in increased numbers of adhering and non-adhering cells with a higher viability, improved maintenance of $CD34^+$ progenitor cells and expansion of CFUs into the erythroid lineage.

Upon cell seeding onto the heparinized and non-heparinized crosslinked collagen films, we found that a fraction of the cells adhered to the surfaces. When using substrates that mimic the BM in a perfused system the cells need to be retained, which could be achieved by cell adhesion. However, 1h after cell seeding only 11 % of the cells adhered to the heparinized surfaces. To the non-heparinized crosslinked collagen films only 7 % adhered. During 2 wks of HPC culture on the collagen substrates, the percentage of adhering cells remained the highest on the heparinized films (11 %). A higher heparin content of the films (34 instead of 23 μg heparin per mg of collagen) resulted in increased numbers of cells adhering to these substrates.

Adhesion of HPCs to heparinized crosslinked collagen surfaces is in accordance with literature. Previous studies showed that GAGs, especially heparan sulfate and heparin, function as adhesion ligands for HPCs [15,25,26]. Mac-1 (CD11b/CD18) and CD45 are reported to be adhesion molecules capable of binding to heparan sulfate and heparin [27]. Additionally, L-selectin and a phosphatidyl-inositol-linked adhesion molecule have been reported as adhesion molecules involved in hematopoietic cell binding to heparan sulfate [26,28]. Although cytokines are known to regulate the

processes of cell migration and adhesion [29,30], $CD34^+$ cells can also directly adhere to several immobilized cytokines, including IL-3 [31]. Therefore, the increased adhesion of $CD34^+$ cells to heparinized crosslinked collagen may also be explained in terms of cell adhesion to heparin-bound IL-3.

During 2 wks of cell culture the total number of viable cells expanded 5-fold when using the heparinized crosslinked collagen substrates. The viability of the cells remained constant. During this period the total number of $CD34^+$ cells decreased when using crosslinked collagen films, but heparinized collagen films showed a slower decrease compared to the non-heparinized films.

Heparinization of crosslinked collagen could have improved the cell culture results by two mechanisms. First, the functioning of cytokines in the medium could have been improved by the binding of these cytokines by heparin. Stroma-derived immobilized heparan sulfate has been shown to improve the activity of IL-3 [24]. The addition of heparan sulfate to HPC cultures has been reported to increase the maintenance of primitive cells [14, 15,16]. These results were explained by the co-localization of heparin-binding cytokines and $CD34^+$ cells as well as activation of the heparin-bound cytokines due to mechanisms like dimerization [15,16]. The poor performance of crosslinked collagen films can be explained by the strong adsorption of IL-3 to crosslinked collagen, which was probably accompanied by conformational changes in the cytokine. Heparinized crosslinked collagen also adsorbed a significant amount of IL-3. However, a part of the IL-3 bound to heparinized films was adsorbed reversibly, probably in a conformation that could stimulate HPC adhesion.

Second, the higher numbers of adherent cells on heparinized crosslinked collagen as compared to non-heparinized collagen could have supported the expansion of non-adherent primitive cells. The higher amount of adhering cells was probably caused by either direct binding of the cells to heparin or to heparin-bound IL-3, as discussed above. Though the number of adhering cells increased with increased heparin content, we did not find an improved HPC proliferation or maintenance.

A $CD34^+$ progenitor expansion using heparinized surfaces was not achieved in this system. In prior work, the $CD34^+$ progenitor expansion (15-fold in 2 wks) on heparinized surfaces achieved by Madihally *et al.* was performed using cord blood progenitors [22]. The different stem cell source used could explain the different results, since cord blood progenitors are documented to have a greater expansion

capacity than the cells obtained from peripheral blood after mobilization by G-CSF treatment [34].

In conventional serum-free peripheral blood cell cultures, not using heparinized surfaces, the $CD34^+$ expansion factors range from a temporary recovery or small expansion [34,35] to an expansion of 6 ± 2 times (medium supplemented with 100 ng/ml FL and SCF, and 20 ng/ml TPO) and 18 ± 4 times (FL, TPO, SCF, 20 ng/ml IL-3 and 20 ng/ml IL-6) after 1 wk [36]. As claimed by these and other authors [37], the cells remain primitive only up to three divisions *in vitro* (8-fold expansion), as shown by the ability of the cells to engraft NOD/SCID mice. These data show a large variety in expansion results, but also that the medium used in our experiments can still be optimised in order to achieve higher $CD34^+$ cell expansion. In our case, the addition of IL-6 or more TPO to the medium is expected to give improved $CD34^+$ expansion results.

During 2 wks of cell culture the number of CFUs among $10^3$ cells transiently expanded 3 times when using heparinized crosslinked collagen films, on which about 10% of the CFUs adhered. Using TCPS as culture substrate, the number of CFUs in culture were 2 to 4 times lower compared to heparinized collagen. Since no decrease in viability was noted during cell culture on TCPS, the CFUs must have differentiated faster into cells not able to form colonies. Thus, CFUs remained more primitive in cell cultures on heparinized crosslinked collagen films compared to TCPS. The numbers of CFUs in cultures using non-heparinized crosslinked collagen surfaces were negligible. Almost no CFUs adhered to crosslinked collagen, though it has been reported that myeloid and erythroid CFUs adhere to collagen type I [32]. This could be due to the use of a cell-line (MO7) in that study, as cell-lines can have different adhesion behaviour [33].

In conclusion, heparinization of crosslinked collagen for HPC culture resulted in increased numbers of adhering and non-adhering cells and improved $CD34^+$ maintenance. During 2 wks of culture CFUs expanded mainly into the erythroid lineage. This heparinized surface can be loaded with cytokines and/or chemokines to further mimic the hematopoietic microenvironment. The chemistry employed to heparinize the surface can also be used to immobilize specific GAGs isolated from the BM. Moreover, before modification, collagen can be processed into different morphologies to provide a three-dimensional environment for the culture of HPCs.

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## 6. References

1. Wang TY, Wu JHD. A continuous perfusion bioreactor for long-term bone marrow culture. Ann N Y Acad Sci 1992;665:274-284.
2. Wang TY, Brennan JK, Wu JHD. Multilineal hematopoiesis in a 3-dimensional murine long-term bone-marrow culture. Exp Hematol 1995;23:26-32.
3. Mantalaris A, Keng P, Bourne P, Chang AYC, Wu JHD. Engineering a human bone marrow model: a case study on ex vivo erythropoiesis. Biotechnol Prog 1998;14:126-133.
4. Bagley J, Rosenzwieg M, Marks DF, Pykett MJ. Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device. Exp Hematol 1999;27:496-504.
5. Meissner P, Schroder B, Herfurth C, Biselli M. Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. Cytotechnology 1999;30:227-234.
6. Tomimori Y, Takagi M, Yoshida T. The construction of an in vitro three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers. Cytotechnology 2000;34:121-130.
7. Banu N, Rosenzwieg M, Kim H, Bagley J, Pykett M. Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine 2001;13:349-358.
8. Sasaki T, Takagi M, Soma T, Yoshida T. 3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics. Cytotherapy 2002;4:285-291.
9. Kim HS, Lim JB, Min YH, Lee ST, Lyu CJ, Kim ES, Kim HO. Ex vivo expansion of human umbilical cord blood CD34+ cells in a collagen bead-containing 3 dimensional culture system. Int J Hematol 2003;78:126-132.
10. Xiong F, Chen Z, Liu H, Xu Z, Liu X. Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system. Biotechnol Lett 2002;24:1421-1426.
11. Zhang J, Niu C, Ye L, Huang HY, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li LH. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-841.
12. Yin T, Li LH. The stem cell niches in bone. J Clin Invest 2006;116:1195-1201.
13. Gupta P, McCarthy JB, Verfaillie CM. Stromal fibroblast heparan sulfate is required for cytokine-mediated ex vivo maintenance of human long-term culture-initiating cells. Blood 1996;87:3229-3236.

14. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche. Blood 1998;92:4641-4651.
15. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O- sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood 2000;95:147-155.
16. Gordon MY, Riley GP, Watt SM and Greaves MF. Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 1987;326:403-405.
17. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis. Nature 1988;332:376-378.
18. Tanaka Y, Kimata K, Adams DH, Eto S. Modulation of cytokine function by heparan sulfate proteoglycans: sophisticated models for the regulation of cellular responses to cytokines. P Assoc Am Physician 1998;10:118-125.
19. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, Feijen J. Immobilization of heparin to EDC/NHS-crosslinked collagen. characterization and in vitro evaluation. Biomaterials 2001;22:151-163.
20. Loke WK, Khor E. Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valve application by differential scanning calorimetry. Biomaterials 1995;16:251-258.
21. Wang CL, Miyata T, Wecksler B, Rubin AL, Stenzel KH. Collagen induced platelet aggregation and release. I. Effects of side-chain modifications and role of argynyl residues. Biochim Biophys Acta 1978;544:555-567.
22. Madihally SV, Flake AW, Matthew HWT. Maintenance of CD34 expression during proliferation of CD34(+) cord blood cells on glycosaminoglycan surfaces. Stem Cells 1999;17:295-305.
23. Hatton MWC, Berry LR, Machovich R, Regoeczi E. Titration of commercial heparins, by reaction with NaB3H4: chemical analysis and biological properties of the product. Anal Chem 1980;106:417-426.
24. Alvarez-Silva M, Borojevic R. GM-CSF and IL-3 activities in schistosomal liver granulomas are controlled by stroma-associated heparan sulfate progeoglycans. J Leukoc Biol 1996;59:435-441.
25. Siczkowski M, Clarke D, Gordon, MY. Binding of primitive hematopoietic progenitor cells to marrow stoma cell involves heparan sulfate. Blood 1992;80:912-919.
26. Netelenbos T, Van den Born J, Kessler FL, Zweegman S, Huijgens PC, Drager AM. In vitro model for hematopoietic progenitor cell homing reveals endothelial heparan sulfate proteoglycas as direct adhesive ligands. J Leukoc Biol 2003;74:1035-1044.
27. Coombe DR, Watt SM, Parish CR. Mac-1 (CD11b/CD18) and CD45 mediate the adhesion of hematopoietic progenitor cells to stromal cell elements via recognition of stromal heparan sulfate. Blood 1994;84:739-752.
28. Gordon MY, Lewis J, Marley SB, Grand FH, Goldman JM. Stromal cells negatively regulate haemopoietic progenitor cell activation via a phosphatidylinositol-anchored cell adhesion/signalling mechanism. Br J Haematol 1997;96:647-653.
29. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. The chemokine SDF-1 is a chemotractant for human CD34+ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34+ progenitors to peripheral blood. J Exp Med 1997;185:111-120.
30. Strobel ES, Mobest D, Von Kleist S, Dangel M, Ries S, Mertelsmann R, Henschler R. Adhesion and Migration are Differentially Regulated in Hematopoietic

Progenitor Cells by Cytokines and Extracellular Matrix. Blood 1997;90:3524-3532.

31. Long MW, Briddell R, Walter AW, Bruno E, Hoffman R. Human Hematopoietic Stem Cell Adherence to Cytokines and Matrix Molecules. J Clin Invest 1992;90:251-255.
32. Koeningsmann M, Griffin JD, DiCarlo J, Cannistra A. Myeloid and Erythroid Progenitor Cells from Normal Bone Marrow Adhere to Collagen Type I. Blood 1992; 79:657-665.
33. Turner ML, Masek LC, Hardy CL, Parker AC, Sweetenham JW Comparative adhesion of human haemopoietic cell lines to extracellular matrix components, bone marrow stromal and endothelial cultures. Br J Haematol 1998; 100:112-122.
34. Tanavde VM, Malehorn MT, Lumkul R, Gao ZG, Wingard J, Garrett ES, Civin CI. Human stem-progenitor cells from neonatal cord blood have greater hematopoietic expansion capacity than those from mobilized adult blood. Exp Hematol 2002;30:816-823.
35. Danet GH, Lee HW, Luongo JL, Simon MC, Bonnet DA. Dissociation between stem cell phenotype and NOD/SCID repopulating activity in human peripheral blood CD34+ cells after ex vivo expansion. Exp Hematol 2001;29:1465-1473.
36. Herrera C, Sanchez J, Torres A, Bellido C, Rueda A, Alvarez MA. Early-acting cytokine driven ex vivo expansion of mobilized peripheral blood CD34+ cells generates post-mitotic offspring with preserved engraftment ability in non-obese diabetic / severe combined immunodeficient mice. Br J Haematol 2001;114:920-930.
37. Glimm H, Eaves CJ Direct Evidence for Multiple Self-Renewal Divisions of Human In Vivo Repopulating Hematopoietic Cells in Short-Term Culture. Blood 1999 94:2161-2168.

# Chapter 4

## Heparinized crosslinked collagen films pre-adsorbed with SDF-1α for hematopoietic progenitor cell culture

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**Abstract**

A system to achieve proliferation and controlled differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great clinical interest and provides a model to investigate hematopoiesis. Previously, we used heparinized crosslinked collagen films as substrates for serum-free HPC culture. Here, we pre-adsorb the heparin-binding chemokine stromal cell-derived factor-1α (SDF-1α) to these films to present or deliver this molecule to the cells cultured on these substrates.

Using an SDF-1α concentration of 2.22 μg/ml, the adsorption of SDF-1α onto heparinized crosslinked collagen films was 25 ± 4 ng per mg of film, with a diameter of 15 mm and a thickness of 50μm, as compared to 18 ± 2 ng per mg onto non-heparinized films. Both from heparinized and non-heparinized crosslinked collagen films, approximately 40 % of the adsorbed SDF-1α was released during 2 wks into culture medium, when employing half medium changes twice a wk.

One h after seeding human $CD34^+$ HPC from G-CSF mobilized peripheral blood, the number of adhering cells increased up to 2-fold as a result of SDF-1α pre-adsorption to the heparinized and non-heparinized crosslinked collagen films. Pre-adsorption of SDF-1α to the heparinized films resulted in a 2-fold increased adhesion of colony-forming units (CFUs) to the surface at d 4 of the cell culture. Pre-adsorbed SDF-1α did not influence proliferation and differentiation of the $CD34^+$ cells during 2 wks of culture.

These interactions between surface material, chemokine and cells may be further exploited to create an artificial three-dimensional environment that could be used in a bioreactor for hematopoietic cell culture.

## 1. Introduction

The expansion and controlled differentiation of hematopoietic stem cells (HSCs) is of great interest for application in bone marrow transplantation. The use of a bioreactor system that mimicks the bone marrow environment is expected to improve the expansion of HSCs and progenitor cells (HPCs). Additionally, such a system provides a model to investigate the mechanisms of hematopoiesis and processes such as stem cell mobilization and homing. There are several reports that claim good stem cell expansion by using a bioreactor [1-8] or a three-dimensional culture system [9,10].

In adults the bone marrow is the principal site where the hematopoietic system produces blood cells. It is generally accepted that the bone marrow contains specific microenvironments in which the HSCs interact with stromal cells, extracellular matrix (ECM) components and cytokines [11,12]. This environment can be mimicked by a porous structure that provides the three-dimensional surroundings for the stem cells. This substrate can be chemically modified in order to further mimic the ECM of the bone marrow.

Collagen type I is a major constituent of the bone marrow ECM. Collagen can be processed into structures like films, beads and sponges, which are suitable as substrates for cell culture. Additionally, collagen can be chemically modified [13] with compounds present in the microenvironments of the bone marrow. This can be done with glycosaminoglycans (GAGs), since these are involved in keeping the ECM in a hydrated state and play an important role in chemokine function [14-19]. Previously, we demonstrated that $CD34^+$ cells can be cultured on heparinized crosslinked collagen films. Heparin served as a model compound for the ECM component heparan sulfate. Heparinization improved $CD34^+$ cell survival, and expansion of colony-forming units (CFUs) in the erythroid lineage. After 2 weeks total viable cell numbers were expanded 5-fold [20].

Heparin is known to bind stromal cell-derived factor-1α (SDF-1α) [21]. The binding of SDF-1α by heparan sulfates in the bone marrow is thought to facilitate HSC homing, adhesion and the development of the marrow in the bone [22-24].

Additionally, this chemokine is produced by HSC-supporting osteoblasts [24] and is reported to play a role in the retention of erythroid progenitors in the bone marrow until they are released when mature [25].

We hypothesized that SDF-1α pre-adsorbed onto a surface can be used for the retention of hematopoietic progenitor cells (HPCs), especially when used in a bioreactor. Moreover, the interactions between this chemokine, ECM components and HPCs can serve as a model for the bone marrow niches. Therefore, we investigated the influence of SDF-1α adsorption onto crosslinked and heparinized crosslinked type I collagen films on HPC adhesion, expansion and differentiation.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Collagen film preparation*

Collagen dispersions were prepared from type I insoluble collagen derived from bovine achilles tendon. Collagen (1 g) was swollen overnight in 0.05 M acetic acid solution (50 ml) at 4 ˚C. The mixture was dispersed with 50 g crushed ice for 4 min in a Philips blender and subsequently homogenized for 30 min at 4 ˚C using an Ultra-Turrax T25 (IKA Labortechnik, Staufen, Germany). The resulting slurry was filtered through a 20 denier nylon filter (average pore size 30 μm) and a 25 μm filter (Cellector screen, Bellco, Feltham, UK) mounted in a disc filter holder. After degassing at 0.1 mbar the suspension was cast onto a poly(ethylene terephthalate) (PET) plate using a casting knife. The films were allowed to dry for 1 wk at ambient conditions in a laminar flowhood.

Subsequently, these films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Fluka, Buchs, Switzerland) and N-hydroxysuccinimide (NHS) [13]. The scaffolds were first incubated in 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) for 30 min at room temperature. The samples were then transferred to the crosslinking solution, containing 2.3 g EDC and 0.56 g NHS in 215 ml MES-buffer per g of collagen (molar

ratio of EDC/NHS = 2.5). After 2 h incubation under gentle shaking, the films were placed in a 0.1M $Na_2HPO_4$ (Merck, Darmstadt, Germany) solution for 2 h to stop the reaction and then rinsed with demineralized water for three times 30 min.

### *2.3. Heparin immobilization*

Crosslinked collagen films were incubated in MES buffer (0.05 M, pH 5.6) for at least 30 min at room temperature. The carboxylic acid groups of heparin (from porcine mucosa, MW 6000-30000, activity>160 IU/mg) were pre-activated for 10 min. This was done by means of 0.43 g EDC and 0.16 g NHS in 188 ml of a 2 (wt)% heparin solution in MES-buffer [13].

The crosslinked collagen (1 g) was heparinized for 2 h in the EDC/NHS-activated heparin solution. Subsequently, the reaction was terminated by immersion of the films in 0.1 M sodium phosphate. The heparinized films were then washed in 2 M NaCl (24 h), 4 M NaCl (4 times 24 h) and MilliQ water (3 times 24 h).

### *2.4. Characterization of films*

The shrinkage temperature ($T_s$) of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC). This temperature, at which the collagen sample undergoes thermal denaturation, is used as a measure of the crosslink density [26]. Before measuring, samples of 5-10 mg were swollen overnight in 50 µl of phosphate buffered saline (PBS, B. Braun Medical, Oss, The Netherlands) in high-pressure capsules. DSC measurements were carried out on a DSC 7 (Perkin Elmer, Wellesley, USA). Samples were heated from 20 ˚C to 90 ˚C at a heating rate of 10 ˚C/min. A sample containing 50 µl of PBS was used as a reference.

The residual number of free primary amino groups in (crosslinked) collagen was determined spectrophotometrically after reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS, Fluka) [27].

The heparin content of the films was determined by a colorimetric method based on the binding of toluidine blue [20].

### *2.5. $^{125}$I-labeling of SDF-1α*

Human recombinant SDF-1α (R&D systems, Minneapolis, USA) was labelled with $^{125}$I using Iodobeads [28]. In short, 3 iodobeads (Pierce, Rockford, USA) were added to 250 µCi $^{125}$I-Na (Amersham Biosciences UK, Little Chalfont, UK) in 100 µl phosphate buffer (100 mM, PH 7.0). After 5 min, SDF-1α solution in phosphate

buffer (1 ml, 10 μg SDF-1α/ml) was added and iodination was carried out under gentle shaking at room temperature. After 15 min, the beads were removed and rinsed in 150 μl phosphate buffer. To the total aliquot of phosphate buffer, 600 μl bovine serum albumin (BSA) solution in phosphate buffer was added, giving a final albumin concentration of 1mg/ml. Residual $^{125}$I was removed from the $^{125}$I-labelled SDF-1α solution by purification over a series of 3 PD10-columns. Thereafter, the radio-labelled SDF-1α solution was alliquoted and stored at –20 ˚C until use.

### *2.6. SDF-1α adsorption and release*

Circular crosslinked collagen films with and without immobilized heparin were fixed in the wells of a 24-well tissue culture polystyrene (TCPS) culture plate (Corning, NY, USA) with Viton O rings. TCPS wells with and without films (d = 15 mm) were incubated overnight at 37 ˚C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml; both from Gibco BRL, Paisley, UK).

After rinsing three times with PBS singular films were incubated overnight at room temperature in 350 μl of a $^{125}$I-labelled SDF-1α solution in PBS (2.22 μg/ml as used in other adhesion studies [23]) containing 0.1 (wt)% BSA. Thereafter, the films were rinsed three times with PBS and the radioactivity of the samples was measured using a Compugamma 1282 γ-counter (LKB, Stockholm, Sweden).

The release of SDF-1α was determined at 37 ˚C by placing heparinized and non-heparinized crosslinked collagen films pre-adsorbed with $^{125}$I-labelled SDF-1α in 0.5 ml culture medium (see below) containing 0.02 (wt)% $NaN_3$. Half of the medium was refreshed twice a wk and the radioactivity of the removed medium was measured. Alternatively, the release of SDF-1α during the first hrs after transfer of the films into culture medium was determined by means of whole media replacements. At the end of the release period (2 wks), the radioactivity remaining associated with the films was measured as well. The amount of SDF-1α released into the medium was also determined using an ELISA (Quantikine, R&D systems).

### *2.7. Hematopoietic cell culture*

Circular crosslinked collagen films (d = 15 mm) with and without immobilized heparin were rinsed with 70 % ethanol. The films were fixed with Viton O rings in the wells of a 24-well tissue culture polystyrene (TCPS) culture plate (Corning, NY, USA) and incubated overnight at 37 ˚C in a solution of 200 U/ml penicillin and 0.2

mg/ml streptomycin. Before cell seeding, the films were incubated once more overnight at room temperature in a PBS solution containing 0.1 (wt)% BSA with or without 2.22 μg/ml SDF-1α.

Human $CD34^+$ cells isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood were obtained from the Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and cultured in a serum-free medium consisting of IMDM (with GlutaMAX I, Gibco), 1 (wt)% BSA, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml recombinant human insulin, 200 μg/ml human partially iron-saturated holo-transferrin, 10 ng/ml TPO, 10 ng/ml IL-3, 100 ng/ml SCF, 50 ng/ml Flt3/Flk2-ligand. All four cytokines were from recombinant human origin (R&D systems). The cells were seeded by adding 0.5 ml of a cell suspension with a density of $1 \cdot 10^5$ cells/ml onto the heparinized and non-heparinized crosslinked collagen films. Wells with Viton O rings but no films were also seeded and used as control. The cells were cultured at 37˚C in a humidified 5% $CO_2$ atmosphere. Half of the medium was exchanged twice a wk.

The effect of surface-adsorbed SDF-1α on cell adhesion was investigated using surfaces without SDF-1α (neither on the surface nor in the medium), SDF-1α pre-adsorbed surfaces and surfaces where SDF-1α was added only to the culture medium at the concentration (50 ng/ml) approximately released into the medium during 1h, as discussed below.

During cell culture two cell fractions were obtained. The cells that did not adhere to the films were removed by gentle pipetting. The adhering cells were obtained after rinsing the surfaces with PBS containing 1 (wt)% BSA and subsequent incubation at room temperature (20 min.) in Cell Dissociation Solution (CDS), a non-enzymatic reagent for the gentle removal of cells from a surface.

Flowcytometric phenotyping was performed using anti-CD45-FITC, anti-CD34-PE and 7AAD vitality dye (all from Immunotech, Marseille, France). Isotype-identical antibodies were used as control and for quantification Flow-Count beads were added to the samples. Cells were counted and analyzed using a Cytomics FC500 flow cytometry system (Beckman Coulter, Fullerton, USA). Moreover, differentiation was evaluated by Colony Forming Unit (CFU) assays. The numbers of CFUs adherent to the heparinized and non-heparinized crosslinked collagen films as well as the non-

adherent CFUs were determined. For quantification of the adherent CFUs the cells were first removed from the surface using CDS. For CFU analysis, samples containing $10^3$ cells were taken from the HSC cultures, subsequently cultured in MethoCult (StemCell Technologies, Meylan, France) and scored after 2 wks.

### *2.8. Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 3 experiments performed in duplicate, except for SDF-1α release which was determined from 2 experiments performed in triplicate. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values < 0.1.

## 3. Results

### *3.1. Collagen film preparation and heparin immobilization*

Compact collagen films with a thickness of 50 μm were obtained (figure 1).

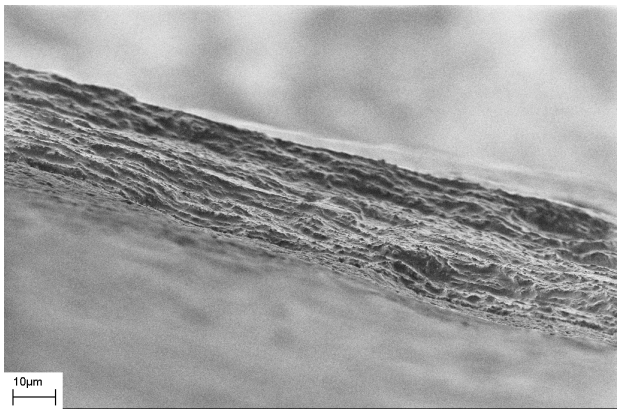


**Figure 1. Scanning electron microscopy (S.E.M.) micrograph of a crosslinked collagen film (original magnification 800x).**

Crosslinking with EDC and NHS resulted in a decrease of the number of free primary amino groups from 27 to 16 per 1000 amino acid residues while the shrinkage temperature increased from 59 ˚C to 76 ˚C, as reported elsewhere [20].

The crosslinked collagen films were heparinized. As previously reported 23 ± 4 μg heparin was immobilized per mg of collagen [20]. Toluidine blue staining was observed throughout the thickness of the films, indicating that heparin was immobilized both on the surface and in the bulk of the film.

### *3.2. SDF-1α adsorption and release*

Under the described conditions 25 ± 4 ng and 18 ± 2 ng SDF-1α (8.0 kDa) bound per mg of heparinized and non-heparinized crosslinked collagen film, respectively. Overnight incubation with a solution of penicillin and streptomycin in PBS did not influence the binding of SDF-1α. To compare the adsorption of SDF-1α to the films and TCPS, the amounts of adsorbed chemokine were expressed per surface area, using the external surface area of the films fixed in the wells. For heparinized and non-heparinized crosslinked collagen this amounted to 59 ± 9 ng and 47 ± 5 ng per $cm^2$, respectively. The adsorption of SDF-1α onto TCPS was 21 ± 1 ng per $cm^2$. This is 20 times lower than the adsorption of BSA onto TPCS from a solution of 15 mg/ml [29].

Because during cell culture half of the medium was changed twice a wk, the release of $^{125}$I-labeled SDF-1α was determined under these conditions (figure 2A). Similar release profiles were obtained with unlabeled SDF-1α when measured with an SDF-1α ELISA (figure 2B). In 2 wks, 40 % of the chemokine was released both from the heparinized and non-heparinized crosslinked collagen films (figure 2C). Although it has been reported that IL-3 can also interact with heparin [15,19,31], the absence of IL-3 (10 ng/ml) from the culture medium did not affect the release of SDF-1α (data not shown).

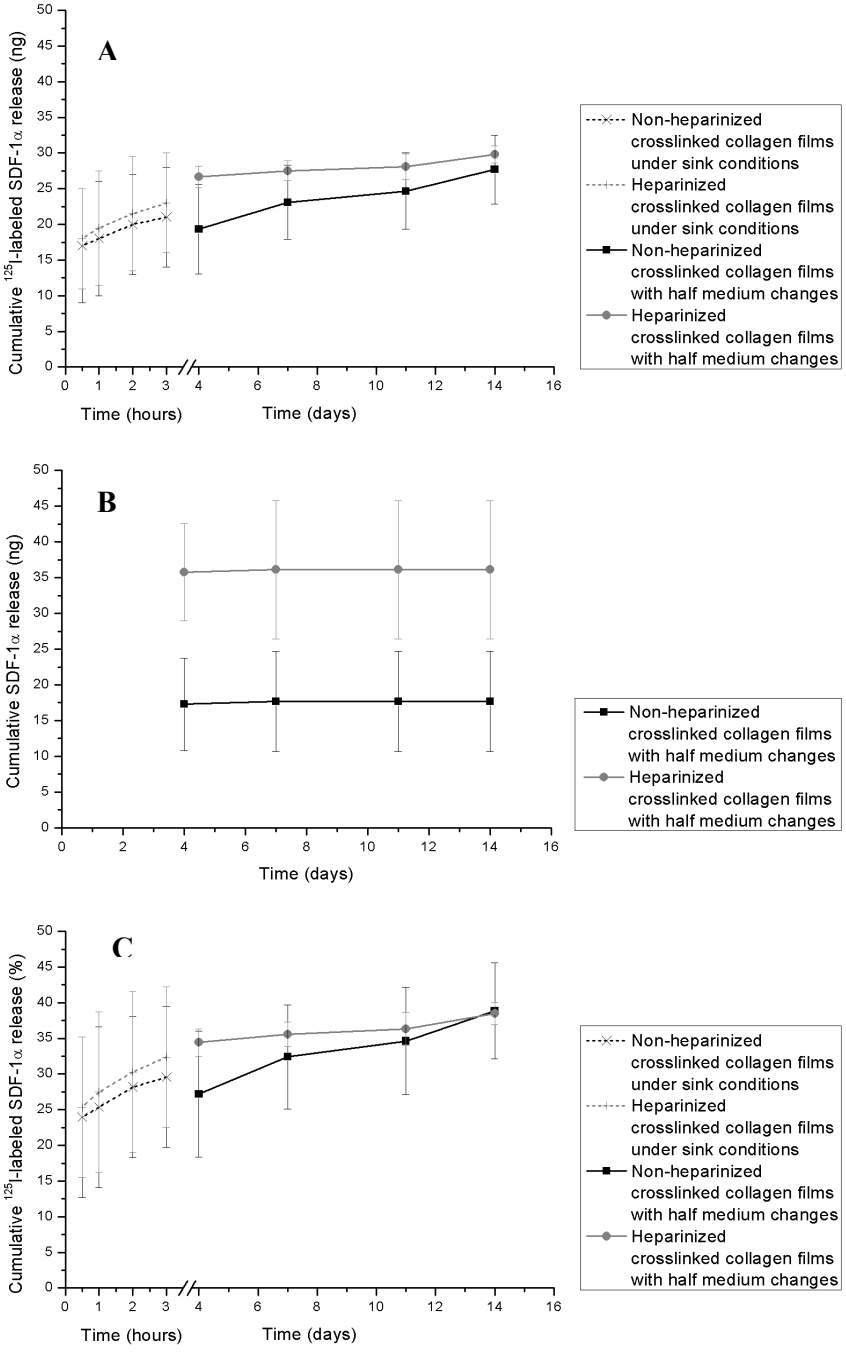


**Figure 2. Release of SDF-1α from heparinized and non-heparinized crosslinked collagen films into 0.5 ml culture medium under cell culture conditions. Circular films with a diameter of 15 mm and a thickness of 50 µm were pre-adsorbed overnight in 350 µl SDF-1α solution (2.22 µg/ml). The SDF-1α concentrations in the supernatant were determined using $^{125}$I-labeled SDF-1α (A&C) or using an SDF-1α-ELISA (B), both from two independent experiments performed in triplicate (± S.D.). The cumulative release of $^{125}$I-labeled SDF-1α was determined both from whole medium changes (A &C the first hours) and when employing half medium changes twice a week (A &C day 4 to 14).**

By measuring the radioactivity of the films after 2 wks, it was confirmed that both in case of crosslinked collagen and heparinized crosslinked collagen 60 % of the SDF-1α remained associated with the films. This SDF-1α could later be desorbed using a 4M NaCl solution (data not shown).

The SDF-1α concentration in the medium during culture conditions is shown in figure 3. At 1 h the concentration of SDF-1α in the culture medium after release from heparinized crosslinked collagen films was 39 ng/ml (47 ng/ml determined with ELISA). This concentration was in the same range as the concentration of SDF-1α when this was added to the culture medium (50 ng/ml). The release of SDF-1α into the culture medium from TCPS pre-adsorbed with the chemokine was only determined after 1 h and amounted to 8 ng/ml (data not shown).

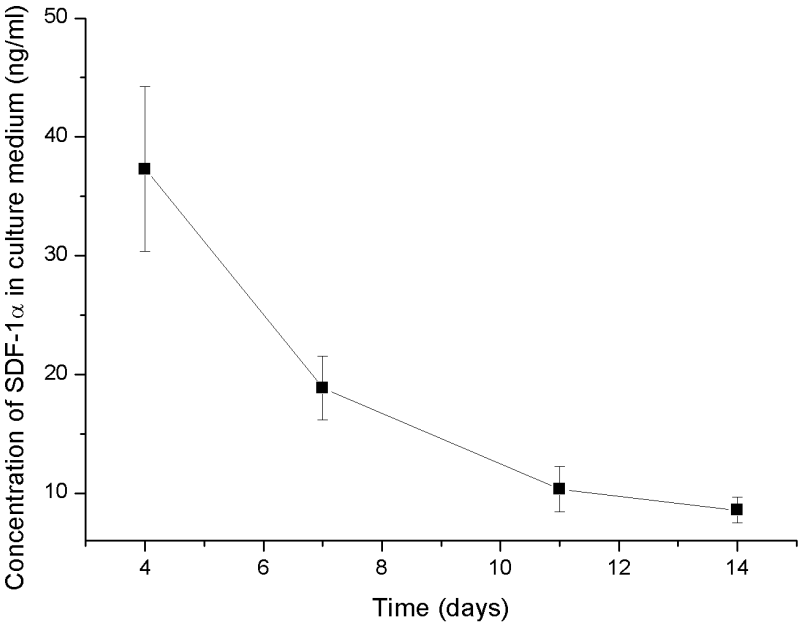


**Figure 3. Concentration of SDF-1α in culture medium (ng/ml) under cell culture conditions, employing half medium changes twice a week. The concentration was determined using $^{125}$I-labeled SDF-1α after release from heparinized crosslinked collagen films**

### *3.3. Cell seeding*

As previously reported [20], heparinization of crosslinked collagen films resulted in increased adhesion of $CD34^+$ enriched (>90 %, on average 95 %) HPCs with an improved viability. Pre-adsorption of SDF-1α onto crosslinked collagen films resulted in higher numbers of adhering cells, especially $CD34^+$ cells, compared to films

without the chemokine and with the chemokine present in the medium (figures 4 and 5). This was also found for heparinized crosslinked collagen films, although the number of cells adhering to this surface showed a larger variation between cell batches.

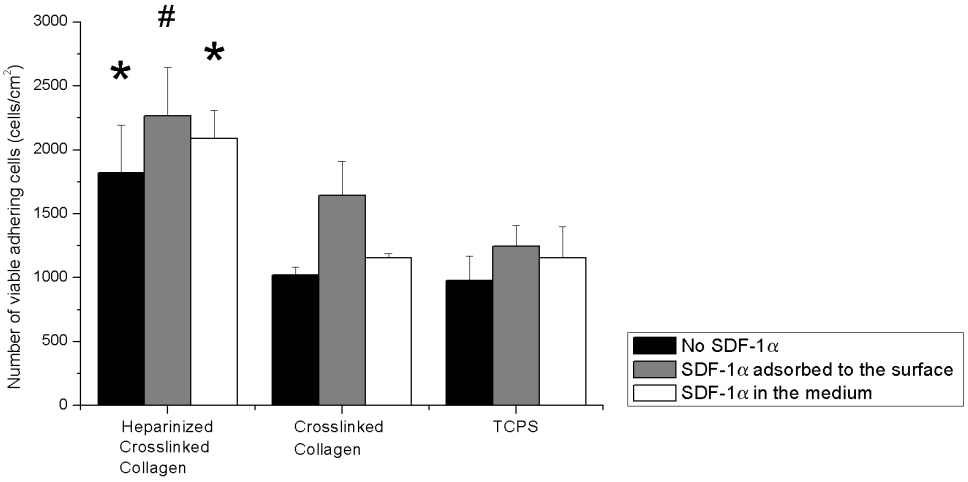


**Figure 4. Number of viable cells (7AAD$^-$ and CD45$^+$) adhering to the surfaces 1 h after seeding, determined from three independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to both crosslinked collagen films and TCPS surfaces. # Indicates a significant difference compared to TCPS.**

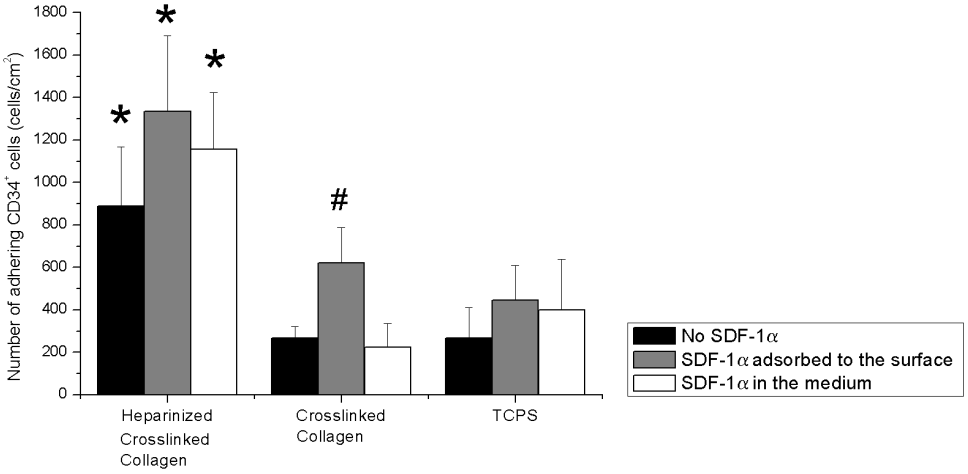


**Figure 5. Number of CD34$^+$ hematopoietic progenitor cells (7AAD$^-$, CD45$^+$ and CD34$^+$) adhering to the surfaces 1 h after seeding, determined from three independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to both crosslinked collagen films and TCPS and # indicates a significant difference compared to crosslinked collagen films without SDF-1α and crosslinked collagen films with SDF-1α only added to the medium.**

### *3.4. Cell culture*

As previously reported [20], HPCs did not proliferate on crosslinked collagen films. This was not improved by pre-adsorption of SDF-1α. Therefore, data regarding HPC expansion and differentiation during cell culture are only shown for heparinized crosslinked collagen films, with TCPS as reference surface.

During 2 wks of cell culture, HPC expansion was not significantly influenced by the presence of SDF-1α. On SDF-1α-adsorbed heparinized crosslinked collagen the total viable cell expansion was 7-fold after 2 wks of culture (figure 6), while no expansion of $CD34^+$ cells was achieved (figure 7), as reported before for heparinized crosslinked collagen films without pre-adsorbed SDF-1α [20].

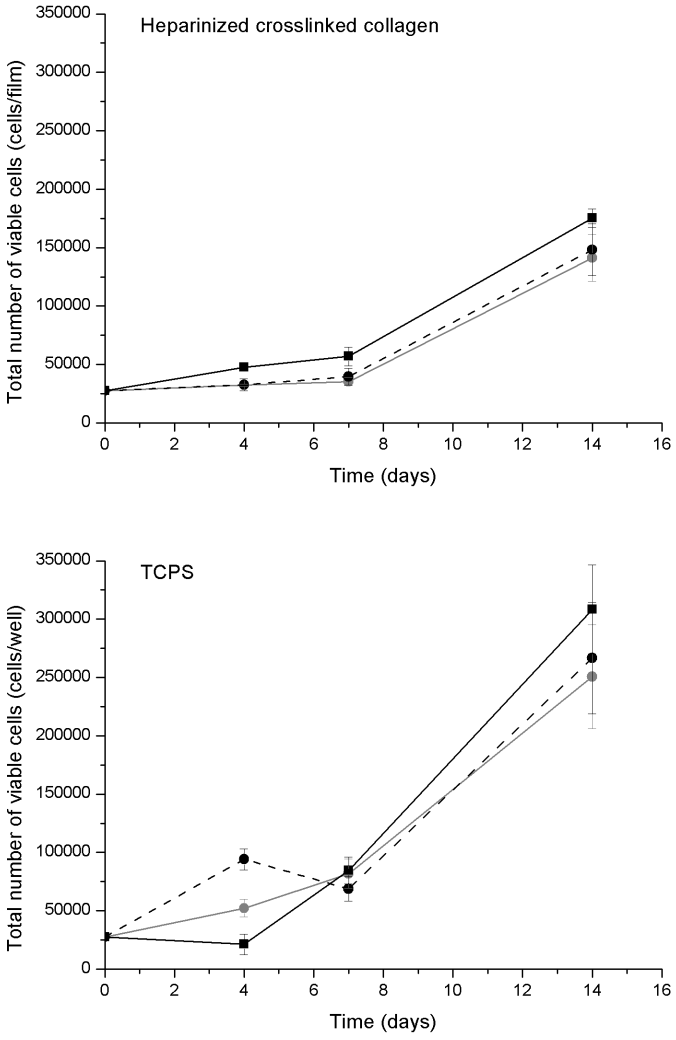


**Figure 6. Total number of viable cells (the sum of adherent and non-adherent cells ($7AAD^-$ and $CD45^+$)), determined from three independent experiments performed in duplicate (± S.E.M.) —■— SDF-1α-adsorbed surfaces, - -●- - SDF-1α in the medium, —●— without SDF-1α.**

The addition of SDF-1α to the culture medium during HPC culture using heparinized crosslinked collagen films had no significant effect on the total viable cell expansion or the number of $CD34^+$ cells. On TCPS the addition of SDF-1α to the culture medium caused a transient increase in the total and $CD34^+$ cell expansion at d 4 (figures 6 and 7).

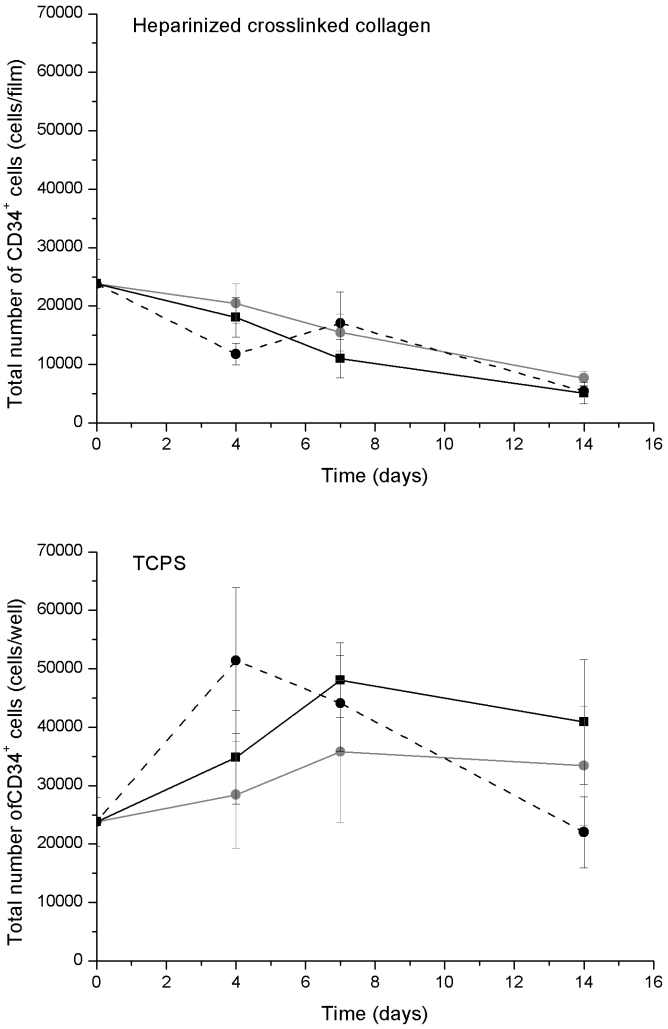


**Figure 7. Total number of $CD34^+$ cells (the sum of adherent and non-adherent cells ($7AAD^-$, $CD45^+$ and $CD34^+$)), determined from three independent experiments performed in duplicate (± S.E.M.). —■— SDF-1α-adsorbed surfaces, - -●- - SDF-1α in the medium, —●— without SDF-1α.**

During 2 wks of cell culture using heparinized crosslinked collagen films, the number of CFUs among the cells in culture increased as previously reported [20]. Pre-adsorption of SDF-1α onto heparinized crosslinked collagen films resulted in a slightly lower expansion of CFUs among the cells in culture (table 1). The same was found when SDF-1α was added to the culture medium (data not shown).

**Table 1. Colony-forming units (CFUs) per $10^3$ non-adhering cells in cultures using SDF-1α pre-adsorbed heparinized crosslinked collagen films. The total number of CFUs per $10^3$ non-adhering cells in cultures using heparinized films without SDF-1α is shown between brackets.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 0* | 4 | 34 | 0 | 50 | 88 |
| Day 4 | 1 (2) | 66 (109) | 15 (21) | 67 (82) | 149 (213) |
| Day 7 | 0 (0) | 32 (24) | 29 (36) | 71 (83) | 132 (143) |
| Day 14 | 0 (0) | 4 (14) | 55 (71) | 9 (16) | 68 (102) |

* Cells from the same cell batch as used in the cell cultures on the different substrates but immediately seeded into MethoCult™.

Without SDF-1α, the number of CFUs among the cells in culture using TCPS were lower than found for heparinized crosslinked collagen surfaces [20]. However, pre-adsorption of SDF-1α onto TCPS resulted in an increased expansion of CFUs among the cells in culture (table 2). The same increase was found when SDF-1α was added to the culture medium (data not shown). This increase in CFU expansion corresponds to the increases in $CD34^+$ cell expansion obtained at d 4 when SDF-1α was pre-adsorbed to TCPS or added in the medium during the HPC cultures on TCPS (figure 7).

**Table 2. Colony-forming units (CFUs) per $10^3$ non-adhering cells in cultures using SDF-1α pre-adsorbed TCPS. The total number of CFUs per $10^3$ non-adhering cells in cultures using TCPS without SDF-1α is shown between brackets.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| Day 0* | 4 | 34 | 0 | 50 | 88 |
| Day 4 | 1 (1) | 159 (59) | 7 (9) | 66 (27) | 234 (95) |
| Day 7 | 0 (0) | 18 (19) | 14 (13) | 11 (9) | 43 (41) |
| Day 14 | 0 (0) | 1 (3) | 11 (15) | 4 (6) | 16 (23) |

* Cells from the same cell batch as used in the cell cultures on the different substrates but immediately seeded into MethoCult™.

During 2 wks of cell culture, comparable numbers of cells adhered to surfaces with and without pre-adsorbed SDF-1α (figure 8). Interestingly, when SDF-1α was added to the culture medium the numbers of adhering cells were significantly lower than in the former two cases. The same was found for the $CD34^+$ cell population (data not shown). Therefore, prolonged exposure of HPCs to SDF-1α present in the culture medium appears to have an adverse effect on the number of adhering cells.

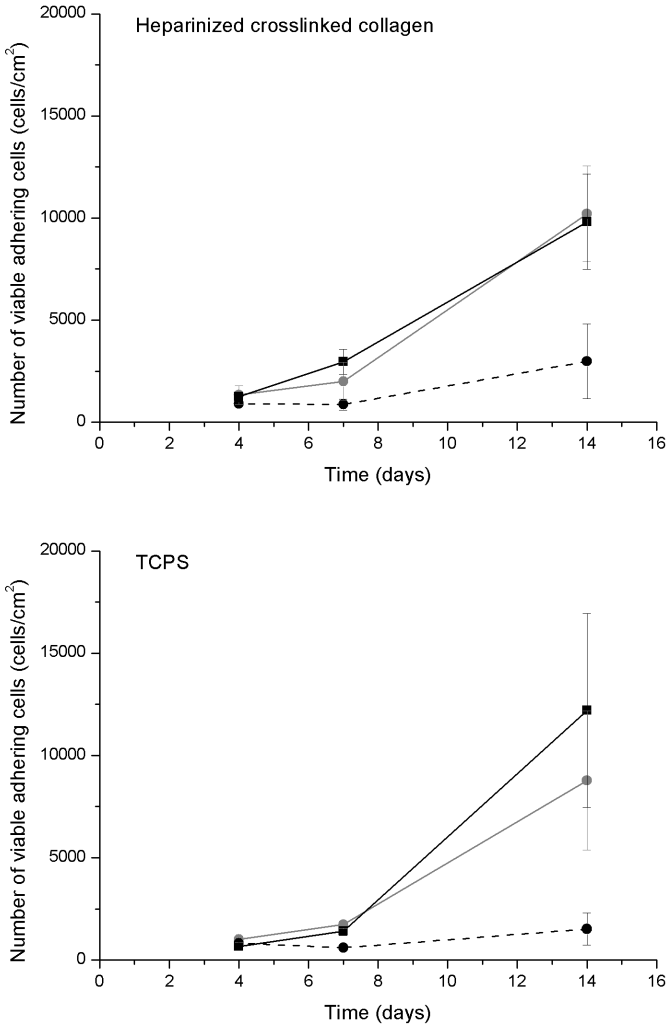


**Figure 8. Number of viable cells (7AAD$^-$ and CD45$^+$) adhering to the surfaces during cell culture, determined from three independent experiments performed in duplicate (± S.E.M.). —■— SDF-1α-adsorbed surfaces, - -●- - SDF-1α in the medium, —●— without SDF-1α.**

The number of CFUs among the cells adhering at d 4 to the heparinized crosslinked collagen films increased when these films were pre-adsorbed with SDF-1α and decreased when SDF-1α was added to the culture medium (table 3). The number of CFUs adhering to TCPS did not change when this surface was pre-adsorbed with SDF-1α (data not shown).

**Table 3. Colony-forming units (CFUs) per $10^3$ cells adhering to heparinized crosslinked collagen films at day 4.**

| | CFU-GEMM | BFU-E | CFU-E | CFU-G/M | Total CFU |
|---|---|---|---|---|---|
| No SDF-1α | 0 | 3 | 0 | 4 | 7 |
| SDF-1α pre-adsorbed | 0 | 6 | 2 | 4 | 12 |
| SDF-1α in the medium | 0 | 2 | 1 | 1 | 4 |

During the remainder of the 2 wks culture period using heparinized crosslinked collagen films, either with or without pre-adsorbed SDF-1α, CFU numbers increased to 50 per $10^3$ adhering cells. On TCPS the number of CFUs increased to about 10-20 per $10^3$ adhering cells. Among the CFUs adhering to TCPS or heparinized crosslinked collagen films 70-85 % was erythroid. The continuous presence of SDF-1α in the culture medium resulted in a lower amount of CFUs adhering to TCPS or heparinized crosslinked collagen films (1 and 11 CFUs per $10^3$ adhering cells, respectively). In the case of the latter substrate 86% of the adhering CFUs were erythroid.

## 4. Discussion

The previously described heparinized crosslinked collagen films for HPC culture [20] were pre-adsorbed with the chemokine SDF-1α. We found that the adsorption of SDF-1α onto crosslinked collagen films was slightly improved by heparinization of the films. The amount of adsorbed SDF-1α increased from 18 ± 2 ng to 25 ± 4 ng per mg of film. It is generally accepted in protein adsorption studies that about 0.4 μg/cm$^2$ can be considered a monolayer. The chemokine did not form such a monolayer on the surfaces, because maximally 21 to 59 ng/cm$^2$ SDF-1α was adsorbed. It is most probable that the chemokine also adsorbed in the bulk of the films, as its molecular weight (8 kDa) is in the molecular weight range of the used heparin (6-30 kDa), which was immobilized both on the surface and in the bulk of the crosslinked collagen films [13, 20].

The relatively small contribution of immobilized heparin to the extent of SDF-1α binding can be explained by the required oligosaccharide size for SDF-1α binding to heparin. The affinity of SDF-1α to heparin is similar to that of the well-studied heparin-binding molecule bFGF [32, 33]. However, bFGF requires only 4-6 saccharide units, while SDF-1α requires 8-14 [34]. The availability of relatively large oligosaccharide sequences and their necessary flexibility [21] could be restricted by covalent coupling of heparin to collagen. Covalent ‘side-on’ binding of heparin to

crosslinked collagen reduced the accessibility of the AT-III binding site of heparin and resulted in decreased thrombin inactivation [13]. The published high affinity of SDF-1α towards immobilized heparin was determined with end group biotinylated heparin bound to strepavidin-functionalized surfaces [21,32]. End-on immobilization of heparin to collagen is less feasible due to electrostatic interactions between negatively and positive charged groups on heparin and collagen, respectively.

In total approximately 40% of the adsorbed SDF-1α was released from both the heparinized and non-heparinized crosslinked collagen films during 2 wks, when employing half medium changes twice a wk. The remaining SDF-1α could be removed with 4M NaCl, indicating the presence of relatively strong ionic interactions, possibly accompanied by changes in the conformation of adsorbed SDF-1α.

When $CD34^+$ cells were cultured on these substrates the number of cells adhering to the substrates was higher at 1 h after cell seeding when the substrates were pre-adsorbed with SDF-1α. This is in accordance with a previous report showing that coating of poly(styrene) (PS) with both the cell adhesion molecule VCAM-1 and SDF-1α significantly improved adhesion of $CD34^+$ cells compared to adhesion to VCAM-1-coated PS with SDF-1α in the medium [22]. It was also documented that SDF-1α bound to GAGs on stromal cells and that loading the stroma with SDF-1α increased the adhesion of $CD34^+$ cells [23]. However, the number of adhering cells as percentage of the total number of seeded cells remains low, even on SDF-1α pre-adsorbed heparinized crosslinked collagen films (12 %).

We found that the adsorption of SDF-1α on crosslinked collagen and heparinized crosslinked collagen gave a similar increase in $CD34^+$ cell adhesion. This suggests that this effect is caused by the local higher concentration of SDF-1α at the surface and not by changes in chemokine conformation induced by specific binding to heparin. However, heparinization of the collagen substrates is necessary for the culture of $CD34^+$ cells as reported before [20]. The increased number of CFUs among the cells adhering to SDF-1α pre-adsorbed heparinized crosslinked collagen surfaces at d 4 could be a consequence of the higher number of $CD34^+$ cells adhering to these surfaces at 1 h after cell seeding and their subsequent differentiation into more committed progenitors.

The continuous presence of SDF-1α in the cell culture medium, however, resulted in a dramatically lower number of cells adhering to the surfaces. Shen *et al.* proposed that

SDF-1α-stimulated adhesion occurs rapidly and is due to changes in integrin avidity mediated by cytoskeletal reorganization [35]. Peled *et al.* reported that prolonged treatment of $CD34^+$ cells with SDF-1α deformed the cells and reduced their VLA-4-dependent adhesion to a level below that of intact cells [22]. Therefore, over-stimulation with SDF-1α could have disrupted the cytoskeletal arrangements necessary for adhesion, leading to a lower number of cells adhering to the surfaces when SDF-1α was continuously present in the medium. Because the release of SDF-1α from the films took place within the first days, the cells were not continuously stimulated by the chemokine and therefore the adhesion was not negatively influenced.

The expansion of the total and $CD34^+$ cell population on heparinized crosslinked collagen was not influenced by SDF-1α, either pre-adsorbed to the surface or added to the culture medium. When using TCPS the total and $CD34^+$ cell expansion increased transiently at d4 when SDF-1α was added to the culture medium. Additionally, more CFUs were found among the cells in culture at d4 when using SDF-1α pre-adsorbed TCPS and when SDF-1α was added to the medium during cell culture on TCPS. During $CD34^+$ culture using heparinized crosslinked collagen films where SDF-1α was pre-adsorbed or added to the culture medium, the number of CFUs among the cells in culture decreased. These results differ from data reported by Glimm et al. about the serum-free culture of human cord blood HPCs for 48 h in medium containing SCF, TPO and 100 ng/ml SDF-1α, where no effect of SDF-1α on CFU expansion was reported [36].

These results shows that pre-adsorption of SDF-1α to heparinized crosslinked collagen films can be used to increase HPC adhesion upon cell seeding, without negatively influencing cell adhesion as found when the chemokine was added to the culture medium. In general, these results show that hematopoietic factors pre-adsorbed to heparinized crosslinked collagen films can be used to influence the HSC cultures performed on these substrates. This can be extended to other hematopoietic factors while specific GAGs isolated from the bone marrow could be used instead of heparin. Before modification, the collagen can be processed into other morphologies to provide a three-dimensional environment for the culture of HSCs.

## 5. Acknowledgements

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## 6. References

1. Wang TY, Wu JHD. A continuous perfusion bioreactor for long-term bone-marrow culture. Ann N Y Acad Sci 1992;665:274-284.
2. Wang TY, Brennan JK, Wu JHD. Multilineal hematopoiesis in a 3-dimensional murine long-term bone-marrow culture. Exp Hematol 1995;23:26-32.
3. Mantalaris A, Keng P, Bourne P, Chang AYC, Wu JHD. Engineering a human bone marrow model: a case study on ex vivo erythropoiesis. Biotechnol Prog 1998;14:126-133.
4. Bagley J, Rosenzwieg M, Marks DF, Pykett MJ. Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device. Exp Hematol 1999;27:496-504.
5. Meissner P, Schroder B, Herfurth C, Biselli M. Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells. Cytotechnology 1999;30:227-234.
6. Tomimori Y, Takagi M, Yoshida T. The construction of an in vitro three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers. Cytotechnology 2000;34:121-130.
7. Banu N, Rosenzwieg M, Kim H, Bagley J, Pykett M. Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix. Cytokine 2001;13:349-358.
8. Sasaki T, Takagi M, Soma T, Yoshida T. 3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics. Cytotherapy 2002;4:285-291.
9. Kim HS, Lim JB, Min YH, Lee ST, Lyu CJ, Kim ES, Kim HO. Ex vivo expansion of human umbilical cord blood CD34+ cells in a collagen bead-containing 3 dimensional culture system. Int J Hematol 2003;78:126-132.
10. Xiong F, Chen Z, Liu H, Xu Z, Liu X. Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system. Biotechnol Lett 2002;24:1421-1426.
11. Zhang J, Niu C, Ye L, Huang HY, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li LH. Identification of the haematopoietic stem cell niche and control of the niche size. Nature 2003;425:836-841.
12. Fuchs E, Tumbar T, Guasch G. Socializing with the neighbors: stem cells and their niches. Cell 2004;116:769-778.
13. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, Feijen J. Immobilization of heparin to EDC/NHS-crosslinked collagen. characterization and in vitro evaluation. Biomaterials 2001;22:151-163.

14. Gupta P, McCarthy JB, Verfaillie CM. Stromal fibroblast heparan sulfate is required for cytokine-mediated ex vivo maintenance of human long-term culture-initiating cells. Blood 1996;87:3229-3236.
15. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche. Blood 1998;92:4641-4651.
16. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. Human LTC-IC can be maintained for at least 5 wks in vitro when interleukin-3 and a single chemokine are combined with O- sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins. Blood 2000;95:147-155.
17. Gordon MY, Riley GP, Watt SM and Greaves MF. Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 1987;326:403-405.
18. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM. Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis. Nature 1988;332:376-378.
19. Tanaka Y, Kimata K, Adams DH, Eto S. Modulation of cytokine function by heparan sulfate proteoglycans: sophisticated models for the regulation of cellular responses to cytokines. P Assoc Am Physician 1998;10:118-125.
20. Siebum B, Grijpma DW, Poot AA, Vermes I, Feijen J. Heparinized crosslinked collagen films for hematopoietic stem cell culture. Chapter 3 of this thesis.
21. Sadir R, Baleux F, Grosdidier A, Imberty A, Lortat-Jacob H. Characterization of the stromal cell-derived factor-l alpha - heparin complex. J Biol Chem 2001;276:8288-8296.
22. Peled A, Grabovsky V, Habler L, Sandbank J, Arenzana-Seisdedos F, Petit I, Ben-Hur H, Lapidot T, Alon R. The chemokine SDF-1 stimulates integrin-mediated arrest of $CD34^+$ cells on vascular endothelium under shear flow. J Clin Invest 1999;104:1199-1211.
23. Netelenbos T, Van den Born J, Kessler FL, Zweegman S, Merle PA, Van Oostveen JW, Zwaginga JJ, Huijgens PC, Drager AM. Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells. Leukemia 2003;17:175-184.
24. Taichman RS. Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 2005;105:2631-2639.
25. Majka M, Ratajczak J, Lee B, Honczarenko M, Douglas R, Kowalska MA, Silberstein L, Gewirtz AM, Ratajczak MZ. The role of HIV-related chemokine receptors and chemokines in human erythropoiesis in vitro. Stem Cells 2000;18:128-138.
26. Loke WK, Khor E. Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valva application by differential scanning calorimetry. Biomaterials 1995;16:251-258.
27. Wang CL, Miyata T, Wecksler B, Rubin AL, Stenzel KH. Collagen induced platelet aggregation and release. I. Effects of side-chain modifications and role of argynyl residues. Biochim Biophys Acta 1978;544:555-567.
28. Markwell MAK. A new solid-state reagent to iodinate proteins: conditions for efficient labelling of antiserum. Anal Biochem 1982;125:427-432.
29. Bos GW. Phd Thesis, Chapter 3, Immobilization of albumin and albumin-heparin conjugate on gas plasma modified polystyrene. University of Twente, 1998:45-70.
30. Alvarez-Silva M, Borojevic R. GM-CSF and IL-3 activities in schistosomal liver granulomas are controlled by stroma-associated heparan sulfate progeoglycans. J Leukoc Biol 1996;59:435-441.
31. Amara A, Lorthioir O, Valenzuela A, Magerus A, Thelen M, Montes M, Virelizier JL, Delepierre M, Baleux F, Lortat-Jacob H, Arenzana-Seisdedos F. Stromal

Cell-derived Factor 1alpha associates with heparan sulfates through the first beta-strand of the chemokine. J Biol Chem 1999;274:23916-23925.

32. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, Feijen J. Binding and release of basic fibroblast growth factor from heparinized collagen matrices. Biomaterials 2001;22:2291-2299.
33. Capila I, Linhardt RJ. Heparin - protein interactions. Angew Chem Int Ed 2002;41:391-412.
34. Shen W, Bendall LJ, Gottlieb DJ, Bradstock KF. The chemokine receptor CXCR4 enhances integrin-mediated in vitro adhesion and facilitates engraftment of leukemic precursor-B cells in the bone marrow. Exp Hematol 2001;29:1439-1447.
35. Glimm H, Tang P, Clark-Lewis I, Von Kalle C, Eaves C. Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice. Blood 2002;99:3454-3457.

# Chapter 5

## IL-3 pre-adsorbed heparinized crosslinked collagen films for hematopoietic progenitor cell culture

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**Abstract**

A successful *in vitro* expansion and differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great clinical interest. This may be achieved by culturing these cells under conditions that mimic the hematopoietic niches found in the bone marrow. In this study human peripheral blood derived $CD34^+$ cells were expanded using heparinized and non-heparinized crosslinked collagen type I films, which were pre-adsorbed with IL-3.

Circular collagen films (d = 15 mm, thickness = 50 μm, weight ~ 3 mg) adsorbed a relatively large amount (~2.4 ng) of IL-3 when incubated overnight with IL-3 containing serum-free culture medium (0.5 ml, 10 ng/ml IL-3), whereas under the same conditions heparinized crosslinked collagen films with the same dimensions adsorbed about 2.0 ng IL-3.

When heparinized crosslinked collagen films were exposed to serum-free culture medium (0.5 ml) at 37˚C with regular whole medium changes at fixed time points, 8% of the pre-adsorbed IL-3 was released after 8 hrs, whereas non-heparinized films only released 1 % of the pre-adsorbed IL-3, indicating that a large part of the IL-3 was irreversibly bound onto the films.

Heparinized crosslinked collagen films were also exposed to a PBS solution containing IL-3 (300 μl, 1 μg/ml IL-3, 0.1 (wt)% BSA) at room temperature overnight, resulting in the adsorption of 111 ± 30 ng IL-3 per film. After incubation with culture medium (0.5 ml, 37˚C) about 22 ng (20%) of the pre-adsorbed IL-3 was

released within 4 days and no further release of IL-3 was observed after subsequent half-medium changes twice a week.

The use of heparinized crosslinked collagen films pre-adsorbed with 111 ± 30 ng IL-3 as substrate for $CD34^+$ cell culture using serum-free medium, initially not containing IL-3, resulted in a relatively large number of adhering cells, a 1.4-fold $CD34^+$ cell expansion during the first wk and a 16-fold total cell expansion after 2 wks. Crosslinked collagen films were pre-adsorbed with IL-3, in a similar way as for the heparinized films. The use of IL-3 pre-adsorbed crosslinked collagen films as a substrate for $CD34^+$ cell culture using serum-free medium, initially not containing IL-3, did not result in the expansion of HPCs.

The use of heparinized crosslinked collagen films pre-adsorbed with 111 ± 30 ng IL-3 as culture substrate for $CD34^+$ cell culture also resulted in more CFUs differentiated in the erythroid lineage than in cultures performed on heparinized films without pre-adsorbed IL-3, but with IL-3 added to the culture medium (10 ng/ml).

## 1. Introduction

The bone marrow is the principal site where the adult hematopoietic system produces blood cells. It is expected that the *in vitro* expansion of hematopoietic progenitor cells (HPCs) will be facilitated by the use of a culture system that mimics the bone marrow microenvironment. Additionally, such a systems will provide a model to investigate mechanisms involved in hematopoiesis. Bone marrow microenvironments consist of stromal cells, extracellular matrix (ECM) components and molecules such as cytokines [1-3]. The binding and release of cytokines, chemokines and growth factors by stromal cells and ECM is an important mechanism regulating hematopoiesis inside these microenvironments [1,4,5].

Previously, we demonstrated the use of heparinized crosslinked collagen films as substrate for culturing of human $CD34^+$ cells obtained from G-CSF mobilized peripheral blood. Collagen type I is one of the major constituents of the bone marrow ECM and can be processed into structures suitable for HPC culture [6-8] and erythropoiesis [9-11]. We used heparin as a model compound for heparan sulfate, a glycosaminoglycan that plays an important role in the bone marrow as it mediates the function of molecules such as cytokines [12-16]. Heparinized crosslinked collagen

films showed improved $CD34^+$ cell survival, a 6-fold expansion of CFUs during the first wk and a 5-fold total cell expansion after 2 wks, whereas no HPC expansion was obtained when using non-heparinized crosslinked collagen films [17]. In a later study SDF-1α was pre-adsorbed to the these films to increase cell adhesion, resulting in an increased number of HPCs adhering onto SDF-1α pre-adsorbed films upon cell seeding as compared to films that did not contain SDF-1α [18]. $CD34^+$ cell survival, total viable cell expansion and the CFU expansion were not influenced by SDF-1α pre-adsorbed onto both heparinized and non-heparinized films.

The cytokine interleukin-3 (IL-3), which was present in the medium in the previous studies, promotes HSC and HPC maintenance under serum-free culture conditions [19]. Using culture plates coated with IL-3, it was shown that IL-3 can function as adhesive ligand for $CD34^+$ HPCs [20]. IL-3 added to the culture medium increases HPC adhesion and migration on immobilized ECM components [21, 22]. Moreover, it has been reported that IL-3 binds to immobilized heparan sulfate and dissolved heparin, which increases its ability to promote proliferation of an IL-3-dependent myeloid cell line [23]. In a previous study, we found that both heparinized and non-heparinized crosslinked collagen films adsorb IL-3 from serum-free HPC culture medium [17].

The aim of the present study is first to investigate IL-3 adsorption onto heparinized and non-heparinized crosslinked collagen films and to investigate the role of IL-3 and its interactions with these films on HPC adhesion, proliferation and differentiation.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Preparation of heparinized crosslinked collagen films*

Collagen dispersions were prepared from type I insoluble collagen derived from bovine achilles tendon. Collagen (1 g) was swollen overnight in 0.05 M acetic acid solution (50 ml) at 4 ˚C. The mixture was dispersed with 50 g crushed ice for 4 min in a Philips blender and subsequently homogenized for 30 min at 4 ˚C using an Ultra-

Turrax T25 (IKA Labortechnik, Staufen, Germany). The resulting slurry was filtered through a 20 denier nylon filter (average pore size 30 μm) and a 25 μm filter (Cellector screen, Bellco, Feltham, UK) mounted in a disc filter holder. After degassing at 0.1 mbar the suspension was cast onto a poly(ethylene terephthalate) (PET) plate using a casting knife. The films were allowed to dry for 1 wk at ambient conditions in a laminar flowhood.

Subsequently, these films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Fluka, Buchs, Switzerland) and N-hydroxysuccinimide (NHS) [24]. The scaffolds were first incubated in 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) for 30 min at room temperature. The samples were then transferred to the crosslinking solution, containing 2.3 g EDC and 0.56 g NHS in 215 ml MES-buffer per g of collagen (molar ratio of EDC/NHS = 2.5). After 2 h incubation under gentle shaking, the films were placed in a 0.1 M $Na_2HPO_4$ (Merck, Darmstadt, Germany) solution for 2 h to stop the reaction and then rinsed with demineralized water for three times 30 min.

Crosslinked collagen films were subsequently heparinized [24]. The films were first incubated in MES buffer for at least 30 min at room temperature. The carboxylic acid groups of heparin (from porcine mucosa, MW 6000-30000, activity > 160 IU/mg) were pre-activated for 10 min. This was done by means of 0.43 g EDC and 0.16 g NHS in 188 ml of a 2 (wt)% heparin solution in MES-buffer.

Crosslinked collagen films (1 g) were heparinized for 2 h in the EDC/NHS-activated heparin solution. Subsequently, the reaction was terminated by immersion of the films in 0.1 M sodium phosphate. The heparinized films were then washed in 2 M NaCl (24 h), 4 M NaCl (4 times 24 h) and MilliQ water (3 times 24 h).

### *2.3. Characterization of films*

The shrinkage temperature ($T_s$) of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC), as described before [17]. This temperature, at which the collagen sample undergoes thermal denaturation, is used as a measure of the crosslink density [25].

The residual number of free primary amino groups in (crosslinked) collagen was determined spectrophotometrically after reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fluka) [26].

The heparin content of the samples was determined by a colorimetric method based on the binding of toluidine blue [27].

### *2.4. IL-3 adsorption and release*

Circular crosslinked collagen films (d = 15 mm, thickness = 50 μm, weight ~ 3 mg) with and without immobilized heparin were fixed with Viton O rings in the wells of a 24-well tissue culture polystyrene (TCPS) culture plate (Corning, NY, USA). These were incubated overnight at 37 ºC in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml; both from Gibco BRL, Paisley, UK). After rinsing 3 times with PBS the films were either incubated at 37 ºC in 0.5 ml of culture medium containing 10 ng/ml IL-3 (see below) or at room temperature in 300 μl of a solution of IL-3 (1 μg/ml) in PBS containing 0.1 (wt)% BSA. After overnight incubation, the concentration of IL-3 in the solutions was determined using an ELISA (Quantikine, R&D systems, Minneapolis, USA).

The release of IL-3 from films was determined at 37 ˚C. After rinsing with PBS, 0.5 ml culture medium without cytokines, containing 0.02 (wt)% $NaN_3$, was added to the heparinized and non-heparinized crosslinked collagen films pre-adsorbed with IL-3. The release of IL-3 during 1 wk from samples that were pre-incubated with IL-3-containing culture medium was determined by completely refreshing the cytokine-free medium at pre-determined time points after which the concentration of IL-3 in the removed medium was measured with the ELISA.

The release of IL-3 during 2 wks from samples that were pre-incubated with the IL-3 solution in PBS was determined by refreshing half of the cytokine-free culture medium twice a wk after which the concentration of IL-3 in the removed medium was measured with the ELISA.

### *2.5. Hematopoietic cell culture*

Circular films cut out of heparinized and non-heparinized crosslinked collagen films (d = 15 mm) were rinsed with 70 % ethanol. The films were fixed with Viton O rings in the wells of a 24-well TCPS culture plate and incubated overnight at 37 ˚C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml). Before cell seeding, the films were incubated once more overnight at room temperature in 300 μl of PBS containing 0.1 (wt)% BSA with or without 1 μg/ml IL-3 (R&D systems).

Human $CD34^+$ cells, isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood, were obtained from the Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and subsequently cultured in medium consisting of IMDM (with GlutaMAX I), 1 (wt)% BSA, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml recombinant human insulin, 200 μg/ml human partially iron-saturated holo-transferrin, 10 ng/ml TPO, 100 ng/ml SCF, 50 ng/ml Flt3/Flk2-ligand, with or without 10 ng/ml IL-3. All four cytokines were from recombinant human origin (R&D systems). The cells were seeded by adding 0.5 ml of a cell suspension with a density of $1 \cdot 10^5$ cells/ml onto the films. The cells were cultured at 37 ˚C in a humidified 5 % $CO_2$ atmosphere. Half of the medium was exchanged twice a wk.

During cell culture two cell fractions were obtained. The cells that did not adhere to the films were removed by gentle pipetting. The adhering cells were obtained after rinsing the surfaces with PBS containing 1 (wt)% BSA and subsequent incubation in Cell Dissociation Solution (CDS) for 20 min at room temperature.

Flowcytometric phenotyping was performed using anti-CD45-FITC, anti-CD34-PE, anti-CD34-ECD, anti-CD36-FITC, anti-CD235A-PE and 7AAD vitality dye (all from Immunotech, Marseille, France). Isotype-identical antibodies were used as control and for quantification Flow-Count beads were added to the samples. Cells were counted and analyzed using a Cytomics FC500 flow cytometry system (Beckman Coulter, Fullerton, USA). Moreover, cell differentiation was evaluated by Colony-Forming Unit (CFU) assays. For CFU analysis, samples containing $10^3$ cells were taken from the HPC cultures, subsequently cultured in MethoCult (StemCell Technologies, Meylan, France) and scored after 2 wks. The number of CFUs adhering to the heparinized and non-heparinized crosslinked collagen films as well as the number of non-adherent CFUs were determined. For quantification of adherent CFUs, the cells were first removed from the films using CDS.

### *2.6. Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 2 experiments performed in duplicate. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values $< 0.05$.

## 3. Results and discussion

### *3.1. Preparation of heparinized collagen films*

Collagen films with a thickness of 50 μm [17] were first crosslinked and subsequently heparinized using EDC and NHS. Crosslinked collagen films had a shrinkage temperature of 76 ˚C and the number of primary amino groups per 1000 amino acid residues was 16. For non-crosslinked collagen these values were 59˚C and 27 primary amino groups per 1000 amino acid residues, respectively. After heparinization of the crosslinked collagen films the shrinkage temperature did not decrease any further, while the number of primary amino groups per 1000 amino acid residues further decreased to about 15. As reported previously, 23 ± 4 μg heparin was immobilized per mg of crosslinked collagen film [17]. Toluidine blue staining throughout the thickness of the films indicated that heparin was immobilized both on the surface and in the bulk of the films.

### *3.2. Pre-adsorption and release of IL-3*

Heparinized and non-heparinized crosslinked collagen films (d = 15mm, thickness = 50 μm, weight ~ 3 mg) adsorbed 2.0 ± 0.3 ng and 2.4 ± 0.3 ng IL-3, respectively from 0.5 ml of culture medium with an initial concentration of 10 ng IL-3 per ml after overnight exposure at 37 ˚C.

The release of IL-3 from films that were pre-incubated with IL-3 containing culture medium was determined using whole medium changes. Heparinized and non-heparinized crosslinked collagen films only released about 8 % and 1 %, respectively of the adsorbed IL-3 during the first 8 hrs (figure 1). This means that about 164 and 22 pg IL-3 were released from heparinized and non-heparinized crosslinked collagen films, respectively into the culture medium. This indicates that the interaction of IL-3 with crosslinked collagen is relatively strong, probably leading to conformational changes, whereas a small fraction of IL-3 adsorbed onto the heparinized films could be desorbed. Most probably this fraction of IL-3 has interactions with the heparin in such a way that the conformation of IL-3 remains preserved.

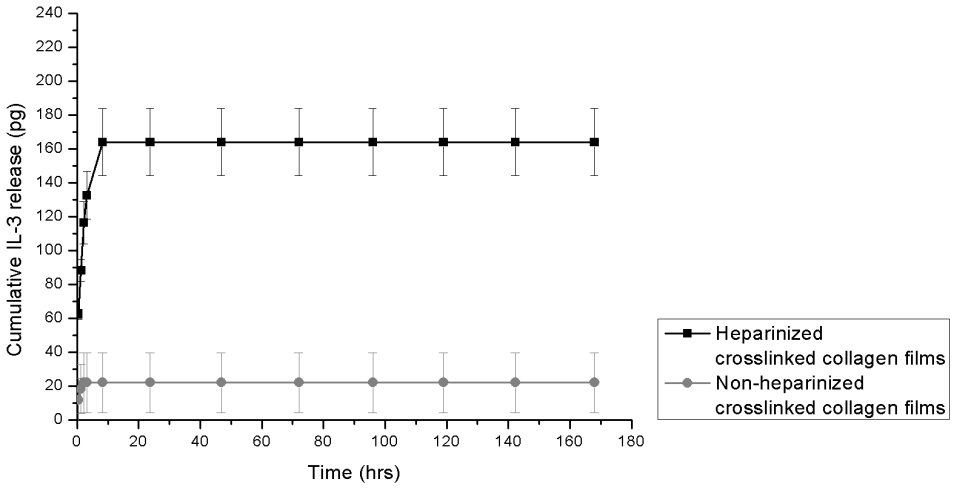


**Figure 1. Release of IL-3 from heparinized and non-heparinized crosslinked collagen films into 0.5 ml cytokine-free culture medium at 37˚C, determined after whole medium changes. Circular films with a diameter of 15 mm and a thickness of 50 µm were pre-adsorbed with IL-3 overnight in 0.5 ml culture medium containing IL-3 (10 ng/ml). The IL-3 concentration in the supernatant was determined using an IL-3-ELISA.**

The IL-3 fraction of 8 % that was released from the heparinized crosslinked collagen films was most probably bound to heparin present in the collagen matrix. The relatively fast partial release of IL-3 from heparinized and non-heparinized crosslinked collagen films was similar to the release pattern of SDF-1α from these films [18]. It has been reported [28] that heparinized films pre-incubated with a basic Fibroblast Growth Factor (bFGF) solution in PBS (0.25 ml, 0 – 320 ng/ml bFGF, 1 mg/ml BSA) adsorbed more bFGF as compared to non-heparinized films. When bFGF pre-adsorbed heparinzed films were exposed to serum-containing culture medium, bFGF release into the medium was sustained for days [28]. The large amount of bFGF adsorbed onto heparinized crosslinked collagen films was most probably facilitated by the small oligosaccharide size required for specific binding of bFGF to heparin [29]. In the present study the covalent coupling of heparin to collagen could have restricted the availability of a sufficient large oligosaccharide sequence for efficient IL-3 binding, as suggested for the binding of AT-III [24] and SDF-1α [18]. More IL-3 might have been specifically adsorbed onto the heparinized films when heparin was immobilized end-on using different chemistry.

Heparinized crosslinked collagen films for HPC culture were incubated overnight at 37˚C with 300 µl of an IL-3 solution in PBS (1 µg/ml) containing 0.1 (wt)% BSA during which 111 ± 30 ng IL-3 per film was adsorbed. Because during cell culture

half of the medium was exchanged twice a wk, the release of IL-3 from heparinized crosslinked collagen films was also determined using these conditions (figure 2). About 22 ng (equivalent to 20 %) of pre-adsorbed IL-3 was released into 0.5 ml culture medium before the first half-medium change at d 4. During further incubation with culture medium, the remaining 80 % of the pre-adsorbed IL-3 remained bound to the films. Thus, only a small fraction of IL-3 pre-adsorbed onto the heparinized films, either from culture medium or from PBS, could be released after exposure of the pre-adsorbed films to culture medium.

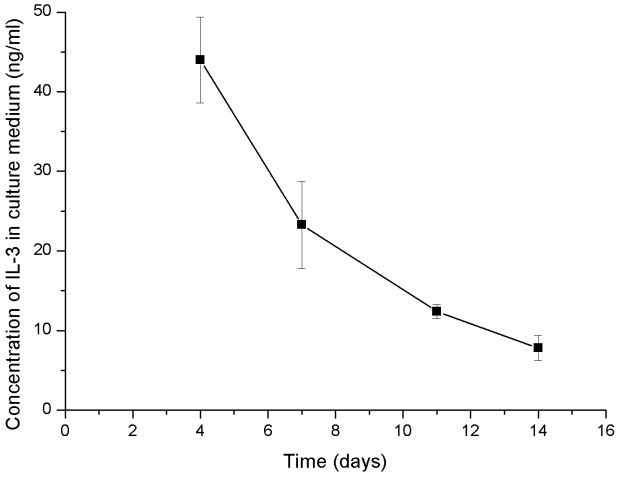


**Figure 2. Concentration of IL-3 in culture medium during desorption of IL-3 from heparinized crosslinked collagen films (d = 15 mm, thickness = 50 µm), that were pre-incubated overnight with 300 µl of an IL-3 solution in PBS (1 µg/ml) containing 0.1 (wt)% BSA at room temperature, employing half medium changes twice a week. The IL-3 concentration in the (initially cytokine-free) supernatant was determined using an IL-3-ELISA**

### *3.3.Pre-adsorbed IL-3 and HPC adhesion*

$CD34^+$ selected cells (~ 90 % $CD34^+$) were seeded onto heparinized and non-heparinzed crosslinked collagen films that were pre-incubated with 300 µl PBS containing IL-3 (1 µg/ml) and BSA (1 mg/ml). After 1 wk of cell culture the number of viable cells adhering to the surface of IL-3 pre-adsorbed heparinized crosslinked collagen films was about 5 times higher than the number of cells adhering to these films without pre-adsorbed IL-3 and no IL-3 added to the culture medium (figure 3). At the same time point, the films without pre-adsorbed IL-3, but with IL-3 added to the culture medium had about 2 times more adhering cells than the films without any IL-3. The number of adhering viable cells on IL-3 pre-adsorbed non-heparinized

films, without IL-3 added to the culture medium, was comparable to the number of cells adhering to heparinized films without pre-adsorbed IL-3 and no IL-3 added to the culture medium (data not shown).

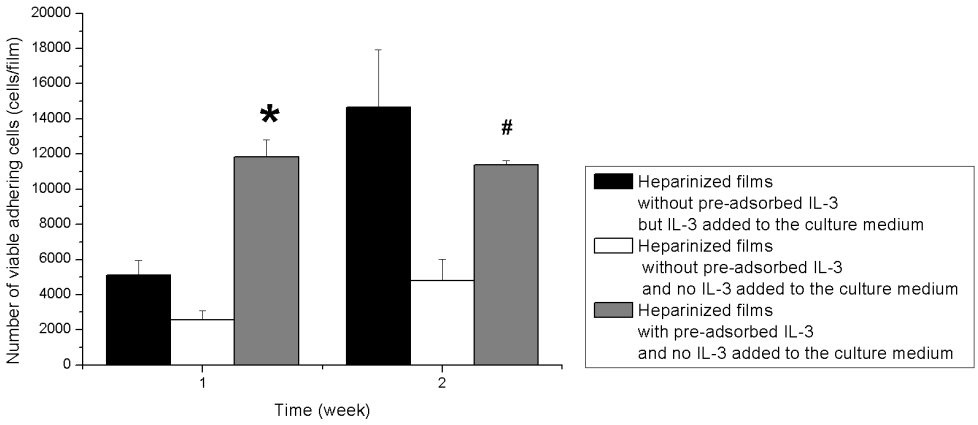


**Figure 3. Number of viable cells (7AAD$^-$ and CD45$^+$) adhering to the surfaces during cell culture using heparinized crosslinked collagen films with or without pre-adsorbed IL-3, determined from two independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to heparinized films without pre-adsorbed IL-3, with and without IL-3 added to the culture medium. # Indicates a significant difference compared to heparinized films without pre-adsorbed IL-3 and no IL-3 added to the culture medium.**

These results demonstrate that the presence of IL-3 increases the number of cells adhering to the heparinized films. After 1 wk the highest number of adhering cells was found on IL-3 pre-adsorbed heparinized crosslinked collagen films, whereas after 2 wks there was no difference between heparinized films with pre-adsorbed IL-3 without IL-3 added to the culture medium and heparinized films without pre-adsorbed IL-3 but with IL-3 added to the culture medium.

The number of CD34$^+$ cells adhering to the IL-3 pre-adsorbed heparinized films, as percentage of the total number of adhering cells, decreased from 63 % after 1 wk to 7 % after 2 wks. Similar data were obtained with heparinized films without pre-adsorbed IL-3, either with or without IL-3 added to the culture medium (data not shown). The influence of pre-adsorbed IL-3 on the number of CD34$^+$ cells adhering to non-heparinized crosslinked collagen could not be determined, due to the very low number of CD34$^+$ cells adhering to these films.

The number of CFUs adhering to IL-3 pre-adsorbed heparinized films did not change much during 2 wks of HPC culture, whereas the number of CFUs adhering to heparinized films without pre-adsorbed IL-3 but with 10 ng/ml IL-3 added to the

culture medium increased (table 1). No CFUs adhered to the non-heparinized films, both with and without pre-adsorbed IL-3 during 2 wks of culture.

**Table 1. Number of Colony Forming Units per surface area (CFU/cm$^2$) adhering to IL-3 pre-adsorbed heparinized crosslinked collagen films without IL-3 added to the culture medium, as compared to heparinized crosslinked collagen films without pre-adsorbed IL-3 but with IL-3 (10 ng/ml) added to the culture medium [17].**

| | IL-3 pre-adsorbed heparinized films, without IL-3 added to the culture medium | | Heparinized films without pre-adsorbed IL-3, with IL-3 added to the culture medium. | |
|---|---|---|---|---|
| | CFUs | Erythroid CFUs | CFUs | Erythroid CFUs |
| Week 1 | 142 | 94 % | 17 | 67 % |
| Week 2 | 152 | 92 % | 158 | 71 % |

In case of adsorption of 2 ng IL-3 from the culture medium onto the heparinized crosslinked collagen films, the resulting concentration of IL-3 in the culture medium was 6 ng/ml. The concentration of IL-3 in the culture medium increased from 6 ng/ml at day 4 to 9.5 ng/ml after 2 wks, due to half medium refreshments. On the other hand, after adsorption of 111 ng IL-3 from the IL-3 solution in PBS onto the heparinized films , 22 ng IL-3 was released into culture medium without added IL-3, resulting in a decreasing IL-3 concentration from 44 ng/ml after 4 d to 5.5 ng/ml after 2 wks due to half medium exchanges with IL-3-free culture medium. During the 2 wk culture period, 89 ng IL-3 remained bound to these films. The relatively high number of cells and CFUs adhering to the films after 1 wk in the latter case, could be due to the high surface concentration of IL-3 as well as the high IL-3 concentration in the medium. Surface-immobilized IL-3 can function as adhesive ligand for CFUs [20]. Moreover, IL-3 in serum-free culture medium (10-100 U/ml) can stimulate HPC adhesion onto ECM components [21].

### *3.4. Pre-adsorbed IL-3 and HPC expansion and differentiation*

The strong interaction between IL-3 and non-heparinized crosslinked collagen, possibly accompanied by conformational changes of the cytokine, was previously proposed as explanation for the poor performance of these films as substrate for serum-free HPC culture [17]. Under similar conditions, HPCs proliferated when using heparinized crosslinked collagen films pre-incubated with the IL-3 solution in PBS (1 μg/ml) as culture substrate (figure 4), but not when using non-heparinized crosslinked collagen films, even after pre-adsorption of the films with IL-3 (data not shown). The use of heparinized crosslinked collagen films pre-adsorbed with IL-3 as substrate during $CD34^+$ culture in IL-3-free culture medium, improved the total viable cell

expansion 4-fold, as compared to cultures using heparinized films with IL-3 added to the culture medium. Cultures performed using heparinized films without pre-adsorbed IL-3 and no IL-3 added to the culture medium, showed no cell expansion.

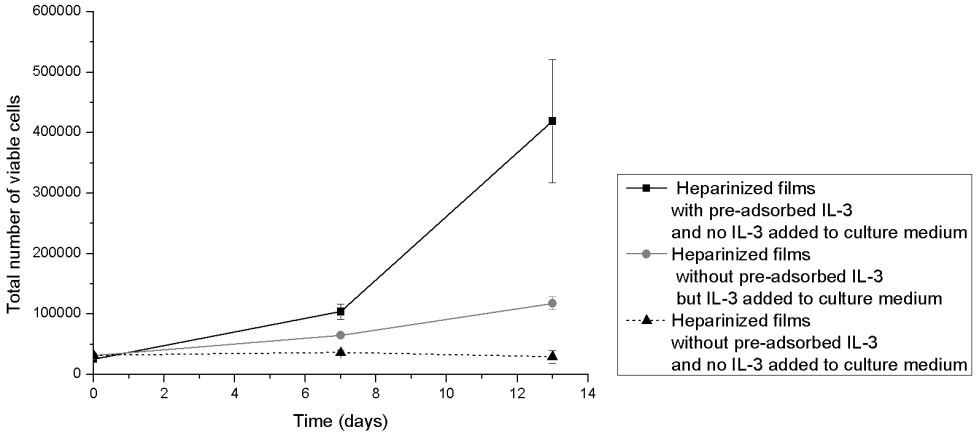


**Figure 4. Total number of viable cells (sum of adherent and non-adherent cells (7AAD$^-$ and CD45$^+$)) during cell culture using heparinized crosslinked collagen films with or without pre-adsorbed IL-3, determined from two independent experiments performed in duplicate (± S.E.M).**

When using IL-3 pre-adsorbed heparinized crosslinked collagen films as culture substrate, the total number of cells expanded 16 times while the CD34$^+$ cells transiently expanded 1.4 times. In contrast, the number of CD34$^+$ cells decreased when using heparinized films that were not pre-adsorbed with IL-3, with or without IL-3 added to the culture medium (figure 5). The number of CFUs among $10^3$ cells expanded transiently, when using heparinized films with or without pre-adsorbed IL-3 (table 2).

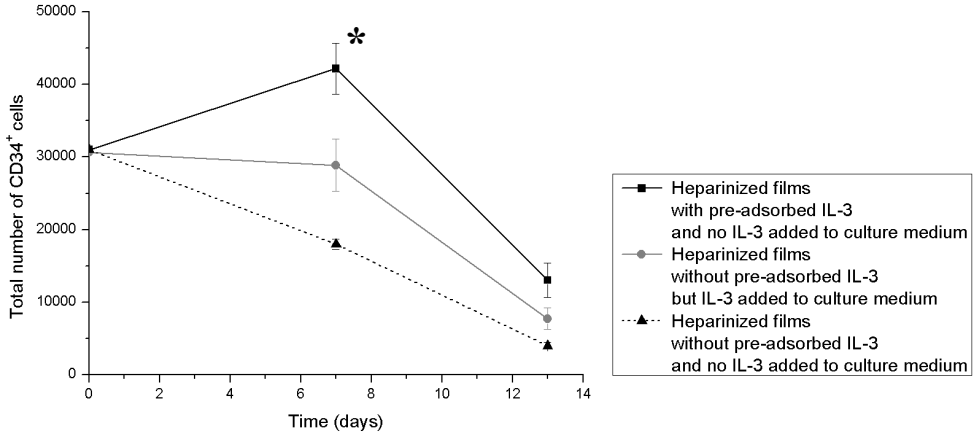

**Figure 5. Total number of $CD34^+$ cells (the sum of adherent and non-adherent cells ($7AAD^-$, $CD45^+$ and $CD34^+$)) during cell culture using heparinized crosslinked collagen films with or without pre-adsorbed IL-3, determined from two independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to heparinized films without pre-adsorbed IL-3 and no IL-3 in the medium.**

**Table 2. Colony Forming Units (CFUs) per $10^3$ non-adhering cells in cultures using heparinized crosslinked collagen films with or without pre-adsorbed IL-3. At cell seeding, the cell population contained 88 CFUs per $10^3$ cells, of which 39-46 % were erythroid CFUs (either BFU-E or CFU-E).**

| | Week 1 | | Week 2 | |
|---|---|---|---|---|
| | CFUs | Erythroid CFUs | CFUs | Erythroid CFUs |
| Heparinized films without pre-adsorbed IL-3, with IL-3 added to the culture medium | 146 | 62 % | 88 | 83 % |
| IL-3 pre-adsorbed heparinized films, without IL-3 added to the culture medium | 111 | 93 % | 32 | 92 % |

The high total cell expansion combined with the relatively low number of CFUs among $10^3$ cells after 2 wks of culture using IL-3 pre-adsorbed heparinized crosslinked collagen films is indicative of cell differentiation. This could have been caused by the relatively high amount of IL-3 released into the culture medium from the IL-3 pre-adsorbed films. It has been reported that relatively high concentrations of IL-3 in serum-free culture medium increase HPC differentiation [19].

The 89 ng IL-3 that remained bound to the IL-3 pre-adsorbed heparinized films during 2 wks of cell culture, could have contributed to the high total and transient $CD34^+$ cell expansion. It has also been reported that IL-3 bound to immobilized heparan sulfate promotes the proliferation of HPCs [23]. However, the relative contribution of released IL-3 or IL-3 that remained bound to the heparinized films to the improved HPC expansion, when using heparinized crosslinked collagen films pre-adsorbed with IL-3, could not be determined from the current results.

The use of IL-3 pre-adsorbed heparinized crosslinked collagen films resulted in a higher percentage of erythroid progenitors among the CFUs, as compared to these films without pre-adsorbed IL-3 but with IL-3 added to the culture medium (table 2). The involvement of IL-3 in erythropoiesis has been reported before. *In vitro*, the absence of IL-3 during erythropoiesis delays the differentiation of HSCs into erythropoietic progenitors [30]. *In vivo*, the number of nucleated red blood cells in the blood, associated with a variety of severe diseases, was correlated with the IL-3 levels [31], indicating increased erythropoiesis with high IL-3 levels.

## 4. Conclusions

Crosslinked collagen films (d = 15 mm, thickness = 50 μm, weight ~ 3 mg) adsorbed a significant amount (ca. 2.4 ng per film) of IL-3 from 0.5 ml culture medium containing 5 ng IL-3 at 37˚C. The adsorption of IL-3 from culture medium onto heparinized films was 2 ng per film. Heparinized films subsequently released 8 % of the adsorbed IL-3 into cytokine-free culture medium, which was 8 times more than the amount released from non-heparinized films. This indicates that part of the adsorbed IL-3 was reversibly bound to the heparinized films, while IL-3 adsorbed onto crosslinked collagen was almost entirely bound in an irreversible way.

The use of non-heparinized crosslinked collagen films, pre-incubated with an IL-3 solution (1 μg/ml), as substrate for $CD34^+$ cell culture with IL-3 free medium did not result in cell proliferation. These results may be explained by the strong interaction between IL-3 and the crosslinked collagen films, possibly accompanied by conformational changes of the cytokine. Upon incubation with the IL-3 solution, the adsorption of IL-3 onto heparinized crosslinked collagen films was 111 ± 30 ng per film. About 20 % of the adsorbed IL-3 was subsequently released from the heparinized films. This release took place before the first medium refreshment at d 4. The use of heparinized crosslinked collagen films pre-adsorbed with IL-3 as substrate for the culture of $CD34^+$ cells with IL-3-free culture medium resulted in a relatively large number of adhering cells and a transient 1.4-fold $CD34^+$ expansion during the first wk of culture and a 16-fold viable cell expansion after 2 wks. These results show that the binding of IL-3 by heparinized crosslinked collagen films improves HPC expansion. Additionally, by using these films, an increased differentiation of CFUs into the erythroid lineage was found.

## 5. Acknowledgements

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## 6. References


1. Taichman RS. *Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche*. Blood 2005;105:2631-2639.
2. Fuchs E, Tumbar T, Guasch G. *Socializing with the neighbors: stem cells and their niches*. Cell 2004;116:769-778.
3. Zhang J, Niu C, Ye L, Huang HY, He X, Tong WG, Ross J, Haug J, Johnson T, Feng JQ, Harris S, Wiedemann LM, Mishina Y, Li LH. *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature 2003;425:836-841.
4. Ruoslahti E. *Proteoglycans in cell regulation*. J Biol Chem 1989;264:13369-13372.
5. Gordon MY, Riley GP, Watt SM and Greaves MF. *Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment*. Nature 1987;326:403-405.
6. Kim HS, Lim JB, Min YH, Lee ST, Lyu CJ, Kim ES, Kim HO. *Ex vivo expansion of human umbilical cord blood CD34+ cells in a collagen bead-containing 3 dimensional culture system*. Int J Hematol 2003;78:126-132.
7. Xiong F, Chen Z, Liu H, Xu Z, Liu X. *Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system.* Biotechnol Lett 2002;24:1421-1426.
8. Meissner P, Schroder B, Herfurth C, Biselli M. *Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells*. Cytotechnology 1999;30:227-234.
9. Wang TY, Wu JHD. *A continuous perfusion bioreactor for long-term bone-marrow culture*. Ann N Y Acad Sci 1992;665:274-284.
10. Wang TY, Brennan JK, Wu JHD. *Multilineal hematopoiesis in a 3-dimensional murine long-term bone-marrow culture*. Exp Hematol 1995;23:26-32.
11. Mantalaris A, Keng P, Bourne P, Chang AYC, Wu JHD. *Engineering a human bone marrow model: a case study on ex vivo erythropoiesis*. Biotechnol Prog 1998;14:126-133.
12. Gupta P, McCarthy JB, Verfaillie CM. *Stromal fibroblast heparan sulfate is required for cytokine-mediated ex vivo maintenance of human long-term culture-initiating cells*. Blood 1996;87:3229-3236.
13. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. *Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche*. Blood 1998;92:4641-4651.
14. Gupta P, Oegema TR, Brazil JJ, Dudek AZ, Slungaard A, Verfaillie CM. *Human LTC-IC can be maintained for at least 5 wks in vitro when interleukin-3 and a single chemokine are combined with O- sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins*. Blood 2000;95:147-155.
15. Roberts R, Gallagher J, Spooncer E, Allen TD, Bloomfield F, Dexter TM. *Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis.* Nature 1988;332:376-378.
16. Tanaka Y, Kimata K, Adams DH, Eto S. *Modulation of Cytokine Function by Heparan Sulfate Proteoglycans: Sophisticated Models for the Regulation of Cellular Responses to Cytokines*. P Assoc Am Physician 1998;10:118-125.
17. Siebum B, Grijpma DW, Poot AA, Vermes I, Feijen J. *Heparinized crosslinked collagen films for hematopoietic stem cell culture*. Chapter 3 of this thesis.
18. Siebum B, Grijpma DW, Poot AA, Vermes I, Kruijer W, Feijen J. *Heparinized crosslinked collagen films pre-adsorbed with SDF-1α for hematopoietic stem cell culture*. Chapter 4 of this thesis.
19. Ivanovic Z. *Interleukin-3 and ex vivo maintenance of hematopoietic stem cells: facts and controversies*. Eur Cytokine Netw 2004; 15: p.6-13.

20. Long MW, Briddell R, Walter AW, Bruno E, Hoffman R. Human *Hematopoietic Stem Cell Adherence to Cytokines and Matrix Molecules*. J Clin Invest 1992;90:251-255.
21. Strobel ES, Mobest D, Von Kleist S, Dangel M, Ries S, Mertelsmann R, Henschler R. *Adhesion and Migration are Differentially Regulated in Hematopoietic Progenitor Cells by Cytokines and Extracellular Matrix*. Blood 1997;90:3524-3532.
22. Aiuti A, Webb IJ, Bleul C, Springer T, Gutierrez-Ramos JC. *The chemokine SDF-1 is a chemotractant for human CD34$^+$ hematopoietic progenitor cells and provides a new mechanism to explain the mobilization of CD34$^+$ progenitors to peripheral blood*. J Exp Med 1997;185:111-120.
23. Alvarez-Silva M, Borojevic R. *GM-CSF and IL-3 activities in schistosomal liver granulomas are controlled by stroma-associated heparan sulfate progeoglycans*. J Leukoc Biol 1996;59:435-441.
24. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, and Feijen J. *Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation*. Biomaterials 2001;22:151-163.
25. Loke WK, Khor E. Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valva application by differential scanning calorimetry. Biomaterials 1995;16:251-258.
26. Wang CL, Miyata T, Wecksler B, Rubin AL, Stenzel KH. Collagen induced platelet aggregation and release. I. Effects of side-chain modifications and role of argynyl residues. Biochim Biophys Acta 1978;544:555-567.
27. Madihally SV, Flake AW, Matthew HWT. Maintenance of CD34 expression during proliferation of CD34(+) cord blood cells on glycosaminoglycan surfaces. Stem Cells 1999;17:295-305.
28. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, Feijen J. *Binding and release of basic fibroblast growth factor from heparinized collagen matrices*. Biomaterials 2001;22:2291-2299.
29. Capila I, Linhardt RJ. Heparin - protein interactions. Angew Chem Int Ed 2002;41:391-412.
30. Bohmer RM. *Erythropoiesis from adult but not fetal blood-derived CD133+ stem cells depends strongly on interleukin-3*. Growth Factors. 2004;22:45-50.
31. Stachon A, Bolulu O, Holland-Letz T, Krieg M. *Association between nucleated red blood cells in the blood and the level of erythropoietin, interleukin 3, interleukin 6, and interleuking 12p70*. Shock 2005;24:34-39.

# Appendix

## Hematopoietic progenitor cell culture on bFGF pre-adsorbed heparinized and non-heparinized crosslinked collagen films: preliminary results

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**Abstract**

In this appendix, the use of heparinized and non-heparinized crosslinked collagen films pre-adsorbed with basic Fibroblast Growth Factor (bFGF) as substrate for the culture of $CD34^+$ hematopoietic progenitor cells (HPCs) is presented.

Heparinized and non-heparinized crosslinked collagen films (d = 15 mm, thickness = 50 μm, weight ~ 3 mg) were pre-incubated with 0.3 ml of a solution of bFGF (1 μg/ml) in PBS. Peripheral blood derived $CD34^+$ cells were seeded onto these films and after 2 wks of serum-free culturing in medium containing Flt3/Flk2-ligand, IL-3, TPO and SCF, a large number of cells adhered to the films (50000-60000 cells/film). On bFGF pre-adsorbed heparinized films the total cell expansion was 10 times after 2 wks, while on bFGF pre-adsorbed non-heparinized films the total cell population expanded 2-fold. $CD34^+$ cells only expanded on heparinized films pre-adsorbed with bFGF, reaching an expansion of 1.5 times after 1 wk. However, the number of CFUs among the expanded cells did not increase. The differentiation of CFUs into the erythroid lineage was not significantly stimulated by bFGF pre-adsorbed to heparinized films.

These results show that culturing of HPCs on heparinized and non-heparinized crosslinked collagen films pre-adsorbed with bFGF, mainly led to an increase in the number of adhering and non-adhering cells, as compared to films which did not contain bFGF.

## 1. Introduction

In the previous chapters, heparinized and non-heparinized crosslinked collagen films were either pre-adsorbed with the cytokine interleukin-3 (IL-3) or the chemokine stromal cell-derived factor-1α (SDF-1α). When culture medium was added to films pre-adsorbed with one of these proteins, subsequent release took place within a day [1,2]. In contrast to this burst-release, the release of basic Fibroblast Growth Factor (bFGF) from these films has been reported to be sustained for days when using 5 ml serum-containing release medium that was replaced every day [3].

To investigate the effects of a slow releasing growth factor, we pre-adsorbed heparinized and non-heparinized films with bFGF and used these films as substrate for the culture of $CD34^+$ hematopoietic progenitor cells (HPCs) obtained from G-CSF mobilized peripheral blood. The interactions of bFGF with ECM components [4] and heparin [3, 4] have been widely studied. This growth factor is involved in the proliferation and differentiation of numerous cell types and is thought to play an important role in hematopoiesis [5-10]. In has been reported that bFGF stimulates myelopoiesis and HPC adhesion [6], modulates erythropoiesis [7, 8] and mediates the expansion of murine hematopoietic stem cells (HSCs) [9, 10].

Therefore, we did some preliminary investigations into the effects of bFGF, pre-adsorbed onto heparinized and non-heparinized crosslinked collagen films, on HPC expansion, cell adhesion and erythroid differentiation.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Heparinized crosslinked collagen film preparation*

Collagen films were prepared from type I insoluble collagen and subsequently crosslinked, heparinized and characterized as described before [1].

### *2.3. Hematopoietic cell culture*

Circular films (d = 15 mm, thickness = 50 μm, weight ~ 3 mg) were cut out of heparinized and non-heparinized crosslinked collagen films and rinsed with 70 % ethanol. The circular films were fixed with Viton O rings in wells of a 24-well TCPS culture plate (Corning, NY, USA) and incubated overnight at 37 ˚C with a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml; both from Gibco BRL, Paisley, UK). Before cell seeding, the films were incubated once more overnight at room temperature with 300 μl of a phosphate-buffered saline solution (PBS, B. Braun Medical, Oss, The Netherlands) containing 0.1 (wt)% BSA with or without 1 μg/ml bFGF (R&D systems, Minneapolis, USA).

Human $CD34^+$ cells, isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood, were obtained from the Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and subsequently seeded onto the heparinized and non-heparinized films, cultured and characterized as described before [1].

### *2.6. Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 2 experiments performed in duplicate. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values $< 0.05$.

## 3. Results

### *3.1. Preparation of heparinized collagen films*

Collagen films were crosslinked and heparinized using EDC and NHS and characterized as previously described [11]. After crosslinking, the increased shrinkage temperature of the collagen films (from 59 ˚C to 76 ˚C) and the decreased number of primary amino groups per 1000 amino acid residues (from 27 to 16) indicated that the collagen was crosslinked. As reported previously, 23 ± 4 μg heparin was immobilized per mg of crosslinked collagen film [11]. Toluidine blue staining throughout the

thickness of the films indicated that the heparin was immobilized both on the surface and in the bulk of the films.

### *3.2. Pre-adsorbed bFGF and HPC adhesion*

$CD34^+$ selected cells (ca. 90 % $CD34^+$) were seeded onto heparinized and non-heparinzed crosslinked collagen films that were incubated overnight at room temperature with 300 µl PBS containing bFGF (1 µg/ml). The number of cells adhering to bFGF pre-adsorbed films increased dramatically during the second wk of $CD34^+$ cell culture (figures 1 and 2).

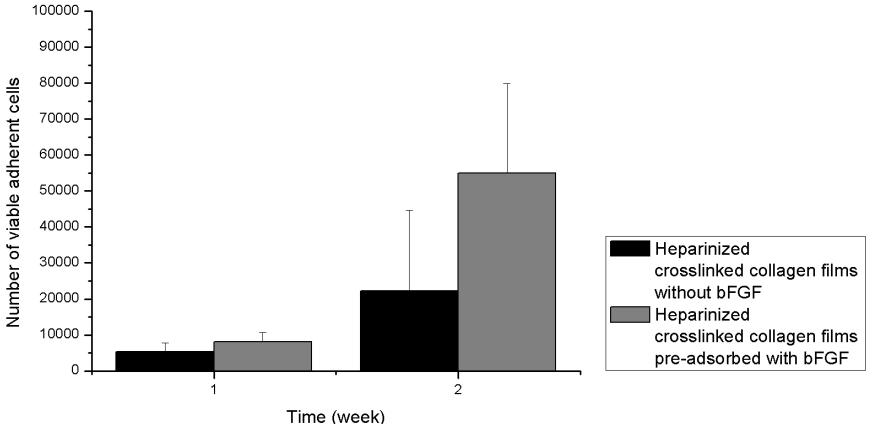


**Figure 1. Number of viable cells ($7AAD^-$ and $CD45^+$) adhering to the surfaces during cell culture using heparinized crosslinked collagen films with or without pre-adsorbed bFGF, determined from two independent experiments performed in duplicate (± S.E.M.).**

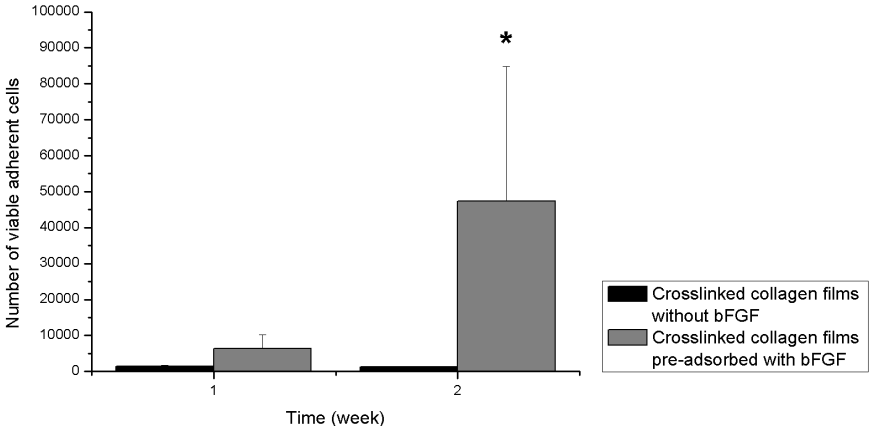


**Figure 2. Number of viable cells ($7AAD^-$ and $CD45^+$) adhering to the surfaces during cell culture using crosslinked collagen films with or without pre-adsorbed bFGF, determined from two independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to the culture on crosslinked collagen films without pre-adsorbed bFGF.**

Less cells adhered to non-heparinized crosslinked collagen films as compared to heparinized films, especially when the films were not pre-adsorbed with bFGF.

The number of $CD34^+$ cells among the cells adhering to the heparinized and non-heparinized crosslinked collagen films during cell culture on bFGF pre-adsorbed films was not higher, as compared to films without bFGF (50 – 60 % after 1 wk and 5 - 8 % after 2 wks).

### *3.3.Pre-adsorbed bFGF and HPC expansion*

Under the described conditions, the total number of cells increased 10-fold and-6 fold during $CD34^+$ cell culture on heparinized films with and without pre-adsorbed bFGF, respectively (figure 3).

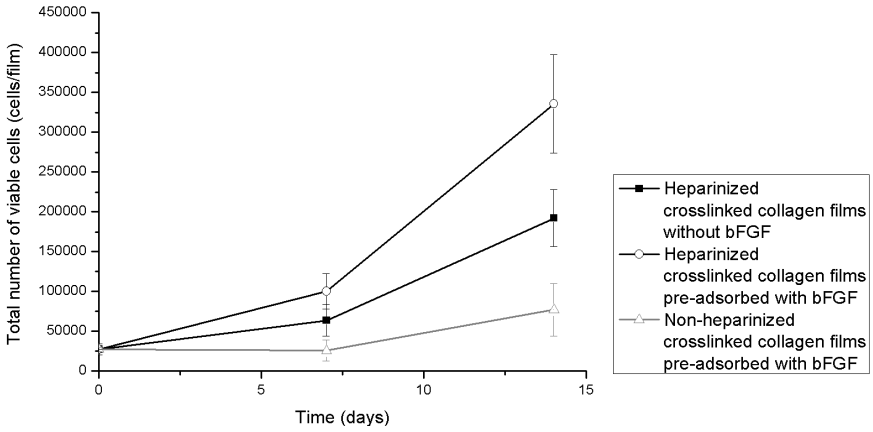


**Figure 3. Total number of viable cells (sum of adherent and non-adherent cells ($7AAD^-$ and $CD45^+$)) during cell culture using heparinized crosslinked collagen films with or without pre-adsorbed bFGF, determined from two independent experiments performed in duplicate (± S.E.M.).**

The use of bFGF pre-adsorbed crosslinked collagen films as substrate for the culture of $CD34^+$ cells resulted in a 2-fold expansion of viable cells after 2 wks (figure 3), but no expansion of $CD34^+$ cells (data not shown).

When using heparinized films pre-adsorbed with bFGF as culture substrate, the $CD34^+$ cells expanded 1.5 ± 0.4 times during the first wk of HPC culture. This is a small improvement compared to the use of heparinized films without pre-adsorbed bFGF, as no $CD34^+$ cell expansion was obtained in that case [11]. However, the number of CFUs among the non-adherent cells in culture decreased when using bFGF pre-adsorbed heparinized films instead of heparinized films without bFGF (table 1). The use of bFGF pre-adsorbed non-heparinized films resulted in a small number of

CFUs among the cells after 1 wk (table 1), in contrast to non-heparinized films without bFGF, where no CFUs were found (data not shown).

**Table 1. Colony Forming Units (CFUs) per $10^3$ non-adhering cells in cultures using heparinized crosslinked collagen films with or without pre-adsorbed bFGF. At cell seeding, the cell population contained 88 CFUs per $10^3$ cells, of which 39-46 % were erythroid CFUs (either BFU-E or CFU-E). Among the erythroid CFUs the percentage of BFU-E colonies was about 90-100% at cell seeding.**

| | Week 1 | | | Week 2 | | |
|---|---|---|---|---|---|---|
| | CFUs | Erythroid CFUs | BFU-E | CFUs | Erythroid CFUs | BFU-E |
| Heparinized films, without bFGF | 146 | 62 % | 29 % | 88 | 83 % | 11 % |
| bFGF pre-adsorbed heparinized films | 105 | 72 % | 11 % | 57 | 84 % | 19 % |
| bFGF pre-adsorbed non-heparinized films | 23 | 91 % | 5 % | 0 | - | - |

### *3.4.Pre-adsorbed bFGF and HPC differentiation*

During cell expansion using heparinized films the percentage of erythroid CFUs was only slightly increased during the first week of culture by pre-adsorbed bFGF (table 1). Additionally, after 2 wks the same number of $CD36^+$ cells as percentage of the total cell population was found when using heparinized films with or without pre-adsorbed bFGF (about 50 %). A higher percentage of BFU-E cells remained after 2 wks among the erythroid CFUs when heparinized crosslinked collagen films pre-adsorbed with bFGF were applied (table 1).

## 4. Discussion and conclusions

In this study, we present the use of heparinized and non-heparinized crosslinked collagen films either pre-adsorbed with bFGF or not as substrate for the culture of $CD34^+$ HPCs. During the second wk of $CD34^+$ cell culture on these substrates the number of adhering cells increased dramatically. It has been reported that the number of hematopoietic cells adhering to stroma in long-term bone marrow cultures was increased by bFGF added to the culture medium [6]. To relate the concentration of bFGF in the culture medium used in he present study to cell adhesion, the release rate of bFGF into the medium under culture conditions needs to be investigated.

On bFGF pre-adsorbed heparinized films the total cell expansion was 10 times after 2 wks, while on bFGF pre-adsorbed non-heparinized films the total cell population expanded 2-fold. Using heparinized films, $CD34^+$ cell expansion during the first wk

was only slightly improved (1.5 times) by pre-adsorption of bFGF. Previously, non-heparinized films never supported the culture of hematopoietic cells [11], not even after pre-adsorption of SDF-1α or IL-3 [1, 2]. Although $CD34^+$ cell-survival did not improve, the total cell expansion on bFGF pre-adsorbed crosslinked collagen films illustrates that these films, under certain conditions, could be used as culture substrate for hematopoietic cells. However, heparinization is still required in order to support $CD34^+$ cell expansion.

The presence of bFGF in the culture medium was expected to influence erythroid differentiation [7, 8] rather than to expand progenitor cells, as this has only been reported for murine HSCs [9, 10]. The number of CFUs among the expanded cells did not increase by using heparinized films pre-adsorbed with bFGF as culture substrate. The differentiation of CFUs into the erythroid lineage was only partially stimulated by bFGF pre-adsorbed onto heparinized films, as a slightly higher percentage of BFU-E cells remained after 2 wks. This would indicate that only the early stages of erythropoiesis, but not further differentiation, were stimulated by bFGF, corresponding to literature data [8]. In short, these results show that culturing of HPCs on heparinized and non-heparinized crosslinked collagen films pre-adsorbed with bFGF, mainly led to an increase in the number of adhering and non-adhering cells, as compared to films which did not contain bFGF. The expansion of $CD34^+$ cells and erythroid differentiation during HPC culture were not significantly improved by pre-adsorbed bFGF.

## 5. References

1. Siebum B, Grijpma DW, Poot AA, Vermes I, Kruijer W, Feijen J. *Heparinized crosslinked collagen films pre-adsorbed with SDF-1α for hematopoietic stem cell culture*. Chapter 4 of this thesis.
2. Siebum B, Grijpma DW, Poot AA, Vermes I, Kruijer W, Feijen J. *IL-3 pre-adsorbed heparinized crosslinked collagen films for hematopoietic progenitor cell culture*. Chapter 5 of this thesis.
3. Wissink MJB, Beernink R, Pieper JS, Poot AA, Engbers GHM, Beugeling T, Van Aken WG, Feijen J. *Binding and release of basic fibroblast growth factor from heparinized collagen matrices.* Biomaterials 2001;22:2291-2299.
4. Capila I, Linhardt RJ. *Heparin - Protein interactions*. Angew Chem Int Ed Engl 2002;41:391-412.
5. Allouche M, Bikfalvi A. *The role of Fibroblast Growth Factor-2 (FGF-2) in hematopoiesis*. Prog Growth Factor Res 1995;6:35-48.

6. Wilson EL, Rifkin DB, Kelly F, Hannocks MJ, Gabrilove JL. *Basic Fibroblast Growth Factor Stimulates Myelopoiesis in Long-Term Human Bone Marrow Cultures*. *Blood* 1991;77:954-960.
7. Burger PE, Lukey PT, Coetzee S, Wilson EL. *Basic Fibroblast Growth Factor Modulates the Expression of Glycophorin A and c-kit and Inhibits Erythroid Differentiation in K562 Cells*. J Cell Physiol 2002; 190: p.83-91.
8. Kashiwakura, I, Takahashi TA. *Basic fibroblast growth factor-stimulated ex vivo expansion of haematopoietic progenitor cells from human placental and umbilical blood*. Br J Haematol 2003;122:479-488.
9. Yeoh JSG, Van Os R, Weersing A, Ausema A, Dontje B, Vellenga E, De Haan G. *Fibroblast growth factor-1 and-2 preserve long-term repopulating ability of hematopoietic stem cells in serum-free cultures.* Stem Cells 2006;24:1564-1572.
10. De Haan G, Weersing E, Dontje B, Van Os R, Bystrykh LV, Vellenga E, Miller G. *In Vitro Generation of Long Term Repopulating Hematopoietic Stem Cells by Fibroblast Growth Factor-1*. Dev Cell 2003;4:241-251.
11. Siebum B, Grijpma DW, Poot AA, Vermes I, Feijen J. *Heparinized crosslinked collagen films for hematopoietic stem cell culture*. Chapter 3 of this thesis.

# Chapter 6

## Heparinized crosslinked collagen beads for culturing of hematopoietic progenitor cells

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**Abstract**

A bioreacor-based system to achieve proliferation and controlled differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great clinical interest and provides a model to investigate hematopoiesis. In the present study, a packed bed of SDF-1α pre-adsorbed heparinized crosslinked collagen beads was compared with SDF-1α pre-adsorbed heparinized crosslinked collagen films as culture substrate for the expansion of $CD34^+$ cells.

Beads were prepared from soluble type I collagen and subsequently crosslinked and heparinized, both by means of EDC/NHS chemistry. Heparin was immobilized to an extent of 19 ± 3 μg per mg of beads. At an SDF-1α concentration of 1.11 μg/ml, the adsorption of SDF-1α was 77 ± 9 ng per mg of heparinized crosslinked collagen beads. During 2 wks 70 % of the chemokine was released when employing half medium changes twice a wk.

$CD34^+$ cells were cultured serum-free on SDF-1α pre-adsorbed heparinized crosslinked collagen beads. Pre-adsorption of SDF-1α to the beads did not significantly improve cell retention in the packed bed of beads. Serum-free cultures using these beads gave a 3-fold expansion of $CD34^+$ cells, whereas these cells did not expand on SDF-1α pre-adsorbed heparinized crosslinked collagen films. Total cell expansion was similar for both substrates. The Colony Forming Units (CFUs) in cell cultures using beads remained more primitive and showed less preference for the

erythroid lineage compared to the cultures using films. The improved expansion of $CD34^+$ cells in cultures using SDF-1α pre-adsorbed heparinized crosslinked collagen beads can be further exploited by developing a bioreactor for hematopoietic cell culture.

## 1. Introduction

The *in vitro* proliferation and controlled differentiation of hematopoietic stem and progenitor cells (HSCs and HPCs) is of great interest for application in bone marrow transplantation. The use of bioreactors containing three-dimensional structures to mimic the bone marrow is expected to improve the expansion of these hematopoietic cells. In adults, the bone marrow is the principle site of hematopoiesis. It is generally accepted that the bone marrow contains microenvironments for HSCs in which these cells interact with stromal cells, extracellular matrix (ECM) components and cytokines [1,2]. There are several reports that claim good HPC expansion by using a bioreactor [3-10] or a three-dimensional culture system [11-13].

Collagen type I is one of the important components of the bone marrow ECM. It can be processed into structures like films, beads and sponges, which are suitable for cell culture. Additionally, collagen can be chemically modified [14] with compounds present in the microenvironments of the bone marrow. This can be done with glycosaminoglycans (GAGs), since these are involved in keeping the ECM in a hydrated state and play an important role in chemokine function [15-20].

Previously, we demonstrated that $CD34^+$ HSCs can be cultured on heparinized crosslinked collagen films. Heparin served as a model compound for the ECM component heparan sulfate. Heparinization improved $CD34^+$ cell survival and expansion of Colony Forming Units (CFUs) in the erythroid lineage. After 2 wks, total viable cell expansion was 5-fold [21]. Heparin and heparan sulfate are known to bind the chemokine stromal cell-derived factor-1α (SDF-1α, 8 kDa) [22,23]. The binding of SDF-1α by heparan sulfate is thought to facilitate HSC homing and adhesion [23]. SDF-1α plays a role in the development of the marrow in the bone and is produced by HSC supporting osteoblasts [24]. SDF-1α also plays a role in the retention of erythroid progenitors in the bone marrow until they are released when mature [25]. Pre-adsorption of SDF-1α on heparinized crosslinked collagen films resulted in a 2-fold increase in the number of adhering cells 1 h after $CD34^+$ cell

seeding. Improved cell adhesion upon seeding was followed by a higher number of CFUs among the adhering cells after 4 d of culturing [26]. An expansion of $CD34^+$ cells, however, was not achieved using heparinized crosslinked collagen films with or without pre-adsorbed SDF-1α.

Here we compare a three-dimensional packed bed of heparinized crosslinked collagen beads with two-dimensional heparinized crosslinked collagen films as culture substrates for the expansion of $CD34^+$ cells.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Collagen bead preparation*

Solid collagen beads were prepared by emulsification [27]. Acid-soluble collagen type I derived from calf skin (Elastin Products Co. Inc., Missouri, USA) was dissolved overnight in 0.5 M acetic acid solution (0.05 g in 5 ml) at 4 ˚C. The collagen solution was neutralized using a NaOH solution and added dropwise to stirred paraffin oil at 37 ˚C. The emulsion was further stirred for 2 h at 1000 rpm to let the collagen drops reconstitute. Water was added and the beads were removed from the aqueous phase. Subsequently, the beads were washed with ethanol (5 min) and water (30 min). Collagen films were prepared from type I insoluble collagen derived from bovine achilles tendon as previously described [21].

The beads and films were crosslinked using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Fluka, Buchs, Switzerland) and N-hydroxysuccinimide (NHS) [14]. The structures were first incubated in 2-morpholinoethane sulfonic acid (MES) buffer (0.05 M, pH 5.5) for 30 min at room temperature. They were then transferred to the crosslinking solution, containing 2.3 g EDC and 0.56 g NHS in 215 ml MES-buffer per g of collagen (molar ratio of EDC/NHS = 2.5). After 2 h incubation under gentle shaking, the structures were placed in a 0.1 M $Na_2HPO_4$ solution (pH 9.2, Merck, Darmstadt, Germany) for 2 h to stop the reaction and then rinsed with demineralized water for three times 30 min.

### *2.3. Heparin immobilization*

Crosslinked beads and films were subsequently heparinized. The structures were first incubated in MES buffer (0.05 M, pH 5.6) for at least 30 min at room temperature. The carboxylic acid groups of heparin (from porcine mucosa, MW 6000-30000, activity>160 IU/mg) were pre-activated for 10 min. This was done by means of 0.43 g EDC and 0.16 g NHS in 188 ml of a 2 (wt)% heparin solution in MES-buffer [14].

The crosslinked collagen (1 g) was heparinized for 2 h in the EDC/NHS-activated heparin solution. Subsequently the reaction was terminated by immersion of the beads and films in 0.1 M sodium phosphate. The heparinized structures were then washed in 2 M NaCl (24 h), 4 M NaCl (4 times 24 h) and MilliQ water (3 times 24 h).

Before cell culture beads with a diameter <500 μm were obtained by sieving (ASTM E11 nr 35, Endecotts Ltd). Micrographs (magnification 5x) of the beads suspended in phosphate buffered saline (PBS, B. Braun Medical, Oss, The Netherlands) were used to measure the size of the beads in order to calculate the average size and size distribution. Size measurements and calculations were done with Scion Image™ computer software.

### *2.4. Characterization of materials*

The shrinkage temperature ($T_s$) of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC) as described before [21]. This temperature, at which the collagen sample undergoes thermal denaturation, is used as a measure of the crosslink density [28].

The residual number of free primary amino groups in (crosslinked) collagen was determined spectrophotometrically after reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fluka) [29].

The heparin content of the samples was determined by a colorimetric method based on the binding of toluidine blue and by using $^{3}$H-labelled heparin [21].

### *2.5. SDF-1α loading and release*

Human recombinant SDF-1α (R&D systems, Minneapolis, USA) was labelled with $^{125}$I using Iodobeads [26] in order to study the binding of SDF-1α and subsequent release from heparinized and non-heparinized crosslinked collagen beads.

Crosslinked collagen beads (4 mg, non-fractionated) with and without immobilized heparin were incubated overnight at 37 °C in a solution of penicillin (200 U/ml) and

streptomycin (0.2 mg/ml; both from Gibco BRL, Paisley, UK). After rinsing three times with PBS the samples were incubated overnight at room temperature in 1 ml of a $^{125}$I-labelled SDF-1α solution (1.11 μg/ml) in PBS containing 0.1 (wt)% BSA. Thereafter, the samples were rinsed 3 times with PBS and their radioactivity was measured using a Compugamma 1282 γ-counter (LKB, Stockholm, Sweden).

The release of SDF-1α was determined at 37 °C by placing heparinized and non-heparinized crosslinked collagen beads pre-adsorbed with $^{125}$I-labelled SDF-1α in 0.5 ml culture medium (see below) containing 0.02 (wt)% $NaN_3$. Half of the medium was refreshed twice a wk, after which the radioactivity of the removed medium was measured. At the end of the release period (2 wks), the radioactivity remaining associated with the beads was measured as well.

### *2.6. Hematopoietic cell culture*

Heparinized crosslinked collagen films (d = 15 mm) and beads (<500 μm) were rinsed with 70 % ethanol. The circular films were fixed with Viton O rings in the wells of a 24-well tissue culture polystyrene (TCPS) culture plate (Corning, NY, USA), yielding a surface area of 1.5 $cm^2$. Packed beds of 4 mg of crosslinked collagen beads consisted of at least two layers and had an approximate volume of 0.19 $cm^3$, a surface area of 7.4 ± 0.8 $cm^2$ and porosity of 70 % (calculated from the number of beads per ml and the average volume of the beads).

The beads and fixed films were incubated overnight at 37 ˚C in a solution of 200 U/ml penicillin and 0.2 mg/ml streptomycin. Before cell seeding, the films and beads were incubated once more overnight at room temperature in a PBS solution containing 0.1 (wt)% BSA and 1.11 μg/ml SDF-1α. The beads were transferred to the wells of a 24-well ultra low adherence culture plate (Corning, NY, USA).

Human $CD34^+$ cells isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood were obtained from the Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and subsequently cultured in a serum-free medium consisting of IMDM (with GlutaMAX I, Gibco), 1 (wt)% BSA, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml recombinant human insulin, 200 μg/ml human partially iron-saturated holo-transferrin, 10 ng/ml TPO, 10 ng/ml IL-3, 100 ng/ml SCF, 50 ng/ml Flt3/Flk2-ligand. All four cytokines were from

recombinant human origin (R&D systems). The cells were seeded by adding 0.5 ml of a cell suspension with a density of $1 \cdot 10^5$ cells/ml onto the heparinized collagen structures. In order to pack the cell-seeded beads, they were covered with a Transwell membrane insert (polycarbonate membrane with 8 μm pores, Corning, NY, USA). The cells were cultured at 37 ˚C in a humidified 5 % $CO_2$ atmosphere. Half of the medium was exchanged twice a wk. The first medium exchange for the cultures containing beads was replaced by adding 0.5 ml fresh culture medium in the Transwell insert. Thereafter, 0.5 ml medium in the insert was refreshed twice a wk.

During cell culture two cell fractions were obtained. The cells that did not adhere to the films were removed by gentle pipetting. Non-adherent cells and beads were separated by filtration (MACS pre-separation filters, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After rinsing the surfaces with PBS containing 1 (wt)% BSA and subsequent incubation in Cell Dissociation Solution (CDS), for 20 min at room temperature, the adhering cells were obtained. Dissociated cells were separated from the beads by filtration as described.

Flowcytometric phenotyping was performed using anti-CD45-FITC, anti-CD34-PE and 7AAD vitality dye (all from Immunotech, Marseille, France). Isotype-identical antibodies were used as control and for quantification Flow-Count beads were added to the samples. Cells were counted and analyzed using a Cytomics FC500 flow cytometry system (Beckman Coulter, Fullerton, USA). Moreover, differentiation was evaluated by Colony-Forming Unit (CFU) assays. For CFU analysis, samples containing $10^3$ cells were taken from the HPC culture, subsequently cultured in MethoCult (StemCell Technologies, Meylan, France) and scored after 2 wks.

### *2.7 Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 2 experiments performed in duplicate. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values $< 0.05$.

## 3. Results and discussion

### *3.1 Heparinized crosslinked collagen bead preparation*

The collagen beads obtained after emulsification had a smooth surface and diameters ranging from 0.1 to 1 mm (figure 1).

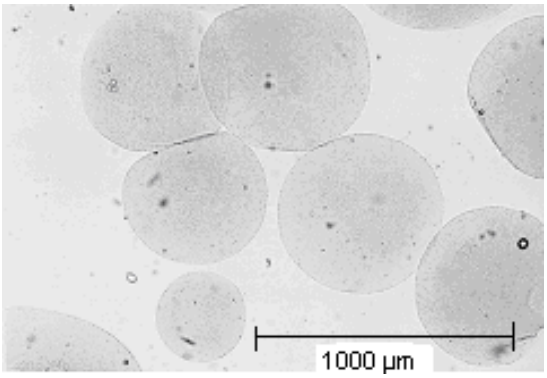


**Figure 1: Lightmicroscopy image of collagen beads in PBS.**

After sieving, the beads had an average diameter of 405 ± 83 µm. The shrinkage temperature of the collagen beads increased from 53 °C to 72 °C after crosslinking. The number of free primary amino groups per 1000 amino acid residues decreased from 27 to 17. The decrease in the number of free primary amino groups and increase in shrinkage temperature demonstrate that the collagen was crosslinked.

The toluidine blue assay indicated that 19 ± 3 µg heparin was immobilized per mg of crosslinked collagen beads. This was confirmed with radio-labelled heparin, of which 17 ± 4 µg was immobilized per mg of collagen. Furthermore, 23 ± 4 µg heparin was immobilized per mg of crosslinked collagen film [21]. Toluidine blue staining was observed throughout the volume of the beads and films, demonstrating that heparin was immobilized both on the surface and in the bulk. These results are in agreement with previously reported data [14].

### *3.2. SDF-1α adsorption and release*

At the described conditions the heparinized crosslinked beads bound 28 ± 3 % of $^{125}$I-labelled SDF-1α while non-heparinized crosslinked collagen beads bound 21 ± 2 %. This corresponds to 77 ± 9 ng and 58 ± 5 ng SDF-1α per mg of beads, respectively. Overnight incubation with a solution of penicillin and streptomycin in PBS did not influence the binding of SDF-1α. As previously reported, 25 ± 4 ng and 18 ± 2 ng SDF-1α adsorbed per mg of heparinized and non-heparinized crosslinked collagen films, respectively [26].

Upon heparinization of the crosslinked collagen beads and films, the increase in the amount of adsorbed SDF-1α was comparable (33 % and 39 % respectively). The amount of bound SDF-1α did not double after heparinization, as reported for bFGF binding to heparinized crosslinked collagen films [30]. Previously, we explained the

relatively small contribution of immobilized heparin to the binding of SDF-1α in terms of unfavourable heparin conformations [26]. This explanation probably also applies for the beads. In contrast to the collagen films, the beads were prepared from soluble collagen. The more gel-like structure of the collagen beads can probably accommodate more SDF-1α than the dense collagen films.

Because during cell culture half of the medium was changed twice a wk, the release of $^{125}$I-labelled SDF-1α was determined accordingly (figure 2A).

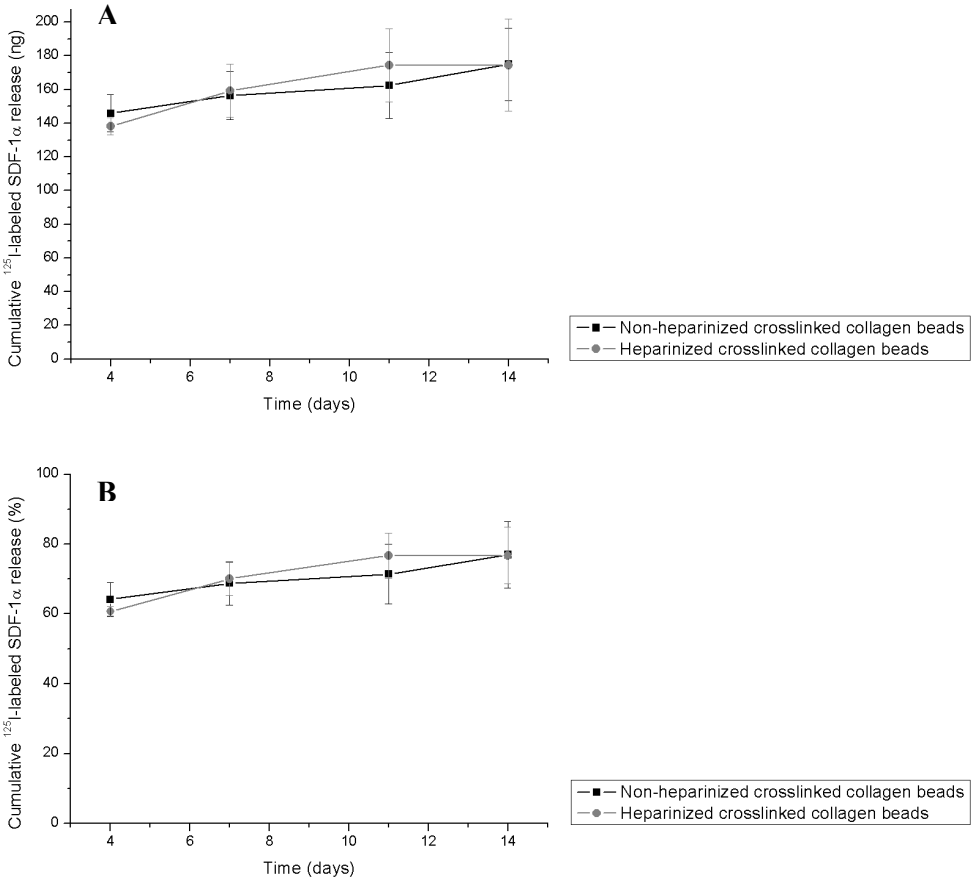


**Figure 2. Release of SDF-1α from heparinized and non-heparinized crosslinked collagen beads into 0.5 ml culture medium, employing half medium changes twice a week. Beads (4 mg) were pre-adsorbed overnight in 1 ml SDF-1α solution (1.11 µg/ml). The SDF-1α concentrations were determined using $^{125}$I-labelled SDF-1α.**

During 2 wks 77 % of the chemokine was released both from the heparinized and non-heparinized crosslinked collagen beads (figure 2B). This is more than from the films (40 % SDF-1α released after 2 wks) and is probably also due to the swollen gel-like structure of the beads.

After 2 wks of release, the amount of SDF-1α remaining on the non-heparinized crosslinked collagen beads (13.4 ± 5.5 ng per mg of beads) was comparable to the amount remaining on the non-heparinized crosslinked collagen films (11.0 ± 0.3 ng per mg film). This indicates a similar interaction of SDF-1α with crosslinked beads made of soluble collagen and crosslinked films made of non-soluble collagen. Additionally, the amounts of the chemokine remaining on heparinized crosslinked collagen beads and films after 2 wks of release were also comparable. The SDF-1α remaining on the beads is probably strongly adsorbed, as previously found for the films [26].

It was expected that the release of SDF-1α from heparinized crosslinked collagen beads would be slower compared to that from the non-heparinized beads. However, as with the films [26], this was not observed. This might be due to a relatively large amount of SDF-1α bound to the heparin and/or collagen via non-specific electrostatic interactions.

### *3.3.1. Hematopoietic cell adhesion*

Serum-free culturing of $CD34^+$ cells was performed using SDF-1α pre-adsorbed heparinized crosslinked collagen films and beads. During the first wk of cell culture, the density of cells adhering to SDF-1α pre-adsorbed heparinized crosslinked collagen films and beads was similar (figure 3).

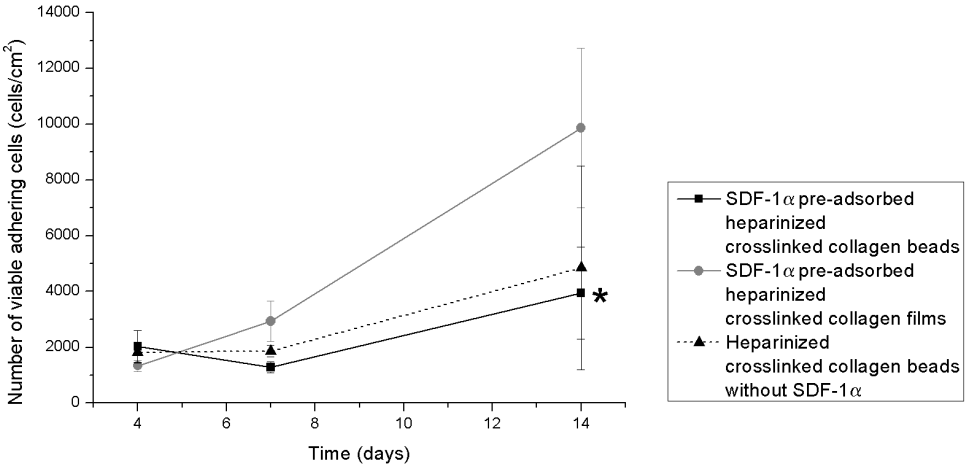


**Figure 3. The density of viable cells adhering to heparinized crosslinked collagen beads and films, determined from two independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to SDF-1α pre-adsorbed heparinized crosslinked collagen films.**

During wk 2, the number of adhering cells per $cm^2$ increased more on the films than on the beads, indicating that the dense surface of the heparinized crosslinked collagen films was most suitable for adhesion of HPCs. During the 2 wk culture period, the total number of cells adhering on the heparinized crosslinked collagen beads with or without SDF-1α was higher than on the films, due to the 5-times larger surface area of the beads.

Moreover, pre-adsorption of SDF-1α onto the beads did not increase the number of adhering cells during cell culture (figure 3). As reported before [26], we found that SDF-1α pre-adsorption increased the number of adhering cells on the crosslinked collagen substrates only upon cell seeding (data not shown). During cell culture the number of cells adhering to the beads never exceeded 20-30 %. Therefore, a packed bed of beads in a bioreactor requires a barrier to retain the remaining cells that do not adhere within the scaffold.

Only during the first wk after cell seeding, a significant amount of $CD34^+$ cells adhered to the heparinized crosslinked collagen beads. The density of $CD34^+$ cells was lower on heparinized crosslinked collagen beads than on the films. SDF-1α pre-adsorption did not influence the surface density of these cells (data not shown).

CFU's were present among the cells adhering to the heparinized crosslinked collagen beads and films. At day 4, the number of CFUs adhering per surface area was highest on the beads (table 1).

**Table 1. Number of colony-forming units per surface area (CFUs/$cm^2$) at day 4 among the adhering cells in cultures using heparinized crosslinked collagen beads and films.**

| | CFU-GEMM | BFU/CFU-E | CFU-G/M | Erythroid % |
|---|---|---|---|---|
| Heparinized collagen beads pre-adsorbed with SDF-1α | 0 | 80 | 8 | 91 % |
| Heparinized collagen beads without pre-adsorbed SDF-1α | 0 | 72 | 20 | 78 % |
| Heparinized collagen films pre-adsorbed with SDF-1α | 0 | 12 | 5 | 69% |

However, during wk 2 the number of adhering CFUs decreased on the beads and increased on the films, resulting in a higher amount of adhering CFUs per surface area on the films at d 14 (table 2). Interestingly, SDF-1α pre-adsorbed heparinized crosslinked collagen beads maintained a higher amount of adhering erythroid CFU's, compared to the beads without the chemokine (table 2). The CXCR4 receptor for SDF-1α is functional on erythroid progenitors and is thought to be involved in their

retention in the bone marrow [31]. Therefore, SDF-1α could have improved erythroid CFU adhesion to the beads.

**Table 2. Number of colony-forming units per surface area (CFUs/cm$^2$) at day 14 among the adhering cells in cultures using heparinized crosslinked collagen beads and films.**

| | CFU-GEMM | BFU/CFU-E | CFU-G/M | Erythroid % |
|---|---|---|---|---|
| Heparinized collagen beads pre-adsorbed with SDF-1α | 0 | 32 | 16 | 67 % |
| Heparinized collagen beads without pre-adsorbed SDF-1α | 0 | 10 | 20 | 33 % |
| Heparinized collagen films pre-adsorbed with SDF-1α | 0 | 119 | 20 | 86% |

### *3.3.2. Hematopoietic cell expansion*

After culturing CD34$^+$ cells using SDF-1α pre-adsorbed heparinized crosslinked collagen beads the total viable cell expansion after 2 wks (10-fold) was comparable to the cell expansion obtained with SDF-1α pre-adsorbed heparinized crosslinked collagen films (figure 4).

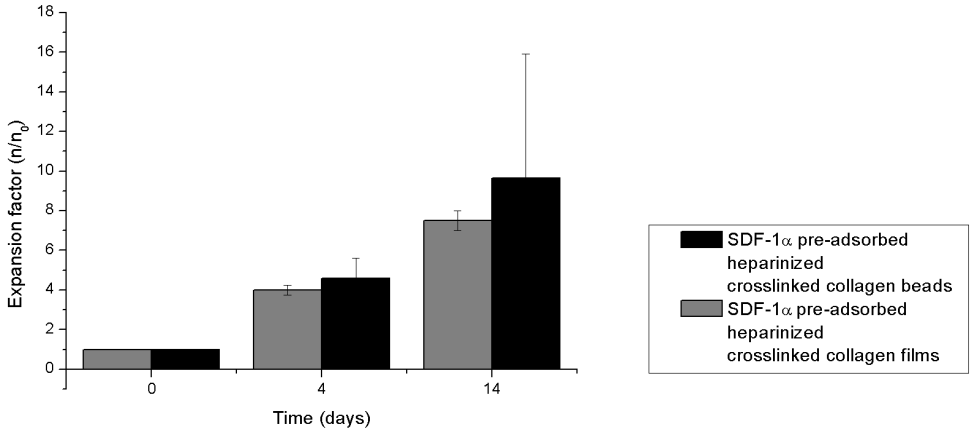


**Figure 4. Total viable cell expansion (the sum of adherent and non-adherent cells (7AAD$^-$ and CD45$^+$)), determined from two independent experiments performed in duplicate (± S.E.M.).**

Under the employed serum-free conditions only cell cultures using beads showed a transient 3-fold expansion of primitive CD34$^+$ cells (figure 5). The expansion of CD34$^+$ cells takes mainly place among the non-adhering cells. In the cultures using SDF-1α pre-adsorbed heparinized crosslinked collagen films the number of CD34$^+$ cells steadily decreased. CD34$^+$ cell expansions of 10-times have been reported for HPC cultures on collagen beads, but these were performed with cells from cord blood with either stroma cells present [12] or serum in the medium [11].

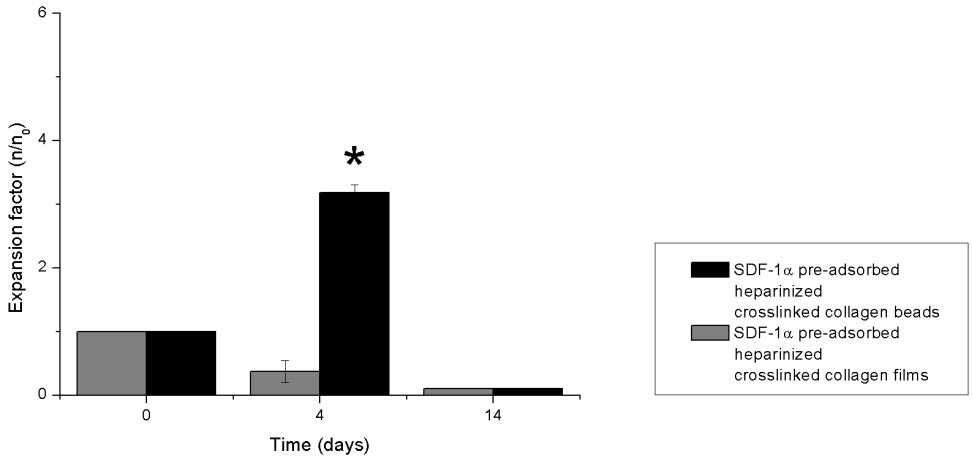


**Figure 5. Expansion of CD34$^+$ cells (the sum of adherent and non-adherent cells (7AAD$^-$, CD45$^+$ and CD34$^+$)), determined from two independent experiments performed in duplicate (± S.E.M.). * Indicates a significant difference compared to SDF-1α pre-adsorbed heparinized crosslinked collagen films.**

Here the use of a three- instead of a two-dimensional culture substrate resulted in an 8 times higher CD34$^+$ expansion at d 4 (3.2 vs 0.4, respectively). This is higher than the 3-5 times reported in literature for CD34$^+$ cell expansion using two- and three-dimensional culture substrates [9,12]. The improved expansion of CD34$^+$ cells could be explained by reduced cellular crowding among the non-adhering cells, due to the increased surface area provided by the beads. For peripheral blood derived mononuclear cultures the high density of non-adherent cells has been reported to lead to an inhibitory local environment [32]. SDF-1α has been reported to slightly, but not significantly, increase CD34$^+$ expansion [26, 33]. Therefore, the relatively high concentration of this chemokine in the culture medium due to release from the beads, could also have contributed to the obtained expansion.

During 2 wks the numbers of CFU colonies were lower in the cultures using the beads as compared to the films (table 3 and 4). When using beads the numbers of CFUs in culture expanded from day 4 to 14. The numbers of CFUs in the cultures using films decreased. Despite a decrease in CFU-G/M numbers, the composition of the CFU population on the films changed from more primitive CFU-GEMM and BFU-E colonies into less primitive colonies (CFU-E + CFU-G/M, table 3 and 4). Using the beads the CD34$^+$ population expanded transiently. Additionally, in the cell cultures using beads the CFUs expanded and remained more primitive, as shown by a higher number of BFU-E colonies (table 3 and 4). Kim et al. also found CFU expansion

when using collagen beads, but at higher total cell expansion ratios due to the use of cord blood cells and serum-containing medium [11].

**Table 3. Number of colony-forming units (CFUs) at day 4 among adhering and non-adhering cells in cultures using heparinized crosslinked collagen substrates pre-adsorbed with SDF-1α. At seeding, the cell population contained about 4400 CFUs, of which 43% were erythroid (either BFU-E or CFU-E).**

| | CFU-GEMM | BFU -E | CFU-E | CFU-G/M/GM | % Erythroid |
|---|---|---|---|---|---|
| Heparinized collagen beads | 0 | 7176 | 1053 | 3520 | 72% |
| Heparinized collagen films | 114 | 16120 | 3720 | 7520 | 73% |

**Table 4. Number of colony-forming units (CFUs) at day 14 among adhering and non-adhering cells in cultures using heparinized crosslinked collagen substrates pre-adsorbed with SDF-1α. At seeding, the cell population contained about 4400 CFUs, of which 43% were erythroid (either BFU-E or CFU-E).**

| | CFU-GEMM | BFU -E | CFU-E | CFU-G/M/GM | % Erythroid |
|---|---|---|---|---|---|
| Heparinized collagen beads | 0 | 9506 | 625 | 6520 | 61% |
| Heparinized collagen films | 0 | 1452 | 17428 | 2880 | 87% |

CFU analysis indicated that at the maximal $CD34^+$ expansion at d 4 using the beads, the progenitor cells were mainly erythroid as was also found for the cultures using the films (table 3). After 2 wks of culture using the beads, the expanded cells were differentiated into both erythroid and granulocyte/macrophage lineages (61% erythroid), while the CFUs in the cultures with the films were mainly differentiated into the erythroid lineage (87 % as shown in table 4).

## 4. Conclusions

The use of a packed bed of smooth heparinized crosslinked collagen beads as three-dimensional substrate for $CD34^+$ cell culture is presented. The beads were made from materials that mimic the compounds found in the bone marrow.

Collagen beads prepared from type I soluble collagen were crosslinked and heparinized using EDC/NHS chemistry. Heparin was immobilized to an extent of 19 ± 3 μg per mg of beads. At an SDF-1α concentration of 1.11 μg/ml, the adsorption of SDF-1α was 77 ± 9 ng SDF-1α per mg of heparinized beads. During 2 wks 70 % of the chemokine was released when employing half medium changes twice a wk.

$CD34^+$ cells obtained from peripheral blood were cultured serum-free in a packed bed of heparinized crosslinked collagen beads and on films, both pre-adsorbed with SDF-1α. Using the described culture conditions, the cultures with beads gave a 3-fold expansion of the $CD34^+$ population, in contrast to the cultures using films where the

$CD34^+$ population was not expanded. Total viable cell proliferation in the cultures using beads was similar tot that of the cultures using films. CFU differentiation appears to proceed slower in the cultures using beads and with slightly less preference for the erythroid lineage. Pre-adsorption of the heparinized crosslinked collagen beads with SDF-1α resulted in a better retention of erythroid CFUs adhering to the beads during cell culture.

A packed bed of heparinized crosslinked collagen beads can be used as culture substrate for HPC expansion. The use of a packed bed of beads results in a large surface area per volume of culture medium, which is favourable for large-scale hematopoiesis in a bioreactor system. Due to the small number of adhering cells as percentage of the total number of cells in culture, such a system will require a barrier to retain the non-adhering cells in the system.

## 5. Acknowledgements

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## 6. References

1. Zhang, J., Niu, C., Ye, L., Huang, H.Y., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., Harris, S., Wiedemann, L.M., Mishina, Y. and Li, L.H., *Identification of the haematopoietic stem cell niche and control of the niche size.* Nature, 2003;425:836-841.
2. Fuchs, E., Tumbar, T., and Guasch, G., *Socializing with the neighbors: Stem Cells and Their Niches.* Cell 2004;116:769-778.
3. Wang, T.Y. and Wu, J.H.D., *A Continuous Perfusion Bioreactor For Long-Term Bone-Marrow Culture.* Ann N Y Acad Sci 1992;665:274-284.
4. Wang, T.Y., Brennan, J.K., and Wu, J.H.D., *Multilineal Hematopoiesis in a 3-Dimensional Murine Long-Term Bone-Marrow Culture.* Exp Hematol 1995;23:26-32.
5. Mantalaris, A., Keng, P., Bourne, P., Chang, A.Y.C. and Wu, J.H.D., *Engineering a human bone marrow model: A case study on ex vivo erythropoiesis.* Biotechnol Prog 1998;14:126-133.

6. Bagley, J., Rosenzwieg, M., Marks, D.F. and Pykett, M.J., *Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device.* Exp Hematol 1999;27:496-504.
7. Meissner, P., Schroder, B., Herfurth, C. and Biselli, M., *Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells.* Cytotechnology 1999;30:227-234.
8. Tomimori, Y., Takagi, M., and Yoshida, T., *The construction of an in vitro three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers.* Cytotechnology 2000;34:121-130.
9. Banu, N., Rosenzwieg, M., Kim, H., Bagley, J. and Pykett, M., *Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix.* Cytokine 2001;13:349-358.
10. Sasaki, T., Takagi, M., Soma, T. and Yoshida, T., *3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics.* Cytotherapy 2002;4:285-291.
11. Kim, H.S., Lim, J.B., Min, Y.H., Lee, S.T., Lyu, C.J., Kim, E.S. and Kim, H.O., *Ex Vivo Expansion of Human Umbilical Cord Blood CD34+ Cells in a Collagen Bead-Containing 3 Dimensional Culture System.* Int J Hematol 2003;78:126-132.
12. Xiong, F., Chen, Z., Liu, H., Xu, Z. and Liu, X., *Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system.* Biotechnol Lett 2002;24:1421-1426.
13. Li, Y., Ma, T., Kniss, D. A., Yang, S. T. and Lasky, L. C., *Human cord cell hematopoiesis in three-dimensional nonwoven fibrous matrices: In vitro simulation of the marrow microenvironment*. J Hematot Stem Cell Res 2001;10:355-368.
14. Wissink, M.J.B., Beernink, R., Pieper, J.S., Poot, A.A., Engbers, G.H.M., Beugeling, T., Van Aken, W.G. and Feijen, J., *Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation.* Biomaterials 2001;22:151-163.
15. Gupta, P., McCarthy, J.B., and Verfaillie, C.M., *Stromal fibroblast heparan sulfate is required for cytokine- mediated ex vivo maintenance of human long-term culture- initiating cells.* Blood 1996;87:3229-3236.
16. Gupta, P., Oegema, T.R., Brazil, J.J., Dudek, A.Z., Slungaard, A. and Verfaillie, C.M., *Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche.* Blood 1998;92:4641-4651.
17. Gupta, P., Oegema, T.R., Brazil, J.J., Dudek, A.Z., Slungaard, A. and Verfaillie, C.M., *Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O- sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins.* Blood 2000;95:147-155.
18. Gordon, M.Y., Riley, G.P., Watt, S.M. and Greaves, M.F., *Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment.* Nature 1987;326403-405.
19. Roberts, R., Gallagher, J., Spooncer, E., Allen, T.D., Bloomfield, F. and Dexter, T.M., *Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis.* Nature 1988;332:376-378.
20. Tanaka, Y., Kimata, K., Adams, D.H. and Eto, S., *Modulation of Cytokine Function by Heparan Sulfate Proteoglycans: Sophisticated Models for the Regulation of Cellular Responses to Cytokines.* Proc Assoc Am Physicians 1998;10:118-125.
21. Siebum B., Grijpma D.W., Poot A.A., Vermes I., Feijen J., *Heparinized crosslinked collagen films for hematopoietic stem cell culture.* Chapter 3 of this thesis.
22. Sadir R, Baleux F, Grosdidier A, Imberty A, Lortat-Jacob H. Characterization of the stromal cell-derived factor-l alpha - heparin complex. J Biol Chem 2001;276:8288-8296.

23. Netelenbos, T., Van den Born, J., Kessler, F.L., Zweegman, S., Merle, P.A., Van Oostveen, J.W., Zwaginga, J.J., Huijgens, P.C. and Drager, A.M., *Proteoglycans on bone marrow endothelial cells bind and present SDF-1 towards hematopoietic progenitor cells.* Leukemia 2003;17:175-184.
24. Taichman, R.S., *Blood and bone: two tissues whose fates are intertwined to create the hematopoietic stem-cell niche.* Blood 2005;105:2631-2639.
25. Majka, M., Ratajczak, J., Lee, B., Honczarenko, M., Douglas, R., Kowalska, M.A., Silberstein, L., Gewirtz, A.M. and Ratajczak, M.Z., *The role of HIV-related chemokine receptors and chemokines in human erythropoiesis in vitro.* Stem Cells 2000;18:128-138.
26. Siebum B., Grijpma D.W., Poot A.A., Vermes I., Kruijer W., Feijen J., *Heparinized crosslinked collagen films pre-adsorbed with SDF-1α for hematopoietic stem cell culture.* Chapter 4 of this thesis.
27. Hsu, F. Y., Chueh, S. C. and Wang, Y. J. *Microspheres of hydroxyapatite/reconstituted collagen as supports for osteoblast cell growth*. Biomaterials 1999;20:1931-1936.
28. Loke, W.K. and Khor, E., *Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valva application by differential scanning calorimetry.* Biomaterials 1995;16:251-258.
29. Wang, C.L., Miyata, T., Wecksler, B., Rubin, A.L. and Stenzel, K.H., *Collagen induced platelet aggregation and release. I. Effects of side-chain modifications and role of argynyl residues.* Biochim. Biophys. Acta 1978;544:555-567.
30. Wissink, M.J.B., Beernink, R., Pieper, J.S., Poot, A.A., Engbers, G.H.M., Beugeling, T., Van Aken, W.G. and Feijen, J., *Binding and release of basic fibroblast growth factor from heparinized collagen matrices.* Biomaterials 2001;22:2291-2299.
31. Majka, M., Ratajczak, J., Lee, B., Honczarenko, M., Douglas, R., Kowalska, M.A., Silberstein, L., Gewirtz, A.M. and Ratajczak, M.Z., *The Role of HIV-Related Chemokine Receptors and Chemokines in Human Erythroipoiesis in Vitro*. Stem Cells 2000;18:128-138.
32. Patel, S.D., Miller, W.M., Winter, J.N. and Papoutsakis, E.T., *Cell density-dependent proliferation in frequently-fed peripheral blood mononuclear cell cultures*. Cytotherapy 2000;2:267-280.
33. Glimm, H., Tang, P., Clark-Lewis, I., Von Kalle, C. and Eaves C., *Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice*. Blood 2002;99:3454-3457.

# Chapter 7

## A bioreactor containing a packed bed of heparinized crosslinked collagen beads for the expansion and erythroid differentiation of $CD34^+$ progenitor cells

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**Abstract**

A bioreactor-based system to expand and differentiate erythroid progenitors is of great clinical interest. Previously, we reported on the expansion of $CD34^+$ cells in a packed bed of heparinized crosslinked collagen beads pre-adsorbed with SDF-1α. In the current study, packed beds of beads were used in a bioreactor system for the expansion and erythroid differentiation of $CD34^+$ progenitor cells.

Human peripheral blood derived $CD34^+$ cells were cultured in a membrane-enclosed packed bed of SDF-1α pre-adsorbed beads in a bioreactor in which 50 ml of serum-free medium containing IL-3, Flt3/Flk2-ligand, TPO and SCF was perfused around the membrane compartment. A 4-fold total cell expansion was obtained after 2 wks, with 50 % of the cells expressing CD36 and none of the cells expressing glycophorin A. During the first wk, $CD34^+$ progenitor cells and CFUs expanded 1.6- and 9-fold, respectively. Of the expanded CFUs 80-90 % were erythroid.

$CD34^+$ cells were also cultured with the same medium in membrane-enclosed packed beds of heparinized crosslinked collagen beads placed in the wells of a culture plate, with half medium refreshments twice a week, resulting in a 2-fold total cell expansion and a 6-fold CFU expansion after 2 wks, without $CD34^+$ cell expansion. The enhanced HPC expansion obtained in the bioreactor can be explained by a better maintenance of the culture medium composition (notably the pH) inside the PVDF membrane compartment as compared to the wells of a culture plate.

Differentiation of $CD34^+$ cells in the bioreactor system with modified serum-free culture medium containing EPO and dexamethasone resulted in a 3.6-fold total cell expansion and a 16-fold CFU expansion in 2 wks. The CFUs were mainly late erythroid progenitors. About 70 % of the expanded cells expressed CD36 and 50 % expressed glycophorin A, indicating differentiation into erythroid precursor cells.
These results show that serum- and stroma-free culturing of $CD34^+$ cells in a membrane-enclosed packed bed of heparinized crosslinked collagen beads is feasible. The bioreactor allows the sequential use of culture media and can be modified for large-scale cell culture. Moreover, this system provides a model to study the role of extra-cellular matrix components and molecules like cytokines in the hematopoietic process.

## 1. Introduction

Erythropoiesis comprises the process by which Hematopoietic Stem Cells (HSCs) differentiate through different stages of Hematopoietic Progenitor Cells (HPCs) into precursor cells and finally mature red blood cells. In adults, erythropoiesis results in the daily production of $2 \cdot 10^{11}$ red blood cells. Cells produced during large scale *in vitro* erythropoiesis might be used for transfusion applications. Additionally, the *in vitro* expansion and controlled differentiation of HPCs is of great interest for application in bone marrow transplantation.
There are several reports claiming successful erythropoiesis [1-3] and HPC expansion [4-8] in a bioreactor containing three-dimensional culture substrates. It is expected that the expansion of HPCs can be improved by culturing HPCs in a bioreactor system in which the bone marrow environment is mimicked. Furthermore, the mechanisms of hematopoiesis and processes such as stem cell mobilization and homing can be investigated using such bioreactors. Collagen type I is an important component of the bone marrow extracellular matrix (ECM). This protein can be processed into structures like beads, crosslinked and subsequently used for HPC culture [9, 10]. Additionally, crosslinked collagen can be modified with compounds present in the microenvironments of the bone marrow [11]. This can be done with glycosaminoglycans (GAGs), since these are involved in keeping the ECM in a hydrated state and play an important role in chemokine function [12-17].

Previously, we presented the results of the serum-free culturing of $CD34^+$ HPCs on heparinized crosslinked collagen films and beads. The use of heparinized crosslinked collagen films with and without pre-adsorbed SDF-1α as substrate for $CD34^+$ cell culture resulted in a 5-fold total cell expansion after 2 wks, no expansion of $CD34^+$ cells and a 6-fold expansion of colony-forming units (CFUs), mainly into the erythroid lineage [18]. SDF-1α was pre-adsorbed onto the films to increase cell adhesion. Upon cell seeding this resulted in a higher number of cells adhering onto heparinized films pre-adsorbed with SDF-1α than on heparinized films that were not pre-adsorbed with this chemokine [19]. When heparinized crosslinked collagen beads were used as cell culture substrate instead of heparinized crosslinked collagen films, a 3-fold $CD34^+$ cell expansion was obtained [20].

In this study, we compare the use of membrane-enclosed packed beds of heparinized crosslinked collagen beads as cell culture substrate in a bioreactor system with culture medium perfusion around the membrane, with the use of membrane-enclosed packed beds of these beads in a culture plate employing half medium refreshments twice a wk. This comparison is done for both HPC expansion and the differentiation of HPCs into erythroid precursor cells.

## 2. Materials and methods

### *2.1. Materials*

Unless otherwise indicated, all chemicals and biochemicals were obtained from Sigma-Aldrich (St. Louis, USA). Solvents were of analytical grade (Biosolve, Valkenswaard, The Netherlands).

### *2.2. Heparinized collagen bead preparation*

Solid collagen beads were prepared from type I soluble collagen (Elastin Products Co. Inc., Missouri, USA) and subsequently crosslinked and heparinized using N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC; Fluka, Buchs, Switzerland) and N-hydroxysuccinimide (NHS) as previously described [20].

### *2.3. Characterization of materials*

The shrinkage temperature ($T_s$) of (crosslinked) collagen was determined using Differential Scanning Calorimetry (DSC) as described before [18]. This temperature,

at which the collagen sample undergoes thermal denaturation, is used as a measure of the crosslink density [21].

The residual number of free primary amino groups in (crosslinked) collagen was determined spectrophotometrically after reacting with 2,4,6-trinitrobenzenesulfonic acid (TNBS; Fluka) [22].

The heparin content of the samples was determined by a colorimetric method based on the binding of toluidine blue [18].

### *2.4. Hematopoietic cell culture*

Circular films (d = 15 mm) were cut out of regenerated cellulose and polyvinylidene fluoride (PVDF) dialysis membranes (MWCO 25 and 1000 kDA, respectively; both Spectrum Laboratories Inc., Rancho Dominguez, USA). These films were used to investigate the suitability of the materials for enclosing the cells. Enclosure is necessary as during previous serum-free culturing of $CD34^+$ cells on SDF-1α pre-adsorbed beads under similar circumstances the number of cells adhering to the beads was low and even after pre-adsorption of SDF-1α on the beads never exceeded 20-30 % of the cells available [20]. Dialysis membranes have been used before to retain hematopoietic cells in a bioreactor [1-3]. The circular films were washed with 70 % ethanol, fixed with Viton O rings in the wells of a 24-well cell culture plate (Corning, NY, USA) and incubated overnight at 37 ˚C in a solution of penicillin (200 U/ml) and streptomycin (200 μg/ml; both from Gibco BRL, Paisley, UK).

Human $CD34^+$ cells isolated using immuno-magnetic beads (MACS, Miltenyi Biotech, Auburn, USA) from G-CSF mobilized peripheral blood were obtained from the Medical Spectrum Twente Hospital. The procedures followed were in accordance with the policies of the Institutional Ethical Review Board of the Hospital Group (ECOM). The cryo-preserved cells were thawed and subsequently cultured in serum-free medium consisting of IMDM (with GlutaMAX I), 1 (wt)% BSA, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 10 μg/ml recombinant human insulin, 200 μg/ml human partially iron-saturated holo-transferrin, 20 ng/ml TPO, 10 ng/ml IL-3, 100 ng/ml SCF, 50 ng/ml Flt3/Flk2-ligand (medium 1). All added cytokines were from recombinant human origin (R&D systems, Minneapolis, USA). The cells were seeded by adding 0.5 ml of a cell suspension with a density of $1 \cdot 10^5$ cell/ml onto the films and cultured for 1 wk at 37˚C in a humidified 5 % $CO_2$ atmosphere. Half of the medium was exchanged at d 4.

The cell cultures in membrane-enclosed packed beds of beads were performed in tubes made from PVDF dialysis membrane (MWCO 1000 kDa). The tubes were washed with 70 % ethanol and incubated overnight at 37 ˚C in a solution of penicillin (200 U/ml) and streptomycin (200 µg/ml), rinsed with PBS and incubated once more overnight at 37˚C with PBS containing 1 (wt)% BSA (66 kDa) and 200 µg/ml human partially iron-saturated holo-transferrin (80 kDa). Before use, the tubes were rinsed with PBS.

Heparinized crosslinked collagen beads were rinsed with 70 % ethanol and incubated overnight at 37˚C in a solution of penicillin (200 U/ml) and streptomycin (200 µg/ml). Before cell seeding, the beads were incubated once more overnight at room temperature in a PBS solution containing 0.1 (wt)% BSA with 1,11 µg/ml SDF-1α [20]. The beads were subsequently rinsed with PBS and IMDM (with Glutamax I, Gibco) before 0.25 ml of beads in medium (ca. 4 mg of beads, with $d < 500$ µm) was transferred into the PVDF tubes. The loosely packed bed of heparinized crosslinked collagen beads inside the PVDF tubes had an approximate volume of 0.19 $cm^3$, a surface area of 7.4 $cm^2$ and a porosity of about 70 % (calculated from the number of beads per ml and the average volume of the beads).

The serum-free culture medium used for expanding progenitor cells in membrane-enclosed packed beds of beads was the same as described above for the culturing on films (medium 1). For erythroid differentiation, the serum-free medium consisted of IMDM (with GlutaMAX I), 1 (wt)% BSA, 10 µg/ml insulin, 120 µg/ml human partially iron-saturated holo-transferrin, 90 ng/ml $Fe(NO_3)_3$, 900 ng/ml $FeSO_4$, $10^{-6}$ M dexamethasone, 5 ng/ml IL-3, 100 ng/ml SCF and 3 IU/ml Epo (medium 2) [23].

Cells were seeded by adding 0.25 ml of a cell suspension with a density of $2 \cdot 10^5$ cells/ml to 0.25 ml of beads present inside the PVDF tubes. The cells were cultured at 37 ˚C in a humidified 5 % $CO_2$ atmosphere. The tubes containing the cell-seeded beads were placed either in: A) a bioreactor unit (Minucells and Minutissue Vertriebs GmbH, Germany) or B) the wells of a 24-well tissue culture plate (Corning, NY, USA), as illustrated in figure 1.

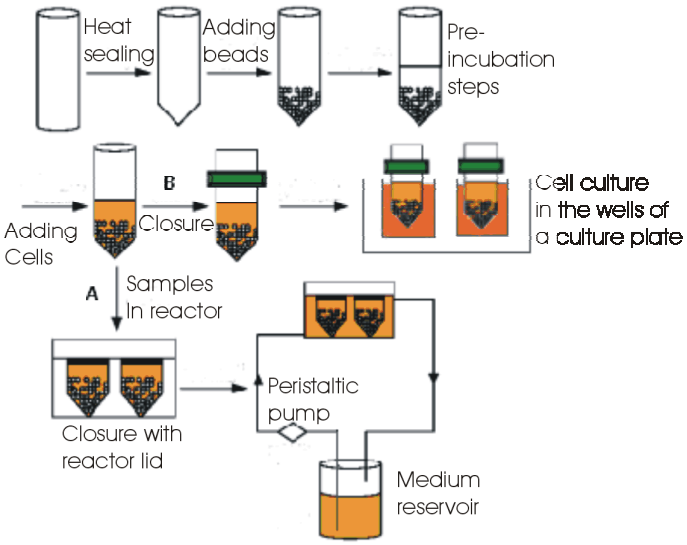


**Figure 1. Schematic representation of A) the bioreactor system containing packed beds of beads enclosed in PVDF membranes and B) the culture in PVDF-enclosed packed beds of beads in a culture plate.**

The tubes with cell-seeded beads placed in a culture plate were immersed in 0.5 ml of culture medium. Half of the culture medium in the culture plates was exchanged twice a wk. Two tubes with cell-seeded beads were placed in the bioreactor, one sample for each time point during cell culture. The bioreactor contained 50 ml of culture medium, which was circulated around the tubes with cell-seeded beads with a flow rate of 0.05 ml per min. The culture medium in the bioreactor was refreshed after 1 wk.

### *2.5. Characterization of hematopoietic cells*

During cell culture using the films of the dialysis membranes and membrane enclosed beads 2 cell fractions (adherent and non-adherent cells) were obtained. The cells that did not adhere to the films were removed by pipetting. The adhering cells were obtained after rinsing the films with PBS containing 1 (wt)% BSA and subsequent incubation in Cell Dissociating Solution (CDS) for 20 min at room temperature.

Non-adherent cells and beads with adhering cells were first separated by filtration (MACS pre-separation filters, mesh size 30 µm, Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After rinsing the beads with PBS containing 1 (wt)% BSA and subsequent incubation in Cell Dissociation Solution (CDS), for 20 min at room temperature, the fraction of adhering cells was obtained. Cells dissociated from the beads were separated from the beads by filtration as described above.

Flowcytometric phenotyping was performed using anti-CD45-FITC, anti-CD34-PE, anti-CD34-ECD, anti-CD36-FITC, anti-CD235A-PE and 7AAD vitality dye (all from Immunotech, Marseille, France). Isotype-identical antibodies were used as control and for quantification Flow-Count beads were added to the samples. Cells were counted and analyzed using a Cytomics FC500 flow cytometry system (Beckman Coulter, Fullerton, USA). Moreover, differentiation of the cells was evaluated by Colony-Forming Unit (CFU) assays. For CFU analysis, samples containing $10^3$ cells were taken from the HPC culture, subsequently cultured in MethoCult (StemCell Technologies, Meylan, France) and scored after 2 wks. The number of CFUs adhering to the beads as well as the number of non-adherent CFUs were determined. For quantification of the adherent CFUs, the cells were first removed from the films using CDS.

### *2.6 Statistical analysis*

Data represent mean ± standard error of the mean (S.E.M.) of 3 experiments. Statistical analyses were performed using an unpaired two-tailed t-test. Results were considered significantly different at p values $< 0.05$.

## 3. Results and discussion

### *3.1 Cell adhesion and retention*

Collagen beads were prepared as previously reported [20]. Crosslinking of the beads increased the shrinkage temperature from 54 ˚C to 76 ˚C and decreased the number of primary amino groups per 1000 amino acid residues from 27 to 17. After immobilization of heparin onto the crosslinked beads, the beads contained 19 ± 3 μg heparin per mg of crosslinked collagen, as determined by the toluidine blue assay. These results are in agreement with previously reported data [11, 20].

$CD34^+$ cell culture using films cut out of PVDF membranes with a MWCO of 1000 kDa as culture substrate showed that no cells adhered to this membrane. Furthermore, the total cell expansion obtained when using PVDF films as culture substrate (data not shown) was comparable to the expansion obtained when using heparinized crosslinked collagen films as culture substrate [18]. Films of regenerated cellulose membranes gave a similar cell expansion, but with a fraction of the cells adhering to

the membrane (data not shown). Therefore, PVDF membranes were used to enclose the beads and cells for subsequent use in the bioreactor and culture plate.

### *3.2. Progenitor cell expansion*

The total cell expansion (adhering and non-adhering cells) obtained during 2 wks of $CD34^+$ cell culture with medium 1 in PVDF-enclosed packed beds of SDF-1α pre-adsorbed heparinized crosslinked collagen beads placed in the wells of a culture plate was 2-fold, whereas cultures with the same beads without the PVDF membrane gave a 10-fold expansion [20]. The total viable cell expansion obtained after 2 wks of culturing $CD34^+$ cells with medium 1 in PVDF-enclosed packed beds of heparinized crosslinked collagen beads pre-adsorbed with SDF-1α was not significantly improved when the PVDF-enclosed packed beds were used in the bioreactor instead of in the wells of a culture plate (figure 2).

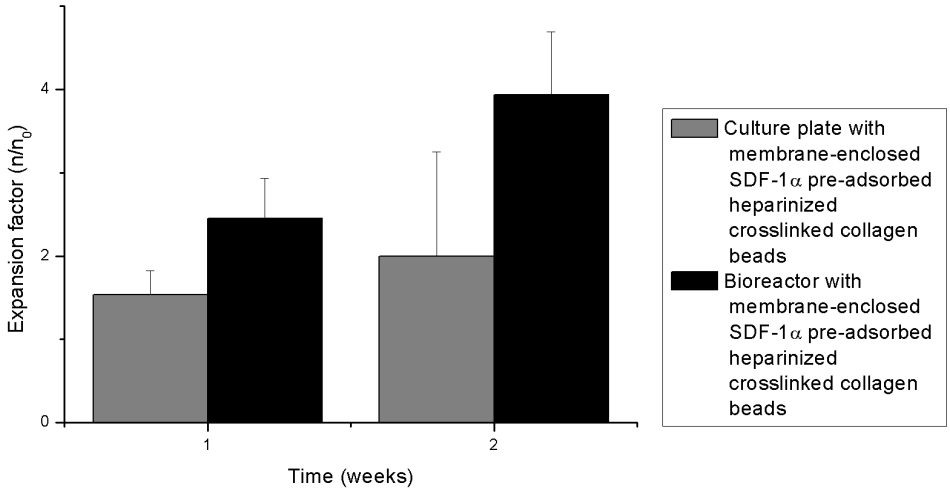


**Figure 2. Total viable cell expansion (the sum of adherent and non-adherent cells ($7AAD^-$ and $CD45^+$)), during cell culture in packed beds of beads with culture medium for HPC expansion (medium 1), determined from three independent experiments ( ± S.E.M.).**

The 1.6-fold expansion of $CD34^+$ cells after 1 wk in PVDF-enclosed packed beds of heparinized crosslinked collagen beads, placed in a bioreactor, was significantly higher than the expansion obtained using the same PVDF-enclosed beads placed in the wells of a culture plate (figure 3).

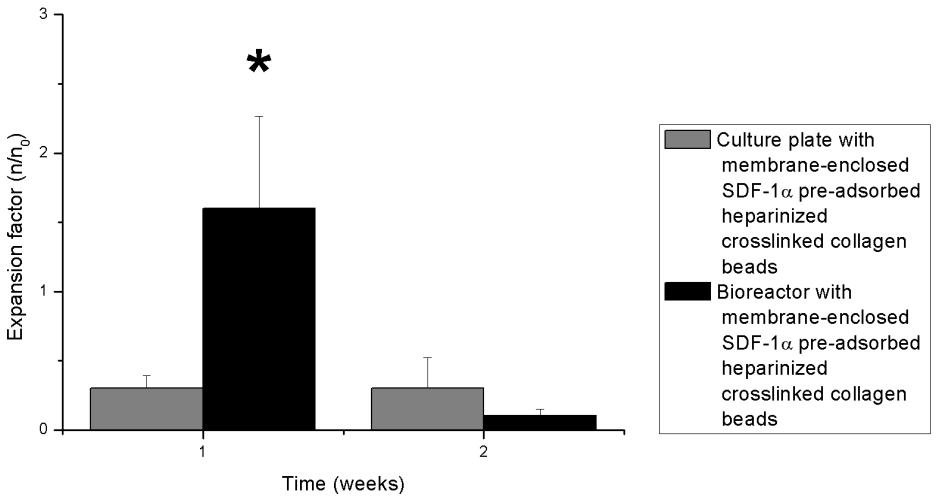


**Figure 3. Expansion of CD34$^+$ cells (the sum of adherent and non-adherent cells (7AAD$^-$, CD45$^+$ and CD34$^+$)), during cell culture in packed beds of beads with culture medium for HPC expansion (medium 1), determined from three independent experiments ( ± S.E.M.). * Indicates a significant difference compared to the culture in PVDF-enclosed packed beds of beads performed in a culture plate.**

Whereas CD34$^+$ cells did not expand using PVDF enclosed beads placed in the wells of a culture plate, previous cultures under similar conditions but without the PVDF membrane led to a 3-fold CD34$^+$ cell expansion [20]. The results described above show that the presence of the PVDF membrane during cell culture leads to inferior results compared to cultures without the enclosing membrane. This may have been caused by the limited diffusion of oxygen, cytokines, nutrients and metabolites through the PVDF membrane. It has been reported that a low oxygen concentration improves HPC expansion [24]. On the other hand, a low pH due to accumulation of metabolites like lactate inhibits proliferation [25]. During culture in the wells of the culture plate, the color of the medium inside the PVDF compartment turned yellow, indicating a relatively low pH.

The higher expansion rate obtained during cell culture in membrane-enclosed packed beds of beads used in the bioreactor was probably caused by improved diffusion of essential compounds through the membrane. The 4-fold total viable cell expansion obtained in the bioreactor was higher than the 2 to 3-fold expansion reported for a bioreactor system containing membrane-enclosed collagen beads seeded with bone marrow mononuclear cells [1-3].

After 2 wks about 9-17 % of the cells adhered to the beads inside the PVDF compartment when used in a culture plate. During cell culture the density of cells

adhering to the SDF-1α pre-adsorbed heparinized crosslinked collagen beads remained stable at approximately 1500 cells/cm$^2$, comparable to the densities previously found on beads not enclosed in a PVDF membrane [20]. The number of CD34$^+$ cells and CFUs among the cells adhering to the SDF-1α pre-adsorbed heparinized beads was not influenced by the presence of the PVDF membrane (data not shown).

In the bioreactor system the number of cells adhering onto the beads enclosed by the PVDF membrane and the number of CD34$^+$ cells among these cells was comparable as when the membrane-enclosed beads were used in a culture plate [20]. In the bioreactor, the number of erythroid CFUs, as percentage of the total number of adhering CFUs, was 85 % after 1 wk, but only 51 % after 2 wks. The relatively low number of erythroid progenitors among the CFUs adhering to the SDF-1α pre-adsorbed beads in the bioreactor system could have been caused by the large volume of medium that was circulated around the packed bed, enhancing the release of SDF-1α from the beads, thereby reducing the effect of this chemokine on the adhesion of erythroid CFUs. When packed beds of heparinized beads pre-adsorbed with SDF-1α were used as culture substrate in a culture plate without PVDF membrane, the amount of erythroid CFUs remaining among the adhering CFUs after 2 wks decreased to 67 %, as compared to 33 % when the beads were not pre-adsorbed with SDF-1α [20]. This shows that the number of erythroid CFUs that adhere to the heparinized beads during cell culture is influenced by the amount of SDF-1α adsorbed to the beads as well as the resulting concentration in the culture medium.

The CFU expansion in packed beds of beads used in culture plates was not affected by the presence of PVDF membranes. During 2 wks of culturing CD34$^+$ cells in packed beds of beads in culture plates, the numbers of non-adhering CFUs in the membrane-enclosed packed beds (tables 1 and 2) were similar to the numbers of CFUs in packed beds in culture plates without PVDF membrane [20]. When PVDF-enclosed packed beds of beads were used in the bioreactor system instead of in culture plates, the expansion of non-adhering CFUs was 3-fold higher after 1 wk (table 1). After 2 wks the number of non-adhering CFUs among the cells were lower in the bioreactor than in the culture plate (table 2).

**Table 1. Number of colony-forming units (CFUs) after 1 wk among the non-adhering cells in cultures in a PVDF-enclosed packed bed of heparinized crosslinked collagen beads, when using culture medium for HPC expansion (medium 1). The number of erythroid colonies (BFU-E and CFU-E) as percentage of the total number of CFUs is shown between brackets. At seeding, the cell population contained about 4400 CFUs, of which 43% were erythroid (either BFU-E or CFU-E).**

| | Erythroid CFUs | G/M/GM CFUs |
|---|---|---|
| Culture plate | 9618 (83%) | 1986 |
| Bioreactor system | 34008 (88%) | 4668 |

**Table 2. Number of colony-forming units (CFUs) after 2 wks among the non-adhering cells in cultures in a PVDF-enclosed packed bed of heparinized crosslinked collagen beads, when using culture medium for HPC expansion (medium 1). The number of erythroid colonies (BFU-E and CFU-E) as percentage of the total number of CFUs is shown between brackets. At seeding, the cell population contained about 4400 CFUs, of which 43% were erythroid (either BFU-E or CFU-E).**

| | Erythroid CFUs | G/M/GM CFUs |
|---|---|---|
| Culture plate | 14379 (83%) | 3006 |
| Bioreactor system | 11932 (81%) | 2725 |

During 2 wks of cell expansion in culture plates, the number of erythroid progenitors (CFU-E and BFU-E) as percentage of the total number of CFUs in PVDF-enclosed packed beds of beads was 83 % (tables 1 and 2). Compared to previous cell cultures using these packed beds of beads in culture plates without PVDF membrane [20], the number of erythroid progenitors as percentage of the total number of non-adhering CFUs was 10 - 20 % higher. This could have been caused by a lower oxygen concentration inside the PVDF bag, as compared to culture medium without PVDF enclosure, which improves erythroid CFU expansion [24]. However, the lower pH inside the PVDF compartment, as indicated by yellow coloured culture medium, could have reduced this effect [25, 26]. In the bioreactor, the relatively large volume of medium flowing around the membrane-enclosed packed beds prevented a large pH change, as indicated by the colour of the culture medium, resulting in a high transient erythroid CFU expansion.

The number of CD36$^+$ cells as percentage of the total cell population increased during wk 1 of cell culture, both in the membrane-enclosed packed beds of beads used in a culture plate and in the bioreactor (figure 4). In the bioreactor system the percentage of CD36$^+$ cells among the cells in culture remained constant during wk 2, while it decreased when the culture was performed in a culture plate. In both cases glycophorin A was not significantly expressed by the expanded cells. Thus, CD36$^+$ erythroid progenitors in a membrane-enclosed packed bed of beads show improved

proliferation and survival in the bioreactor system as compared to the wells of a culture plate.

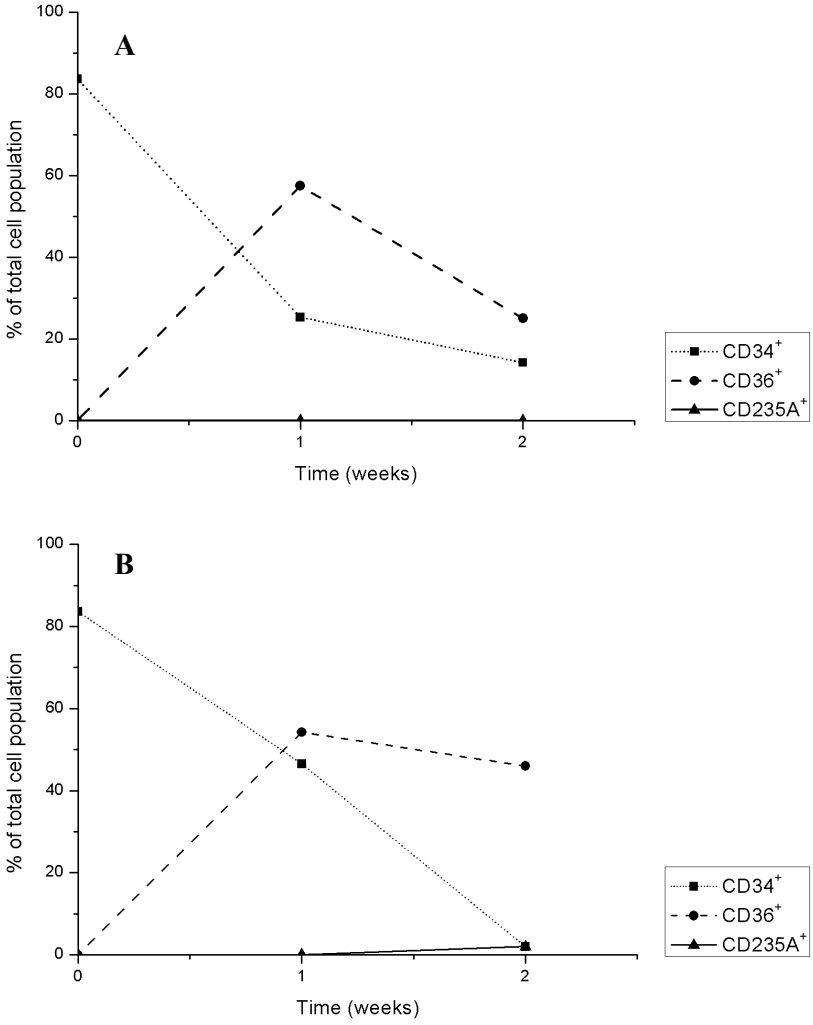


**Figure 4. Number of cells expressing HPC and erythroid antigens as percentage of the total cell population during 2 wks of cell culture with medium for HPC expansion (medium 1). The PVDF-enclosed cell cultures were performed in culture plates (A) and in the bioreactor (B).**

In short, the circulation of medium in the bioreactor system compensated for the reduction in total and $CD34^+$ cell expansion caused by the membrane required to retain the cells in the reactor. Moreover, the bioreactor culture in a membrane-enclosed packed bed of beads resulted in a large expansion of erythroid CFUs. The increased number of cells expressing CD36 and the absence of glycophorin A (CD235A) expression indicate that the expanded erythroid progenitors did not differentiate further into erythroblasts or more mature erythroid precursor cells.

### *3.3. Erythroid differentiation*

Additionally, CD34$^+$ cells were cultured serum-free with medium containing Epo and dexamethasone (medium 2) in PVDF-enclosed packed beds of SDF-1α pre-adsorbed heparinized crosslinked collagen beads, using either a culture plate with half medium refreshments twice a week or a bioreactor system with medium perfusion around the membrane-enclosed packed beds. The total viable cell expansion after 2 wks of cell culture was higher in the bioreactor as compared to the culture plate (figure 5). CD34$^+$ cells did not expand using this medium (figure 6).

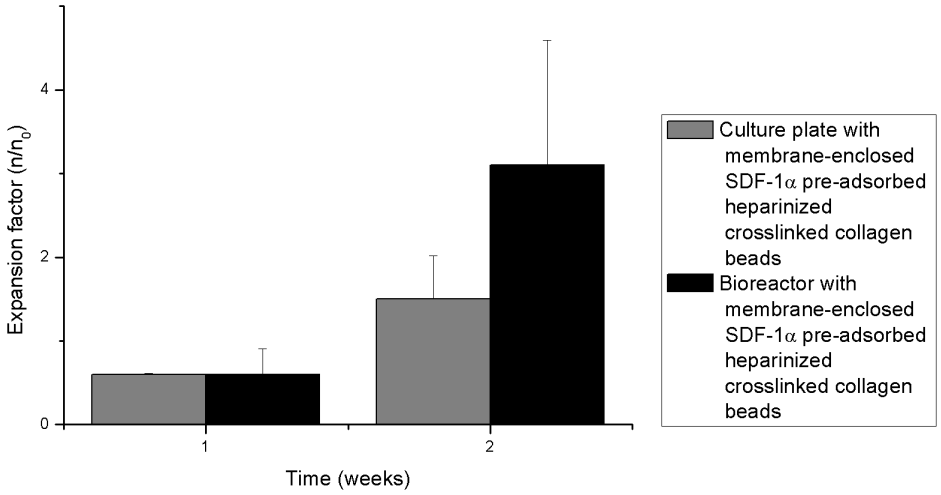


**Figure 5. Total viable cell expansion (the sum of adherent and non-adherent cells (7AAD$^-$ and CD45$^+$)), during cell culture in packed beds of beads with culture medium containing Epo and dexamethasone (medium 2), determined from three independent experiments ( ± S.E.M.).**

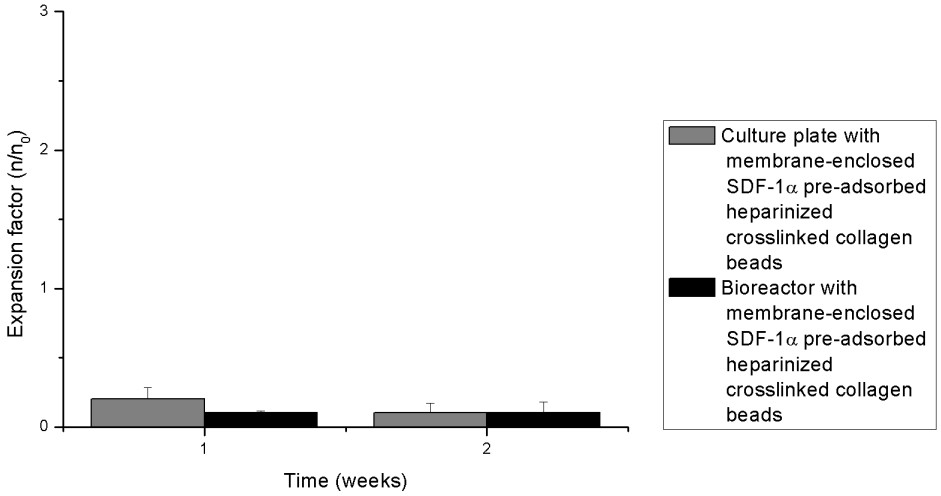


**Figure 6. Expansion of CD34$^+$ cells (the sum of adherent and non-adherent cells (7AAD$^-$, CD45$^+$ and CD34$^+$)), during cell culture in packed beds of beads with culture medium containing Epo and dexamethasone (medium 2), determined from three independent experiments ( ± S.E.M.).**

The absence of $CD34^+$ expansion using medium 2 was expected, since with this medium HPCs should differentiate instead of expand. The total cell expansion was lower than that obtained during HPC expansion with medium 1, as described above. The lower total cell expansion obtained with erythroid differentiation medium, as compared to HPC expansion medium, was also found for cultures on TCPS culture plates without beads (data not shown). The expansions during erythroid differentiation reported in literature are higher than the expansions obtained here. However, in those studies either $CD36^+$ enriched cells [27] or co-cultures on stroma [23] were used.

Cells adhered to the beads during HPC culture with medium containing Epo and dexamethasone. As before, the percentage of adhering cells never exceeded 30 % [20]. The phenotype of the adhering cells was similar to that of the non-adhering cells and the low numbers of CFUs that adhered were from the same lineages as the non-adherent CFUs (data not shown).

Total number of non-adhering CFUs in the packed beds was higher in cultures performed in the bioreactor system than in culture plates (table 3). An average 16-fold expansion was obtained after 2 wks of culturing in the bioreactor. The majority of CFUs in the packed beds was erythroid, both in the bioreactor and in the culture plates. The highest number of erythroid CFUs was found in the bioreactor system, where 90 % of the CFUs were erythroid after 2 wks of culturing. Most of the CFUs expanded in the bioreactor system were later stage CFU-E.

**Table 3. Number of colony-forming units (CFUs) among the non-adhering cells in cultures in a PVDF-enclosed packed bed of heparinized crosslinked collagen beads, when using the culture medium for erythroid differentiation (medium 2). The number of erythroid colonies (BFU-E and CFU-E) as percentage of the total number of CFUs is shown between brackets. At seeding, the cell population contained about 4400 CFUs, of which 43% were erythroid (either BFU-E or CFU-E).**

| | | Erythroid CFUs | G/M/GM CFUs |
|---|---|---|---|
| Week 1 | Culture plate | 7029 (83%) | 1446 |
| | Bioreactor system | 6419 (89%) | 826 |
| Week 2 | Culture plate | 11522 (78%) | 3273 |
| | Bioreactor system | 62956 (90%) | 7250 |

The percentage of $CD34^+$ cells among the total cell population decreased during 2 wks of cell culture. During the same period, the percentage of $CD36^+$ cells increased and reached about 60 % in cultures performed in a culture plate and 70 % in the bioreactor system (figure 7).

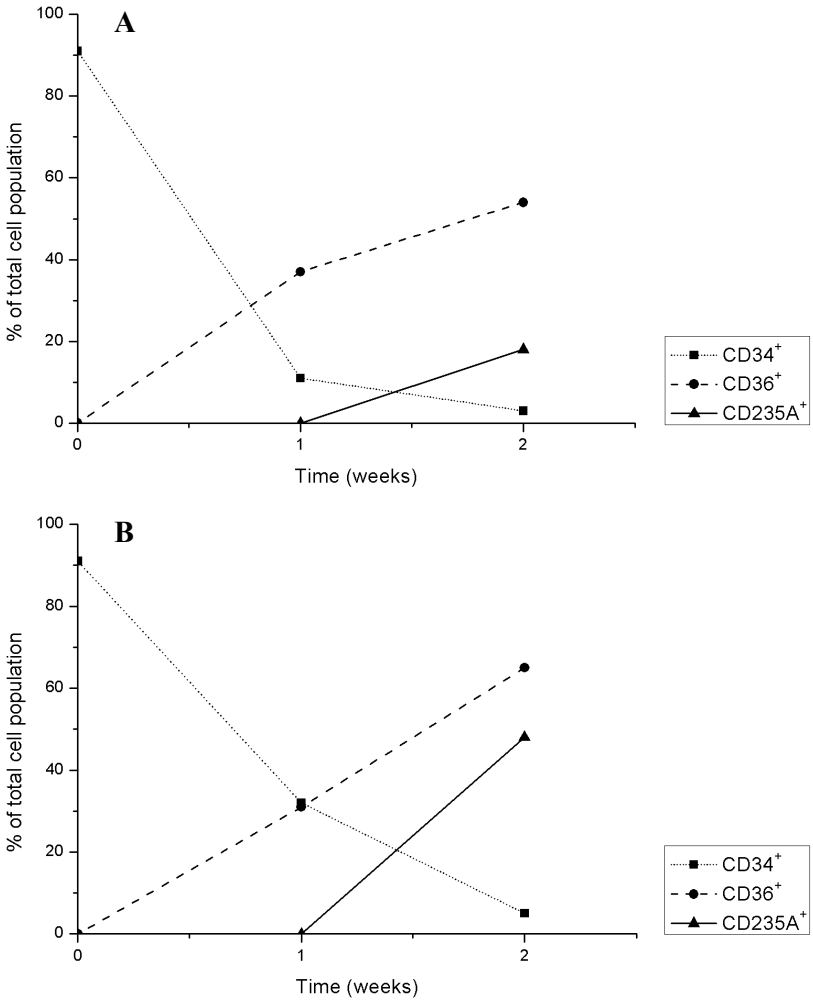


**Figure 7. Number of cells expressing HPC and erythroid antigens as percentage of the total cell population during 2 wks of culturing in medium containing Epo and dexamethasone (medium 2). The PVDF-enclosed cell cultures were performed in culture plates (A) and in the bioreactor (B).**

After 1 wk the cells also started to express glycophorin A. The percentage of cells expressing glycophorin A reached about 20 % in cultures performed in a culture plate and 50 % in the bioreactor. The expression of glycophorin A (CD235A) indicates that the CFU-E cells started to differentiate into (pro)erythroblasts. About 7 % of $CD36^-$ and glycophorin A positive cells were detected, which could indicate reticulocytes. However, May-Giemsa-Grünwald stained cells on cytospin slides did not show enucleated cells (figure 8).

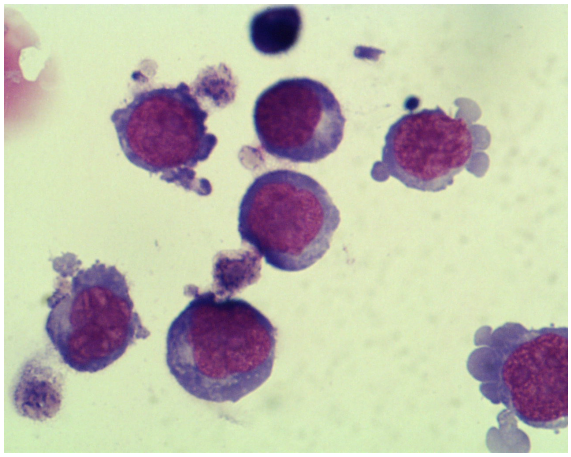

**Figure 8. Representative May-Giemsa-Grünwald stained cytospin of cells obtained after 2 wks of cell culture in a PVDF-enclosed packed bed of beads in the bioreactor system using culture medium for erythroid differentiation (medium 2, photograph taken at original magnification x 1000).**

It has been reported that enucleated cells can be produced using bioreactors in which full bone marrow, including stromal cells, is cultured on collagen beads with medium containing serum and Epo [1-3]. In the present study, the absence of enucleation was expected since terminal differentiation of erythrocytes usually requires supporting stroma [23] or macrophage cells [28]. Moreover, terminal erythroid differentiation requires the removal of proliferation factors such as SCF and dexamethasone [29].

Here we presented the erythroid differentiation of $CD34^+$ cells on heparinized crosslinked collagen beads without stroma and therefore lacking terminal differentiation. However, the absence of stroma and serum in this system allows further investigation of the role of ECM components and cytokines in the production of erythroid progenitors and precursors.

## 4. Conclusions

A bioreactor system for the expansion and erythropoietic differentiation of $CD34^+$ cells in a packed bed of heparinized crosslinked collagen beads is presented. This system provides a large surface area for culturing HPCs and is made from materials that mimic some of the compounds present in bone marrow. This bioreactor system was used to produce erythroid progenitors as well as more differentiated erythroid precursors. These two procedures could be combined, as this bioreactor system facilitates the sequential use of different culture media without disturbing the cells in

culture. After one wk of expanding HPCs with serum-free culture medium containing IL-3, SCF, TPO and Flt3/Flk2-ligand, the medium could be changed for culture medium containing Epo and dexamethasone for further erythroid differentiation. Moreover, the use of serum-free media and the absence of a supporting cell line in this system allows more detailed studies into the influence of ECM components and cytokines on erythropoiesis.

## 5. Acknowledgements

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## 6. References

1. Wang, T.Y. and Wu, J.H.D., *A Continuous Perfusion Bioreactor For Long-Term Bone-Marrow Culture.* Ann N Y Acad Sci 1992;665: 274-284.
2. Wang, T.Y., Brennan, J.K., and Wu, J.H.D., *Multilineal Hematopoiesis in a 3-Dimensional Murine Long-Term Bone-Marrow Culture.* Exp Hematol 1995;23:26-32.
3. Mantalaris, A., Keng, P., Bourne, P., Chang, A.Y.C. and Wu, J.H.D., *Engineering a human bone marrow model: A case study on ex vivo erythropoiesis.* Biotechnol Prog 1998;14:126-133.
4. Bagley, J., Rosenzwieg, M., Marks, D.F. and Pykett, M.J., *Extended culture of multipotent hematopoietic progenitors without cytokine augmentation in a novel three-dimensional device.* Exp Hematol 1999;27:496-504.
5. Meissner, P., Schroder, B., Herfurth, C. and Biselli, M., *Development of a fixed bed bioreactor for the expansion of human hematopoietic progenitor cells.* Cytotechnology 1999;30:227-234.
6. Tomimori, Y., Takagi, M., and Yoshida, T., *The construction of an in vitro three-dimensional hematopoietic microenvironment for mouse bone marrow cells employing porous carriers.* Cytotechnology 2000;34:121-130.
7. Banu, N., Rosenzwieg, M., Kim, H., Bagley, J. and Pykett, M., *Cytokine-augmented culture of haematopoietic progenitor cells in a novel three-dimensional cell growth matrix.* Cytokine 2001;13:349-358.

8. Sasaki, T., Takagi, M., Soma, T. and Yoshida, T., *3D culture of murine hematopoietic cells with spatial development of stromal cells in nonwoven fabrics.* Cytotherapy 2002;4:285-291.
9. Kim, H.S., Lim, J.B., Min, Y.H., Lee, S.T., Lyu, C.J., Kim, E.S. and Kim, H.O., *Ex Vivo Expansion of Human Umbilical Cord Blood CD34+ Cells in a Collagen Bead-Containing 3 Dimensional Culture System.* Int J Hematol 2003;78:126-132.
10. Xiong, F., Chen, Z., Liu, H., Xu, Z. and Liu, X., *Ex vivo expansion of human umbilical cord blood hematopoietic progenitor cells in a novel three-dimensional culture system.* Biotechnol Lett 2002;24:1421-1426.
11. Wissink, M.J.B., Beernink, R., Pieper, J.S., Poot, A.A., Engbers, G.H.M., Beugeling, T., Van Aken, W.G. and Feijen, J., *Immobilization of heparin to EDC/NHS-crosslinked collagen. Characterization and in vitro evaluation.* Biomaterials 2001;22:151-163.
12. Gupta, P., McCarthy, J.B., and Verfaillie, C.M., *Stromal fibroblast heparan sulfate is required for cytokine- mediated ex vivo maintenance of human long-term culture- initiating cells.* Blood 1996;87:3229-3236.
13. Gupta, P., Oegema, T.R., Brazil, J.J., Dudek, A.Z., Slungaard, A. and Verfaillie, C.M., *Structurally specific heparan sulfates support primitive human hematopoiesis by formation of a multimolecular stem cell niche.* Blood 1998;92:4641-4651.
14. Gupta, P., Oegema, T.R., Brazil, J.J., Dudek, A.Z., Slungaard, A. and Verfaillie, C.M., *Human LTC-IC can be maintained for at least 5 weeks in vitro when interleukin-3 and a single chemokine are combined with O-sulfated heparan sulfates: requirement for optimal binding interactions of heparan sulfate with early-acting cytokines and matrix proteins.* Blood 2000;95:147-155.
15. Gordon, M.Y., Riley, G.P., Watt, S.M. and Greaves, M.F., *Compartmentalization of a heamatopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment.* Nature 1987;326:403-405.
16. Roberts, R., Gallagher, J., Spooncer, E., Allen, T.D., Bloomfield, F. and Dexter, T.M., *Heparan sulphate bound growth factors: a mechanism for stromal cell mediated haematopoiesis.* Nature 1988;332:376-378.
17. Tanaka, Y., Kimata, K., Adams, D.H. and Eto, S., *Modulation of Cytokine Function by Heparan Sulfate Proteoglycans: Sophisticated Models for the Regulation of Cellular Responses to Cytokines.* Proceedings of the Association of American Physicians 1998;10:118-125.
18. Siebum B., Grijpma D.W., Poot A.A., Vermes I., Feijen J., *Heparinized crosslinked collagen films for hematopoietic stem cell culture.* Chapter 3 of this thesis.
19. Siebum B., Grijpma D.W., Poot A.A., Vermes I., Kruijer W., Feijen J., *Heparinized crosslinked collagen films pre-adsorbed with SDF-1α for hematopoietic stem cell culture.* Chapter 4 of this thesis.
20. Siebum B., Lelivelt H., Grijpma, D.W., Poot, A.A., Vermes, I., Kruijer, W., Feijen J., *Heparinized crosslinked collagen beads for culturing hematopoietic progenitor cells.* Chapter 6 of this thesis.
21. Loke, W.K., Khor, E., *Validation of the shrinkage temperature of animal tissue for bioprosthetic heart valva application by differential scanning calorimetry.* Biomaterials 1995;16:251-258.

22. Wang, C.L., Miyata, T., Wecksler, B., Rubin, A.L., Stenzel, K.H., *Collagen induced platelet aggregation and release. I. Effects of side-chain modifications and role of argynyl residues.* Biochim Biophys Acta 1978;544:555-567.
23. Giarratana, M. C., Kobari, L., Lapillonne, H., Chalmers, D., Kiger, L., Cynober, T., Marden, M. C., Wajcman, H., Doay, L.. *Ex Vivo generation of fully mature human red blood cells from hematopoietic cells.* Nature biotechnol 2005;23:69-74.
24. Ivanovic, Z., Hermitte F., De la Grange P.B., Dazey B., Belloc F., Lacombe F., Vezon G., Praloran V. *Simultaneous Maintenance of Human Cord Blood SCID-Repopulating Cells and Expansion of Committed Progenitors at Low $O_2$ concentration (3%).* Stem Cells 2004;22:716-724.
25. Patel, S.D., Papoutsakis, E.T., Winter, J.N. and Miller, W.M., *The lactate issue revisited: Novel feeding protocols to examine inhibition of cell proliferation and glucose metabolism in hematopoietic cell cultures.* Biotechnol Prog 2000;16:885-892.
26. McAdams, T.A., Miller, W.M. and Papoutsakis, E.T., *pH is a potent modulator of erythroid differentiation.* Br J Haematol 1998;103:317-325.
27. Freyssinier, J.M., Lecoq-Lafon, C., Picard, F., Ducrocq, R. and Mayeux, P. *Purification, amplification and characterization of a population of human erythroid progenitors*. Br J Haematol 1999;106:912-922.
28. Malik, P., Fisher, T.C., Brasky, L.L.W., Zeng, L., Izadi, P., Hiti, A., Weinberg, K.I., Coates, T.D., Meiselman, H.J. and Kohn, D.B. *An In Vitro Model of Human Red Blood Cell Production from Hematopoietic Progenitor Cells*. Blood 1998;91:2664-2671.
29. Von Lindern, M., Zauner, W., Mellitzer, G., Steinlein, P., Fritsch, G., Huber, K., Lowenberg, B. and Beug, H. The glucocorticoid receptor cooperates with the erythropoietin receptor and c-Kit to enhance and sustain proliferation of erythroid progenitors in vitro. Blood 1999;94:550-559.

# Summary

It is generally accepted that bone marrow contains niches that regulate the activity of hematopoietic stem cells (HSCs). These cells are the source of all different blood cells. In their niche the HSCs are localized in a specific microenvironment, where they interact with stromal cells, extracellular matrix (ECM) components and molecules like cytokines, chemokines and growth factors.

The objective of the studies presented in this thesis was to develop a bioreactor system, in which the bone marrow is mimicked, for the controlled expansion and differentiation of HSCs and hematopoietic progenitor cells (HPCs). Such a system is expected to facilitate an effective expansion of HSCs and to provide a model for investigating the mechanisms of hematopoiesis and processes such as stem cell mobilization and homing. The *in vitro* expansion and controlled differentiation of HSCs and HPCs is of great interest for application in bone marrow transplantation. Additionally, controlled differentiation of these cells can be used to produce blood cells for transfusion. The focus will be on the expansion of HPCs and their differentiation into erythroid cells.

An introduction to the bone marrow environment, hematopoiesis, erythropoiesis and different approaches to *in vitro* hematopoietic cell culture is presented in **chapter 2**. The chemical and cellular components of the bone marrow microenvironments are discussed and an overview of the literature concerning *in vitro* hematopoietic cell culture and bioreactor systems for hematopoiesis is presented.

The serum- and stroma-free culturing of $CD34^+$ selected cells, obtained from G-CSF mobilized peripheral blood, on heparinized crosslinked collagen films is introduced in **chapter 3**. Collagen type I, a major constituent of the bone marrow ECM, was processed into crosslinked films. Chemical modification using a water-soluble carbodiimide was used to immobilize heparin onto the collagen films, to an extent of 23 ± 4 μg per mg of collagen film. Heparin was used as a model compound for heparan sulfates, which are expected to play an important role in hematopoietic microenvironments by modulating the function of growth factors, chemokines and cytokines. Cell expansion was only achieved during cell culture using heparinized crosslinked collagen films as substrate. The poor performance of non-heparinized

films might have been caused by the adsorption of interleukin-3 (IL-3) to this material, which was possibly accompanied by conformational changes of the cytokine. Using heparinized crosslinked collagen films as culture substrate, the total cell population was expanded 5-fold after 2 weeks, but expansion of $CD34^+$ cells was not obtained. Analysis of the colony-forming units (CFUs) showed that during 2 wks these progenitors expanded 6 times and were mostly (~ 84 %) from the erythroid lineage.

Application of heparinized crosslinked collagen films pre-adsorbed with the chemokine stromal cell-derived factor-1α (SDF-1α) as substrate for $CD34^+$ cell culture is presented in **chapter 4**. The use of heparinized films slightly increased the amount of SDF-1α adsorbed to the films, from 18 ± 2 to 25 ± 4 ng SDF-1α per mg of film. The release of the pre-adsorbed chemokine into the culture medium took place during the first hours of cell culture. Both from heparinized and non-heparinized films approximately 40 % of the pre-adsorbed chemokine was released. Upon $CD34^+$ cell seeding, the adsorbed and/or released SDF-1α increased the number of HPCs adhering to the substrate up to 2-fold. The number of CFUs adhering to heparinized films was 2 times higher at day 4 when these films were pre-adsorbed with SDF-1α, probably caused by differentiation of the initially higher number of $CD34^+$ cells adhering to these films. During 2 weeks of culture the pre-adsorbed chemokine did not influence cell proliferation and differentiation.

The adsorption and subsequent release of IL-3 from heparinized and non-heparinized crosslinked collagen films and the use of IL-3 pre-adsorbed films as culture substrate for the expansion of $CD34^+$ cells are described in **chapter 5**. Crosslinked collagen films adsorbed a significant amount (~ 2.4 ng) of the IL-3 from culture medium containing 5 ng IL-3. Under the same conditions, the amount of IL-3 adsorbed from culture medium onto heparinized crosslinked collagen films was about 2 ng. When the films were heparinized, they subsequently released 8 % of the adsorbed IL-3 into the supernatant, which was 8 times more than released from non-heparinized films, indicating that part of the adsorbed cytokine was reversibly bound to the heparinized films. The use of non-heparinized crosslinked collagen films pre-incubated with 0.3 ml of an IL-3 solution in PBS (1 μg/ml) did not result in the expansion of HPCs when cultured in IL-3-free medium. This was probably caused by IL-3 adsorption to crosslinked collagen films, which possibly induced conformational changes in the cytokine. Heparinized crosslinked collagen films pre-adsorbed with IL-3 under the

same conditions, supported HPC expansion. Using this substrate, culturing with IL-3-free medium resulted in a relatively large number of adhering cells, a 1.4-fold $CD34^+$ cell expansion during the first wk of culture and a 16-fold viable cell expansion after 2 wks. Additionally, the use of heparinized films pre-adsorbed with IL-3 as culture substrate in IL-3 free medium resulted in a 6-fold CFU expansion with differentiation of 93 % of the CFUs into the erythroid lineage.

In an **appendix** to chapter 5 it is shown that the use of heparinized and non-heparinized crosslinked collagen films pre-adsorbed with bFGF as substrate for $CD34^+$ cell culture, resulted in a large number of adhering and non-adhering cells, while the expansion of HPCs and erythroid differentiation were not significantly improved.

**Chapter 6** deals with the use of packed beds of heparinized crosslinked collagen beads as culture substrates for the expansion of HPCs. $CD34^+$ cells were cultured in packed beds of SDF-1α pre-adsorbed heparinized crosslinked collagen beads and on SDF-1α pre-adsorbed heparinized crosslinked collagen films. The use of heparinized beads pre-adsorbed with SDF-1α did not significantly improve cell retention in the packed bed of beads, as compared to beads not pre-adsorbed with this chemokine. Culturing of HPCs using SDF-1α pre-adsorbed heparinized beads, resulted in a 3-fold expansion of $CD34^+$ cells, whereas these cells did not expand on SDF-1α pre-adsorbed heparinized films. The increased surface area provided by the beads probably caused the improved HPC expansion. Total cell expansion was similar for both substrates (~ 6 times). CFUs in the cell cultures using packed beds of beads as culture substrate expanded 4-fold, whereas the CFUs expanded 6-fold in the cultures using films. After 2 wks of culturing 61 % of the CFUs in the packed beds were erythroid, while this was about 84 % when the films were used.

The packed beds of SDF-1α pre-incubated heparinized crosslinked collagen beads were used in a bioreactor to expand HPCs into erythroid progenitor cells and differentiate these cells into more committed erythroid precursor cells like erythroblasts, as presented in **chapter 7**. $CD34^+$ cells were cultured serum- and stroma-free in a packed bed of these beads in a bioreactor. The use of the bioreactor, in which 50 ml of culture medium was continuously circulated around a membrane enclosing the cells and beads, compensated for the reduction in total and $CD34^+$ cell expansion probably caused by limited diffusion through the membrane. The total viable cell expansion was 4 times after 2 wks of culturing in the bioreactor system in

medium containing IL-3, Flt3/Flk2-ligand, TPO and SCF. About 50% of the expanded cells expressed CD36, whereas glycophorin A was not expressed. During the first wk, the $CD34^+$ cells and CFUs transiently expanded 1.6- and 9-fold, respectively. The expanded CFUs were 81 – 88 % erythroid. Differentiation of $CD34^+$ cells in the bioreactor system with modified serum-free culture medium, containing EPO and dexamethasone, resulted in a 3.6-fold total cell expansion, no $CD34^+$ cell expansion and a 16-fold CFU expansion in 2 wks. The expanded CFUs were 89 – 90 % erythroid. Moreover, 70% of the total expanded cells expressed CD36 and 50% expressed glycophorin A, indicating differentiation into erythroid precursor cells.

These results show that the serum- and stroma-free expansion and differentiation of $CD34^+$ HPCs in a packed bed of heparinized crosslinked collagen beads used in a bioreactor is feasible. The bioreactor allows the sequential use of different culture media and can be modified for large-scale cell culture. Moreover, this system provides a model to study the role of ECM components and molecules like IL-3 and SDF-1α in the hematopoietic process.

# Samenvatting

Het beenmerg bevat niches die de activiteit van hematopoietiche stamcellen (HSCs) reguleren. De HSCs zijn de voorlopers van de verschillende bloedcellen. In hun niche worden de HSCs omringt door een specifieke (micro)omgeving, waar ze interacties hebben met stroma cellen, extracellulaire matrix (ECM) componenten en moleculen zoals cytokines, chemokines en groeifactoren.

Het doel van de studies die hier beschreven staan was het ontwikkelen van een bioreactor, waarin het beenmerg is nagemaakt, om daarin de HSCs en de iets meer gespecialiseerde voorlopercellen (HPCs) te vermeerderen en gecontroleerd te laten differentieren. In zo'n systeem zouden HSCs en HPCs effectief vermeerderd kunnen worden. Tegelijketijd kan een dergelijk systeem functioneren als een model voor het bestuderen van hematopoiese en processen zoals stamcel mobilizatie en homing. Het *in vitro* vermeerderen en gecontroleerd differentieren van HSCs en HPCs is van groot belang voor het toepassen van deze cellen in beenmerg transplantaties. Bovendien kan gecontroleerde differentiatie gebruikt worden om bloedcellen te produceren voor transfusie. In dit onderzoek wordt bij het differentieren gekeken naar de productie van rode bloedcellen (erythropoiese).

In **hoofdstuk 2** worden het beenmerg, hematopoiese en erythropoiese beschreven en wordt een overzicht gegeven van literatuur over het kweken van hematopoietische cellen en het gebruik van bioreactoren voor *in vitro* hematopoiese.

Het serum- en stroma-vrij kweken van $CD34^+$ HPCs op gecrosslinkte collageen films die met heparine gemodificeerd zijn, wordt in **hoofdstuk 3** geïntroduceerd. Collageen type I, een belangrijk onderdeel van de ECM in het beenmerg, werd verwerkt tot films. Chemische modificatie met een wateroplosbare carbodiimide werd gebruikt om heparine te immobilizeren op de collageen films, hierbij werd $23 \pm 4$ μg heparine per mg collageen bereikt. Heparine werd gebruikt als model materiaal voor heparan sulfaten, waarvan wordt verwacht dat deze een belangrijke rol spelen in het beenmerg omdat ze chemokines, cytokines en groeifactoren binden en presenteren aan de cellen. De HPCs vermeerderden zich alleen op de geheparinizeerde films. Op de alleen maar gecrosslinkte films zouden de slechte resultaten veroorzaakt kunnen zijn door de binding van IL-3 aan het collageen, waardoor waarschijnlijk conformatie

veranderingen in de deze cytokine zijn opgetreden. Als geheparinizeerde films gebruikt werden nam de totale celpopulatie 5 maal toe tijdens 2 weken, terwijl het aantal $CD34^+$ cellen niet toenam. Bestudering van de kolonie vormende cellen (CFUs) liet zien dat tijdens de kweek het aantal van deze voorloper cellen 6 maal was toegenomen en dat deze HPCs voornamelijk (ca 84%) erythroid waren.

Het gebruik van de geheparinizeerde films, waaraan eerst de chemokine SDF-1α gebonden was, als substraat voor de kweek van $CD34^+$ cellen is gepresenteerd in **hoofdstuk 4**. Door heparinizatie van de films nam de hoeveelheid SDF-1α die aan de films bond toe van 18 ± 2 tot 25 ± 4 ng per mg film. De afgifte van de aan de films gebonden chemokine naar het kweekmedium vond plaats tijdens de eerste uren van de celkweek. De SDF-1α afgifte van de geheparinizeerde en de niet-geheparinizeerde films was ongeveer 40 % van de gepreadsorbeerde hoeveelheid. Tijdens het zaaien van de $CD34^+$ cellen werd de hoeveelheid cellen die aan het materiaal binden verdubbeld door de gepreadsorbeerde of afgegeven SDF-1α. Het aantal CFUs dat aan het materiaal bond was verdubbeld na vier dagen, waarchijnlijk door differentiatie van het aanvankelijk hogere aantal adhererende $CD34^+$ cellen. Gedurende de twee weken van de celkweek beïnvloedde de gepreadsorbeerde chemokine de vermeerdering en differentiatie van de cellen niet.

De adsorptie en afgifte van de cytokine IL-3 van geheparinizeerde en gecrosslinkte collageen films en het gebruik van deze IL-3 gepreadsorbeerde films als kweek substraat voor de vermeerdering van $CD34^+$ cellen wordt in **hoofdstuk 5** beschreven. Gecrosslinkte collageen films adsorberen een significante hoeveelheid (ca. 2,4 ng) IL-3 uit 0,5 ml kweekmedium dat 5 ng IL-3 bevat. Onder dezelfde omstandigheden adsorbeerden de geheparinizeerde films ongeveer 2 ng IL-3 uit het kweekmedium. Geheparinizeerde films gaven vervolgens 8 % van de gebonden IL-3 af in vers medium, dit was 8 keer zo veel als niet-geheparinizeerde films afgaven. Dit duidt erop dat een deel van de geadsorbeerde cytokine reversibel aan de geheparinizeerde films was gebonden. Het gebruik van niet-geheparinizeerde films die met 0,3 ml van een IL-3 oplossing in PBS (1 μg/ml) gepreïncubeerd zijn resulteerde niet in een expansie van HPCs als deze met IL-3-vrij medium gekweekt werden. Dit kwam waarschijnlijk door de IL-3 adsorptie aan gecrosslinkt collageen, welke waarschijnlijk conformatie veranderingen in de cytokine veroorzaakt. Onder dezelfde omstandigheden was het wel mogelijk om de HPCs te vermeerderen met geheparinizeerde films, die in dezelfde hoeveelheid IL-3 gepreïncubeerd waren, als kweeksubstraat. Met dit

substraat resulteerden de kweken met IL-3-vrij medium in een 1,4 voudige vermeerdering van de $CD34^+$ cellen tijdens de eerste week van de celkweek en een 16 voudige vermeerdering van de totale celpopulatie tijdens twee weken. Bovendien werden de CFUs 6 maal vermeerderd, waarbij deze voornamelijk (93 %) in de erythroide lineage differentieerden.

In een **appendix** bij hoofdstuk 5 worden de voorlopige resultaten gepresenteerd van $CD34^+$ celkweken op geheparinizeerde en gecrosslinkte collageen films die daarvoor in 0,3 ml van een bFGF oplossing in PBS (1 μg/ml) gepreïncubeerd zijn. Deze kweken resulteerden in grote hoeveelheden adhererende en niet adhererende cellen, terwijl de vermeerdering van HPCs en erythroide cellen niet significant verbeterde.

**Hoofdstuk 6** gaat over het gebruik van opeengepakte bedden van geheparinizeerde en gecrosslinkte collageen bolletjes als kweek substraat voor de vermeerdering van HPCs. $CD34^+$ cellen werden gekweekt in een gepakt bed van geheparinizeerde bolletjes die met SDF-1α gepreadsorbeerd waren. Het gebruik van SDF-1α gepreadsorbeerde bolletjes leide niet tot een significante verbetering van de cel retentie in het gepakte bed, ten opzichte van bolletjes die niet met deze chemokine gepreadsorbeerd waren. Het kweken van HPCs met SDF-1α gepreadsorbeerde bolletjes resulteerde in een drievoudige toename van het aantal $CD34^+$ cellen, terwijl deze cellen zich niet vermeerderden op SDF-1α gepreadsorbeerde films. Deze verbetering werd waarschijnlijk door de toename van het kweekoppervlak veroorzaakt. De toename van het totale aantal cellen in het gepakte bed was vergelijkbaar met die op de films (ca 6 keer). De CFUs in de kweken met gepakte bedden vermeerderden zich slechts 4 keer, terwijl deze zich 6 maal vermeerderden in kweken waarbij films als substraat werden gebruikt. Na twee weken was 61 % van de CFUs in de gepakte bedden erythroid, terwijl dit 84 % was bij de kweken met films.

Gepakte bedden van geheparinizeerde bolletjes met gepreadsorbeerd SDF-1α zijn in een bioreactor gebruikt om HPCs te expanderen en te differentieren naar rijpere erythroide voorloper (precursor) cellen, zoals in **hoofdstuk 7** staat beschreven. $CD34^+$ cellen werden serum- en stroma-vrij gekweekt en in een gepakt bed geplaatst in een bioreactor. In de reactor werd 50 ml kweekmedium continu rond een membraan die de bollen en de cellen omringt, gepompt. Het gebruik van deze reactor compenseerde voor de afname in de vermeerdering van het totale aantal cellen en $CD34^+$ cellen, Deze afname was waarschijnlijk veroorzaakt door de beperkte diffussie door het membraan. Het totale aantal cellen vermeerderde zich 4 maal tijdens twee weken

kweken in de bioreactor met kweek medium met IL-3, Flt3/Flk-2 ligand, TPO en SCF. Ongeveer 50 % van de cellen was $CD36^+$ en negatief voor glycophorine A. Tijdens de eerste week van de celkweek vermeerderden de $CD34^+$ cellen en CFUs zich respectievelijk 1,6 en 9 keer. De vermeerderde HPCs waren 81 – 88 % erythroid. Differentiatie van $CD34^+$ cellen met een gemodificeerd serum-vrij medium met Epo en dexamethason, resulteerde in een toename van het aantal cellen van 3,6 keer, geen toename van het aantal $CD34^+$ cellen en een vermeerdering van de CFUs van 16 keer. De vermeerderde CFUs waren 89 – 90 % erythroid. Bovendien was 70 % van de vermeerderde cellen $CD36^+$ en 50 % van de cellen was ook positief voor glycophorine A. Deze antigenen geven aan dat de cellen gedifferentieerd zijn in eythroide precursor cellen.

De beschreven resultaten tonen aan dat de serum- en stroma-vrije vermeerdering en differentiatie van $CD34^+$ HPCs in een gepakt bed van geheparinizeerde collageen bolletjes in een bioreactor haalbaar is. De bioreactor maakt het mogelijk om achtereenvolgens verschillende kweekmedia te gebruiken. Tevens is een reactor systeem beter geschikt voor grotere hoeveelheden cellen. Dit systeem kan een goed model bieden voor de bestudering van de rol van ECM materialen en moleculen zoals IL-3 en SDF-1α tijdens hematopoiese.

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# Biography

Bastiaan Siebum was born on 28th December 1975 in Enschede, The Netherlands. After graduating from secondary school, he continued his studies at Twente University. There he studied Chemical Engineering, with a major in polymerchemistry and biomaterials. His master research was performed in the group of prof. Jan Feijen, on the synthesis, processing and characterization of copolymers of polylactide and trimethylene carbonate for tissue engineering. He also studied Philosophy of Science, Technology and Society. His master scripture about language and material in the development of biomedical technologies was supervised by prof. Hans Achterhuis. After graduating in 2002 he continued as PhD student in the group of Prof. Jan Feijen. The results of his research are described in this thesis.