

**CULTURED BONE ON BIOMATERIAL SUBSTRATES:
A Tissue Engineering Approach to Treat Bone Defects**

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Cultured bone on biomaterial substrates:

A tissue engineering approach to treat bone defects

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**CULTURED BONE ON BIOMATERIAL SUBSTRATES:
A TISSUE ENGINEERING APPROACH TO TREAT BONE DEFECTS**

DISSERTATION

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To Gert and my parents

Table of contents

Chapter 1

| | |
|--|----|
| General Introduction | 11 |
| Bone | 12 |
| Materials for Osseous Reconstruction | 13 |
| <i>Autologous bone</i> | 13 |
| <i>Allogeneic and xenogeneic bone</i> | 14 |
| <i>Synthetic biomaterials</i> | 14 |
| <i>Biomaterials with intrinsic osteoinductivity</i> | 15 |
| Novel Strategies for Bone Repair and Regeneration | 16 |
| <i>Chemical stimulation of bone healing</i> | 17 |
| <i>Cell therapy approaches for bone reconstruction</i> | 19 |
| Aims of the Thesis | 28 |

Chapter 2

| | |
|---|----|
| A Preliminary Study on the In Vivo Bone Formation by Human Bone Marrow Stromal Cells: Effect of Osteogenic Culture Supplements | 39 |
|---|----|

Chapter 3

| | |
|---|----|
| Human Bone Marrow Stromal Cells for Bone Tissue Engineering: In Vitro and In Vivo Characterisation | 47 |
|---|----|

Chapter 4

| | |
|--|----|
| Bone Tissue Engineered Implants Using Human Bone Marrow Stromal Cells: Effect of Culture Conditions and Donor Age | 65 |
|--|----|

| | |
|--|-----|
| Chapter 5 | |
| Temporal Expression of Stro-1, Alkaline Phosphatase and Osteocalcin in Cultures of Whole Human Bone Marrow During Differentiation | 81 |
| Chapter 6 | |
| A Reliable Method to Predict the In Vivo Osteogenic Potential of Human Bone Marrow Stromal Cells | 99 |
| Chapter 7 | |
| A Cultured Living Bone Equivalent Enhances Bone Formation When Compared to a Cell Seeding Approach | 117 |
| Chapter 8 | |
| Evaluation of Two Biodegradable Polymeric Systems as Substrates for Bone Tissue Engineering | 131 |
| Chapter 9 | |
| General Discussion and Concluding Remarks | 153 |
| General Discussion | 154 |
| Concluding Remarks | 163 |
| Summary | 167 |
| Samenvatting | 168 |
| Acknowledgments | 169 |
| Curriculum Vitae | 170 |

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

BONE

Bone is a complex dynamic tissue that is constantly being remodelled throughout adult life (resorbed and re-deposited). It is a natural composite material, mainly composed of mineral (60% in weight), an organic matrix (30% in weight) and water (10% in weight) [1]. The mineral part of bone confers stiffness to the tissue and consists of calcium phosphates, from which the major component is hydroxyapatite [2]. The organic matrix of bone confers tensile strength and is composed of a well organised network of proteins, from which collagen type I is the main constituent. The non collagenous proteins include osteonectin, osteopontin, bone sialoprotein, osteocalcin, decorin and biglycan [2-3].

Bone has mainly three functions: (i) It is a major organ for calcium homeostasis and it stores phosphate, magnesium, potassium and bicarbonate; (ii) it is the most abundant site of hematopoiesis in the human adult and (iii) it provides mechanical support for soft tissue and attachment sites for the muscles [4-5]. To fulfil these functions bone is constantly being remodelled. In adult life, physiological remodelling consists of bone resorption followed by bone deposition in approximately the same location. Bone resorption is accomplished by multinucleated giant cells of hematopoietic origin, named osteoclasts, while bone deposition occurs via osteoblasts, which are from stromal origin [2].

Bone exists in two forms, cortical and trabecular. The cortical bone, also called compact bone, is rigid, dense, anisotropic and plays a major role in mechanical support. It comprises the outer shell of the long bones, as well as the outer surface of small and flat bones. Trabecular or cancellous bone is less dense than cortical bone but it is metabolically more active. It occurs near the ends of long bones, in the interior of small bones and between the surfaces of flat bones [4-6].

Bone formation occurs by either of two processes, intramembranous or endochondral. In the intramembranous process, mesenchymal progenitors condense and differentiate directly into osteoblasts, while in the endochondral ossification process the same progenitors first form a cartilage template that is later replaced by bone. Intramembranous ossification is mainly responsible for the development of flat bones from the skull and for the addition of bone on the periosteal surfaces of long bones. Endochondral ossification occurs in the formation of long bones, vertebrae and fracture repair [5-6]. Besides the different processes of bone formation, also distinct embryonic lineages are involved in forming the different parts

of the skeleton. Craniofacial tissues are from ectodermal origin, while postcranial limb, rib, skull base and appendicular skeletons are from mesodermal origin [6].

MATERIALS FOR OSSEOUS RECONSTRUCTION

Bone tissue regeneration remains an important challenge in the field of orthopaedic and oral-maxillofacial surgery. Spinal fusion, augmentation of fracture healing and reconstruction of bone defects resulting from trauma, tumour, infections, biochemical disorders and abnormal skeletal development are some of the clinical situations in which surgical intervention is required. The type of graft materials available to treat such problems essentially include autologous, allogeneic and xenogeneic bone, as well as a wide range of synthetic biomaterials such as metals, ceramics, polymers and composites.

Autologous bone

Currently the use of autologous (host) bone grafts is broadly considered as the golden standard therapy for bone repair and regeneration [5, 7-10]. Besides lacking immunogenicity, autologous bone possesses a range of intrinsic properties that make it an optimal implant material to achieve bone healing. These grafts are osteogenic, osteoinductive and osteoconductive. The osteogenic potential of autologous grafts is provided by bone forming cells present in the bone marrow, which are directly delivered at the implant site [11-12]. The grafts are also osteoinductive, that is, they are able to recruit mesenchymal cells located near the implant or from blood vessels and induce them to differentiate into osteogenic cells, through the exposure of osteoinductive growth factors of which the bone morphogenetic proteins (BMP's) are the most commonly studied [7, 9, 12-13]. Finally, the three-dimensional structure of the bone matrix, mainly composed of hydroxyapatite and collagen, allows for the infiltration of osteogenic cells that establish direct contact with the material (osteoconductivity) [9, 12, 14]. The usual donor site to harvest bone is the iliac crest since bone obtained from this location has shown to contain the highest osteogenic potential [9, 15]. Bone from tibia, rib, fibula and trochanter is also used, however, to a lesser extent.

Although autologous bone grafting has the requirements for optimal bone regeneration, its use is also associated with serious drawbacks. The harvest of the graft implies an extra and invasive surgical procedure and the removal of bone often causes morbidity at the donor site [7, 9-10, 11-12]. Post-operative continuous pain [9, 15-17], hypersensitivity [9], pelvic instability [15-16, 18], infection [12, 17, 19] and paresthesia [9, 12] are other possible complications associated with autologous bone grafting which affect 10 to 30% of the

patients [11, 17, 19]. The limited amount of bone that can be collected constitutes another disadvantage of these grafts. In young patients with small donor sites or in situations in which the amount of bone required is very large this may constitute serious problems. Additionally, in patients with osteoporosis the graft material may be of inferior quality [20].

Allogeneic and xenogeneic bone

The use of allogeneic (donor) bone for osseous reconstruction can solve some of the problems associated with autologous grafts since the harvest procedure is eliminated and the quantity of available tissue is no longer an issue. Nevertheless, these types of grafts present a poor degree of cellularity, less revascularisation and a higher resorption rate as compared to autologous grafts [9, 12], which may be responsible for the slower rate of new bone tissue formation observed in several studies [15, 21-23]. In addition, the immunogenic potential of these grafts and the risks of virus transmission to the recipient constitute serious disadvantages [10, 22, 24]. Processing techniques such as demineralisation, freeze-drying and irradiation have shown to reduce the patient's immune response, however, processing also alters the structure of the graft and reduces its potential to induce bone healing, while the possibility of disease transmission still remains [9].

Xenogeneic (cross-species) bone has also been tested as a grafting material. Although partial deproteinisation can decrease the severe antigenic response associated with these implants, it also removes the osteoinductive proteins [25]. In general, xenogeneic bone grafts do not induce bone formation when implanted into hard or soft tissues [9].

Synthetic biomaterials

Due to the limitations associated with bone derived grafts, several synthetic biomaterials are currently available, or under investigation, to be used as bone replacements. Four main classes biomaterials can be distinguished: metals, ceramics, polymers and composites. For many years, metal implants, mainly titanium and titanium alloys, have been used in orthopaedic and dental surgery for load bearing bone replacement. In joint replacement surgery, particularly total hip arthroplasty, these types of implants have achieved good clinical results, restoring patient mobility and providing pain relief [26]. These implants have high mechanical performance and do not evoke major adverse tissue responses. Nevertheless, they also present low bonding strength with bone, which can result in osteolysis if micro movements occur [26-29].

Ceramic materials have been widely studied as bone grafts substitutes. Among them, hydroxyapatite (HA) and tricalcium phosphate (TCP) have received the most attention due to their similarity to the inorganic component of bone and teeth [9-10, 30-33]. TCP is

reported to possess greater biodegradation rate as compared to HA but its mechanical properties are, however, inferior [9, 11, 30]. Blends of the two components allow to obtain biphasic calcium phosphates with a wide range of mechanical properties and resorbable rates, that can be tailored according to the specific application [11, 30, 34]. Extensive studies demonstrated that these materials are non toxic and do not evoke immunologic responses [33-35]. In addition, they promote bone ingrowth and form a strong intimate bond with bone [26, 32-35]. Due to those advantageous properties calcium phosphates have found applications in orthopaedic, dental and cranio-maxillofacial fields [9, 36-37]. Nevertheless, their relatively poor mechanical performance restricts their use to non load bearing applications [38]. Calcium phosphates are also used as coatings on metallic and polymeric substrates to promote a direct bond between bone and the implant, which results in improved osseointegration and firm implant fixation [28, 39]. Additionally, HA powder is commonly used as a polymeric filler aiming to obtain composites with higher mechanical performance [40-41].

To date several polymeric materials have been suggested as bone graft substitutes. Among the non biodegradable polymers, ultra high molecular weight polyethylene (UHMWPE) and poly (methyl methacrylate) (PMMA) have been extensively used. The main application of UHMWPE consists on the manufacture of acetabular cups, while PMMA has been used as bone cement and dental prosthesis [26]. Synthetic biodegradable polymers have also been proposed as bone grafts substitutes. These materials are “easily” processed into highly porous and complex three dimensional shapes. In addition, their degradation and mechanical properties can be tailored by adjusting the composition and molecular weight of the polymers. To date the polymeric systems that have been investigated for bone repair include poly(α -hydroxy esters) [10, 42-45], poly(dioxanone) [46], poly(propylene fumarate) [26, 47], poly(ethylene glycol) [48], poly(urethanes) [49], starch based systems [41] and copolymers of poly(ethylene glycol)-terephthalate and poly(butylene terephthalate) [50-51].

Biomaterials with intrinsic osteoinductivity

Although successful results have been achieved when using biomaterial approaches, none of the materials in the four above mentioned classes (metals, ceramics, polymers and composites) possess osteogenic properties. Additionally, it is generally agreed that they lack intrinsic osteoinductivity. As a consequence, their clinical application is restricted to relatively small osseous defects and their performance is inferior as compared to autologous bone grafts. Nevertheless, during the last decade, increasing evidence pointed out that specific calcium phosphate ceramics induced bone formation after implantation in soft tissues. In 1969 Winter and Simpson [52] reported bone induction by macroporous sponges of

polyhydroxyethyl methacrylate after subcutaneous implantation in pigs and, a few decades later, Ripamonti [53] found bone in hydroxyapatite ceramics after intramuscular implantation in baboons. Since then, several studies demonstrated that a number of porous calcium phosphate ceramics and cements, as well as glass ceramics, were capable of inducing osteogenesis when implanted in ectopic (non bony) sites [54-59]. Results suggested that osteoinduction was material related and the specific chemical and structural characteristics of the materials, including their microstructure, were very important factors playing a determinant role on their osteoinductive capacity. Additionally, both Yuan et al. [60] and Ripamonti [61] reported the osteoinductivity of porous calcium phosphate ceramics to be strongly dependent on the animal species. With regard to the mechanism of bone induction, Ripamonti [61] has suggested that the adsorption of bone morphogenetic proteins on the materials surface after implantation was the main reason for their osteoinductive properties. In addition, Yang et al. [54] observed that bone formation induced by calcium phosphates mainly occurred at the porous surfaces where microvessels were abundantly present and, therefore, proposed that pericytes from microvessels were the precursor cells that would differentiate towards osteoblasts and form bone. Nevertheless, recent studies by Yuan et al. [62-63], using a calcium phosphate ceramic loaded with a monoclonal antibody against bone morphogenetic proteins (BMP's) 2 and 4, indicated that, although BMP's may play a role in osteoinduction by calcium phosphates, they are not the sole reason for this phenomena. Moreover, results from one of the above mentioned studies [63] suggested that pericytes from microvessels are not the exclusive precursors of bone forming cells since the combination of the materials with an angiogenic factor did not enhance bone induction as compared to control samples.

In summary, materials with intrinsic osteoinductivity do exist and are excellent candidates as grafts for bone reconstruction. However, a better understanding of the biological mechanisms of osteoinduction, as well as further insight on the required biomaterial characteristics are still needed. In addition, factors related to the animal species variability observed in bone induction are not yet understood and the time required for bone formation, often 2 to 3 months, is also a limiting factor.

NOVEL STRATEGIES FOR BONE REPAIR AND REGENERATION

In 1993 Langer and Vacanti [64] defined tissue engineering as an 'Interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function'. With regard to bone tissue engineering, mainly two strategies have been implemented to generate new tissue: (I)

Chemical stimulation of bone formation through the use of bone inducing substances and (II) The construction of hybrid implants composed of osteogenic cells/tissue and a biomaterial scaffold.

Chemical stimulation of bone healing

Bone tissue contains peptide regulator molecules generally named growth factors that are capable of modulating bone cell activity. Bone growth factors are mainly produced by osteoblasts and are incorporated into the extracellular matrix during the process of bone formation. These factors are known to stimulate neighbouring cells to proliferate and increase protein synthesis (paracrine effect) and also act on the osteoblasts themselves inducing higher metabolic activity (autocrine effect) [65]. Numerous in vitro studies have reported that bone growth factors have several regulating effects on cells from the osteoblastic lineage and in vivo studies have demonstrated that some factors can induce bone formation and/or stimulate healing. Therefore, these agents became an area of intensive investigation. To date numerous growth factors have been identified and produced by recombinant gene technology, among those are bone morphogenetic proteins (BMP's), transforming growth factors β (TGF's β), fibroblast growth factors (FGF's), platelet derived growth factors (PDGF's) and insuline growth factors (IGF's).

In 1965 Urist [13] demonstrated that demineralised bone matrix free of viable cells could induce bone formation when implanted subcutaneously. Bone induction was attributed to a substance which had the property of inducing undifferentiated mesenchymal cells to differentiate towards osteoprogenitors. Later on this substance was identified as a protein, which Urist et al. [66] named bone morphogenetic protein. Since then 12 different bone morphogenetic proteins have been identified (BMP 1-12). The BMP's belong to the transforming growth factor β superfamily and are so far the only growth factors that can stimulate the differentiation of mesenchymal stem cells into the chondro and osteoblastic direction [12, 65-66]. Bone formation induced by BMP's recapitulates the process of endochondral ossification [12]. In vitro studies demonstrate that these proteins stimulate the differentiation of pluripotent cell lines and bone marrow stromal cells, from human and animal origin, into the osteogenic lineage in a dose dependant manner [67-71]. In vivo these proteins were found to induce ectopic bone formation in several animal models [72-73]. In addition, numerous studies reported the capability of BMP's to heal bone defects and /or induce orthotopic (osseous location) bone formation in a wide range of animal species including rats [74], rabbits [75], dogs [76] and baboons [73]. These proteins have also been successfully used for spinal fusions [71] and augmentation of alveolar bone [76].

Nevertheless, the dosage required for such treatment is strongly dependent on the animal model and a direct relation is observed between the amount of BMP's required and the size of the animal [73-74]. Additionally, when implanted alone these proteins diffuse too rapidly for bone induction to occur successfully, therefore, the success of BMP's in bone reconstruction is dependent on the existence of an appropriate carrier to maintain their activity at minimal dosage, preferably allowing a controlled release. Possible carriers tested for BMP's delivery include demineralised bone matrix [76], collagen [69, 76], calcium phosphate ceramics [67, 73-75], hyaluronan [77] and various synthetic polymers [72, 76-77]. With regard to the clinical use of BMP's in humans, few studies have been performed and in those reports very high physiological doses of protein, ranging from 1.8 to 3.4 mg, were used [78-79]. As a consequence, important safety questions were raised, especially because these agents are capable of inducing ectopic bone formation in regions neighbouring but external to their carrier [80]. Moreover, these proteins are not specific modulators of hard tissue, for example, the central nervous system is reported to contain BMP receptors [81]. In summary, prior to the clinical use of BMP's for bone reconstruction, the establishment of the proper dosage has to be further investigated, as well as the possible secondary effects that may result from their use.

Transforming growth factors β (TGF's β) are cytokines with a wide range of activities in bone, connective tissue and in the immunological system [65]. In general, they stimulate cells of the mesenchymal origin having profound effects on osteogenic cell proliferation, differentiation and matrix synthesis [65]. Although TGF's β are reported as potent mitogens for bone marrow stromal cells [65, 71, 82-83] their effects on bone cell differentiation are controversial. Collagen type I synthesis is stimulated by TGF β [71], while alkaline phosphatase activity and expression, as well as matrix mineralisation, are inhibited [71, 82]. With regard to osteocalcin synthesis, studies have demonstrated either an inhibitory [71] or a lack of effect [82] when bone marrow cells are exposed to TGF β . The effects of these factors in bony sites are contradictory and appear to vary with the set up of the specific study. Sumner et al. [84] demonstrated that TGF β enhanced bone ingrowth of implants inserted in trabecular bone in dogs. On the contrary, in a study by Aspenberg et al. [74], using a bone conduction chamber with porous hydroxyapatite in rats tibiae, it was shown that the bone ingrowth distance had a trend towards inhibition in implants treated with TGF β , as compared to controls. Additionally, a negative correlation between the TGF β dosage and bone ingrowth distance was found.

To date, two fibroblast growth factors (FGF's) were identified, acidic (aFGF) and basic (bFGF) [12, 65]. bFGF increases mitogenesis on fibroblastic, chondrogenic and osteogenic

cells [12, 65]. In bone marrow stromal cell cultures, it enhances cell growth, while maintaining the cells in an immature state [85]. In vivo studies also suggest that bFGF exerts a stimulatory effect on proliferation of osteoblastic cells, however, excess dosage may also result in reduced cell growth [86]. In addition, FGF's are also angiogenic factors stimulating revascularisation during bone healing [12, 65].

Both platelet derived growth factors (PDGF's) and insulin growth factors (IGF's) stimulate osteogenic cell proliferation [65]. PDGF's have been detected in osteogenic cells during fracture repair and are thought to play an important role in the regenerative process [12]. IGF's have also been shown to participate in fracture repair and bone formation [12].

Cell therapy approaches for bone reconstruction

The solution to the problems associated with bone replacement may lie in the creation of a vital autologous bone substitute using patient own osteogenic cells in association with a biomaterial. The biomaterial besides of providing volume, will function both as a carrier for the transplanted cells/tissue and as a scaffold for the formation of new bone tissue. The goal is, therefore, to develop an alternative to the traditional autologous bone graft that achieves similar success in bone regeneration, but without the limitations inherent to autologous grafting. Although an extra surgical procedure will still be needed to harvest the osteogenic cells, this will be much less invasive as compared to the collection of bone and it will not bear the post-operative complications associated with autologous bone grafting. Additionally, large quantities of osteogenic cells/tissue can be obtained from small biopsies after culture expansion. In this approach, factors such as cell source, cell proliferation and osteogenic differentiation, as well as the material scaffold are of extreme importance to successfully engineer bone tissue. With regard to cell source, various cell types from several tissues and locations have been investigated. These include calvarial [87-88] and periosteal cells [89-90], osteoblasts of trabecular bone from various locations [91-92], chondrocytes [93] and even vascular pericytes [94] and cells from extramedullary adipose tissue [95]. Nevertheless, the most widely used source of osteogenic cells is bone marrow and the rationale for its choice is both scientific and practical. Bone marrow has long been recognised as a source of osteoprogenitor cells that can differentiate towards bone forming cells when cultured under adequate conditions [96-99]. In addition, bone marrow has been claimed to be the most abundant source of osteoprogenitors, which possess high proliferative ability and great capacity for differentiation [100-101]. From a practical point of view, bone marrow is the most accessible source of osteogenic cells since it can be collected using a relatively simple aspiration procedure, which is much less invasive than collecting bone, cartilage or another type of tissue.

Bone marrow stromal cells (BMSCs)

Bone marrow is a complex tissue composed of two main cellular systems: hematopoietic and stromal. The stromal tissue consists of a network of cells with very little extracellular matrix that provides mechanical support for hematopoietic cells. The bone marrow stroma also expresses cell signalling factors that participate in the development of blood cells, while hematopoietic cells are also known to influence the activity of the stromal compartment [102-103]. The cell types comprising the stromal system include reticular cells, smooth muscle cells, endothelial cells, adipocytes and cells from the osteogenic lineage [104].

Friedenstein et al. [96] and Owen [98] performed pioneering studies in the characterisation of BMSCs using both in vitro culture systems and in vivo models. In these studies, when bone marrow stromal cells were plated in culture at low densities they readily adhered and formed fibroblastic colonies, each derived from a single precursor cell, the colony forming unit fibroblast (CFU-F) [105]. When marrow cells were plated at high densities, the colonies merge and the cells grew as monolayers. It has been demonstrated that CFU-F are heterogeneous in size (reflecting various growth rates), morphology and potential for differentiation, suggesting that they originate from progenitors at various stages of differentiation [98, 105]. The high proliferative ability of some of the CFU-F together with the known regenerative capacity of BMSCs led Friedenstein [96] to propose the existence of stromal stem cells that give rise to committed progenitors for different cell types. Stem cells were then defined as able to self-renew, multipotential and capable of regenerating tissue after injury [105]. This hypothesis was consistent with results from a study in which single colony derived mouse BMSCs were implanted on ectopic sites in syngeneic hosts. Approximately 15% of the implanted colonies produced bone, adipose and marrow reticular tissue with the establishment of hematopoiesis by host cells. Another 15% of the transplanted colonies formed bone without associated marrow and the rest either gave rise to fibrous tissue formation or did not form any tissue [104, for review]. This experiment suggested the existence, among the CFU-F population, of both multipotential cells and precursors with a more limited potential. Similar results were also obtained in a more recent study performed by Muraglia et al. [106] using clonal cultures of human BMSCs. Since the early studies from Friedenstein and Owen, numerous reports have provided evidence that bone marrow tissue contains progenitor cells that after extended culture, are capable of giving rise to several phenotypes, including adipocytic, chondrogenic and osteogenic lineages [106-109].

Osteogenic cell differentiation

With regard to osteogenic cell differentiation, the existence of a lineage hierarchy in which a multipotential precursor cell gives rise to cells with a more restricted potential and these ultimately originate monopotential progenitors has been proposed [107]. The osteogenic differentiation process may be characterised by the sequential acquisition and/or loss of specific extracellular matrix molecules and cell surface markers (fig. 1). Four maturational stages in osteoblast development have been identified in bone in situ: the preosteoblast, osteoblast, osteocyte and bone lining cell [6]. The preosteoblast is the immediate precursor of the osteoblast and it is localised in the adjacent cell layers from the bone producing osteoblasts. These cells possess alkaline phosphatase (ALP) activity and limited capacity for proliferation [6]. Osteoblasts are postproliferative cells with cuboidal morphology and strong ALP activity. These cells synthesise bone matrix proteins, some hormone receptors, cytokines and growth factors. Osteoblasts produce bone tissue and line the matrix at sites of active matrix production [4, 6]. Bone lining cells present a flat, thin and elongated morphology and are thought to be inactive osteoblasts [4, 6]. When osteoblasts become incorporated in the newly formed bone matrix they are termed osteocytes. These cells are considered the most mature stage of the osteoblastic lineage and present a decreased ALP activity as compared to osteoblasts [4, 6].

Expression of the kidney/bone/liver isoform of ALP is directly related with bone formation, and it is widely accepted that an increase in ALP activity in a population of osteogenic cells corresponds to a shift to a more differentiated state [6, 85, 95, 97, 110-112]. ALP is present in both preosteoblasts and osteoblasts and studies suggest that its expression is detected in differentiating osteoblastic cells preceding the expression of the non collagenous proteins [113]. Although the exact role of ALP is unknown, studies suggest that it is involved in the mineralisation process since an inhibition of ALP activity inhibits bone matrix mineralisation [6]. Collagen type I (coll-I) constitutes approximately 90% of the total organic matrix in bone and although synthesised by many cell types it is intensively produced by osteoblasts being, therefore, considered as a characteristic marker of the osteoblast phenotype [6, 97, 114-117]. This protein is also expressed in preosteoblasts [6]. Osteopontin (OPN) is synthesised by osteoblastic cells, however, it is also produced by many cells of non skeletal tissues [6]. On the contrary, bone sialoprotein (BSP) is almost exclusively produced by hypertrophic chondrocytes, preosteoblasts, osteoblasts and osteocytes [6]. In a recent study by Cooper et al. [117], it was suggested that the expression of BSP but not osteocalcin in human bone marrow stromal cell cultures preceded histological evidence of in vivo bone formation. Osteocalcin (OCN) or bone gla protein is undetectable in preosteoblasts but highly expressed in mature osteoblasts. This protein is considered the latest of the expression

markers along the process of osteogenic differentiation [6]. In addition to the bone matrix proteins mentioned, osteoblasts also secrete other proteins such as osteonectin, decorin and CD44 [6]. A wide list of hormones, growth and transcription factors have also been reported to regulate osteogenic activity and/or differentiation. Among those, osteogenic cells are known to possess receptors for parathyroid hormone (PTHrP, PTH-R1) and basic fibroblastic growth factor (FGFR-1) [6]. Additionally, the transcription factor *cbfa1* is known to play an important role in osteoblast development [118].

To better characterise and identify the osteogenic cell differentiation process in the bone marrow stromal cell system, the isolation of a subset of cells with the highest proliferative ability and great capacity for osteogenic differentiation would be of utmost importance. Although several monoclonal antibodies are reported to bind with BMSCs at early stages of differentiation, including SH2, SH3, SH4 [109, 119] and HOP-26 [120], the IgM monoclonal antibody Stro-1 is the most widely used [121-126]. It recognises a specific population of human BMSCs, in which osteoprogenitors appear to reside [121-122, 124]. Although within the stromal compartment there are cells with the Stro-1 epitope which are not CFU-F's, all detectable CFU-F's are exclusively present on the Stro-1 positive population [121]. Using this antibody in combination with an antibody against the kidney/bone/liver isoform of ALP it has been possible to identify osteogenic cells at three different stages of differentiation, supposedly stromal precursors, osteoprogenitors and mature osteoblasts [125]. In addition, the expression of the transcription factor *cbfa1* was found to be restricted to fractions expressing Stro-1 and/or ALP [125].

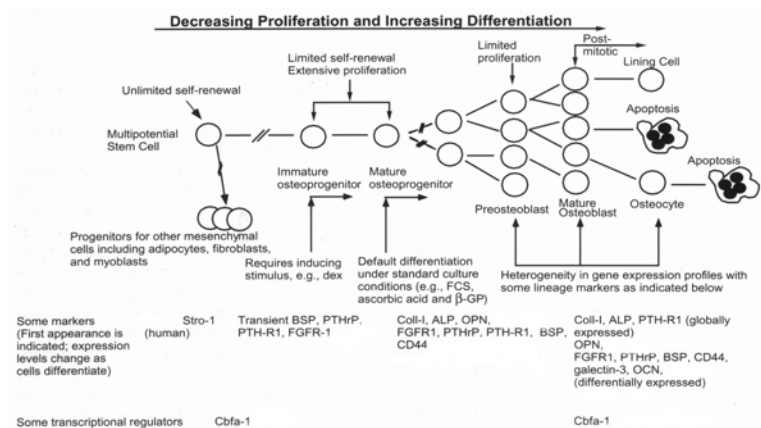


Figure 1 – Proposed steps in the osteoblastic lineage, implying recognisable stages of proliferation and differentiation.

Markers of the osteoblast phenotype and their expression during differentiation. Adapted from Aubin [107].

Plasticity of bone marrow stromal cells

As described above, bone marrow stromal cells can form various tissues, including bone, cartilage and fat. Another, extremely interesting characteristic of these cells is that they present a certain degree of plasticity with regard to lineage commitment. In 1991, Bennett et al. [127] showed that differentiated marrow adipocytes could differentiate *in vitro* back to a more proliferative stage and then form osteogenic tissue *in vivo*. Another example of cell commitment plasticity was reported by Galotto et al. [128], in a study in which fully differentiated chondrocytes have shown to dedifferentiate during culture and then, express the osteoblastic phenotype. These studies clearly reveal that, during culture, the lineage commitment of bone marrow stromal cells is reversible, whether this plasticity also occurs in an *in vivo* situation is still unclear.

Osteogenic character of BMSCs *in vitro*

In 1988 Maniopoulos et al. [97] cultured BMSCs from the femora of adult rats and reported that these cells differentiated along the osteogenic lineage, as revealed by their ability to form mineralised nodules in which the extracellular matrix was mainly composed of collagen type I and also contained osteonectin and osteocalcin. In addition, cells associated with the nodules exhibited high ALP activity. Since then, numerous studies have described the osteogenic character of BMSCs both from animal and human origin using similar criteria in defining osteogenic potential, that is, expression and/or synthesis of bone matrix proteins, ALP and capacity to form a mineralised tissue [85, 111, 112, 114-117, 129-133]. Nevertheless, the osteogenic character of the cultured cells and tissue has shown to be dependent on the culture conditions. The mostly widely known bioactive factors that have an influence the proliferation and differentiation of cultured bone marrow stromal cells are: serum, ascorbic acid, inorganic phosphate and glucocorticoids. The selected batch of serum added to the culture medium was shown to be extremely important for both the growth and osteogenic differentiation of BMSCs [134]. Ascorbic acid (vitamin C) was found essential for collagen synthesis and secretion. It also increases the levels of procollagen mRNA during culture [97, 111]. For mineralisation to occur, the culture medium must contain an inorganic source of phosphate which is normally obtained by the addition of sodium β -glycerophosphate to the culture medium [97, 111]. Glucocorticoids when administered *in vivo*, especially at high dosage, are known to suppress bone formation and stimulate bone resorption, inducing osteoporosis [135-136]. Nevertheless, they exert a powerful influence on BMSCs osteogenic differentiation during culture. Dexamethasone (dex) has been extensively reported to stimulate osteogenic differentiation in cultures of BMSC's from animal and human origin [85, 111, 115-116, 130-131, 137-141]. Signs of differentiation

induced by dexamethasone include morphological changes from an elongated to a more cuboidal cell shape [85, 111, 137, 139] and an increase in the expression and/or activity of ALP [111, 115-116, 130-131, 137-138, 140-141]. Additionally, this bioactive factor has been reported as essential for the mineralisation of BMSCs cultures [111, 115, 131, 137]. With regard to the effect of dexamethasone on the expression and/or synthesis of osteocalcin and osteopontin, both stimulatory [130, 140, 141] and inhibitory [115-116, 137] effects have been reported. These discrepancies may be a result of different culture conditions and experimental set-ups.

Several other biologically active factors such as BMP's, TGF's β , FGF's, PDGF's and IGF's are also known to affect the proliferation and/or osteogenic character of BMSCs (*see above: chemical stimulation of bone healing*).

Osteogenic character of BMSCs in vivo

Although the in vitro phenotype of BMSCs cultures provides valuable information on their osteogenic character, the behaviour of these cells after implantation gives the ultimate answer on whether these cells can form bone tissue. However, several factors may affect the outcome of the studies, such as species origin of the cells, culture conditions prior to implantation and implantation model. With regard to ectopic implantation models, both diffusion chambers and open systems have been used to test the osteogenic potential of BMSCs. Diffusion chambers allow for the diffusion of nutrients from the host but isolate the implanted cell population from invasion by recipient cells. As a consequence, vascularisation does not occur in the transplanted cells and the tissues formed are from donor origin [102]. In studies using cultured human BMSCs cultured without ascorbic acid and dex, both Haynesworth et al. [142] and Gundle et al. [92] reported the absence of bone or cartilage tissue after implantation in diffusion chambers in nude mice. Additionally, both types of tissue were detected when cells were cultured in the presence of ascorbic acid and dex prior to implantation [92]. Moreover, in the above mentioned study by Haynesworth et al. [142] in vivo bone formation was obtained, using the same cell preparations, when implantation was performed in an open system, using a porous calcium phosphate as a scaffold material. These results suggest that open systems are more sensitive in identifying the in vivo osteogenic potential of cells, which may be related to the lack of vascularisation in diffusion chambers.

Bone formation by rat BMSCs was widely investigated by subcutaneous implantation in nude mice or syngeneic hosts, using several porous calcium phosphate ceramics as biomaterial scaffolds [99, 143-147]. In this type of implants, bone formation was shown to start on the surface of the ceramic, advancing towards the centre of the pores. Ohgushi et

al. [143] reported bone formation in both HA and TCP ceramics combined with fresh bone marrow, after 4 weeks of implantation. At the end of 8 weeks survival, the extent of bone in the implants significantly increased and in some pores regeneration of bone marrow was detected. Yoshikawa et al. [144] also showed bone formation by cultured rat BMSCs on porous HA but only in samples where cells had been treated with dexamethasone. In this study, during the entire implantation period (1, 2, 3, 4 and 8 weeks) cartilage formation was not detected and therefore the process of bone formation was considered to be intramembranous. On the contrary, de Bruijn et al. [146] reported the formation of both bone and cartilaginous tissue by rat cells continuously cultured in the presence of dex, after 4 weeks of implantation. Nevertheless, cartilage like tissue was only found in samples with high cell seeding densities. Additionally, in a study by Dennis et al. [145], the culture of rat BMSCs in the presence of dex was not required to obtain in vivo formation of bone. Riley et al. [148] did report ectopic bone formation by rat BMSC cultured on poly(DL-lactic-co-glycolic acid) foams. Bone was formed as early as one week post implantation. Nevertheless, the maximum penetration of bone into the sponges was approximately 250 μ m after 4 weeks of implantation.

Mouse BMSC were also found to form bone and bone marrow when subcutaneously implanted in combination with a wide range of material scaffolds, such as collagen sponges and matrices, polyvinyl sponges and HA/TCP blocks and powder [149].

Rabbit BMSCs have demonstrated the capacity to produce bone tissue in ectopic sites when seeded both on calcium phosphate ceramics [150] and hyaluronic acid-based polymers [151].

Finally, the in vivo osteogenic potential of goat BMSCs cultured on porous HA has also been proven after subcutaneous implantation in immunodeficient mice. Results demonstrated that the ability of these cell populations to produce bone in vivo was not dependent on the presence of factors such as ascorbic acid, sodium β -glycerophosphate or dex in the culture medium [146].

With regard human BMSCs, several investigators have demonstrated the ability of these populations to form bone in ectopic sites [99, 142, 146, 149, 152-154]. Nevertheless, bone formation by human BMSCs did not consistently occur with all tested cultures. Ohgushi et al. [99] reported bone formation by human BMSC cultures loaded in porous HA from 2 of the 6 donors tested, after an implantation period of 4 weeks at subcutaneous sites in immunodeficient mice. In the same study, fresh human bone marrow from 5 of the 7 assessed donors exhibited in vivo osteogenic potential. In a similar study using cultured BMSCs from 11 donors, Haynesworth et al. [142] reported bone formation in most of the

biphasic calcium phosphate ceramics subcutaneously implanted with cells in nude mice. However, cultured cells from one of the donors did not form bone during the implantation periods tested (3 and 6 weeks). In studies by Krebsbach et al. [149] and Kuznetsov et al. [152], the in vivo osteogenic potential of cultured human BMSCs seeded on to various scaffold materials was tested. Cells seeded on calcium phosphate materials (hydroxyapatite/tricalcium phosphate powder and blocks) consistently formed bone, while cells seeded on collagen sponges or gelatin produced bone sporadically but only when cultured with dex. In addition, bone formation was never observed in polyvinyl sponges and poly (L-lactic acid). The capacity of human BMSCs to induce the formation of bone marrow like tissue was also established in some of the above mentioned studies [146-147, 149, 152].

In vivo ectopic osteogenesis, although providing valuable information on the osteogenic potential of the cells, does not simulate the microenvironment of an osseous site, which they will encounter if used in bone reconstruction. Few studies have used orthotopic (bone site) models for the implantation of culture expanded BMSCs. Porous HA/TCP scaffolds seeded with cultured BMSCs, from both rat [155] and human origin [156], were found to heal clinically relevant segmental bone defects in rat femora while defects filled with the scaffold alone did not heal. In those studies the extent of bone present on the implants was significantly increased by the presence of the cultured cells. Accordingly, in critical size segmental bone defects in dogs [157] union did not occur when the defects were left empty, while it was established in both defects filled with HA/TCP cylinders and HA/TCP cylinders loaded with cultured BMSCs. Nevertheless, the amount of bone present on the samples loaded with cells was significantly greater as compared to cell free implants. The use of cells seeded on calcium phosphate ceramics has also been reported to improve healing of critical size segmental bone defects in sheep [158-159].

Cell therapies for bone reconstruction: different strategies

At present, in the bone tissue engineering field three different strategies make use of patient own bone marrow cells to engineer autologous osteogenic grafts. One of these strategies consists in BMSC harvest, followed by cell seeding on a biomaterial scaffold and immediate implantation into the defect site (fig. 2, I); In other approach, the harvested cells are first culture expanded and then seeded on a suitable scaffold shortly before implantation (fig. 2, II). In the third strategy, after harvesting, the cell numbers are expanded in culture and, when a sufficient number of cells is obtained, they are seeded on a biomaterial scaffold, in which cells are further cultured to promote the formation of a bone-like tissue layer on the implant prior to implantation (fig. 2, III).

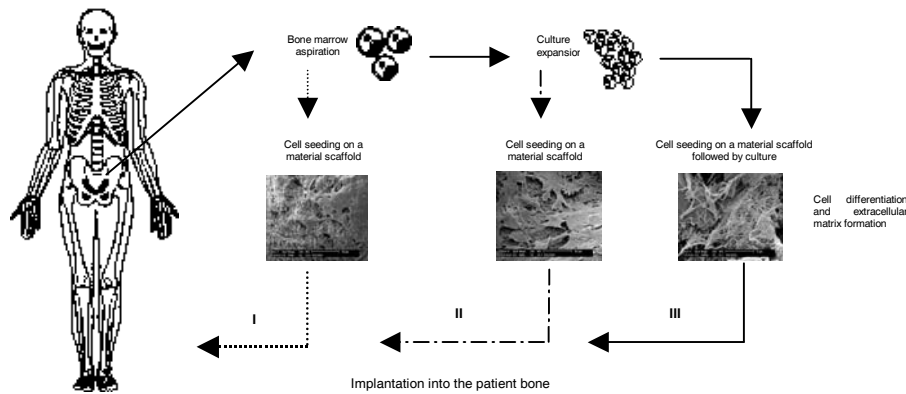


Figure 2 – Different strategies for bone tissue engineering.

The first above mentioned approach (implantation of the total bone marrow cell population) has clearly logistic advantages since it is possible to collect a bone marrow aspirate shortly before the reconstructive procedure takes place. The bone marrow sample is then seeded on the biomaterial scaffold that can be immediately implanted into the patient defect site. Nevertheless, with this strategy BMSC numbers will be limited and higher quantities of aspirate will be required, which besides of may posing a problem to the patient, it is known to increase contamination by peripheral blood and decrease the final concentration of osteoprogenitors in the sample [101]. Results from animal studies using this strategy are somewhat contradictory. For example, in an above mentioned study by Kandiyala et al. [155], using critical size segmental bone defects in rats femora, the addition of fresh bone marrow to the biomaterial implants did not induce differences in the rate and extent of bone formation as compared to the cell free implants, while being significantly lower than on implants seeded with culture expanded cells. Accordingly, Boden et al. [75] using a rabbit model reported that HA seeded with fresh bone marrow was not an acceptable bone graft substitute for posterolateral spine fusion. However, Louisia et al. [160] have reported that HA combined with fresh bone marrow was able to bridge osteoperiosteal gaps in rabbits after two months, while HA alone could not produce union.

With regard to the second strategy, several investigators have reported the ability of culture expanded BMSCs to form bone in ectopic sites when seeded in a biomaterial shortly before implantation [92, 99, 142-143, 145, 149-152]. In this approach, BMSCs are seeded on the biomaterials either in the presence or absence of fetal bovine serum. When serum free cell suspensions are used, investigators utilise proteins such as fibronectin and fibrin to stimulate cell adhesion to the biomaterial substrate [154, 158].

In vivo bone formation by hybrid constructs composed of biomaterial covered with a layer of in vitro formed bone-like tissue was also demonstrated in several studies [144, 146-148, 161]. This last approach appears to present some significant advantages since the cells have already started to produce bone matrix in vitro, which is expected to accelerate in vivo bone formation. In addition, the in vitro formed bone matrix may contain several proteins and growth factors that can enhance bone formation. To our knowledge, a study comparing the in vivo osteogenic potential of these two strategies has not yet been reported.

AIMS OF THE THESIS

The main goal of this thesis was to identify and optimise parameters that affect the osteogenic character of BMSCs, aiming at the application of these cells in the treatment of large bone defects. In such an approach the growth and differentiation characteristics of the cells, which are affected by external stimuli during culture, as well as the model design used for the construction of engineered tissue are of utmost importance. Additionally, the choice of the biomaterial scaffold that will support cell growth, differentiation and the formation of bone will affect the final osteogenic potential of the implants. Therefore, several studies were performed with the following objectives:

- (1) - To identify and test bioactive factors that affect the proliferation characteristics and osteogenic potential of human BMSCs, aiming to optimise in vitro culture conditions;
- (2) - To evaluate whether human BMSCs characteristics are dependent on the donor and, if so, to determine which donor related parameters influence the cultures both at an proliferation and differentiation level;
- (3) - To characterise the development of the osteogenic lineage during human BMSCs in vitro culture;
- (4) - To identify which features are displayed by human BMSCs during culture and which subset of cells would be determinant for bone formation after implantation;
- (5) - To characterise the role of the extracellular matrix formed by the cells during in vitro culture on the osteogenic capacity of the implants;
- (6) - To evaluate different biomaterials as scaffolds for bone tissue engineering.

In chapter 2, a preliminary study was set up to determine the effect of differentiation factors, added to the culture medium, on the capacity of the human cultures to produce bone after implantation. The effect of several growth factors on human BMSC

proliferation was assessed in chapter 3. In this chapter, phenotypic characterisation of the cultures was performed, as well as preliminary attempts to identify the distinct cell subpopulations present in culture. In chapter 4, the effect of dexamethasone on the in vivo bone forming capacity of human BMSCs was characterised in further detail and the influence of donor age on both proliferation and bone forming capacity was investigated. The temporal expression of bone cell related markers to identify subpopulations of cells at different stages of osteogenic maturation was assessed in chapter 5 and the results were related to the ability of the cultures to form bone after implantation into ectopic sites. In chapter 6, the problem of identification and quantification of early osteoprogenitors in human BMSC cultures was addressed and an experimental method was developed to quantify early osteoprogenitor cell numbers. The results were then related to the in vivo osteogenic potential of the cultures. In chapter 7, the role of the extracellular matrix present on the tissue engineered constructs prior to implantation was assessed with regard to in vivo bone formation and, in chapter 8, two biodegradable polymeric materials were evaluated as scaffold materials for bone tissue engineering. Finally, chapter 9 contains a general discussion and conclusions from the performed studies.

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CHAPTER 2

***A PRELIMINARY STUDY ON THE IN VIVO BONE FORMATION BY
HUMAN BONE MARROW STROMAL CELLS: EFFECT OF
OSTEOGENIC CULTURE SUPPLEMENTS***

A PRELIMINARY STUDY ON THE IN VIVO BONE FORMATION BY HUMAN BONE MARROW STROMAL CELLS: EFFECT OF OSTEOGENIC CULTURE SUPPLEMENTS

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Abstract

Bone marrow is known to contain a population of osteoprogenitor cells that can go through complete differentiation when cultured in medium containing appropriate bioactive factors. In this study, porous particles of a calcium phosphate material were seeded with second passage adult human bone stromal marrow cells (HBMSC). After an additional culture period of one week in the particles, the samples were subcutaneously implanted in nude mice for a period of 4 weeks. The cell seeding density used was 200,000 cells per particle and the cell culture system was designed to investigate the single and combined effects of dexamethasone and recombinant human bone morphogenetic protein 2 (rhBMP-2). After 4 weeks survival, the implants were processed for histology and the amount of de novo formed bone was quantified by histomorphometric techniques. The relative percentage of mineralised bone on the implants reached a maximal value of 19.8 ± 5.1 for samples in which cells were cultured in the presence of rhBMP-2. In this study, rhBMP-2 proved to be an essential bioactive factor to obtain in vivo bone formation by HBMSC. The results presented herein demonstrate the capacity of adult HBMSC to form bone after transplantation into an ectopic site.

Introduction

In bone reconstructive surgery, the repair of critical size bone defects is a major problem since the current therapies do not always provide an effective treatment. At present the use of autologous bone grafts is one of the most successful means of reconstruction. It avoids complications related with foreign body responses, while providing bioactive molecules and cells that will allow effective regeneration. However, orthopaedic surgeons face substantial problems: bone is only available in limited quantities and the harvest procedure has associated health risks such as donor site morbidity and pain. These drawbacks motivated research activities from which the bone tissue engineering technology has emerged. This approach aims at the treatment of bone defects without the limitations of the traditional therapies. Briefly, cells are obtained from a small bone marrow biopsy, expanded in vitro and then seeded onto a biomaterial specially designed for this purpose. Afterwards, the cells

are induced to follow osteogenic differentiation and finally transplanted into a patient bone defect to create new bone tissue.

The biomaterial to be used as scaffold for the cells and/or tissue must fulfil several requirements. It should be biocompatible and allow for the attachment of cells, providing an adequate environment for their proliferation and for the ingrowth of vascular tissue, ensuring the survival of the transplanted cells.

A suitable site to harvest osteogenic cells is bone marrow, as marrow tissue has long been recognised as a source of osteoprogenitor cells that can be induced to differentiate along the osteoblastic lineage, when cultured under conditions permissive for the osteogenic development [1-4]. Furthermore, it has been claimed that marrow tissue contains osteogenic cells with more proliferative ability and greater capacity for differentiation than those originated from other skeletal sites [5].

To date several investigators have demonstrated that cells grown from non human marrow sources can be induced to osteogenic differentiation in response to various bioactive factors including the synthetic glucocorticoid dexamethasone [1-2, 6-9] and rhBMP-2 [6, 10-11]. Moreover, it was found that dexamethasone enhances the effect of rhBMP-2 on the differentiation of rat bone marrow cells and rat calvaria cells [6,12-13]. A drawback from these studies, however, is that the results are difficult to extrapolate to humans. In addition, several in vivo experiments [14-16] indicate that only animal and non adult human bone marrow stromal cells are able to form bone tissue.

The current investigation was designed to study the effect of the osteogenic culture supplements, dexamethasone and rhBMP-2, on the in vivo bone formation capacity of adult human bone marrow stromal cells (HBMSC). After proliferation, the cells were further cultured for one week in a porous ceramic biomaterial to allow bone matrix formation and cell differentiation. Following this period, the samples were subcutaneously implanted into the back of nude mice for 4 weeks.

Materials and methods

Materials

Porous granules of coralline hydroxyapatite (Pro-Osteon 500) were obtained from Interpore. The interconnected pores had a median diameter of 435µm and the size of the implanted particles was approximately 3×2×2mm.

Human bone marrow stromal cell (HBMSC) isolation and culture

Cells were obtained from a 66-year-old female patient undergoing total hip arthroplasty. After the removal of the femoral head, cancellous bone plugs of approximately 1cm³ were removed and transported in cold culture medium. Prior to further processing, the marrow cells were isolated by placing the plugs in 50ml syringes, followed by repeated washing with culture medium until the bone plugs changed colour from red to whitish. The cell suspensions were passed through a 20G needle and then centrifuged, for 10 minutes, at 500g. The resulting cell pellet was resuspended in minimum essential medium (α -MEM) supplemented with 10% of foetal bovine serum (FBS) and antibiotics (culture medium) and finally plated in T75 flasks (one plug per flask). At near confluence, cells were enzymatically lifted from the flask using a 0,25% trypsin solution and counted. The cells were then concentrated by centrifugation at 500g, during 10 minutes, and the resulting pellet was resuspended in culture medium. Aliquots of 100 μ l of cell suspension containing 200,000 cells were seeded in Pro-Osteon particles, placed in 24 wells bacteriological grade plates. The cells were allowed to settle for 3 hours, after which an additional 2ml of culture medium, supplemented with 50 μ g/ml ascorbic acid and 10mM β -glycerophosphate, was added to each well. In order to evaluate the effect of osteogenic supplements, dexamethasone (dex, 10⁻⁸M) and/or rhBMP-2 (1 μ g/ml) were also added to the medium. The cells were cultured for seven days prior to implantation, to allow the production of an in vitro formed extracellular matrix. During that period the culture medium was refreshed once. The cell seeding density used for each condition was 200,000 cells per particle and triplicate samples were used per condition (n=3). In addition, control particles (without cells) were incubated for one week in the several culture media (n=3 per culture condition).

In vivo implantation

Prior to implantation, the samples with cells and controls were soaked in serum free medium and phosphate buffered solution, pre-warmed to 37°C. The nude mice were anaesthetised by an intramuscular injection of a mixture 2:6:7 of atropine (67 μ g/ml), xylazine (8mg/ml) and ketamine (46,7 μ g/ml). The surgical sites were cleaned with 70% ethanol and subcutaneous pockets were created in each side of the spine (two per side), in which the samples were implanted. At the end of the four weeks survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3.

Histological preparation

The fixed samples were dehydrated in increasing ethanol solutions and embedded in methyl methacrylate for sectioning. Approximately 10 μ m thick, undecalcified sections were processed on a histological diamond saw (Leiden microtome cutting system). The sections were stained with basic fuchsin and methylene blue, in order to study bone formation.

Histomorphometry

On all implants the percentage of de novo formed bone was determined using a computerised image analysis system (VIDAS). The percentage of bone formation was calculated as the total surface area of bone in relation to the total surface area of implanted ceramic material. Although this measuring technique is not optimal, in the way that the obtained absolute values do not give information about the amount of formed bone as compared to the amount of pores within the implant, it still provides a valid method to compare bone formation induced by the HBMSC cultured in several different conditions. Furthermore, it allows to measure not only the bone formed within the pores, but also bone formation on the outer surface of the implant.

Results and discussion

After four weeks of implantation, all the samples with cells grown in the presence of rhBMP-2 and dex or rhBMP-2 alone contained osteogenic tissue. Bone was composed of a mineralised matrix with embedded osteocyte cells and layers osteoblasts. For both conditions, ingrowth of vascular tissue was observed adjacent to bone. Moreover, bone marrow, which included blood vessels, fat and hematopoietic cells was also detected in these implants (fig. 1 a and b).

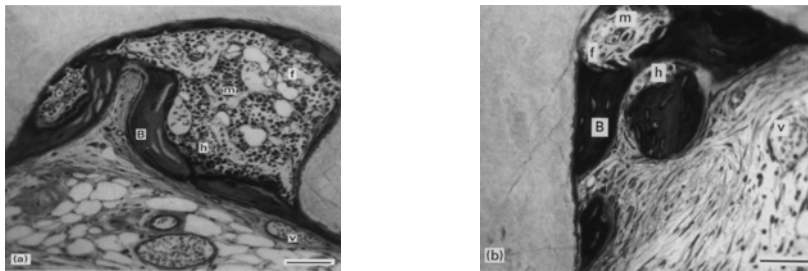


Figure 1 – Bone tissue formed by HBMSC cultured in the presence of dex and rhBMP-2 (a) and rhBMP-2 (b) after subcutaneous transplantation in nude mice for four weeks. New bone shows osteocytes embedded within the matrix (B) and surrounds a bone marrow cavity (m) containing hematopoietic tissue (h) and fat cells (f). Blood vessels (v) were frequently observed near to newly formed bone (bar = 50 μ m).

Control samples, devoid of cultured cells, soaked in medium containing dex and rhBMP-2, infrequently revealed traces of bone tissue. A very thin and discontinuous layer of bone was sometimes detected near to the implant surface (fig. 2). However, marrow tissue was never found and, as proved by the histomorphometric measurements (fig. 3), the amount of bone was substantially less as compared to implants with cultured cells.

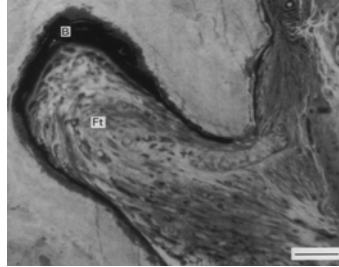


Figure 2 – Bone formed after transplantation of the ceramic material soaked for one week in medium containing rhBMP-2 and dex. The thin bone line (B) formed at the implant surface is surrounded by fibrous tissue (Ft) without bone marrow tissue formation (bar = 50 μ m).

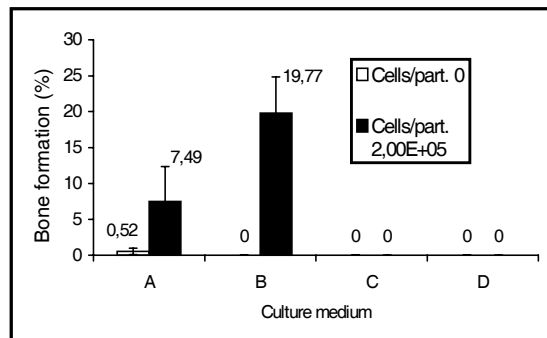


Figure 3 – Bone formation by adult HBMSC: effect of osteogenic supplements (A: +rhBMP-2 +dex; B: +rhBMP-2 –dex; C: –rhBMP-2 +dex; D: –rhBMP-2 –dex).

Several researchers [17-19] have reported ectopic bone formation by rhBMP-2 to which was associated the production of rich bone marrow. However, the concentrations of rhBMP-2 used on those studies were significantly higher than the concentration we used on our work. In this report, the lack of marrow tissue formation in control samples, soaked in medium with rhBMP-2 and dex, may be related to the very small amount of newly formed bone. Therefore this bone is not active enough to induce marrow production in the same time period. Interestingly, samples without cells soaked in medium containing rhBMP-2 but no dex, de novo bone formation was not detected. These findings indicate that the combination of the

two bioactive factors (rhBMP-2 and dex) results in synergetic mechanism with regard to bone induction.

In samples with cells cultured in control media (without rhBMP-2 and Dex) only fibrous tissue was observed (data not shown), revealing that the complete differentiation of osteoprogenitor cells in our system needed to be potentiated by bioactive factors. These findings are in agreement to those of several other authors [2, 17-18, 20-21] who reported rhBMP-2 to have a strong stimulatory effect on the osteogenic differentiation of bone marrow cells from animal and human origin.

In this study, dex appeared to potentiate the effect of rhBMP-2 for control samples (without cells), however, in samples containing cells, the presence of dex tended to decrease the extent of bone formation (fig. 3). These observations may indicate that the amount of bone forming cells was lower in samples cultured in the presence of dex, which can be due to a proliferation delaying effect caused by this factor over the cells. This also would explain the lack of bone formation on implants cultured in the presence of dex and absence of rhBMP-2. Although it has already been reported [22] the in vivo bone formation capacity of HBMSC when cultured in the presence of dex, the cell densities used in those studies were substantially higher. In addition, the cells were obtained from young patients, having therefore a much higher proliferative potential than the adult HBMSC that we describe in this report.

Conclusions

The ability of adult HBMSC to form bone tissue that supports hematopoiesis was established in this study. These results are encouraging and indicate the regenerative potential of tissue engineering technology for bone reconstruction.

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CHAPTER 3

***HUMAN BONE MARROW STROMAL CELLS FOR BONE TISSUE
ENGINEERING: IN VITRO AND IN VIVO CHARACTERISATION***

HUMAN BONE MARROW STROMAL CELLS FOR BONE TISSUE ENGINEERING: IN VITRO AND IN VIVO CHARACTERISATION

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Introduction

Autologous bone grafting is, currently, the standard and the most successful means for bone reconstruction. However, the limited amount of available bone and the donor site morbidity associated with this therapy has led to efforts to develop a bone tissue engineering technology. This approach, which enables the creation of a large autologous bone graft through the culture of a thin layer of bone on a biomaterial scaffold, is expected to address the needs of an increasing number of patients requiring large amounts of bone for skeletal reconstruction.

In the past 10 years, several authors reported bone marrow tissue as a rich source of progenitor stromal cells, which are capable of giving rise to several phenotypic lineages including fibroblastic, reticular, adipocytic, chondrogenic and osteogenic [1-7]. Although these precursor cells have been largely reported as stem cells [8-11], it is still unknown whether they are truly pluripotent and homogeneous or if they constitute subpopulations of cells committed to various lineages of differentiation. The osteoblast precursors in bone marrow are contained in a subpopulation of cells that, when cultured, possess the ability to proliferate and display a fibroblast like morphology [1].

With regard to bone formation, the development of osteoblastic cells from bone marrow stromal precursors, is characterised by a sequence of events involving cell proliferation, expression of bone related proteins (cell differentiation) and synthesis and deposition of a collagenous extracellular matrix [12-13]. The characterisation of these events would provide knowledge about the factors that rule the process and the stages at which external stimulation towards the osteogenic lineage may be implemented.

In the production of tissue-engineered implants, control of these events is essential for the success of the technique. With respect to the cell proliferation step, several growth factors may be used to increase cell proliferation rate, reducing the waiting period for the patient. During the differentiation step, the use of differentiation factors, such as dexamethasone, may also be advantageous since it has shown to stimulate osteogenic differentiation of bone marrow cells [14-15]. Finally, the existence of an extracellular matrix on such implants may be

advantageous to a rapid healing, since it contains a variety of bone related proteins [16-18] that might enhance the osteogenicity of the implant.

In the present investigation we studied the effect of several growth factors on the in vitro proliferation characteristic of human bone marrow stromal cells (HBMSC). Parallel studies were initiated, to identify HBMSC sub-populations by flow cytometry (fluorescence-activated cell sorting, FACS). Following the proliferation step, the cultured HBMSC were seeded and cultured, up to a week, in chamber slides and porous calcium phosphate particles. Immunofluorescence and RTPCR (reverse transcriptase polymerase chain reaction) techniques were used to examine the expression of bone related proteins. The production of extracellular matrix during this period was examined by scanning electron microscopy (SEM) and immunostaining against collagen type I.

With regard to the in vivo osteogenic potential, culture expanded HBMSC were seeded on porous calcium phosphate materials, further cultured for one week and then subcutaneously implanted in nude mice for six weeks. Finally, de novo bone formation was analysed and quantified.

Materials and methods

Human bone marrow stromal cell (HBMSC) collection and culture

Bone marrow aspirates (5-15ml) were obtained from fifteen patients that had given written informed consent. Donor information is summarised in Table 1. The bone marrow aspirates were mixed with minimum essential medium (α - MEM) containing 10% foetal bovine serum (FBS), antibiotics (AB) and 50U/ml heparin. Cells were re-suspended with a 20G needle, plated at a density of 500,000 nucleated cells per cm^2 and cultured in (unless stated otherwise) α - MEM, in which was added 10% FBS, antibiotics, and 0.2mM L-ascorbic acid 2-phosphate (AsAP) (control culture medium). Cells were grown at 37°C and in a humid atmosphere with 5% CO_2 . The culture medium was refreshed twice a week and at near confluence (usually 10-15 days) the adherent cells were washed with phosphate buffered saline solution (PBS) and enzymatically released by means of a 0.25% trypsin – EDTA solution. Subcultured cells were plated at a density of 5,000 cells per cm^2 and subsequent passages were performed when cells were at near confluence, usually 4-5 days later.

Table 1 – HBMSC donors information.

| Donor | Age | Sex | Source of bone marrow |
|-------|-----|-----|-----------------------|
| 1 | 69 | F | iliac crest |
| 2 | 74 | F | iliac crest |
| 3 | 66 | F | trochanter |
| 4 | 67 | F | acetabular fossa |
| 5 | 67 | M | acetabular fossa |
| 6 | 29 | F | acetabular fossa |
| 7 | 68 | F | iliac crest |
| 8 | 52 | M | acetabular fossa |
| 9 | 78 | M | iliac crest |
| 10 | 22 | F | iliac crest |
| 11 | 28 | F | iliac crest |
| 12 | 70 | F | trochanter |
| 13 | 39 | M | acetabular fossa |
| 14 | 52 | M | acetabular fossa |
| 15 | 35 | M | iliac crest |

F = female; M = male

In vitro studies

Effect of several growth factors on the HBMSC proliferation and morphology

Bone marrow cells from three donors were used for these experiments. The HBMSC were plated and cultured as described above but, for each donor, four different types of culture medium were used: the control medium and the same medium to which a growth factor was added. The studied growth factors were basic fibroblastic growth factor (bFGF, 1ng/ml), epidermal growth factor (EGF, 10ng/ml), transforming growth factor β 1 (TGF β 1, 10ng/ml) or β -mercaptoethanol (β ME, 5×10^{-5} M). The concentrations used were a result of either previous optimisation or literature findings [19-20]. When near confluence of one of the culture conditions was reached, all the cells were trypsinised and counted. Cells from one of the donors were also further studied until the first passage. During the entire in vitro period cells were regularly monitored by light microscopy for morphological evaluation.

Fluorescence-activated cell sorting (FACS) analysis of fresh bone marrow and culture expanded HBMSC

Bone marrow cells from three donors were used for these experiments. Fresh bone marrow, primary, first and second passage cells were analysed for each patient. Until first passage cells were cultured in medium containing bFGF, for second passage cells two culture conditions were used: (i) the cells were grown in medium containing bFGF (+ bFGF

medium) or (ii) the same medium to which 10^{-8} M dexamethasone was added (+ dex medium).

Cells were washed twice at 4°C in PBS containing 1% bovine serum albumin and 0.1% sodium azide. To block potential non-specific binding, the cells were incubated in wash buffer containing 5% FBS and 10% human serum, before antibody labelling. The primary antibodies used were against CD34 (IgG1); CD146 (IgG1k); CD166 (IgG1k); SH2 (IgG1, kindly provided by Prof. A. I. Caplan, Case Western University, Cleveland) and Stro-1 (IgM). CD34 was already labelled with PE. CD146 and CD166 were labelled with biotin and AVIDIN-FITC was used for their detection. SH2 was detected with goat anti-mouse FITC ($G\alpha M$ -FITC, IgG/M,) while Stro-1 was detected with goat anti-mouse IgM-FITC ($G\alpha M$ -IgM-FITC). Isotype-matched negative control antibodies were used to delineate the gated negative populations; IgG γ 2aFITCS- γ 1PE; IgM and IgM + $G\alpha M$ -IgM-FITC. In each step, cells were incubated for 25-30 minutes on ice and in the dark. After the final wash, cells were resuspended in buffer and analysed using a FACS Calibur apparatus (Becton Dickinson Immunocytometry systems).

The selection of antibodies was based on their reported reactivity with stromal progenitors. Although CD34, CD146 and CD166 are not specific for stromal precursors, several studies [21-23] demonstrated that, a portion of bone marrow cells that reacted with the above mentioned antibodies contained stromal precursors. In previous investigations [24-25], the SH2 monoclonal antibody was found to be reactive with epitopes on the surface of mesenchymal stem cells. Finally, the antibody Stro-1 has been widely reported to react with stromal precursors, and an association has been made between the expression of Stro-1, in fresh adult bone marrow, and the presence of cells with osteogenic potential [26-30].

Immunofluorescence analysis of culture expanded HBMSC

HBMSC from two donors were cultured in medium containing bFGF until their first passage. Second passage cells were then plated in chamber slides at a density of 5,000 cells/cm². Two culture conditions were then used: (i) + bFGF and (ii) + dex medium. At near confluence (4-6 days), cells were washed, fixed in a 4% solution of paraformaldehyde (PFA) and incubated for, at least, 30min in alcohol 70%. The staining procedure was identical to the one described for FACS analysis. Antibodies against alkaline phosphatase (AP, IgG); pro-collagen I (IgG); osteonectin (ON, IgG1); osteopontin (OP, IgG1k) and osteocalcin (OC, IgG3) were used. All antibodies were detected with Goat anti-mouse-FITC (IgG/M). In each step cells were incubated for 45-60 minutes on ice and in the dark, which was followed by intensive washing. After the final wash, the samples were mounted with an anti fade agent

(Molecular Probe) and analysed by immunofluorescence microscopy. FITC fluorescence was graded from none (-) to very high (+++++).

Expression of bone related proteins: reverse transcriptase polimerase chain reaction (RT-PCR) analysis

The expression of bone related proteins was determined for HBMSC obtained from one donor. First passage cells were seeded on porous hydroxyapatite (HA) particles at a density of 200,000 cells per particle. The granules of HA had interconnected pores with a median diameter of 435 μ m and their size was approximately 3x2x2mm. The cells were cultured for one week both in (i) +bFGF and (ii) + dex medium. At the end of 7 days in vitro culture the expression of parathyroid hormone receptor (PTHr), alkaline phosphatase (AP), osteopontin (OP), osteocalcin (OC) and receptor human bone morphogenetic protein 2 (rhBMP-2) was analysed. Total RNA was isolated from cells using Trizol. For each sample 1 μ g RNA was used in the reverse transcriptase reaction, in a 20 μ l mixture containing strand buffer, 0.05M DTT, 0.5mM dNTPs, 20U RNase inhibitor, 0.025 μ g/ml random prime and 20U superscript enzyme. The RT-PCR was performed in 50 μ l volume reaction mixture containing 10x PCR buffer, 1.5 or 2mM MgCl₂, 20pmol 5' and 3' primers, 0.2mM dNTPs and 1,25U Taq Gold polymerase. Optimisation of the number of cycles for each target was performed in previous experiments (unpublished data). The PCR products were visualised by ethidium bromide staining on a 1% agarose gel. For the semi-quantitative analysis the results of each target were divided by the expression of the housekeeping gene, β -actine, and expressed as a percentage of the positive control.

Extracellular matrix examination

HBMSC (five donors; passage 1-3) were seeded on porous granules of HA (mentioned above) and cultured for 7 days in medium with or without dex. After the in vitro culture period, the possible extracellular matrix formation was examined by scanning electron microscopy (SEM) and identified with immunostaining against collagen type I. For the SEM analysis, samples were fixed, dehydrated, gold coated and examined in a Philips S 525 microscope. Samples for immunostaining were fixed with a 4% PFA solution, placed in alcohol 70% and incubated in PBS containing, 0.1% natrium azide, 5% FBS and 10% human serum, before labelling. The primary antibody used was collagen I (Rb α H α Collagen I) and the second step consisted of G α Rb-IgG-HRPO . In each step, samples were incubated for 1h at room temperature, which was followed by intensive washing. Finally, the

cromogen diaminobenzidine (DAB) was added to the system for 3 min, after which time samples and controls were analysed on a stereo microscope.

In vivo studies

HBMSC (eight donors; passage 1-4) were seeded on porous HA granules (referred above), at a density of 100,000-500,000 cells/particle and cultured for one week prior to implantation. In some cases two culture conditions were used: (i) + bFGF and (ii) + dex medium, both with the addition of 0.01M of β -glycerophosphate (β GP). For other HBMSC cultures only the + dex condition was used, also with the addition of 0.01M of β GP.

Subcutaneous implantation

Prior to implantation, the samples were soaked in α - MEM, washed in PBS and subcutaneously implanted into nude mice for 6 weeks. Control samples incubated in both media, without cultured cells were also implanted. At the end of the survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3.

Histology and histomorphometry

The fixed samples were dehydrated and embedded in methyl methacrylate for sectioning. Approximately 10 μ m thick, undecalcified sections were processed on a histological diamond saw (Leiden microtome cutting system). The sections were stained with basic fuchsin and methylene blue, in order to study bone formation. Samples from three donors were further characterised by histomorphometry. The percentage of bone formation was calculated as the bone area related to the total pore area.

Results

In vitro studies

Effect of several growth factors on the HBMSC proliferation and morphology

After 7-10 days of primary culture, cell colonies could be detected in all conditions. Within these colonies, cells exhibited a fibroblastic shape. During this period, cell proliferation was strongly increased by the addition of bFGF (2.7-2.9 fold, depending on the patient) and EGF (1.7-2.8 fold, depending on the patient) (table 2). Although TGF β 1 stimulated cell growth,

this effect was only significant for one of the patients. The addition of β ME had no positive effect on cell growth (table 2). During first passage, the increase in cell proliferation due to the presence of growth factors, although present, was not so pronounced. In primary cultures, and with regard to cell morphology, clear differences between the several conditions were not detected. However, in first passage cultures, cells grown in the presence of bFGF and EGF maintained the fibroblast-like phenotype, whereas cells cultured in the presence of TGF β 1 became bigger and assumed a more flattened morphology (fig.1a and b). Cells grown in control medium, although still fibroblastic were not so thin and elongated as in primary culture, indicating a gradual lost of their original morphology.

Table 2 - Effect of different growth factors on proliferation of HBMSC.

| Growth medium | Primary culture | First passage |
|---------------|-----------------|----------------|
| Standard | 1x | 1x |
| bFGF | 2.7 - 2.9 | 2.2 |
| EGF | 1.7 - 2.8x | 2.1x |
| TGF β 1 | 1.2 - 2.1x | 1.2x |
| β ME | 0.67 - 1x | not determined |

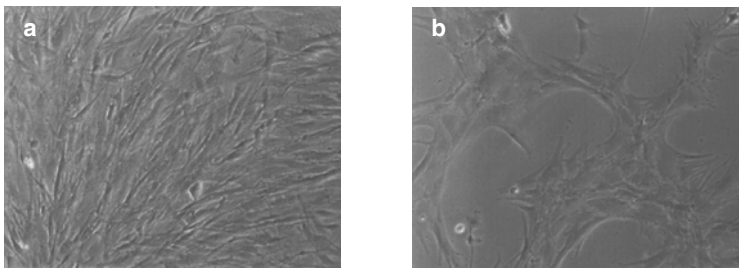


Figure 1 – First passage HBMSC cultured in standard medium with (a) bFGF (1ng/ml) or (b) TGF β 1 (10ng/ml).

Fluorescence-activated cell sorting (FACS) analysis of fresh bone marrow and culture expanded HBMSC

The selection of antibodies for these preliminary studies was based on their reported [21-30] reactivity with stromal progenitors. Table 3 summarises the characterisation performed on HBMSC from one representative patient during several culture periods.

Table 3 – Flow cytometry analysis of bone marrow and cultured HBMSC at several time periods (%).

| Anti-body | Bone marrow | Primary culture | 1 st passage | 2 nd passage (+bFGF) | 2 nd passage (+dex) |
|-----------|-------------|-----------------|-------------------------|---------------------------------|--------------------------------|
| CD34 | 13.8 | 0.01 | 0.71 | 0.28 | 0.67 |
| CD146 | 23.01 | 94.69 | 59.27 | 11.84 | 8.26 |
| CD166 | 15.78 | 97.57 | 99.99 | 99.31 | 99.49 |
| SH2 | 10.49 | 95.01 | 99.76 | 93.14 | 74.38 |
| Stro-1 | 16.66 | 35.73 | 15.28 | 6.56 | 4.16 |

Bone marrow mononucleated cells from all donors have shown to react with all assayed antibodies. In fresh bone marrow the percentage of CD34⁺ cells varied from 2-14%, however, irrespective of the patient, during culture the proportion of CD34⁺ cells was reduced to less than 2%.

With regard to the reactivity for CD146, 6.5-23% positive cells were present in bone marrow and, during culture the amount of reactive cells was increased. However, the addition of dex to the culture system consistently induced a decrease in the proportion of the CD146⁺ sub population.

The ability of cultured cells to bind with CD166 was nearly 100% (>93%) for all cases, and during the entire culture period. Furthermore, the presence or absence of dex in the growth medium was did not affect this reactivity.

More than 93% of all cultured cells stained for SH2, irrespective of the patient and culture period. However, this expression was reduced for cultures in which dex was added.

With respect to the monoclonal antibody Stro-1, and depending on the donor, 13-17 % Stro-1⁺ cells were present in bone marrow. For cultured cells the reactivity was found to be extremely dependent on the donor and time point (6.5-35.7%), and the addition of dex to the cultures resulted in a tendency to decrease the amount of reactive cells.

Immunofluorescence analysis of culture expanded HBMSC

First passage cells from two donors were seeded on chamber slides and further cultured until near confluency (4-6 days) in medium with and without dex. As shown in figure 2, irrespective of the culture medium or donor, cells exhibited very high binding to the antibodies pro-collagen I (PCI) and osteonectin (ON). None of the culture conditions showed reactivity with alkaline phosphatase (AP) or osteocalcin (OC) antibodies (table 4). Osteopontin (OP) expression was detected in all cultures, however, the intensity of expression was dependent on the culture conditions and donor. Cells from both donors, cultured in medium without dex, exhibited low reactivity to this bone protein (++), but while in

cells from one donor, the presence of dex decrease the OP expression, in cells from the other donor the reactivity became moderate (fig. 3).

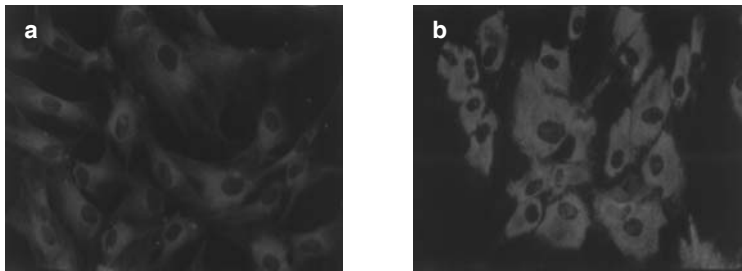


Figure 2 – Expression of (a) pro-collagen I and (b) osteonectin antigens in cultured HBMSC (400x).

Table 4 – Immunoreactivity of cultured HBMSC with bone related antibodies (results obtained from 2 donors).

| Anti-body | Female; 67 years old | | Male; 67 years old | |
|-----------|----------------------|--------------|--------------------|--------------|
| | +bFGF medium | + dex medium | +bFGF medium | + dex medium |
| AP | - | - | - | - |
| PCI | +++++ | +++++ | +++++ | +++++ |
| ON | +++++ | +++++ | +++++ | +++++ |
| OP | ++ | + | ++ | +++ |
| OC | - | - | - | - |

From none (-) to very high (+++++)

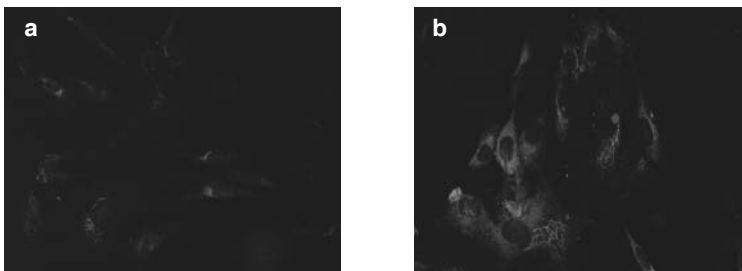


Figure 3 – Immunoreactivity of HBMSC with osteopontin, when cultured in medium (a) with and (b) without dex.

Expression of bone related proteins: reverse transcriptase polimerase chain reaction (RT-PCR) analysis

In order to assess the degree of differentiation of HBMSC previous to implantation, the cells loaded on the calcium phosphate materials and further cultured for one week were analysed through RT-PCR. Cells grown in medium without dex were negative for AP and osteocalcin while cells cultured in + dex medium exhibited RNA for all targets studied (fig. 4). The relative expression of PTHr was substantially higher in the + dex condition, while levels of OP and rhBMP-2 RNA, did not differ much in both culture media. It is worth noting that,

although during the immunocytochemical studies the cultures did not exhibit reactivity to AP or OC, RT-PCR revealed that for this culture, AP and OC mRNA were present in the + dex condition.

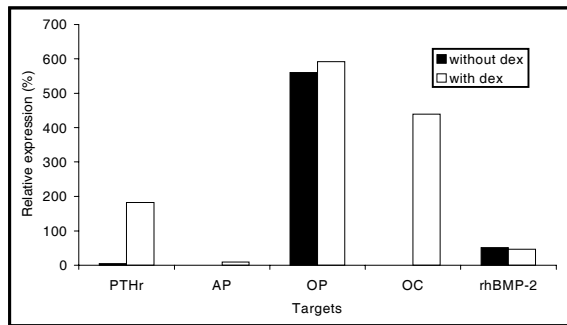


Figure 4 – Relative mRNA levels for HBMSC seeded on CaP particles and grown in medium with and without dex for a week.

Extracellular matrix examination

SEM examination of the cultured samples revealed no substantial differences between the two culture conditions. At the end of the culture period, the material surfaces were covered with multilayered structures of cells embedded within extracellular matrix (fig. 5). The abundant presence of collagen I was proved by an intense reactivity of these samples with a collagen I antibody (fig. 6), indicating that they were not biomaterials with isolated cultured cells, but hybrid constructs of 'material/cultured tissue'.

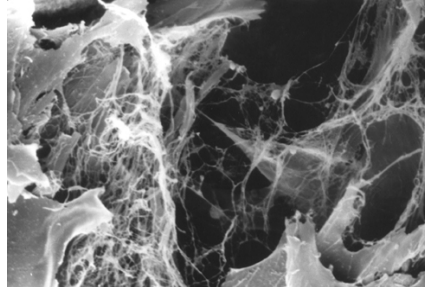


Figure 5 – Scanning electron micrograph illustrating the presence of collagen fibers on the hybrid constructs (1000x).

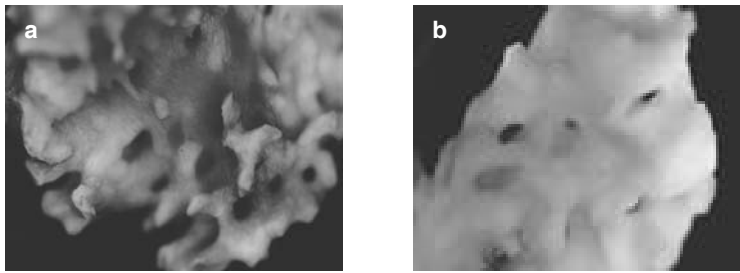


Figure 6 – Light micrographs of HBMSC cultured on porous calcium phosphate particles and stained with (a) collagen type I antibody or (b) secondary HRPO antibody and DAB (control) (49x).

In vivo studies

Histology and histomorphometry

Implants from six of the eight donors induced de novo bone formation, after 6 weeks of subcutaneous implantation in nude mice (table 5). In these implants, bone was formed in all samples with cultured cells, regardless of the culture medium. Within this donor population age, sex and the passage of the seeded cells had no obvious influence on bone formation.

Table 5 – Osteogenesis by HBMSC.

| Bone formation after subcutaneous implantation | | | | | |
|--|-----|-----------|-------------------|----------------|-------|
| Age | Sex | Passage # | Seeding density * | Culture medium | |
| | | | | - dex | + dex |
| 52 | M | 1 | 100,000 | 7/7 | 7/7 |
| 78 | M | 2 | 500,000 | 4/4 | 4/4 |
| 52 | M | 3 | 100,000 | 0/4 | 0/4 |
| 35 | M | 1 | 100,000 | 0/4 | 0/4 |
| 22 | F | 4 | 200,000 | ND | 3/3 |
| 28 | F | 3 | 200,000 | ND | 3/3 |
| 70 | F | 3 | 200,000 | ND | 6/6 |
| 39 | M | 1 | 200,000 | ND | 6/6 |

*per porous calcium phosphate particle, 3x2x2mm, surface area approximately 0.2-0.3 cm²

ND, not determined

De novo formed bone was deposited against the walls of the carrier material and it comprised osteocytes embedded within the bone matrix and a continuous layer of osteoblasts (fig. 7). Ingrowth of vascular tissue was observed adjacent to bone, providing the metabolic requirements of the new tissue. In some of the implants, areas of hematopoietic tissue were observed, closely associated with bone (fig. 8). However, the

development and extent of a marrow cavity were not always correlated with the abundance of osteogenic tissue. Control samples, soaked in both culture conditions but without cells, exhibited abundant growth of fibrous tissue with no signs of bone.

With regard to the extent of osteogenesis, a variable degree of bone formation was observed, depending on the donor. Quantification of the newly formed bone was performed for 3 donors (fig. 9). Although the presence of dex in the culture medium was not essential for bone formation, samples cultured in the presence of dex exhibited a higher degree of osteogenesis. However, this difference only was proved to be statistically significant for one patient.



Figure 7 – Bone formation by HBMSC after subcutaneous implantation. New bone (B) is formed on the surface of the porous calcium phosphate material (CaP). Arrows designate embedded osteocytes and arrow head the layer of osteoblasts; (a) 100x and (b) 200x.

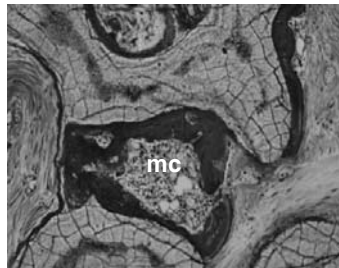


Figure 8 – Marrow cavity (mc) in close association with the newly formed bone, 100x.

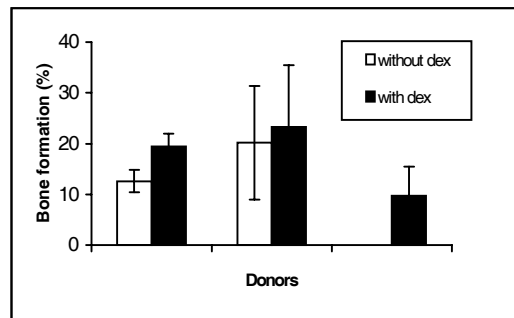


Figure 9 – Extent of bone formation by HBMSC and effect of culture medium.

Discussion

The construction and implantation of a living bone equivalent, using patient own cultured bone in a biomaterial scaffold would provide an innovative and efficient approach for the treatment of large bone defects. Many investigations [10, 20, 31-36] have already shown that cultured adult HBMSC possess *in vitro* and *in vivo* osteogenic potential. In view of these results, in the near future we may predict that the bone tissue engineering therapy will play a major role in bone reconstruction. However, before it can be used in clinical practice, the technology needs to be optimised and standardised, in order to induce consistent bone formation for every patient and reproducibility in the degree of osteogenesis. To achieve these goals, the harvest procedure must be performed in a standard and optimal fashion, supplying a biopsy with the necessary proportion of osteoprogenitor cells. Care should also be taken on the effect of prescribed drugs over the osteogenic potential of the patients' bone marrow. With regard to the culture technology, growth conditions have to be optimised, bone marrow and cultured cells should be characterised to obtain information about the amount of osteoprogenitor cells in the starting population and the factors that rule their complete differentiation into bone forming cells. In an attempt to optimise culture conditions during the proliferation step, we investigated the effect of several growth factors on the proliferation of HBMSC. Our results suggested that, although bFGF, EGF and TGF- β 1 actually participated in the proliferation mechanisms of these cells, bFGF and EGF were the most active in promoting cell growth and in maintaining their fibroblastic like morphology. These findings are in agreement with a recent report by Martin et al.[20], which demonstrates that bFGF and EGF are potent mitogen for HBMSC, particularly during primary culture. With respect to the use of β ME to promote cell growth our data, contrary to the previous report by Triffitt et al [19], indicated no positive effect.

In this report monoclonal antibodies were used to identify subpopulations that would contain osteoprogenitor cells and to monitor their differentiation. The CD34 and CD166 antibodies did not provide any information over the amount of osteoprogenitors in culture, at least when used in single staining procedures. HBMSC reactivity for CD34 was basically lost during culture and the ability of cells to bind with CD166 was always higher than 93% irrespective of the presence or absence of dex in the culture medium. These results indicate that CD166 binds not only to stromal precursors but also to cells that are already in the process of differentiation to a certain lineage. The reactivity of culture expanded cells to CD146, SH2 and Stro-1 tended to decrease for cultures in which dex was added. Although more detailed and wider studies have to be performed to draw conclusions, this loss of expression could be due to the maturation of osteoprogenitors into more differentiated cells. During culture Stro-1 appears to be more selective to detect stromal precursors as compared to CD146 and SH2, since the proportion of Stro-1⁺ cells was always significantly lower. Although SH2 has been reported as a monoclonal antibody directed against mesenchymal stem cells [25], our results show that more than 93% of the cultured cells express SH2 antigen, indicating that SH2 binds to a broader cell population and not exclusively to undifferentiated stem cells. However, the effect of dex in culture was most noted by this antibody, leading to decrease of the SH2⁺ cell population.

The sequential expression of bone proteins depends on the differentiation stage of the producing cells. Several immunoreactivity and RT-PCR studies were performed in order to determine the differentiation stage of HBMSC before implantation. The results demonstrated that cultures were immunoreactive for early markers of the osteoblastic phenotype (PCI, ON and OP). However, neither AP nor OC were functionally active in these cultures. The lack of AP expression is consistent with reports indicating that OP expression precedes that of AP during osteoblast differentiation [37-38]. SEM and immunostaining against collagen I revealed that the tissue engineered implants consisted of cells embedded in an extracellular matrix rich in collagen I. Taken together, these findings indicate that the implanted cells were committed osteoprogenitors, in the process of differentiation towards mature osteoblasts. The RT-PCR data also support this line of thought, since high levels of OC RNA, the only protein specific for mature osteoblasts [39] were detected in the + dex culture. The fact that this culture expressed both high levels of OC mRNA and relatively high levels of PTHr, may indicate the presence of two cell subpopulations in the beginning of the culture: osteoprogenitor cells stimulated further by dex into the osteogenic lineage and also undifferentiated cells recruited by dex into the early stages of differentiation. It was already suggested [33] that the bone forming cells in human marrow were divided into two compartments: undifferentiated cells and committed osteoprogenitors.

Concerning *in vivo* bone formation, 75% of the assayed donors possessed HBMSC with *in vivo* osteogenic potential. In such cases, results have shown that the presence of dex was not a mandatory requirement to obtain *de novo* bone formation, also indicating that the HBMSC population contains a subset of osteoprogenitor cells already committed to the osteogenic lineage. However, when dex was added to the system the extent of bone formation tended to increase. These findings, also in agreement with the RT-PCR results, indicate that dex induces committed osteoprogenitor cells to a further stage of differentiation, leading to an earlier start of bone formation. Moreover, it may recruit undifferentiated cells into the osteoblastic lineage, increasing the number of bone forming cells. HBMSC from two donors failed to induce bone formation after subcutaneous implantation and, it is likely, that these results may be related with an initial bone marrow cell population containing a reduced amount of osteoprogenitor cells, enhancing once again the importance of a standard and optimal biopsy procedure.

In summary, the obtained results demonstrate the potential of the bone tissue engineering technology, in which a living bone equivalent is produced. The engineered implants, constituted by a biomaterial with cultured cells and matrix proved to have *in vivo* osteogenic potential. However, their degree of osteogenicity was dependent on the donor and culture conditions. Experiments to identify and later isolate the actual osteoprogenitor cells within the HBMSC population are also being planned, in order to ensure reproducibility in both osteogenic potential and degree of bone formation.

Acknowledgments

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CHAPTER 4

***BONE TISSUE ENGINEERED IMPLANTS USING HUMAN BONE
Marrow Stromal Cells: Effect of Culture Conditions
AND DONOR AGE***

BONE TISSUE ENGINEERED IMPLANTS USING HUMAN BONE MARROW STROMAL CELLS: EFFECT OF CULTURE CONDITIONS AND DONOR AGE

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Abstract

At present, it is well known that populations of human bone marrow stromal cells (HBMSC) can differentiate into osteoblasts and produce bone. However, the amount of cells with osteogenic potential that is ultimately obtained will still be dependent on both patient physiological status and culture system. In addition, to use a cell therapy approach in orthopaedics, large cell numbers will be required and, as a result, knowledge of the factors affecting the growth kinetics of these cells is needed. In the present study, we analysed both the effect of dexamethasone stimulation on the in vivo bone tissue formation by HBMSC, as well as its influence on donor variability with regard to the extent of osteogenesis. Furthermore, the effect of donor age on the growth rate of the cultures and on their ability to form bone was investigated. In 67% of the assayed patients (8/12), the presence of dexamethasone in culture was not required to obtain in vivo bone tissue formation. However, in cultures without bone forming ability or with a low degree of osteogenesis, dexamethasone increased the bone forming capacity of the cells. During cellular proliferation, a significant age related decrease was observed in the growth rate of cells from donors older than 50 years as compared to younger donors. With regard to the effect of donor age on in vivo bone formation, HBMSC from several donors in all age groups proved to possess in vivo osteogenic potential, indicating that the use of cell therapy in the repair of bone defects can be applicable irrespective of patient age. However, the increase in donor age significantly decreased the frequency of cases in which bone formation was observed.

Introduction

Several synthetic materials are currently available to treat bone defects. However, their therapeutic potential depends on the presence of a sufficient amount of osteoprogenitor cells in the defect site. Therefore, the effectiveness of such implants, especially in large bone defects, may be compromised unless they contain a biological, preferably patient own, component that will provide metabolic activity and biological integration. The construction of a living, autologous bone equivalent using patient own bone cells cultured in a biomaterial

scaffold would provide an innovative and efficient therapy for bone reconstruction. To produce the tissue engineered implants, a suitable site to harvest bone precursor cells is bone marrow, as marrow tissue has been recognised as a rich source of osteoprogenitor cells that can be induced to differentiate along the osteoblastic lineage [1-3]. Furthermore, it was also reported that cell populations from marrow contain osteoprogenitors with more proliferative ability and greater capacity for differentiation than those originated from other skeletal sites [4]. Several investigators have shown that human bone marrow stromal cells (HBMSC) possess *in vivo* bone forming potential when cultured on several biomaterial substrates [5-10]. However, in order to use cell therapy in the repair of bone defects, reproducibility in bone formation and amount of osteogenesis has to be achieved. The definition and optimisation of the culture conditions are of extreme importance and dependent on the harvested bone marrow stromal cell population. One controversial question regarding the use of these cells in clinical applications is whether the harvested precursor cells represent a homogenous population of undifferentiated progenitors or a mixture of cells at different stages of differentiation [6, 8, 11-12]. Osteogenesis involves the recruitment of osteoprogenitor cells, their proliferation and differentiation into bone forming osteoblasts. Several investigators already reported that the treatment of HBMSC cultures with the synthetic glucocorticoid dexamethasone promotes a shift towards osteogenic differentiation *in vitro* [11, 13-15]. Furthermore, in cultures of rat stromal bone marrow cells, dexamethasone was found to be essential for the recruitment and differentiation of osteoprogenitor cells [1]. With regard to HBMSC, various studies have shown that stimulation by dexamethasone was not always required to obtain *in vivo* osteogenesis [8, 10]. However, whether stimulation by this factor will increase the reproducibility of the results with regard to occurrence and degree of bone formation has not been investigated.

Although the production of tissue engineered implants represents an important advance in skeletal tissue repair, extensive *in vitro* expansion is necessary to obtain a sufficient number of cells. Therefore, the effect of patient related parameters, such as age, on the growth kinetics of the cultures needs to be further investigated. Furthermore, age may also affect the bone forming capacity of the cells, and therefore of the implants. It is well known that the process of skeletal aging is associated with a progressive reduction in bone mass and that fracture healing is faster in younger than in older patients [16]. However, the influence of age on the growth properties and osteogenic potential of HBMSC has not been clearly established in humans. In literature, and with regard to growth kinetics, there is a discrepancy among studies, with some reporting an age related decrease [17-20] and others that find no effect of donor age on the proliferation rate of HBMSC [21-23]. Furthermore, it

has also been reported that increasing age is associated with alterations in bone protein expression by HBMSC [24].

In the present study we investigated the effect of dexamethasone stimulation on the in vivo osteogenic potential of HBMSC. After a proliferation step, the cells were seeded and cultured on porous calcium phosphate scaffolds for one week, and then subcutaneously implanted in nude mice for six weeks, in order to evaluate their in vivo bone forming ability. Furthermore, the effect of donor age on the proliferation rate of the cultures and their ability to induce in vivo bone formation was studied.

Materials and methods

Human bone marrow stromal cell (HBMSC) harvest and culture

Bone marrow aspirates (5 -20ml) were obtained from 53 patients that had given written informed consent. Donor information and aspiration sites are summarised in table 1. The bone marrow specimens were mixed with minimum essential medium (α - MEM, Life Technologies, The Netherlands) containing 10% of a selected batch of foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics (AB) and 50U/ml heparin. Cells were re-suspended with a 20G needle, plated at a density of 500,000 nucleated cells per cm^2 and cultured in α - MEM containing 10% FBS, AB, 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands) and 1ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C and in a humid atmosphere with 5% CO_2 . The culture medium was refreshed twice a week and at near confluence the adherent cells were washed with phosphate buffered saline solution and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands). Cells were plated at a density of 5,000 cells per cm^2 and subsequent passages were performed when cells were near confluence (80-90%).

Scaffold material

Porous granules of coralline hydroxyapatite (HA, Pro-Osteon 500, Interpore) with an average surface area of 0.2 – 0.3 cm^2 were used as scaffold material. The interconnected pores had a median diameter of 435 μm and the size of the implanted particles was approximately 3x2x2mm.

Effect of culture medium on in vivo bone formation

HBMSC from 12 donors (1-12; passages 1-6) were seeded on the porous HA granules at a density of 100,000 – 250,000 cells/particle. Prior to implantation, the cells were cultured for a week in two different conditions: (i) α - MEM containing 10% FBS, AB, 0.2mM AsAP and 0.01M β -glycerophosphate (β GP, Sigma, The Netherlands) (- dex medium) and (ii) the same medium with the addition of 10^{-8} M dexamethasone (dex, Sigma, The Netherlands) (+ dex medium).

Effect of donor age on the growth rate of HBMSC

The multiplication rate of HBMSC from 36 donors (2-4, 12-28, 30-36, 38-45, 53) was determined based on different passages (P0 to P3, that is, the cumulative cell numbers of the populations were plotted against time in culture to determine the growth kinetics during expansion).

Effect of donor age on the in vivo osteogenic potential of HBMSC

HBMSC from all the 53 donors were tested. When third passage cells became near confluent, they were trypsinised, seeded on the porous HA scaffolds, at a density of 200,000 cells per particle and further cultured for one week in (+) dex medium. Following this period, the samples were subcutaneously implanted in nude mice for 6 weeks.

In vivo implantation

Prior to implantation, tissue engineered samples from donors 1-53 were soaked in serum free medium and then washed in phosphate buffered solution pre-warmed to 37°C. The nude mice (HsdCpb:NMRI-nu, Harlan, The Netherlands) were anaesthetised by an intramuscular injection of a mixture containing atropine, xylazine and ketamine. The surgical sites were cleaned with ethanol and subcutaneous pockets were created, in which the samples were inserted. At the end of the six-week survival period, the implants (n = 2 to 6 per condition) were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3.

Table 1 – HBMSC donor information and source of bone marrow.

| Donor | Source of bone marrow | Gender | Age | | | | |
|-------|-----------------------|--------|-----|----|------------------|---|----|
| | | | | 30 | Acetabular fossa | F | 42 |
| 1 | Iliac crest | F | 17 | 31 | Iliac crest | M | 75 |
| 2 | Trochanter | F | 43 | 32 | Iliac crest | M | 75 |
| 3 | Trochanter | F | 26 | 33 | Iliac crest | F | 74 |
| 4 | Trochanter | F | 81 | 34 | Spine | M | 44 |
| 5 | Acetabular fossa | F | 60 | 35 | Spine | M | 44 |
| 6 | Iliac crest | F | 71 | 36 | Iliac crest | F | 69 |
| 7 | Iliac crest | F | 28 | 37 | Iliac crest | F | 81 |
| 8 | Iliac crest | F | 30 | 38 | Iliac crest | M | 74 |
| 9 | Unknown | F | 61 | 39 | Iliac crest | M | 61 |
| 10 | Acetabular fossa | F | 67 | 40 | Iliac crest | F | 70 |
| 11 | Acetabular fossa | M | 67 | 41 | Acetabular fossa | M | 86 |
| 12 | Acetabular fossa | F | 37 | 42 | Acetabular fossa | M | 57 |
| 13 | Acetabular fossa | F | 41 | 43 | Iliac crest | F | 51 |
| 14 | Trochanter | F | 70 | 44 | Iliac crest | M | 45 |
| 15 | Iliac crest | F | 56 | 45 | Iliac crest | F | 39 |
| 16 | Femora | F | 66 | 46 | Acetabular fossa | F | 41 |
| 17 | Unknown | M | 54 | 47 | Iliac crest | F | 72 |
| 18 | Iliac crest | F | 80 | 48 | Trochanter | F | 82 |
| 19 | Iliac crest | F | 73 | 49 | Iliac crest | F | 33 |
| 20 | Trochanter | F | 70 | 50 | Iliac crest | F | 59 |
| 21 | Unknown | M | 47 | 51 | Trochanter | M | 61 |
| 22 | Acetabular fossa | F | 76 | 52 | Acetabular fossa | F | 70 |
| 23 | Iliac crest | M | 45 | 53 | Acetabular fossa | F | 56 |
| 24 | Trochanter | F | 63 | | | | |
| 25 | Acetabular fossa | F | 75 | | | | |
| 26 | Acetabular fossa | F | 66 | | | | |
| 27 | Acetabular fossa | F | 59 | | | | |
| 28 | Femora | F | 74 | | | | |
| 29 | Iliac crest | F | 68 | | | | |

F = female M = male

Histology

The fixed samples were dehydrated and embedded in methyl methacrylate or decalcified, dehydrated and embedded in glycol methacrylate. The sections were processed on a histological diamond saw (Leica SP1600, Leica, Germany) or on a microtome (Microm HM3555, Microm GmbH, Germany) and then stained with a 0.3% basic fuchsin solution and/or a 1% methylene blue solution in order to study bone formation. In samples from donors 1 to 12 osteogenesis was blindly semi-quantified by three independent investigators. The following scale was used: (-) no bone formation, (+) traces of bone tissue were found in few sections, (++) bone tissue occupied a small part of each section or of some sections, (+++) bone occupied a significant part of each section, but less than half of the available pore area, (+++++) bone tissue spread over more than half of the pore area.

Statistics

Statistical analysis was performed using unpaired t student tests. Statistical significance was defined as $p < 0.05$.

Results

Effect of culture medium on in vivo bone formation

Three hours after cell seeding on the porous scaffolds, the HBMSC were already attached to the scaffold material and cell spreading had began (fig. 1a). Irrespective of the presence or absence of dexamethasone (dex) in culture, at the end of the in vitro period the material surfaces were completely covered with cell multi-layers, indicating that the implanted samples were 'biomaterial/cultured tissue' hybrids (fig. 1b).

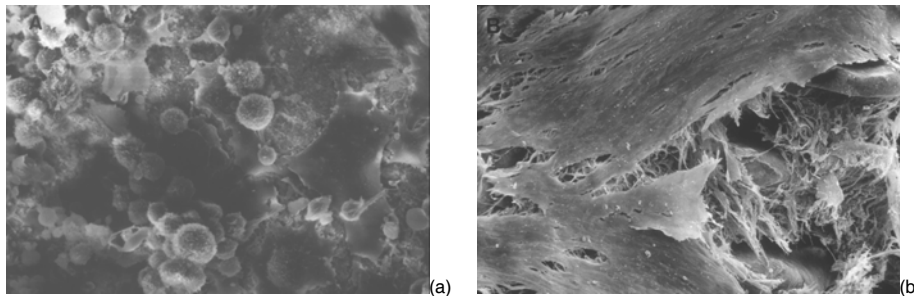


Figure 1 – Scanning electron micrograph of HBMSC grown on porous hydroxyapatite particles for a period of (a) 3 hours (500x) and (b) 7 days (100x). Cell seeding density was 200,000 cells/particle.

With regard to the *in vivo* osteogenic capacity of these constructs, the results revealed that stimulation by dex was not required in HBMSC from eight of the twelve patients assayed (table 2).

Table 2 – *In vivo* bone formation capacity by HBMSC and influence of the culture medium.

| In vivo bone formation | | | | | Culture medium | |
|------------------------|--------|-----|------------------|-----------|----------------|-------|
| Donor | Gender | Age | Seeding density* | Passage # | - dex | + dex |
| 1 | F | 17 | 200,000 | 4 | 4/4 | 4/4 |
| 2 | F | 43 | 200,000 | 6 | 3/3 | 3/3 |
| 3 | F | 26 | 200,000 | 5 | 3/3 | 3/3 |
| 4 | F | 81 | 100,000 | 2 | 4/4 | 4/4 |
| 5 | F | 60 | 100,000 | 3 | 0/2 | 2/2 |
| 6 | F | 71 | 250,000 | 4 | 0/4 | 4/4 |
| 7 | F | 28 | 200,000 | 2 | 0/6 | 6/6 |
| 8 | F | 30 | 200,000 | 3 | 0/4 | 4/4 |
| 9 | F | 61 | 200,000 | 3 | 3/6 | 6/6 |
| 10 | F | 67 | 200,000 | 2 | 6/6 | 6/6 |
| 11 | M | 67 | 200,000 | 2 | 6/6 | 6/6 |
| 12 | F | 37 | 200,000 | 4 | 6/6 | 6/6 |

* Per porous HA particle

F = female M = male

After six weeks of subcutaneous implantation in nude mice, samples with cells cultured either in (–) dex or (+) dex medium, exhibited *de novo* formed bone in direct apposition to the ceramic surfaces. Bone tissue was composed of a mineralised matrix with embedded osteocytes and layers of osteoblasts lining the outer edges of the newly formed bone (fig. 2). Bone formation appeared to progress towards the centre of the pores as osteoblast layers deposited new bone onto already formed bone. In some implants, and for both culture conditions, bone marrow tissue which included blood vessels, fat and hematopoietic cells, was also observed (fig. 3). Fibrous and vascular tissue occupied the remaining pore area of the implants. HBMSC from four of the twelve donors did not induce *in vivo* osteogenesis unless cultured in the presence of dex (table 2). No correlation could be found between the lack of bone formation by cells cultured in the absence of dex and donor age, passage number or seeding density.

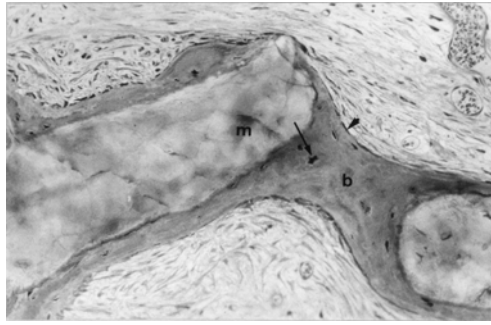


Figure 2 – Representative light micrograph illustrating in vivo formed bone by HBMSC after subcutaneous implantation in nude mice for 6 weeks. De novo formed bone tissue (b) was deposited against the material (m) surfaces. This tissue consisted of a mineralised matrix with embedded osteocytes (arrow) and layers of osteoblasts (arrow head), (200x).

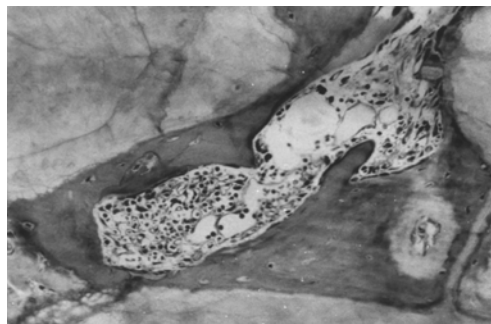


Figure 3 – Light micrograph illustrating bone marrow tissue formed after subcutaneous implantation of tissue engineered samples containing cultured HBMSC. Bone marrow was frequently found surrounded by the newly formed bone tissue, (200x).

With regard to the extent of bone formation, the degree of osteogenesis was strongly dependent on the donor and, in some cases, affected by the culture conditions (table 3). The addition of the differentiation factor, dex, to the culture medium did not affect the amount of newly formed bone by cultures with already high bone forming ability (bone formation score: +++ or higher). However, in samples without bone forming capacity or with a low degree of osteogenesis (- to ++) the addition of dex to the culture medium increased their bone forming capacity, also increasing the reproducibility in the degree of bone formation from patient to patient. Bone tissue was never observed in any of the control samples. These samples consisted of material, without cultured cells, soaked in (+) dex medium for one week.

Table 3 – Extent of bone formation by HBMSC and effect of the culture medium.

| Donor | Extent of bone formation | |
|-------|--------------------------|-------|
| | Culture medium | |
| | - dex | + dex |
| 1 | ++++ | ++++ |
| 2 | +++ | +++ |
| 3 | +++ | +++ |
| 4 | ++ | +++ |
| 5 | - | + |
| 6 | - | + |
| 7 | - | ++ |
| 8 | - | ++ |
| 9 | + | ++ |
| 10 | +++ | +++ |
| 11 | ++ | +++ |
| 12 | ++ | +++ |

The following scale was used: (-) no bone formation, (+) traces of bone tissue were found in few sections, (++) bone tissue occupied a small part of each section or of some sections, (+++) bone occupied a significant part of each section, but less than half of the available pore area, (++++) bone tissue spread over more than half of the pore area.

Effect of donor age on the proliferation rate of HBMSC

Depending on the donor, primary cultures reached confluency between 8 and 20 days of culture. At this point, the amount of cell colonies per cm² varied widely from patient to patient. Within these colonies, cells exhibited a thin and elongated morphology (fig. 4). First passage cultures became near confluent after 3 to 13 days of culture, exhibiting average doubling periods between 1.3 and 7.0 days. Such wide variations in growth rate of HBMSC from different patients were present during the entire growth period. In an attempt to examine whether donor age would affect the growth rate of HBMSC, and therefore contribute to the large variations observed, the patients were divided into five age groups: < 41, 41-50, 51-60, 61-70 and 71-86 years old and the slope of the exponential growth curve was determined for each patient. Figure 5 illustrates the HBMSC proliferation characteristics as a function of age. An age related decrease was observed in the growth rate of cells from donors older than 50 years as compared to younger patients ($p < 0.05$).

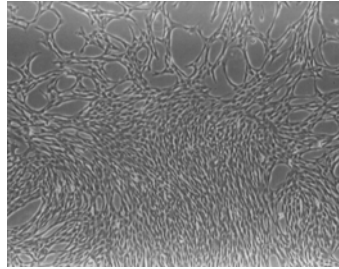


Figure 4 – Representative light micrograph illustrating the morphological appearance of primary HBMSC cultures when near confluent. Cells grew in colonies and within these colonies exhibited a thin and elongated morphology. (40x).

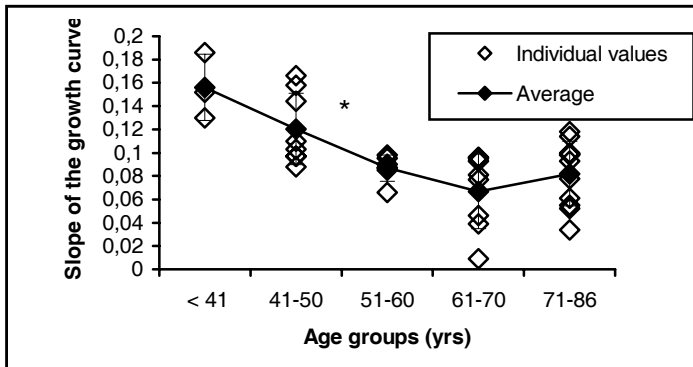


Figure 5 – Growth characteristics of HBMSC as a function of age. *Statistical decrease in the proliferation rate of HBMSC from donors older than 50 years ($p=0.003$).

Effect of donor age on the in vivo osteogenic potential of HBMSC

HBMSC from several donors in all age groups proved to possess in vivo osteogenic potential in the nude mice model, revealing that the use of cell therapy in the repair of bone defects can be applicable irrespective of the patient age. However, as illustrated in figure 6, the increase in donor age significantly decreased the frequency of cases in which the bone tissue engineering approach was not successful, especially after the age of 50 years. HBMSC cultures from all the patients with age inferior to 41 years, had in vivo osteogenic potential. In donors with ages between 41 and 50 years, the frequency of cultures that had the ability to form bone was 67%, while for patients between 51 and 70 years in vivo cell osteogenicity was found in 50% of the cases. Above 70 years the success rate decreased again to 46.7% of the tested donors.

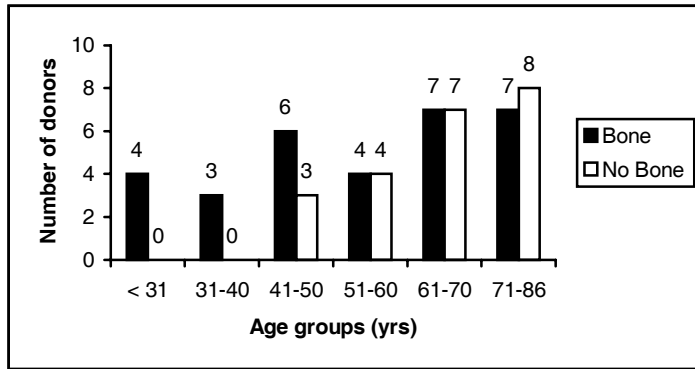


Figure 6 – Effect of donor age on the *in vivo* osteogenic character of HBMSC. Results obtained after six weeks of subcutaneous implantation of the tissue engineered samples in nude mice.

Discussion

The extensive research in the field of bone tissue engineering is leading to the development of an efficient approach to reconstruct large bone defects. The *in vivo* osteogenic potential of adult HBMSC cultured on porous ceramic materials has already been reported [7-10]. However, this potential and the degree of *in vivo* bone formation, besides of strongly dependent on the patient itself, it can be affected by the culture medium composition. In addition, to produce a large autologous bone equivalent, a large number of HBMSC is needed. Thus, the growth kinetics of the cultures, as well as the effect of donor related parameters, such as age, on the growth characteristics need to be established. With regard to the effect of the culture medium, our data revealed that in 67% of the assayed patients, the presence of dex in culture was not required to obtain *in vivo* bone formation by HBMSC. These findings are in agreement with those reported by Martin et. al. [12] and suggest, as proposed Kuznetsov et. al. [8], that the HBMSC population contains subpopulations of both committed osteoprogenitors and undifferentiated cells. Since the relative amounts of these subpopulations appear to vary widely from patient to patient, the use of dex in the culture medium may be advisable to ensure that a sufficient number of HBMSC will differentiate towards the osteoblastic lineage. In addition, dex appeared to contribute to a higher reproducibility in the degree of bone formation from donor to donor, increasing the extent of osteogenesis in samples with low ability to induce bone tissue formation.

In this study, and in agreement with others [20, 23], we reported a donor variation in the growth properties and osteogenic potential of HBMSC. With regard to the growth characteristics, an age related decrease in the proliferation rate was observed for patients

older than 50 years. Although, in a recent report by Phinney et al. [23], no age related effect could be detected on the growth rate of HBMSC, our results do not conflict since in that study the age range investigated was from 19 to 45 years, where we also did not detect statistical differences in cell growth. Several investigators have shown [25-26] that for a given period of time, one proliferative cell from a young donor has the same number of progeny as a proliferative cell from an old patient, therefore the age related decrease found in this study is probably related to a decrease in the number of proliferative precursors present in bone marrow as age increases. This hypothesis is in agreement with findings reported by Bab et. al. [17], in which colony forming unit fibroblasts (CFU-F) from human bone marrow also exhibited an age related decrease.

With regard to the effect of donor age on the *in vivo* osteogenic potential of HBMSC, the results revealed that the bone tissue engineering approach presented herein can be applicable to patients in all age ranges. However, the increase of age especially above 50 years, resulted in a decrease in the success rate of the technology. These findings also point out a reduction in the amount of cells with osteogenic potential in bone marrow, as age increases. Our results agree with findings from animal studies [26] and from reports in humans [20, 25], in which the number of HBMSC colonies expressing alkaline phosphatase decreased during aging. However, it should be noted that the bone marrow aspiration method was already reported to affect the osteoprogenitor cell content of the bone marrow populations [23, 27-28], therefore, in older patients, an optimisation of the aspiration procedure may increase the success rate of the approach. With regard to the nude mice model used in this study to determine cells osteogenicity, although widely accepted [7-10, 29] lacks the capacity to determine the osteogenic potential of the cultures just prior to their implantation in the patient. Consequently, the development of new analysis methods that will allow to predict *in vitro*, and in the early stages of proliferation, the performance of the engineered implant in an *in vivo* situation are of extreme importance. Such method is currently under investigation in our group and is expected to substantially increase the reproducibility of bone formation by allowing to detect cultures with low osteogenic potential, indicating the need for a second biopsy procedure or for the use of e.g. bone growth factors in the culture medium to enhance the osteogenicity of cells.

Conclusions

In these investigations effort was placed on the optimisation of the bone tissue engineering technology by analysing the effect of several donor and culture related variables on the HBMSC proliferation and *in vivo* bone formation. Our data indicated that, with adequate

stimuli it is possible to produce in vitro an implant capable of forming bone tissue in a in vivo situation, revealing a promising future for the autologous cultured tissue therapies in bone reconstruction. Although age proved to be an important factor for the osteogenic character of HBMSC, in vivo bone formation was obtained with patients in all age groups, proving that the present approach is also applicable to elderly patients.

Acknowledgments

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CHAPTER 5

***TEMPORAL EXPRESSION OF STRO-1, ALKALINE PHOSPHATASE
AND OSTEOCALCIN IN CULTURES OF WHOLE HUMAN BONE
MARROW DURING DIFFERENTIATION***

TEMPORAL EXPRESSION OF STRO-1, ALKALINE PHOSPHATASE AND OSTEOCALCIN IN CULTURES OF WHOLE HUMAN BONE MARROW DURING DIFFERENTIATION

S.C. Mendes, J.M. Tibbe, M. Veenhof, S. Both, J.D. de Bruijn and C.A. van Blitterswijk

Abstract

The differentiation of osteogenic cells from their precursors in human bone marrow stromal cell (HBMSC) cultures may be characterised by the sequential acquisition and/or loss of specific bone related markers. The focus of this study was to evaluate the osteogenic potential of HBMSC by analysing the expression of bone cell markers during culture. In addition, the *in vitro* cell differentiation pattern was related to the *in vivo* osteogenic potential of the cultures based on a nude mice model. To determine the developmental stage of cells during culture, they were screened for both Stro-1 and alkaline phosphatase (ALP) expression through a dual labelling procedure using flow cytometry (FACS). The effect of dexamethasone (dex) stimulation on the expression of both markers was also determined, as well as its influence on the growth rate of the cultures. Reverse transcriptase polymerase chain reaction (RT-PCR) was used to evaluate ALP and osteocalcin (OC) mRNA levels during osteogenic differentiation. The temporal pattern of Stro-1 expression showed an initial increase during the preconfluent period, followed by a progressive decline. With respect to ALP expression, the fraction of ALP positive cells increased during culture reaching a maximum value between day 7 and day 9. The results further demonstrated that stimulation by dex induced an increase in the Stro-1 positive fraction in sub and near confluent cultures, while it consistently increased the proportion of ALP positive cells during the entire culture period. The effect of dex on the growth rate of cells was evaluated both in sub and confluent cultures. Results did not show a significant effect of this factor on the growth kinetics of HBMSC. Gene expression of both ALP and OC was detected in (+) dex cultures, throughout the entire tested period. With regard to the *in vivo* results, HBMSC cultures from 4 of the 5 studied donors possessed *in vivo* osteogenic potential, revealing a good agreement between *in vitro* and *in vivo* data. However, although *in vitro* data also indicated osteogenic character, HBMSC cultures from donor 2 did not form bone, indicating the need to define a minimal amount of osteogenic cells required for *in vivo* bone formation and, therefore, the importance of developing methods that allow the quantification of the osteogenic cell fraction in the total cell population.

Introduction

The reconstruction of large bone defects and the revision of hip implants with bone loss are common problems in the orthopaedic field. In such cases, the traditional therapies involve the use of autologous or allogeneic bone, both of which present serious drawbacks. The creation of an autologous bone filler, through the use of cultured, patient own, osteogenic cells in association with a biocompatible material scaffold may provide an alternative approach to solve these problems.

Cells of the osteoblast lineage control the normal growth, development and remodelling of the skeleton. With respect to bone remodelling, this process continuously occurs throughout adult life and, as osteoblasts have a relatively short life span, the existence of precursor cells with great potential for proliferation and further differentiation was postulated [1]. During the last decade, bone marrow tissue has been extensively reported as a source of precursor cells with potential to differentiate into several phenotypes, including the fibroblastic, chondrogenic, adipocytic and osteogenic lineages [1-8]. It is still debatable whether these reports point out to the existence of homogeneous, pluripotent cells with the ability for self-renewal or to the existence of subpopulations of precursor cells committed to several lineages of differentiation [9-10]. The osteogenic potential of bone marrow is attributed to a small population of cells termed colony forming units fibroblast (CFU-F). These cells, when cultured, present a high capacity for proliferation and generate colonies of cells with a fibroblast-like morphology [1, 11-12]. To acquire a better understanding on the differentiation mechanism of osteoprogenitor cells, several studies focused on the immuno-isolation of CFU-F from freshly harvested bone marrow and/or from cultures of human bone marrow stromal cells (HBMSC) [8,12-20]. Although several monoclonal antibodies are reported to bind with cells in marrow stromal colonies, at early stages of differentiation, the IgM monoclonal antibody Stro-1 is the most widely used. It recognizes a cell surface antigen present on a small population of HBMSC that contains virtually all CFU-F [13, 15, 18-22]. Using this antibody and the bone/liver/ kidney isoform of the enzyme alkaline phosphatase, an early marker for cells of the osteoblast phenotype, it was possible to isolate and identify osteogenic cells at different stages of differentiation [18]. However, such isolation studies, although significantly reducing the heterogeneity of the cell population, pose the problem of a restricted availability of source material, especially when considering the use of those cells in bone tissue repair and regeneration. Another approach to reduce the heterogeneity in bone marrow consists in the selection of highly proliferative cells by successive culture and subculture in conditions that promote cell proliferation but not further differentiation. Basic fibroblast growth factor (bFGF) was shown to stimulate the expansion of osteogenic

precursors, while maintaining the osteogenicity of the expanded cells [10, 23]. When a sufficient number of cells is obtained, differentiation may be activated by the use of bioactive factors, such as dexamethasone or bone morphogenetic proteins, which were reported to stimulate osteogenic differentiation of HBMSC [24-32]. However, when considering the use of a cell therapy in bone reconstruction is of extreme importance to investigate the effect of these factors not only on cell differentiation but also on growth kinetics. With regard to the influence of dexamethasone in the growth rate of HBMSC, the published data conflict, with some investigators reporting a stimulatory effect [23-24] while others observe inhibition of cell growth [28,33].

In the present study, HBMSC were grown in conditions promoting cell proliferation until their third passage. Following this period, the cells were trypsinised, reseeded and stimulated to differentiate along the osteogenic lineage. The temporal expression of the developmental markers Stro-1 and ALP was screened during culture by flow cytometry. In addition, the mRNA levels of ALP and osteocalcin were also determined. During these investigations, the effect of dexamethasone on cell growth and differentiation was also evaluated. Finally, the in vivo osteogenic potential of the cultures, grown on porous calcium phosphate scaffolds, was evaluated through subcutaneous implantation in immunodeficient mice.

Materials and methods

Human bone marrow stromal cell (HBMSC) harvest and culture

Bone marrow aspirates (10 -20ml) were obtained from 5 patients that had given written informed consent. Donor information and bone marrow aspiration site are summarised in table 1. The bone marrow specimens were collected in heparinised tubes and transported at room temperature. Cells were re-suspended with a 20G needle, plated at a density of 500,000 nucleated cells per cm² and cultured in minimum essential medium (α - MEM, Life Technologies, The Netherlands) containing 10% foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics (AB), 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands) and 1ng/ml basic fibroblast growth factor (bFGF, InstruChemie, The Netherlands). Cells were grown at 37°C and in a humid atmosphere with 5% CO₂. The culture medium was refreshed twice a week and, at near confluence, the adherent cells were washed with phosphate buffered saline solution and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands). Cells were plated at a density of 5,000 cells per cm² and subsequent passages were performed when cells were near confluence (80-90%).

Table 1 – HBMSC donor information and bone marrow aspiration site.

| Donor | Source of bone marrow | Gender | Age |
|-------|-----------------------|--------|-----|
| 1 | Iliac crest | F | 69 |
| 2 | Acetabular fossa | F | 72 |
| 3 | Iliac crest | F | 70 |
| 4 | Iliac crest | M | 45 |
| 5 | Iliac crest | F | 39 |

F = female M = male

Scaffold material

Porous granules of coralline hydroxyapatite (HA, Pro Osteon 500, Interpore) with an average surface area of 0.2 – 0.3cm² were used as scaffold material. The interconnected pores had a median diameter of 435µm and the size of the particles was approximately 3x2x2mm.

Antibodies

Both Stro-1 monoclonal antibody and the purified anti-ALP (hybridoma B4-78) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA). The control mouse immunoglobulin M (IgM) and G (IgG2a) monoclonal antibodies were obtained from Dako (Denmark). The secondary antibodies goat anti-mouse IgM µ-chain-specific-FITC and rabbit anti-mouse IgG γ-chain-specific-PE were purchased from Zymed (The Netherlands).

Temporal expression of the developmental markers Stro-1 and ALP (flow cytometry)

Fourth passage HBMSC were plated at a density of 5,000 cells per cm² and cultured for 8 to 9 days in two different media: (i) α - MEM containing 10% FBS, AB, 0.2mM AsAP and 0.01M β-glycerophosphate (βGP, Sigma, The Netherlands) (control medium) and (ii) control medium with the addition of 10⁻⁸ M dexamethasone (dex, Sigma, The Netherlands) (+ dex medium). The dual expression of Stro-1 and ALP was evaluated by flow cytometry at several culture periods (from day 1 to day 9). Briefly, after trypsinisation, cells were washed twice at 4°C in PBS containing 1% bovine serum albumin and 0.1% natrium azide (wash buffer). Before antibody labelling, cells were resuspended in PBS containing 5% BSA and 10% human serum and incubated for 30 minutes on ice to block potential non-specific binding. Cells (approx. 0.1-0.3E6 / staining) were then resuspended in blocking buffer containing: (a) control mouse anti-human IgM (1:50 dilution) and control mouse anti-human IgG2a (1:50 dilution); (b) Stro-1 supernatant (1:2 dilution) and control mouse anti-human IgG2a; (c) anti-ALP monoclonal antibody (1:50 dilution) and mouse anti-human IgM; (d) Stro-1 supernatant and anti-ALP monoclonal antibody. Cells were incubated on ice for 45

minutes and then washed twice. Antibody reactivity was detected by suspending the cells with blocking buffer containing goat anti-mouse IgM μ -chain-specific-FITC (1:100 dilution) and rabbit anti-mouse IgG γ -chain-specific-PE (1:100 dilution). Cells were incubated on ice and in the dark for 30 minutes. After washing, cells were resuspended in 200 μ l of FACS-flow/staining and analysed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry systems). For each measurement 10,000 events were collected.

Expression of bone related proteins: reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Fourth passage cells were seeded on porous HA particles, at a density of 200,000 cells per particle, and further cultured for up to 9 days in (+) dex medium. The expression of alkaline phosphatase (ALP) and osteocalcin (OC) was evaluated at several time periods during culture. Total RNA was isolated from cells using Trizol (Sigma, The Netherlands). For each sample 1 μ g RNA was used in the reverse transcriptase reaction, in a 20 μ l mixture containing 5x strand RT-buffer (Life Technologies, The Netherlands), 0.05M dithiothreitol (DTT, Life Technologies, The Netherlands), 0.5mM dNTPs (Pharmacia, The Netherlands), 20U RNase inhibitor (Promega, The Netherlands), 0.025 μ g/ml random prime (Pharmacia, The Netherlands) and 20U superscript enzyme (Perkin Elmer, The Netherlands). The RT-PCR was performed in 50 μ l volume reaction mixture containing 10x PCR buffer, 1.5 or 2mM MgCl₂ (Perkin Elmer, The Netherlands), 20pmol 5' and 3' primers (Life Technologies, The Netherlands), 0.2mM dNTPs and 1,25U Taq Gold polymerase (Perkin Elmer, The Netherlands). Optimisation of the number of cycles for each target was performed in previous experiments (unpublished data). The PCR products were visualized by ethidium bromide (Life Technologies, The Netherlands) staining on a 1% agarose gel, using a Geldoc apparatus (Geldoc 2000). For the semi-quantitative analysis, the results of each target were divided by the expression of the housekeeping gene, β -actin, and expressed as a percentage of this gene.

Effect of dexamethasone (dex) on the growth rate of HBMSC

Fourth passage HBMSC were plated at a density of 5,000 cells per cm² and grown for 7 to 8 days in control and (+) dex medium. During growth, cell numbers were quantified after releasing the cells from the culture flasks by means of trypsin- EDTA digestion. The doubling period of the total cell population was determined for each measurement.

In vivo osteogenic potential of HBMSC

HBMSC (passage 4) were seeded on porous HA granules, at a density of 200,000 cells/particle and cultured for one week in (+) dex medium. Following this period, and prior to implantation, the tissue engineered samples were soaked in serum free medium and washed in phosphate buffered solution pre-warmed to 37°C. The immunodeficient mice (HsdCpb:NMRI-nu) were anaesthetised by an intramuscular injection of a mixture containing atropine, xylazine and ketamine. The surgical sites were cleaned with ethanol and subcutaneous pockets were created, in which the samples were implanted (each pocket contained three samples and from each donor samples were divided over two mice). At the end of the six-week survival period, the implants (n = 6 per donor) were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3. The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed undecalcified on a histological diamond saw (Leica SP1600, Leica, Germany) and then stained with a 0.3% basic fuchsin solution and a 1% methylene blue solution in order to study bone formation.

Statistics

Statistical analysis was performed using an unpaired t student tests. Statistical significance was defined as p (two tail) <0.05.

Results

Temporal expression of the developmental markers Stro-1 and ALP (flow cytometry)

Reactivity with Stro-1 antibody was detected in all HBMSC cultures, irrespective of the presence of dexamethasone (dex) in the culture medium. Additionally, in HBMSC grown in the presence of dex, Stro-1 expression initially increased during culture exhibiting a peak of expression between day 4 and day 7 (fig. 1a). Depending on the donor, the maximum of the Stro-1 positive fraction comprised 24.7 to 93.9% of the total cell population. As illustrated in figure 1a, the pattern of Stro-1 expression was similar between donors, although a wide donor variation was found in the range of individual values. This indicates that the proportion of Stro-1 positive cells in HBMSC cultures is extremely dependent on the donor. With regard to the effect of dex, our data revealed that this differentiation factor increased Stro-1 expression in sub- and near confluent cultures (fig. 1b). After HBMSC had reached confluency, the effect of dex on the proportion of Stro-1 positive cells was mainly donor dependent. In all HBMSC cultures, the fraction of ALP positive cells increased during culture reaching a maximum between day 7 and 9 (fig. 2a).

A wide donor variation was also detected in the expression of ALP, with the maximum of expression ranging from 39.4 and 78.7 % of the total cell population. After the first two days of culture, the proportion of ALP positive cells in the (+) dex condition was significantly higher as compared to the control ($p < 0.05$), revealing that dex stimulation induced an increase in the fraction of committed osteoprogenitor cells (fig. 2b).

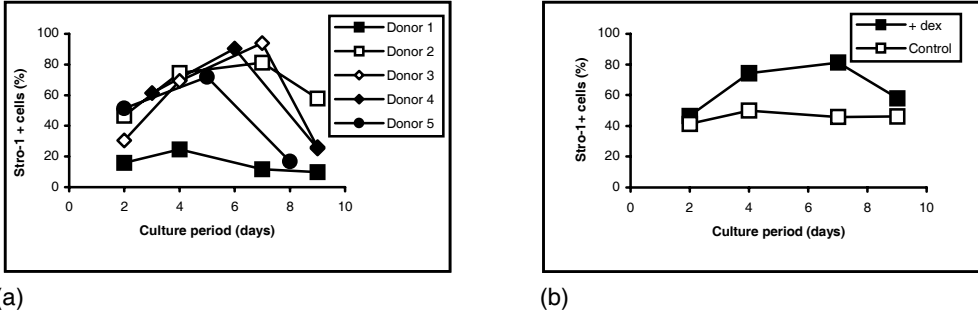


Figure 1 – (a) Development of Stro-1 expression in HBMSC from five donors cultured in the presence of dex. (b) Representative example of the effect of dex stimulation on HBMSC reactivity with Stro-1 antibody. Results expressed as a percentage of the total cell population.

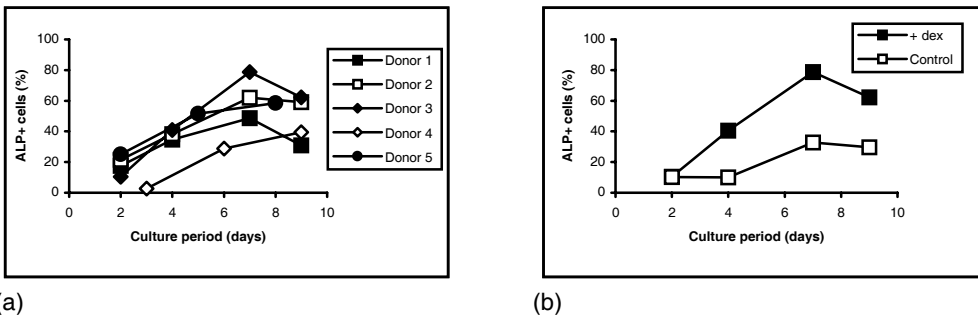


Figure 2 –(a) Development of ALP expression in HBMSC from five donors cultured in the presence of dex. (b) Representative example of the effect of dex stimulation on HBMSC reactivity with ALP antibody. Results expressed as a percentage of the total cell population.

Dual expression of the developmental markers Stro-1 and ALP (flow cytometry)

To obtain more data on the differentiation pattern of the cultures, further analysis was performed defining four different cell populations: (a) Stro-1⁻/ALP⁻, (b) Stro-1⁺/ALP⁻, (c) Stro-1⁺/ALP⁺ and (d) Stro-1⁻/ALP⁺ (fig. 3).

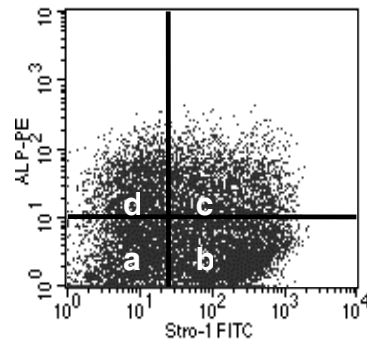


Figure 3 – Representative dot plot illustrating Stro-1 and ALP dual expression. Stro-1 is detected on the x-axis (FITC label) and ALP is detected on the y-axis (PE label). Four different populations can be defined: Stro-1⁻/ALP⁻ (a), Stro-1⁺/ALP⁻ (b), Stro-1⁺/ALP⁺ (c) and Stro-1⁻/ALP⁺ (d). The isotype control of dual labelled cells allowed to set the quadrants, considering that 97.5% of the events were contained within the lower left quadrant. Control samples allowed compensating interference between fluorescence signals.

During culture in the presence of dex, the relative amount of double negative cells sharply declined until confluency was reached (day 5 to 7), indicating that cells that were not potentially osteogenic may have been recruited into this lineage (table 2 and fig. 4). To this decrease was associated an increase in the population expressing ALP, that is, in the double positive fraction and/or in the most differentiated population (Stro-1⁻/ALP⁺) (table 2 and fig. 4). In the absence of dex, the proportion of double negative cells (Stro-1⁻/ALP⁻) was consistently higher, depending on the donor the increase on this population ranged from 1.45 to 6.65x (data not shown). In the post confluence period, an increase in the double negative population was observed, associated to a decrease in the most differentiated fractions, Stro-1⁺/ALP⁺ and/or Stro-1⁻/ALP⁺ (table 2 and fig. 4, day 9). These findings seem to suggest that cells expressing ALP may have gone further in the differentiation process and lost the epitopes for the early osteogenic markers, belonging therefore to the Stro-1⁻/ALP⁻ population. With respect to the development of the ALP⁺ populations, for each donor, the maximum value of expression for the double positive fraction occurred before (between day 4 and day 7) the maximum of the most differentiated fraction (day 8 or 9) (table 2 and fig. 4). Again, the relative proportion of these populations varied widely from donor to donor. The maximum of the Stro-1⁺/ALP⁺ fraction ranged 12.3 to 75.0% of the total cell population, while in the Stro-1⁻/ALP⁺ fraction the value varied between 24.8 to 48.8% (table 2 and fig. 4).

Table 2 – Coexpression expression of Stro-1 and ALP during HBMSC culture in the presence of dex (results obtained by flow cytometry).

| | Culture period (days) | Stro-1 ⁻ /ALP ⁻ (%) | Stro-1 ⁺ /ALP ⁻ (%) | Stro-1 ⁺ /ALP ⁺ (%) | Stro-1 ⁻ /ALP ⁺ (%) |
|---------|-----------------------|---|---|---|---|
| Donor 1 | 2 | 69.7 | 12.8 | 3.0 | 14.5 |
| | 4 | 52.9 | 12.4 | 12.3 | 22.4 |
| | 7 | 46.3 | 5.0 | 6.6 | 42.1 |
| | 9 | 65.1 | 4.0 | 5.9 | 25.0 |
| Donor 2 | 2 | 42.8 | 35.7 | 10.9 | 10.6 |
| | 4 | 17.9 | 44.0 | 30.5 | 7.6 |
| | 7 | 7.8 | 30.2 | 51.0 | 11.0 |
| | 9 | 17.3 | 23.7 | 34.2 | 24.8 |
| Donor 3 | 2 | 61.5 | 28.1 | 2.3 | 8.1 |
| | 4 | 18.8 | 40.7 | 28.6 | 11.9 |
| | 7 | 2.3 | 18.9 | 75.0 | 3.8 |
| | 9 | 29.5 | 8.4 | 17.7 | 44.4 |
| Donor 4 | 3 | 37.9 | 59.3 | 2.0 | 0.8 |
| | 6 | 7.1 | 64.2 | 26.3 | 2.4 |
| | 8 | 31.0 | 64.1 | 4.0 | 0.9 |
| | 9 | 45.7 | 14.9 | 10.3 | 29.1 |
| Donor 5 | 2 | 37.2 | 37.6 | 13.8 | 11.4 |
| | 5 | 12.5 | 35.9 | 35.9 | 15.7 |
| | 8 | 34.5 | 7.0 | 9.7 | 48.8 |

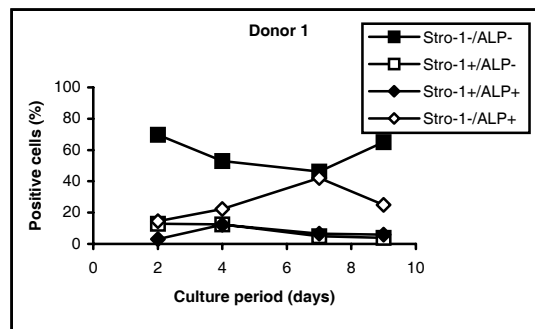


Figure 4 – Coexpression of Stro-1 and ALP by HBMSC cultured in the presence of dex. Representative example of HBMSC cultured up to nine days (results determined by flow cytometry and expressed as a percentage of the total cell population).

Expression of bone related proteins: reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RT-PCR was used to examine the development of ALP and OC mRNA levels during culture. Results revealed that all HBMSC cultures exhibited expression of both earlier (ALP) and late (OC) osteogenic markers, as soon as 24 hours after plating the cells (fig. 5). During culture, the expression for both proteins occurred independently of each other and results did not point any association between ALP and OC mRNA levels. Furthermore, although the level of expression for both targets varied along the culture period, the pattern of expression was inconsistent from donor to donor, indicating that the relative proportion of early osteoprogenitors and more differentiated cells is markedly donor dependent.

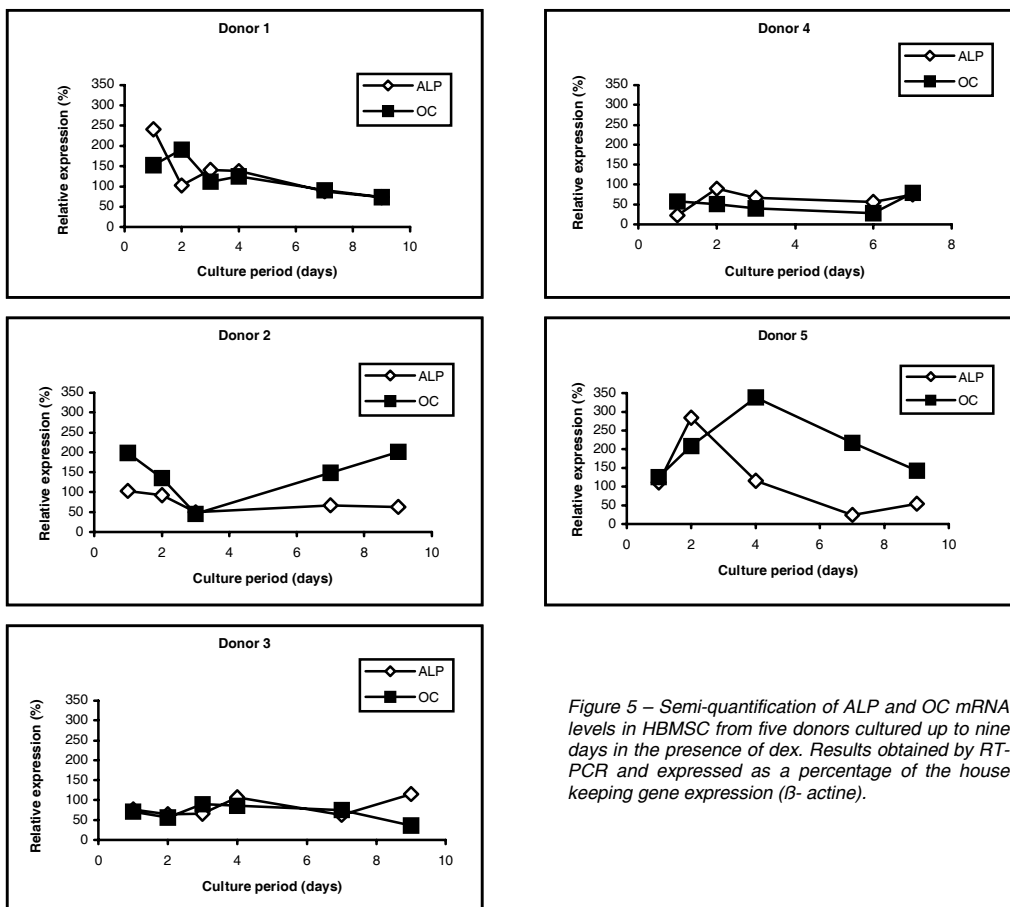


Figure 5 – Semi-quantification of ALP and OC mRNA levels in HBMSC from five donors cultured up to nine days in the presence of dex. Results obtained by RT-PCR and expressed as a percentage of the house keeping gene expression (β -actine).

Effect of dex on the growth rate of HBMSC

The addition of dex to HBMSC in the fourth passage altered their morphology. While in control cultures cells displayed a typical elongated fibroblastic morphology (fig. 6a), in cultures stimulated by dex, cells became more polygonal in shape (fig. 6b). With regard to cell growth, treatment with dex, at the concentration of 10^{-8} M, did not affect the proliferation rate of the cultures (fig. 7). Both in sub- and confluent control cultures, the average doubling period of control cells was very similar to those stimulated by dex ($p=0.23$ for sub-confluent cultures and $p=0.28$ at confluency). These results reveal that the effect of dex on extensively expanded HBMSC mainly concerns differentiation and not proliferation.

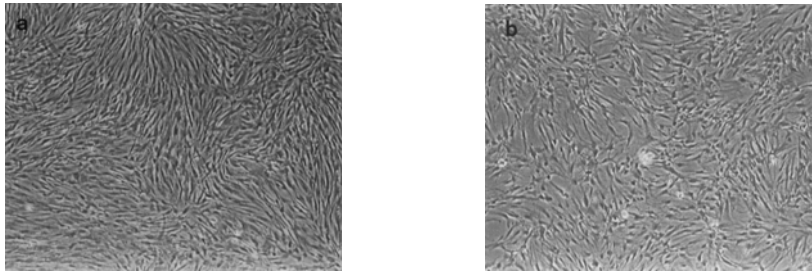


Figure 6 – Light micrograph illustrating the morphology of fourth passage HBMSC cultured in (a) control and (b) + dex medium. (40x).

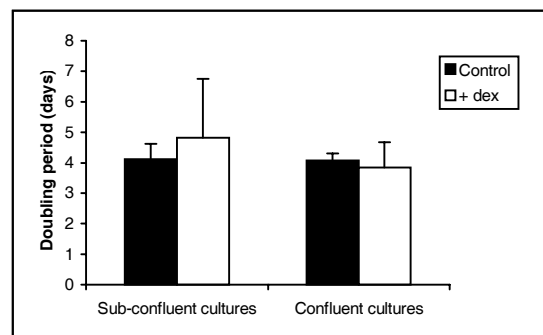


Figure 7 – Effect of dex on the proliferation rate of fourth passage HBMSC both in sub-confluent and confluent cultures. Each bar represents the means of 5 donors \pm SEM. Statistical significance was not detected.

In vivo osteogenic potential of HBMSC

To examine the in vivo osteogenic potential of HBMSC cultures, cells were loaded into porous HA scaffolds and further cultured for one week, in the presence of dex, prior to subcutaneous implantation in immunodeficient mice. Six weeks post implantation, tissue engineered samples from 4 of the 5 donors showed the formation of bone tissue in ectopic

sites. Figure 8 illustrates a representative section from the histological analysis. De novo formed bone, with embedded osteocytes, was observed along the walls of the pores in the ceramic material. For these cultures, the *in vivo* results were in agreement with the *in vitro* data (both FACS and RT-PCR), in which the osteogenic character of the cultures was demonstrated by the expression of several osteogenic markers and by an increase in the fraction of committed osteoprogenitors due to dex stimulation (fig. 2b and fig. 5). However, despite the fact that *in vitro* results revealed both the expression of bone cell markers and reactivity to dex, implants from donor 2 did not induce *in vivo* osteogenesis.

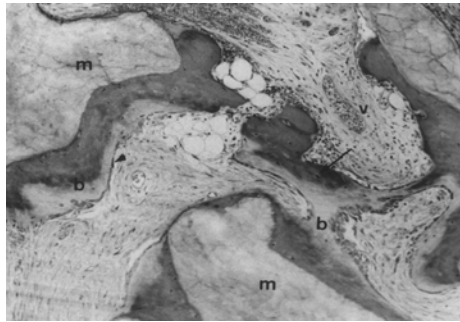


Figure 8 – Light micrograph illustrating a representative histological section. Note the de novo formed bone tissue (b) along the material surface (m), osteocytic cells (arrow) embedded in the bone matrix and an osteoblast layer surrounding the newly formed bone (arrow head). Blood vessels (v) were also found in the vicinity of the newly formed bone tissue (100x).

Discussion

The aim of this study was to characterise the osteogenic character of culture expanded HBMSC. In addition, the *in vitro* cell differentiation pattern was related to the *in vivo* osteogenic potential of the cultures. To determine the developmental stage of cells during culture, they were screened for both Stro-1 and ALP expression through the use of a dual labelling flow cytometric procedure. Since dex is known to have a key role in the differentiation of HBMSC [19, 25, 33-34], its effect on the expression of both Stro-1 and ALP was determined, as well as its influence on the growth rate of the cultures. RT-PCR was also used to evaluate ALP and OC mRNA levels during osteogenic differentiation.

Temporal expression of the developmental markers Stro-1 and ALP (flow cytometry)

Flow cytometric analysis of Stro-1 antigen expression revealed similar developmental patterns between donors. However, the exact proportion of Stro-1+ cells in the total population was markedly donor dependent, which was also reported by Walsh et al. [18, 20].

Within the assayed donors this variation did not appear to be related with age or gender. Besides of the donor physiological status, the bone marrow aspiration procedure, site and volume are known to affect the obtained cell population [35, 36] and, therefore, these parameters most likely contributed for the observed donor variance, pointing out the importance of developing standardised and optimised aspiration procedures. With regard to the temporal pattern of Stro-1 expression, our data showed an initial increase during the preconfluent period, followed by a progressive decline. These findings are consistent to those of Simmons and co-workers [37] in long term HBMSC primary cultures and appear to indicate an initial recruitment of Stro-1⁺ cells into the Stro-1 positive fraction followed, at later stages, by a progressive loss of expression that may be related to the differentiation of the cells into a more mature cell type, therefore lacking the Stro-1 epitope. With respect to ALP expression, the fraction of ALP positive cells increased during culture reaching a maximum value between day 7 and day 9. The results further demonstrated that stimulation by dex increased the Stro-1 positive fraction in sub and near confluent cultures, while consistently increasing the proportion of ALP positive cells during the entire culture period. These effects are in accordance with a model in which dex promotes the recruitment of cells into the osteogenic lineage and further stimulates their maturation [33-34].

Dual expression of the developmental markers Stro-1 and ALP (flow cytometry)

Recent studies by Walsh et al. [18, 20] demonstrated that dual labelling of early passage HBMSC cultures with Stro-1 and ALP allowed to identify osteogenic cells at different stages of differentiation, namely stromal precursors (Stro-1⁺/ALP⁻), osteoprogenitors (Stro-1⁺/ALP⁺) and maturing osteoblasts (Stro-1⁻/ALP⁺). Furthermore, in one of the reports [18] an inverse association was found in the proportion of Stro-1⁺/ALP⁻ cells and that of Stro-1⁻/ALP⁻ and Stro-1⁻/ALP⁺. In our study, and following the same approach, an inverse association was detected between the fraction expressing ALP (Stro-1⁺/ALP⁺ and Stro-1⁻/ALP⁺) and the double negative fraction. However, in subconfluent cultures both Stro-1 and ALP expression were found to increase. In the post confluent period (after 6 to 7 days of culture), the proportion of double negative cells exhibited an increase associated to a decline in the most differentiated populations (Stro-1⁺/ALP⁺ and Stro-1⁻/ALP⁺), suggesting that ALP⁺ cells may have gone further in the maturation process, losing the epitopes for the early osteogenic markers.

Expression of bone related proteins: reverse transcriptase polymerase chain reaction (RT-PCR) analysis

Data on gene expression also confirmed the presence of bone cells in the assayed HBMSC cultures. mRNA for both the early osteogenic marker, ALP, and the osteoblast specific gene, OC [37] were detected in the (+) dex cultures throughout the entire assayed period. The coexpression of ALP and OC in the same culture also points out the existence of a heterogeneous osteogenic population, containing bone cells at different stages of differentiation. This heterogeneity is consistent with results from several other studies [18,22, 25, 38], in which, HBMSC cultures were found to coexpress bone cell related markers associated to different developmental stages [17]. In this study, the pattern and level of mRNA expression for both assessed markers was inconsistent from donor to donor, indicating that the relative proportion of osteoprogenitors and osteoblasts was donor dependent. This large donor variability can, as previously discussed, be related both to donor physiological status and to variances introduced in the bone marrow cell population during the aspiration procedure. Another factor to take into account is that the RT-PCR data presented herein, results from the analysis of one sample per culture period and condition, therefore, variability introduced during RT-PCR procedure could not be measured.

Effect of dex on the growth rate of HBMSC

With regard to the effect of dex stimulation on the growth characteristics of HBMSC, conflicting data has been published with some investigators reporting an increase in cell proliferation [25-27], while others observe inhibition of cell growth [28, 33]. In our study the effect of dex on HBMSC growth rate was evaluated both in sub and confluent cultures and the results did not show a significant effect of this factor on the growth kinetics of the cultures, revealing that dex stimulation on extensively expanded HBMSC mainly concerns differentiation and not proliferation. The discrepancy found between the several published data appears to be related to different cell culture systems and methods of analysis.

In vivo osteogenic potential of HBMSC

With respect to the in vivo results, HBMSC cultures from 4 (donors 1, 3-5) of the 5 studied donors possessed in vivo osteogenic potential, revealing an agreement between in vitro and in vivo data. During in vitro testing HBMSC from donor 2 have shown both to react to dex stimuli and to express several bone cell markers. However, implants containing these cultures failed to induce in vivo osteogenesis. As the in vivo implantation of these cells was performed on different individuals, the hypothesis that individual animal related parameters may have affected the results can be ruled out. Therefore, the conflict between in vitro and

in vivo data may be related not to a lack of osteogenic cells but to an insufficient amount of these cells to induce in vivo bone formation. Our group has previously reported [39], a decrease in the osteogenic capacity of HBMSC cultures from elderly donors. In fact, as donor age increases, the more critical is the optimisation of the bone marrow aspiration procedure for the success of the present technology. In conformity with these results, in this study, bone induction failed to occur in implants containing cells from a 72-year-old donor. An approach that may allow for the indirect quantification of osteoprogenitor cells, and therefore to detect if a second biopsy procedure is required, is the degree of culture stimulation by dex with regard to ALP expression. That is, cultures exhibiting a high fold increase in ALP expression due to dex stimulation most likely contain a higher proportion of osteoprogenitor cells as compared to cultures in which stimulation by dex induces a lower fold increase in ALP expression. In the present study, only cultures from one donor did not form bone in vivo, therefore is not possible to perform a reliable comparison between the degree of culture stimulation in bone forming and non bone forming cultures.

Conclusions

The results presented herein provide evidence that extensively expanded HBMSC possess osteogenic capacity. The differentiation pattern of the cultures could be screened based on the temporal expression of Stro-1 and ALP and it was consistent with a model in which stimulation by dexamethasone increased the recruitment of cells into the osteogenic lineage and further promoted their maturation. These cultures proved to be composed of a heterogeneous cell population containing cells at several developmental stages. In addition, results indicated a large donor variation in the expression of the screened bone cell markers. Finally, our data indicates the need to define a minimal amount of osteogenic cells required to promote in vivo osteogenesis and, therefore, the importance of developing methods that allow the quantification of the osteogenic cell fraction in the total cell population.

Acknowledgments

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CHAPTER 6

***A RELIABLE METHOD TO PREDICT THE IN VIVO OSTEOGENIC
POTENTIAL OF CULTURED HUMAN BONE MARROW STROMAL
CELLS***

A RELIABLE METHOD TO PREDICT THE IN VIVO OSTEOGENIC POTENTIAL OF CULTURED HUMAN BONE MARROW STROMAL CELLS

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Abstract

The use of cell therapies in bone reconstruction has been the subject of extensive research. It is known that human bone marrow stromal cell (HBMSC) cultures contain a population of progenitor cells capable of differentiation towards the osteogenic lineage. Therefore, the quantification of such cell population is of paramount importance to assess the osteogenicity of the cultures. In the present study, a method to indirectly quantify the proportion of osteoprogenitor cells in culture was developed. HBMSC cultures were established from 14 different donors. Fourth passage cells were examined for the expression of alkaline phosphatase (ALP), procollagen I (PCI) and osteopontin (OP), through flow cytometry and the effect of the osteogenic differentiation factor dexamethasone (dex) on this expression was evaluated. In addition, the capacity of the cultures to induce *in vivo* bone formation was analysed by culturing the cells on a hydroxyapatite (HA) scaffolds followed by subcutaneous implantation of these constructs in nude mice. Large donor variability was found on the expression of the bone cell proteins. Dex failed to have a significant effect on the expression of PCI and OP at the evaluated time period. However, during culture, a consistent increase in the relative amount of cells expressing ALP was observed. Furthermore, after dex treatment, the increase in the proportion of cells expressing ALP was shown to be related to the ability of the cultures to form bone *in vivo*, suggesting that the degree of culture response to dex provides a simple method to assess the osteoprogenitor cell content of a given culture. Based on these results, an index was calculated to predict the *in vivo* osteogenic potential of cultured HBMSC.

Introduction

The increasing demands for organ and tissue transplants have motivated many scientists to perform research in the field of tissue engineering. At present, numerous investigators have proposed the use of autologous cultured tissue approaches as an alternative to the traditional bone grafting therapies [1-10]. The engineering of bone tissue is based on the idea of seeding a suitable implant material with patient own cells that, during *in vitro* culture

and prior to transplantation into the defect site, will form a bone tissue coating over the material surface [10-11].

The bone marrow stromal cell population is known to contain progenitors capable of differentiation into mesenchymal lineages such as bone, cartilage, fat and other connective tissues [12-15]. Therefore, they constitute an interesting population of cells for use in cell therapies. Furthermore, bone marrow stromal cells can be easily isolated, extensively expanded and induced to further differentiate into the relevant lineage [15-18]. The *in vitro* and *in vivo* osteogenic potential of adult human bone marrow stromal cells (HBMSC) cultured on porous calcium phosphate scaffolds has already been reported [7, 10, 19-24]. However, in several of these studies, *in vivo* bone formation by HBMSC did not occur in all of the assessed cultures [10, 19, 21, 23]. Moreover, osteogenic potential of the cultures was found to decrease with patient age [19, 25]. Therefore, the development of an analysis method that will allow predicting *in vitro* the performance of the tissue-engineered constructs after implantation is of extreme importance. Such method would allow detecting cultures with low osteogenic potential, indicating the need for a second aspiration procedure or making possible to further enhance the bone forming capacity of the cultures through the use of e.g. bone growth factors or gene therapy [26-28].

Bone tissue contains high levels of type I collagen and several non-collagenous proteins (such as osteopontin, bone sialoprotein and osteocalcin) that distinguish it from other types of tissues [29-31]. However, alkaline phosphatase (ALP) is the most widely recognized marker for osteoblast activity [16, 22, 25, 29, 32-35]. In bone, high levels of ALP are present in pre-osteoblasts and, in culture, osteogenic cells are also known to express high levels of this enzyme [29]. The synthetic glucocorticoid, dexamethasone, has been extensively reported to induce cultures of bone marrow cells to differentiate along the osteogenic lineage [7-8, 10, 16, 19, 32, 34-37]. Signs of differentiation induced by dexamethasone include morphological changes from an elongated to a more cuboidal cell shape and an increase in the expression of osteoblast markers such as ALP [35-38], osteopontin and osteocalcin [39]. The effects of this glucocorticoid on collagen I expression are dependent on the culture conditions and period [36, 38].

The aim of this study was to develop a simple, quantitative and sensitive method capable of predicting the *in vivo* osteogenic potential of cultured HBMSC. To find this correlation between *in vitro* and *in vivo* results, HBMSC were screened for ALP, pro collagen I (PCI) and osteopontin (OP) expression during culture. The degree of cell stimulation caused by the presence of dexamethasone in the medium was measured through the effect of this differentiation factor on the expression of the bone cell markers. Finally, the degree of

stimulation was related to the ability of the cells to form bone after subcutaneous implantation in a nude mice model.

Materials and methods

Human bone marrow stromal cell (HBMSC) harvest and culture

Bone marrow aspirates (10 - 30ml) were obtained from 14 patients that had given written informed consent. Donor information is summarised in table 1. The bone marrow specimens were collected in heparinised tubes and transported at room temperature. Cells were re-suspended with a 20G needle, plated at a density of 500,000 nucleated cells per cm² and cultured in minimum essential medium (α - MEM, Life Technologies, The Netherlands) containing 10% of a selected batch of foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics (AB), 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands) and 1ng/ml basic fibroblast growth factor (bFGF, Instruchemie, The Netherlands). Cells were grown at 37°C and in a humid atmosphere with 5% CO₂. The culture medium was refreshed twice a week and, at near confluence, the adherent cells were washed with phosphate buffered saline solution (PBS, Life Technologies, The Netherlands) and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands). Cells were plated at a density of 5,000 cells per cm² and subsequent passages were performed when cells were near confluence (80-90%).

Table 1 – HBMSC donor information.

| Donor | Source of bone marrow | Gender | Age |
|-------|-----------------------|--------|-----|
| 1 | Iliac crest | M | 75 |
| 2 | Acetabular fossa | M | 86 |
| 3 | Iliac crest | M | 74 |
| 4 | Iliac crest | M | 45 |
| 5 | Iliac crest | F | 39 |
| 6 | Acetabular fossa | F | 54 |
| 7 | Spine | M | 44 |
| 8 | Iliac crest | F | 69 |
| 9 | Iliac crest | M | 74 |
| 10 | Acetabular fossa | F | 72 |
| 11 | Iliac crest | F | 70 |
| 12 | Iliac crest | F | 74 |
| 13 | Acetabular fossa | F | 67 |
| 14 | Spine | M | 44 |

F = female M = male

Scaffold material

Porous granules of coralline hydroxyapatite (HA, Pro-Osteon 500, Interpore) with an average surface area of 0.2 – 0.3cm² were used as scaffold material. The interconnected pores had a median diameter of 435µm and the size of the particles was approximately 3x2x2mm.

Antibodies

The purified anti-ALP (hybridoma B4-78), anti-PCI (M-38) and anti-OP (MPIIB10) were obtained from the Developmental Studies Hybridoma Bank (University of Iowa, USA). The control mouse immunoglobulin G (IgG2a) monoclonal antibody and the secondary antibody goat anti-mouse IgG γ -chain-specific-FITC were purchased from Dako (Denmark).

Expression of PCI and OP

Fourth passage HBMSC (donors 1 to 7) were plated at a density of 5,000 cells per cm² and cultured until confluency in two different types of media: (i) α - MEM containing 10% FBS, AB, 0.2mM AsAP and 0.01M β -glycerophosphate (β GP, Sigma, The Netherlands) (control medium) and (ii) the same medium with the addition of 10⁻⁸ M dexamethasone (dex, Sigma, The Netherlands) (+ dex medium). The expression of PCI and OP was evaluated by flow cytometry. Briefly, after trypsinisation, cells were washed twice at 4°C in PBS containing 1% bovine serum albumin and 0.1% sodium azide (wash buffer). Before antibody labelling, and to block potential non-specific binding, cells were resuspended in PBS containing 5% BSA and 10% human serum and incubated for 30 minutes on ice. Cells (approx. 0.1-0.3E6 / staining) were then resuspended in fixative solution (Fix & Perm kit, Caltag Lab., Burlingame, CA) for 15 minutes, at room temperature, and then washed twice. Afterwards, the cells were resuspended in permeabilization medium (Fix and Perm kit, Caltag Lab., Burlingame, CA) and blocking buffer containing: (a) control mouse anti-human IgG2a (1:5 dilution); (b) anti-PCI (1:5 dilution) and (c) anti-OP (1:5 dilution). Cells were incubated at room temperature, for 15 minutes, and then washed twice. Antibody reactivity was detected by suspending the cells with blocking buffer containing goat anti-mouse IgG γ -chain-specific-FITC (1:5 dilution). Cells were incubated on ice and in the dark for 30 minutes. After washing, the cells were resuspended in 200µl of FACS-flow/staining and analysed using a FACS Calibur flow cytometer (Becton Dickinson Immunocytometry systems). For each measurement 10,000 events were collected.

Temporal expression of ALP

Fourth passage HBMSC (donors 1 to 14) were plated at a density of 5,000 cells per cm² and cultured up to 9 days both in control and (+) dex medium. The expression of ALP was evaluated by flow cytometry at several culture periods (three to four measurements were performed for each culture). Briefly, after trypsinisation, cells were washed twice in wash buffer and blocked against non-specific binding (see above). Cells (approx. 0.1-0.3E6 / staining) were then resuspended in blocking buffer containing: (a) control mouse anti-human IgG2a (1:5 dilution) and (b) ALP monoclonal antibody (1:10 dilution). After incubation on ice for 45 minutes and washing, antibody reactivity and measurements were performed as described above for PCI and OP.

In vivo osteogenic potential of HBMSC

HBMSC (passage 4, donor 1 to 14) were seeded on porous HA granules, at a density of 200,000 cells/particle and cultured for one week in (+) dex medium. Following this period, and prior to implantation, the tissue engineered samples were soaked in serum free medium and washed in phosphate buffered solution pre-warmed to 37°C. Samples (n = 6 per donor) were then implanted into subcutaneous pockets created in the back of immunodeficient mice (HsdCpb:NMRI-nu, Harlan, The Netherlands). Samples of each culture were divided at least over two animals. At the end of the six-week survival period, the implants were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3. The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed undecalcified on a histological diamond saw (Leica SP1600, Leica, Germany) and then stained with basic fuchsin and methylene blue in order to visualise bone formation.

Statistics

Statistical analysis was performed using both t student tests and Mann-Whitney U tests assuming non equal variances. Statistical significance was defined as p<0.05.

Results

Expression of PCI and OP

Expression of intracellular type I collagen was detected in all HBMSC cultures, irrespective of the presence of dexamethasone (dex) in the culture medium. The proportion of cells that stained for PCI was consistently high, comprising $81.8 \pm 21.4\%$ of the total cell population (fig. 1). A high donor variation was found in the values expressed by each individual culture,

which ranged from 45.3 to 99.1% of the total cell amount. The addition of dex to the culture medium did not induce statistically significant changes in the relative proportion of cells expressing intracellular collagen I (fig. 1). With regard to osteopontin expression, positive cells were detected in all confluent cultures, comprising in average 20% of the total cell population (fig. 2). However, the range of individual values was extremely wide (3.2 to 58.9%), indicating that the exact proportion of OP positive cells was strongly donor dependent. In addition, in the majority of the donors tested, the cells that stained positively for OP generated fluorescence signals that were only marginally above control values (data not shown), indicating a low intracellular content of this protein on the positive cells. Dex treatment of the cultures had no stimulatory effect on the relative amount of OP positive cells or on the intensity of their fluorescence signal (fig. 2).

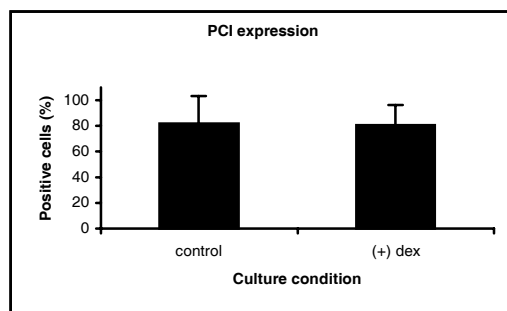


Figure 1 – Pro-collagen I expression by HBMSC cultures: Effect of dexamethasone treatment measured at confluency. Results express the average of cultures established from seven donors (1-7).

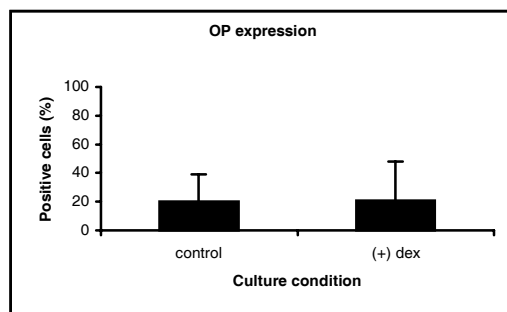


Figure 2 – Osteopontin expression by HBMSC cultures: Effect of dexamethasone treatment measured at confluency. Results express the average of cultures established from seven donors (1-7).

Temporal expression of ALP

In HBMSC cultures from each donor, the pattern of expression of ALP positive cells during time was similar in both culture conditions. However, in cultures treated with dex, the fraction of ALP positive cells was consistently higher as compared to control cultures (fig. 3a). Statistical analysis revealed that after the first two days of culture, the proportion of ALP positive cells in the (+) dex condition was significantly higher as compared to the control ($p < 0.05$), revealing that dex stimulation induced an increase in the fraction of committed osteoprogenitor cells. In the majority of the donors tested (12 of 14), the relative amount of ALP positive cells increased during culture period reaching a maximum value and decreased thereafter. The time period required to achieve the maximum of ALP expression, as well as the value of the maximal fraction of ALP positive cells, was affected by the culture conditions and markedly donor dependent (fig. 3a and b). In HBMSC cultures from 2 of the 14 patients, the percentage of cells expressing ALP was above 80% in the beginning of the culture and decreased thereafter (data not shown).

Quantification of osteoprogenitor cells in culture

An approach that may allow for the indirect quantification of early osteoprogenitor cells is the degree of culture stimulation by dex with regard to the fraction of ALP positive cells. That is, cultures exhibiting a high increase in the amount of cells expressing ALP due to dex treatment most likely contain a higher proportion of osteoprogenitor cells as compared to cultures in which stimulation by dex induces a lower increase in ALP expression. Therefore, for each donor and culture period, the degree of stimulation by dex was measured through the ratio between the fraction of ALP positive cells in the (+) dex and control conditions. Both t-student and Mann-Whitney U tests indicated that, after the first two days in culture, this ratio was time independent for each donor, revealing that the optimal cell response to dex treatment occurred after the first 48 hours. To verify whether the degree of culture response to dex was correlated to the in vivo bone formation ability of the cultures, for each donor the average ratio was determined using the measurements performed from day 3 to day 9 (table 2). This ratio, taken as an indirect measure for the proportion of early osteoprogenitor cells, was then compared to the in vivo osteogenic potential of the cultures.

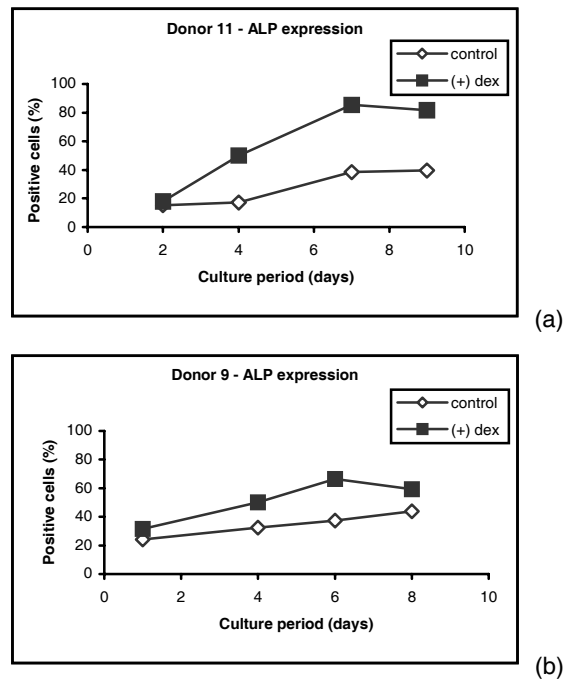


Figure 3 – Temporal expression of ALP in HBMSC cultures: Effect of dexamethasone treatment and variance between donors. (a) Donor 11 and (b) Donor 9.

Table 2 – Degree of dex stimulation measured as the ratio between the fraction of ALP positive cells in the (+) dex and control conditions.

| Donor | Ratio | Log (ratio) | In vivo result |
|-------|-------|-------------|----------------|
| 1 | 1.53 | 0.18 | + |
| 2 | 1.53 | 0.18 | + |
| 3 | 1.40 | 0.15 | - |
| 4 | 2.71 | 0.43 | + |
| 5 | 2.53 | 0.40 | + |
| 6 | 1.52 | 0.18 | - |
| 7 | 1.25 | 0.10 | - |
| 8 | 3.72 | 0.57 | + |
| 9 | 1.56 | 0.19 | - |
| 10 | 2.12 | 0.33 | - |
| 11 | 2.40 | 0.38 | + |
| 12 | 2.56 | 0.41 | + |
| 13 | 1.55 | 0.19 | - |
| 14 | 1.80 | 0.26 | + |

(+) Bone formation; (-) Lack of bone formation

In vivo osteogenic potential of HBMSC

Six weeks post implantation, de novo formed bone was found in all the samples from 8 of the 14 assessed donors (1-2, 4-5, 8, 11-12, 14). Figure 4 illustrates a representative section from the histological analysis. Mineralised bone tissue was observed in direct contact with the ceramic material, indicating that the implanted cells survived and further differentiated into osteoblasts. The bone matrix displayed embedded osteocytes and blood vessels were often observed close to the newly deposited bone. The HBMSC cultures from these donors revealed a good agreement between the in vivo and vitro data, in which the osteogenic character of the cultures was demonstrated by the expression of PCI, OP (donor 1, 2, 4, 5) and by an increase in ALP expression after treatment with dex. However, HBMSC cultures from donors 3, 6, 7, 9, 10 and 13 failed to induce in vivo osteogenesis despite the fact that in vitro testing also indicated expression of PCI, OP (donor 3, 6, 7) and an increase in ALP expression after treatment with dex.

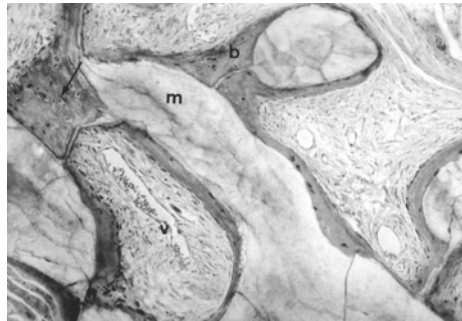


Figure 4 – Light micrograph illustrating a representative histological section of the samples after six weeks of subcutaneous implantation in nude mice. Note mineralised bone matrix (b) with embedded osteocytes (arrow), formed in direct apposition to the scaffold material (m). Blood vessels (v) were present in the vicinity of the newly formed bone, 100x.

In vivo osteogenic potential versus degree of stimulation by dex with regard to ALP expression

The in vivo bone formation capacity of HBMSC could not be related to their in vitro expression of PCI, OP or ALP. However, the relative increase in the proportion of ALP positive cells in culture following dex treatment proved to be related to the in vivo bone formation capacity of the cultures. This indicates that this increase, expressed by the ratio between the fraction of ALP positive cells in (+) dex and control conditions, can be taken as an indirect measurement for the proportion of osteoprogenitor cells in culture. Our data demonstrated that the degree of dex stimulation was higher in bone forming cultures as

compared to cultures that failed to induce osteogenesis (fig. 5 and table 2). Both t student and Mann-Whitney U tests revealed a statistically significant difference between bone forming and non bone forming cultures with regard to the increase on ALP expression after dex treatment ($p = 0.021$, t student test; $p = 0.029$, Mann-Whitney U test).

Following these results, we performed an attempt to define an index to predict in vitro the in vivo performance of the implant. Statistical analysis indicated that this index should be based on the log of the ratio between the proportion of cells expressing ALP in the (+) dex and control condition. This parameter displayed the smallest variance and the best discrimination in the t test ($p = 0.016$ for log ratio and $p = 0.021$ for ratio). Therefore, these values were calculated for each donor (table 2) and the best discriminating index was determined (see table 3 in association with table 2). Sensitivity was defined as correct predictions and specificity as the accuracy in classifying non bone forming cultures. The results revealed that the minimum index should be higher than 0.19, meaning that in order to obtain in vivo bone formation by HBMSC log ratio should be higher than 0.19. This index provided a correct prediction (sensitivity) in 78.6% of the cases and accuracy in classifying non bone forming cultures (specificity) of 83.3% (see table 3 in association with table 2).

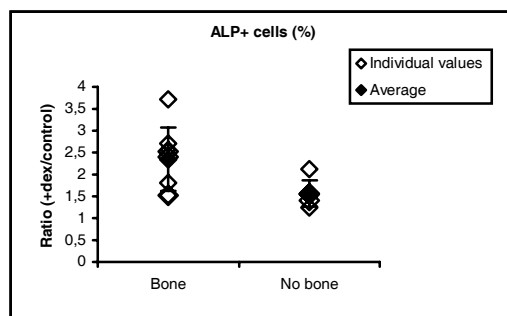


Figure 5 – Relative increase in the fraction of ALP⁺ cells in bone forming and non bone forming cultures, after dex treatment. (◇) Individual values of 14 donors; (◆) Average of each population; (*) Statistical significance was observed: $p = 0.021$ in t test and $p = 0.029$ in Mann-Whitney U test.

Table 3 – Determination of the best discriminating index. Sensitivity was defined as correct predictions and specificity as the accuracy in classifying non bone forming cultures. The values of log (ratio), that is, the index is presented in an ascendant order.

| Log(ratio) > | Sensitivity (%) | Specificity (%) |
|--------------|-----------------|-----------------|
| 0.1 | 64.3% (9/14) | 16.7 % (1/6) |
| 0.15 | 71.4% (10/14) | 33.3% (2/6) |
| 0.18 | 64.3% (9/14) | 50.0% (3/3) |
| 0.19 | 78.6% (11/14) | 83.3% (5/6) |
| 0.26 | 78.6% (11/14) | 83.3% (5/6) |
| 0.33 | 78.6% (11/14) | 100% (6/6) |
| 0.38 | 78.6% (11/14) | 100% (6/6) |
| 0.4 | 71.4% (10/14) | 100% (6/6) |
| 0.41 | 64.3% (9/14) | 100% (6/6) |
| 0.43 | 57.1% (8/14) | 100% (6/6) |
| 0.57 | 50.0% (7/14) | 100% (6/6) |

Discussion

Our results have demonstrated that all HBMSC cultures established from 14 different donors contained a fraction of cells expressing markers of the osteoblast phenotype, such as, PCI, OP and ALP, indicating that each culture contained a population of cells committed to differentiate along the osteogenic pathway. Since a wide donor variability was observed in the expression of the assessed markers, and reactivity with both ALP and OP was detected, these data further supports that the HBMSC cultures are not a uniform population of mesenchymal stem cells, but are composed of an heterogeneous mixture of cells at various stages of differentiation and with distinct osteogenic properties [40-41]. These observations are consistent with a report by Kuznetsov et al. [24], in which it was demonstrated that only 59% of clonally derived human marrow stroma fibroblasts, established from different donors, were able to form bone when implanted in immunodeficient mice. In our study, the strong donor dependency observed, with regard to the fraction of cells expressing PCI, OP and ALP, is in agreement with studies by Jaiswal et al. [16], Stewart et al. [32] and Phinney et al. [42] which also reported a large variability in ALP expression by cultures derived from human bone marrow of different donors. Differences on the physiological status of the donor, as well as the aspiration site and procedure can account for these variations. With regard to the aspiration site, Phinney and coworkers [42] detected a large variation in the expression of ALP enzyme activity in HBMSC cultures from different donors despite the fact that all aspirates were obtained from the iliac crest. Furthermore, they observed clear differences in ALP activity of cultures established from the same donor over a 6 month

period, which indicated that the method of bone marrow harvest plays a major role in producing cellular heterogeneity.

The differentiation of osteogenic cells from their precursors is known to be enhanced by dex. Therefore, the effect of this glucocorticoid on PCI, OP and ALP expression was determined. Our data revealed that dex treatment had no stimulatory effect on the relative proportion of cells expressing PCI or OP. With regard to procollagen I reactivity, conflicting results have been published in literature [36, 38], in which dex was reported to have both an inhibitory and no effect. This discrepancy of results is most likely due to two main factors: the culture conditions and the culture period at which the analysis was performed. With respect to OP expression, the absence of stimulation by dex can be related to the differentiation stage of the cells, since dex would mainly act on early progenitors. This hypothesis is consistent with the fact that dex invariably increased the proportion of cells expressing ALP, an early osteogenic cell marker [32, 38, 43]. In addition, the observed effect of dex over the HBMSC populations is in agreement with numerous studies [16, 22, 25, 32, 35-38, 42] and indicates that this glucocorticoid induces progenitor cells to start the process of osteogenic differentiation.

Although several reports have demonstrated the therapeutic potential of HBMSC cultures in bone repair [7, 10, 19-24], *in vivo* bone formation by these cultures depends on the presence of a sufficient number of early osteoprogenitors on the implant, that can proliferate and further differentiate into osteoblasts. Therefore, the quantification of the osteoprogenitor cell content in the implanted population is of extreme importance. Due to the lack of procedures to isolate early osteoprogenitor cells, we proposed an indirect quantification method based on the hypothesis that after dex stimulation, the increase on the proportion of cells expressing ALP would provide a measurement for the amount of early (and therefore inducible) osteoprogenitor cells in culture. After calculating the degree of stimulation by dex displayed by each culture, the results were compared to their ability to form bone in an *in vivo* situation, using a nude mice model. The data revealed that the degree of stimulation, with regard to ALP expression, was statistically higher in bone forming cultures as compared to the non bone forming ones. These results suggested that the ratio between the proportion of cells positive for ALP in the (+) dex and control conditions provides a simple method to assess the early osteoprogenitor cell content (that is, inducible osteogenic cells) of a given population. Nevertheless, it should be noted that the present method does not take into account osteogenic cells that, previous to dex treatment, had started the process of osteogenic differentiation. Although these cells may also partially contribute to the *in vivo* osteogenic potential of the total population, in this study the relation established between *in vitro* and *in vivo* data was based on the measurement of early osteoprogenitors in culture.

An index was defined to discriminate between bone forming and non bone forming cultures. The index, however, has to be seen with some reservation since the sample number is composed of 14 patients. In future, further analysis of a wider donor population will be performed in order to obtain a more sensitive index. With respect to the nude mice model used to assess in vivo osteogenic potential of HBMSC cultures, it is worth noting that for each donor six tissue engineered samples were implanted divided over at least two animals, and the presence or absence of newly formed bone on the samples was not affected by the animal in question. However, the use of more than one animal per donor is advisable since previous studies in our group showed that it can have an influence in the occurrence of bone formation (data not shown). In addition this model presents some drawbacks since it is difficult to extrapolate results obtained in an ectopic site on a small animal to a clinical relevant situation.

In summary, the findings of this study and, as a result, the method developed can be extremely relevant for the use of HBMSC in bone reconstruction, since it allows the detection of cultures with low osteogenic potential pointing out the need for a second biopsy procedure or for the use of e.g. bone growth factors in the culture medium to enhance the osteoinductivity of cells. This method is, therefore, expected to improve the success rate of tissue engineered devices.

Conclusions

In conclusion, the proportion of bone forming cells in HBMSC cultures proved to be related to the increase in the fraction of cells expressing ALP after dex treatment. This outcome allowed to develop a simple in vitro method that is capable to predict the in vivo osteogenic potential of cultured HBMSC. Such method is, therefore, of extreme importance for the use of a therapeutic cell approach in bone reconstruction.

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CHAPTER 7

***A CULTURED LIVING BONE EQUIVALENT ENHANCES BONE
FORMATION WHEN COMPARED TO A CELL SEEDING APPROACH***

A CULTURED LIVING BONE EQUIVALENT ENHANCES BONE FORMATION WHEN COMPARED TO A CELL SEEDING APPROACH

S.C. Mendes, M. Sleijster, A. van den Muysenberg, J.D. de Bruijn and C.A. van Blitterswijk

Abstract

The development of cell therapy methods to confer osteogenic potential to synthetic bone replacement materials has become common during the last years. At present, in the bone tissue engineering field, two different approaches use patient own cultured osteogenic cells in combination with a scaffold material to engineer autologous osteogenic grafts. One of the approaches consists of seeding cells on a suitable biomaterial, after which the construct is ready for implantation. In the other approach, the seeded cells are further cultured on the scaffold to obtain in vitro formed bone (extracellular matrix and cells), prior to implantation. In the present study, we investigated the in vivo osteogenic potential of both methods through the implantation of porous hydroxyapatite (HA) scaffolds coated with a layer of in vitro formed bone and porous HA scaffolds seeded with osteogenic cells. Results showed that as early as 2 days after implantation, de novo bone tissue was formed on scaffolds in which an in vitro bone-like tissue was cultured, while it was only detected on the cell seeded implants from 4 days onwards. In addition, after 4 days of implantation statistical analysis revealed a significantly higher amount of bone in the bone-like tissue containing scaffolds as compared to cell seeded ones.

Introduction

The regeneration of large bone defects caused by injury, cancer, infection, congenital malformations and fracture non-union, remains a great challenge in orthopaedic surgery. Autologous bone grafting is considered the golden standard in the treatment of such defects. It provides osteoprogenitor cells present in bone marrow and an extracellular matrix containing collagen, hydroxyapatite and a range of osteoinductive growth factors. However, the supply of bone to be harvested is quite limited with this therapy, while its collection is painful and associated with infections and donor site morbidity [1]. Allogenic bone grafting is also a sub-optimal treatment since it can elicit immunological responses and its success in bone regeneration is lower as compared to autologous bone due to the low or absent cellular function of allogeneic bone [2]. To overcome these problems, researchers are testing new ways to replace bone. Although a wide range of biomaterials is currently

available to fill bone defects, the success of these materials is limited due to their general lack of osteogenic and/or osteoinductive properties.

The process of *in vivo* bone formation comprises a sequence of events that involve the recruitment and proliferation of osteoblastic precursors, followed by cell differentiation, matrix formation and, ultimately, mineralisation [3-4]. Growth factors and proteins contained in the bone matrix are involved on the regulation of cell growth, differentiation and mineralisation [3-7].

In recent years, the possibility of *in vitro* engineering an autologous graft with osteogenic properties has been investigated. The goal is to develop an alternative to the traditional autologous bone graft that achieves similar success in bone regeneration. In this approach, a small biopsy of the relevant cells is taken from the patient, cells are then expanded in culture and, finally, combined with a biomaterial. The biomaterial functions as a scaffold for the formation of new bone tissue, as a carrier for the transplanted cells and it also provides volume to better fill the bone defect. Several investigators [8-17] have reported the ability of culture expanded bone marrow stromal cells to form bone in ectopic sites when seeded on a biomaterial shortly before implantation. However, such an approach lacks the existence of an extracellular matrix on the implants, which can be essential to rapid healing since it contains a variety of bone related proteins and growth factors. A second approach, therefore, utilises the culture of a bone-like tissue layer on the scaffolds prior to implantation. In fact, it is known that *in vitro* bone formation by osteogenic cultures is similar to the initial process of bone formation *in vivo* [18-19], which indicates that by culturing osteogenic cells on a suitable biomaterial scaffold an autologous bone equivalent can be obtained [20-22]. Several investigators have widely reported ectopic *in vivo* bone formation induced by such hybrid constructs of cultured bone and biomaterial [23-29]. However, to our knowledge no study has compared the osteogenic potential of the two above mentioned techniques. In summary, two cell therapy approaches are currently investigated in the bone tissue engineering field. One is to seed cultured osteogenic cells on a biomaterial scaffold after which the construct is implanted. The other approach aims at culturing a layer of autologous bone equivalent on the scaffold before implantation. The objective of the current study is to evaluate both methods by investigating whether porous hydroxyapatite scaffolds coated with a layer of *in vitro* formed bone would induce faster bone formation in a ectopic implantation site, as compared to cell seeded hydroxyapatite.

Materials and methods

Isolation and culture of bone marrow cells

Bone marrow cells were obtained from the femora of young adult male F344 rats (150-180g). The marrow cell preparation procedure was described in a previous report [25]. Briefly, femora were removed and washed in an antibiotic solution with a concentration 10 times higher than on culture medium. After the removal of the epiphyses, the bone marrow cells were flushed out with culture medium (see below). The bone marrow obtained from all the rats was pooled and plated in 75cm² flasks at a density equivalent to a femur per flask. The cells were cultured at 37⁰C in a humidified atmosphere of 5% CO₂ and the culture medium consisted of alpha- minimum essential medium (α - MEM, Life Technologies, The Netherlands), 15% foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics, 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands), 0.01M β -glycerophosphate (β GP, Sigma, The Netherlands) and 10 nM dexamethasone (dex, Sigma, The Netherlands). The culture medium was refreshed after 24h and thereafter three times a week. At near confluence, the adherent cells were washed with phosphate buffered saline solution and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands).

Scaffold material

Porous granules of hydroxyapatite (HA, IsoTis NV, The Netherlands) with a porosity of approximately 60% were used as scaffold material. The interconnected pores had a median diameter of 430 μ m and the size of the implanted particles was approximately 3x2x2mm.

Cell seeding and culture on the scaffolds

First passage cells were seeded on the HA particles placed on bacteriological grade plates. Aliquots of 50 μ L of cell suspension were seeded into each scaffold (see cell densities below) and cells were allowed to attach on the HA samples for 4 hours, after which time an additional 2mL of culture medium was added. Four experimental groups were defined as stated in table 1: (I) cells seeded at a density of 100,000 cells per particle followed by an additional culture period of 5 days prior to implantation; (II) cells seeded at a density of 750,000 cells per particle for 16 hours prior to implantation. This seeding density is at least equivalent to the cell number present on the scaffolds seeded with 100,000 cells after 5 days of culture (the number was obtained by extrapolating the results of cell growth rate on tissue culture polystyrene plates); (III) cells seeded at a density of 100,000 cells per particle

for 16 hours prior to implantation. This group was used to analyse the effect of 5 days of cell culture versus cell seeding and implantation for an additional period of 5 days; (IV) control HA particles without cells.

Table 1 – Experimental groups and design.

| Experimental group | Seeding density/scaffold | Seeding/culture time | Implantation times (days) |
|--------------------|--------------------------|----------------------|--------------------------------|
| I | 100,000 | 5 days | 2, 4, 7, 9 and 12 * |
| II | 750,000 | 16 hours | 2, 4, 7, 9 and 12 [#] |
| III | 100,000 | 16 hours | 2, 4, 7, 9 and 12 * |
| IV | 0 | - | 2, 4, 7, 9 and 12 * |

* n = 8 per implantation time.

[#] n = 6 per implantation time.

Light and scanning electron microscopy

Prior to implantation, samples were fixed, dehydrated and either embedded in methyl methacrylate, sectioned on diamond saw (SP1600, Leica, Germany), stained with a 1% methylene blue solution and examined by light microscopy (n=3) or critical point dried (Balzers model CPD 030 Critical Point Drier), sputter coated with carbon (Balzers sputter coater model SCD 004) and examined in a Philips XL30 ESEM-FEG scanning electron microscope (n=3), at an accelerating voltage of 10-15kV.

In vivo implantation

Prior to implantation, tissue engineered samples from the four experimental groups were soaked in serum free medium and then washed in phosphate buffered solution pre-warmed to 37°C. Fifteen male syngeneic F344 rats (300-350g) were anaesthetised, the surgical sites cleaned with ethanol and subcutaneous pockets were created, in which the samples were inserted randomly (2 samples per pocket, 3 to 4 pockets per rat). After 2, 4, 7, 9 and 12 days of implantation, the samples (n = 8 per experimental group and per survival period, except for group II, in which n=6 due to the large cell number required) were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3.

Histology of the implanted samples and extent of bone formation

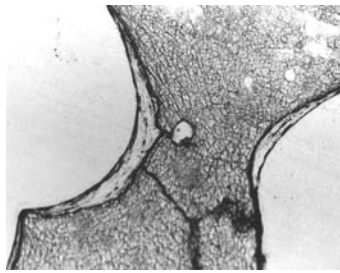
The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed on a histological diamond saw (Leica SP1600, Leica, Germany) and stained with a 1% methylene blue solution and a 0.3% basic fuchsin solution in order to visualise bone formation. Osteogenesis was blindly estimated by three independent investigators (SCM, MS, AM). The following scale was used: (0) no bone formation, (1) first signs of bone

formation in few sections of the sample, (2) bone tissue occupied less than 10% of the pore area, (3) bone occupied between 10 and 20 % of the pore area, (4) bone tissue spread over 20 to 50% of the pore area and (5) Bone occupied more than half of the pore area. For each survival period, the average score for the extent of osteogenesis was calculated for each sample of the three experimental groups (n=6 to 8). Statistical analysis was performed using both the Kruskal-Wallis and the Mann - Whitney U tests, which are appropriated to the non parametric and ordinal nature of the bone formation score. Statistical significance was defined as $p < 0.05$.

Results

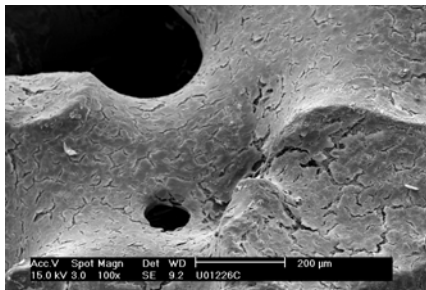
Light and scanning electron microscopy

Light and scanning electron microscopy examination revealed that HA scaffolds seeded with 100,000 cells which were further cultured for 5 days (group I) were entirely covered with multilayers of cells (fig. 1a and b). In between cell layers numerous collagen-like fibres could be observed (fig. 1c).

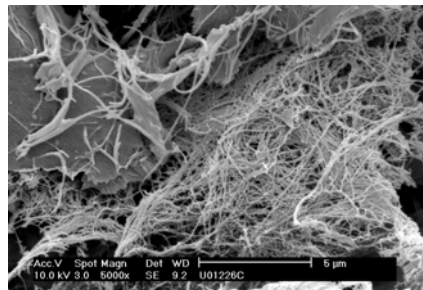


(a)

Figure 1 – (a) Light micrograph (200x), (b) scanning electron micrograph (100x) and (c) scanning electron micrograph (5000x) of rat bone marrow cells grown for 5 days on porous HA particles. Cell seeding density: 100,000 cells/scaffold. Group I. Note the abundant presence of extracellular matrix and the numerous collagen-like fibbers in between cell layers.



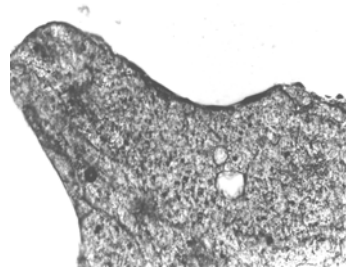
(b)



(c)

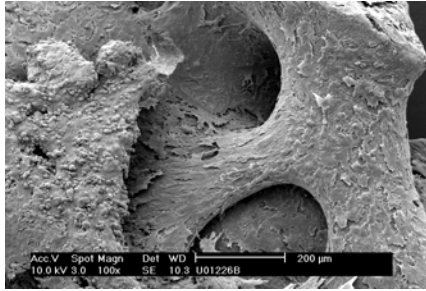
On scaffolds seeded with 750,000 cells for 16 hours (group II), numerous cells were present throughout the porous materials although cells did not cover the entire surface of the scaffold and the presence of extracellular matrix was not detected (fig. 2). In the higher cell

density areas rounded cells were still detected, indicating that cell spreading was still in process.

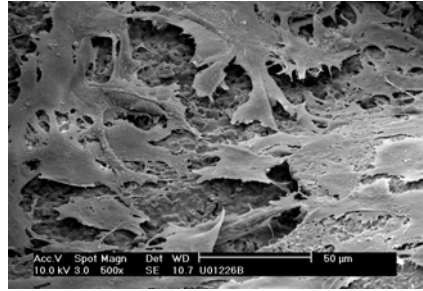


(a)

Figure 2 – (a) Light micrograph (200x), (b) scanning electron micrograph (100x) and (c) scanning electron micrograph (500x) of rat bone marrow cells seeded for 16 hours on porous HA particles. Cell seeding density: 750,000 cells/scaffold. Group II. Note the abundant cell number but the absence of extracellular matrix.

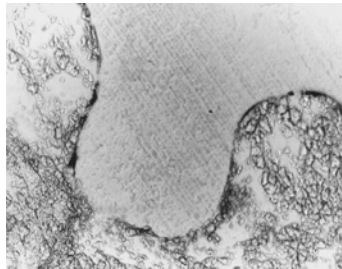


(b)



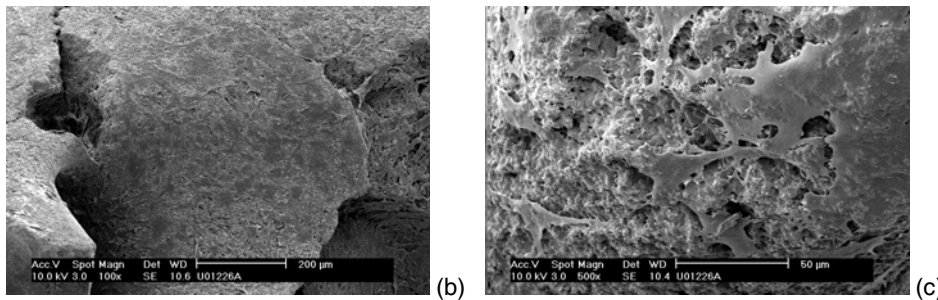
(c)

On samples from group III (HA scaffolds seeded with 100,000 cells for 16 hours), isolated cells were seen, uniformly distributed throughout the porous scaffolds. The degree of cell-to-cell contact was quite low (fig. 3). On these scaffolds cell density was clearly lower as compared to the samples of groups I and II.



(a)

Figure 3 – (a) Light micrograph (200x), (b) scanning electron micrograph (100x) and (c) scanning electron micrograph (500x) of rat bone marrow cells seeded for 16 hours on porous HA particles. Cell seeding density: 100,000 cells/scaffold. Group III. Note the presence of isolated cells equally distributed throughout the scaffold surfaces.



Histology of the implanted samples and extent of bone formation

In control HA samples without osteogenic cells (group IV), bone tissue formation did not occur at any of the survival periods studied. The histological findings in cell containing scaffolds are summarised in table 2. As early as 2 days after implantation, all bone-like matrix containing scaffolds (group I) presented the first signs of *in vivo* bone formation. Cells acquired a more cuboidal shape and, in few areas, osteoid was formed (fig. 4a). Both on high (group II) and low (group III) cell density seeded scaffolds only fibrous tissue was present (fig. 4b), indicating that the culture of cells on HA scaffolds prior to implantation induces faster bone formation as compared to cell seeding only.

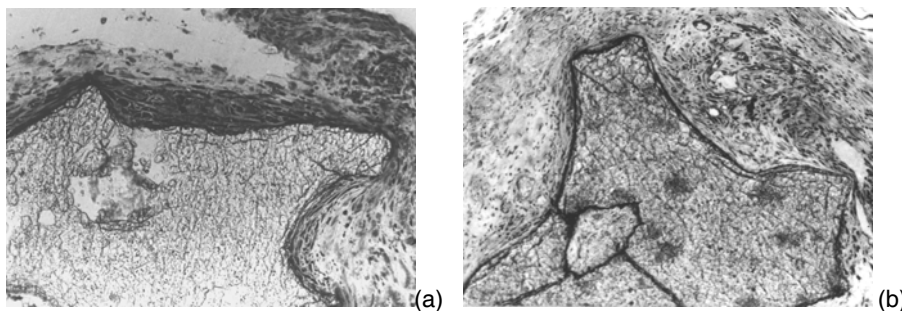


Figure 4 – Light micrographs illustrating representative sections after 2 days of implantation. (a) First signs of *in vivo* bone formation on HA scaffolds in which rat bone marrow cells grown for 5 days, (group I, 200x); (b) Fibrous tissue is present on the cell seeded implants (group II, 100x).

In group I, all implants harvested after 4 days of implantation showed bone tissue, which in average occupied more than 10 and less than 20% of the implant pore area (average bone score 2.2, table 2).

Table 2 – Bone formation in HA scaffolds containing rat bone marrow stromal cells. Effect of cell seeding versus cell seeding and culture.

| Implantation period (days) | Experimental group | Total number of implants (#) | Bone formation score | | | | | Average bone formation score (+/- SD) | |
|----------------------------|--------------------|------------------------------|----------------------|-------|-------|-------|-------|---------------------------------------|---------|
| | | | 0 | 0.1-1 | 1.1-2 | 2.1-3 | 3.1-4 | | 4.1-5 |
| 2 | I | 8 | | 6 | 2 | | | 0.8±0.2 | |
| | II | 6 | 6 | | | | | 0.0±0.0 | |
| | III | 8 | 8 | | | | | 0.0±0.0 | |
| 4 | I | 8 | | | | 8 | | 2.2±0.1 | |
| | II | 6 | 2 | | 4 | | | 1.1±1.0 | |
| | III | 8 | 4 | 4 | | | | 0.1±0.1 | |
| 7 | I | 8 | | | | 4 | 4 | 3.1±0.7 | |
| | II | 6 | | | | 2 | 4 | 2.3±0.6 | |
| | III | 8 | | 6 | 2 | | | 1.0±0.2 | |
| 9 | I | 8 | | | | | 8 | 3.4±0.1 | |
| | II | 6 | | | | 4 | 2 | 3.1±0.7 | |
| | III | 8 | | | 6 | 2 | | 1.9±0.2 | |
| 12 | I | 8 | | | | | 6 | 2 | 3.7±0.4 |
| | II | 6 | | | | 2 | 4 | | 3.4±0.5 |
| | III | 8 | 2 | 2 | 2 | 2 | | | 1.2±1.1 |

Experimental group I: HA scaffolds seeded with 100,000 cells, which were cultured for 5 days prior to implantation.

Experimental group II: HA scaffolds seeded with 750,000 cells for 16 hours prior to implantation.

Experimental group III: HA scaffolds seeded with 100,000 cells for 16 hours prior to implantation.

The following scale was used to estimate bone formation: (0) no bone formation, (1) first signs of bone formation in few sections of the sample, (2) bone tissue occupied less than 10% of the pore area, (3) bone occupied between 10 and 20 % of the pore area, (4) bone tissue spread over 20 to 50% of the pore area and (5) Bone occupied more than half of the pore area.

For the same survival period, 4 of the 6 implants seeded with 750,000 cells for 16 hours (group II) had less than 10% of their pore area filled with bone tissue, while in the remaining 2 implants, osteogenesis had not started (average bone score 1.1, table 2). Also after 4 days of implantation, half of the low cell density seeded implants (group III) did not show

signs of bone tissue, while on the other half the first signs of bone formation appeared (average bone score 0.1, table 2). At this survival period, statistical analysis revealed a significantly higher degree of osteogenesis in group I, as compared to groups II and III ($p=0.032$ and $p=0.019$, respectively), indicating a positive effect of bone-like matrix containing scaffolds with regard to *in vivo* bone formation. With respect to the cell seeding density, the extent of bone tissue at day 4 in the high cell density seeded scaffolds (group II) was not statistically different from the degree of bone formation on the low cell density seeded scaffolds (group III) ($p=0.271$).

At the end of one week survival, bone was detected in all samples from all experimental groups (except control group IV), (fig. 5). The tissue was composed of a mineralised matrix, with embedded osteocytes and a layer of osteoblasts surrounding the outer surface of the newly formed bone. In groups I and II the average bone formation score was 3.1 and 2.3, respectively. The differences between the two groups failed to be statistically significant ($p=0.724$). From day 7 on, bone formation in groups I and II increased with the implantation period (table 2). Although samples from group I exhibited a slightly higher extent of bone formation when compared to samples from group II, the differences were not statistically significant ($p=0.564$, day 9 and $p=0.372$, day 12).

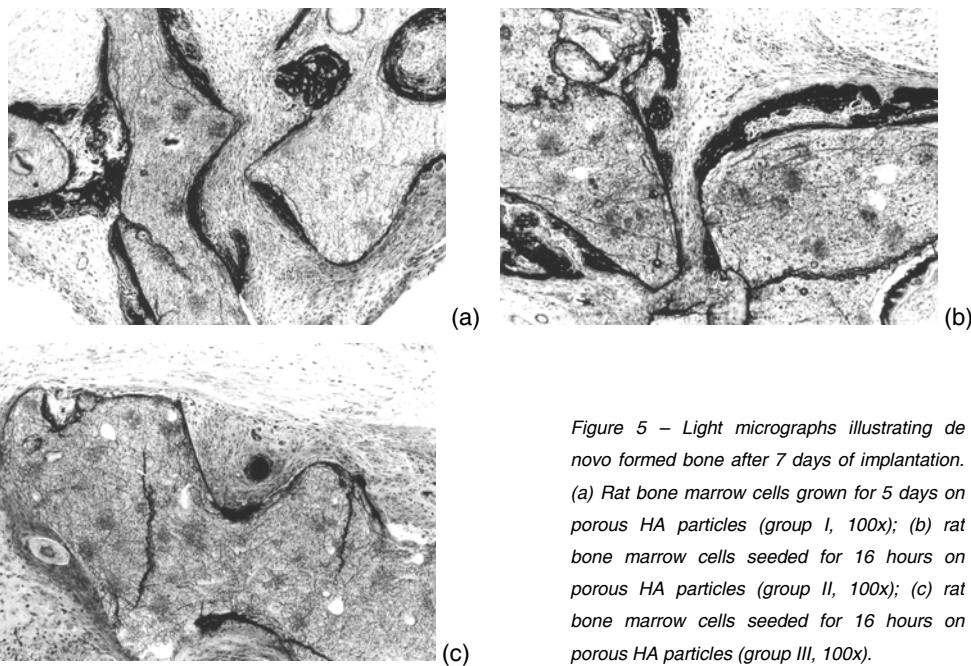


Figure 5 – Light micrographs illustrating de novo formed bone after 7 days of implantation. (a) Rat bone marrow cells grown for 5 days on porous HA particles (group I, 100x); (b) rat bone marrow cells seeded for 16 hours on porous HA particles (group II, 100x); (c) rat bone marrow cells seeded for 16 hours on porous HA particles (group III, 100x).

With respect to the low cell density seeded scaffolds (group III), at day 7, the extent of bone formation varied from less than 10% (score 0.1 to 1, table 2) to between 10 and 20% (score 1.1 to 2, table 2), with an average bone formation score of 1.0 (table 2). At this implantation period, a significant difference was found between this group and the high cell density seeded one ($p=0.034$). This difference was maintained both at 9 and 12 days post implantation, indicating that the extent of newly formed bone was directly proportional to the amount of seeded bone marrow cells.

An interesting analysis is to compare the *in vivo* osteogenic potential of bone-like matrix containing scaffolds (group I) at day 2, 4 and 7 to the lower cell density seeded scaffolds (group III) at day 7, 9 and 12, respectively. On both groups, HA particles were seeded with an equal cell amount, however, in group I cells were cultured for an additional period of 5 days prior to implantation. Therefore, when adding *in vitro* and *in vivo* testing periods samples of group I at day 2, 4 and 7 after implantation can be compared with samples from group III at day 7, 9 and 12 after implantation, respectively. Although no differences in bone formation could be detected between samples from group I at 2 days of implantation and samples from group III at day 7 ($p=0.2381$), group I at day 4 and 7 exhibited significantly higher bone formation scores as compared to group III at day 9 ($p=0.021$) and 12 ($p=0.015$), respectively. This indicates that cell seeding and culture for 5 days prior to implantation seems more efficient than cell seeding followed by an extra implantation period of 5 days.

Discussion

Bone marrow has long been recognised to contain osteoprogenitor cells that are able to differentiate towards the osteogenic lineage when cultured in conditions permissive to osteoblastic development [30]. In the present study, we used rat bone marrow cells to evaluate the potential of two cell therapy approaches used in the development of bone grafts with osteogenic properties. One approach aims at *in vitro* engineering an autologous bone graft through the use of porous scaffolds coated with a layer of bone-like tissue, while the second approach uses porous scaffolds in combination with seeded osteogenic cells. For this purpose four experimental groups were developed (table 1) and studied. On HA scaffolds, in which cells were seeded and cultured for 5 days, light and scanning electron microscopy results revealed the presence of multilayers of cells embedded within extracellular matrix where collagen fibres were abundantly detected. Although, in this study, the identification of collagen was only based on microscopic observations, our group has previously reported the identification of collagen I on this type of constructs using

immunological assays [26], demonstrating the presence of a bone-like tissue on such samples. On the contrary, scaffolds seeded with cells for 16h were extracellular matrix free, consisting of cell/HA constructs. To determine whether porous HA scaffolds containing in vitro formed bone-like matrix would induce faster bone formation as compared to HA scaffolds with seeded osteogenic cells, the samples were subcutaneously implanted in syngeneic rats for periods of 2, 4, 7, 9 and 12 days. Our data indicated that the bone-like matrix containing scaffolds (group I), during the earlier implantation periods (day 2 and day 4), clearly induced faster bone formation as compared to the high cell density seeded scaffolds (group II). Such differences between the two groups may have resulted from several factors. It is likely that the cultured cells were in a further stage in the process of osteogenic differentiation, since they had been in the presence of the differentiation factor dexamethasone for an additional period of 5 days. In addition, and as suggested by Yoshikawa et al. [29], the immediate in vivo bone forming ability of these constructs can be related to bone proteins and growth factors that are present in the formed extracellular matrix and contribute to enhanced osteogenicity of the implants. In fact, previous research in our group [26] revealed that similar constructs, obtained from human bone marrow cells, expressed mRNA for alkaline phosphatase, osteopontin, osteocalcin and receptor human bone morphogenetic protein 2.

For the implantation periods of 7, 9 and 12 days, the average degree of osteogenesis found in group I was slightly higher than in group II. This difference, however, was not statistically significant. Nevertheless, it should be noted that bone turn-over is very fast in rats. In a larger animal, the two types of implants would take longer than 7 days to achieve the same degree of bone formation. Therefore, it is likely that on a clinical relevant situation, such as a bone defect in a large animal, implant stability will be achieved earlier if bone-like tissue is present on the grafts at the time of implantation. These two tissue engineering approaches are currently being tested in a large animal model.

To compare the in vivo osteogenic potential of scaffolds in which cells were cultured for 5 days followed by implantation to scaffolds in which cells were seeded and implanted for an additional period of 5 days (so, identical total test periods), the extent of bone formation on samples from group I at days 2, 4 and 7 of implantation was compared to the extent of bone formation on samples from group III at days 7, 9 and 12. Results demonstrated that scaffolds from group I presented a significantly higher degree of bone tissue at day 4 and 7, as compared to scaffolds from group III at day 9 and 12, respectively. This data indicates that cell seeding and culture for 5 days prior to implantation is more efficient as cell compared to cell seeding followed by an extra implantation period of 5 days. As previously mentioned, these findings maybe related to the longer exposure of the cultured cells to

dexamethasone, resulting in different degrees of cell differentiation in both experimental groups.

Conclusions

The results presented herein demonstrate that scaffolds, which contain an in vitro formed matrix, induce significantly faster bone formation as compared to scaffolds in which cells are only seeded. This suggests that a tissue engineered bone implant is more efficient when cells have already started to form a bone-like tissue in vitro. Moreover, the results indicate that longer implantation periods for the cell seeded implants do not achieve the degree of bone induction found in implants containing an in vitro cultured bone-like matrix.

Acknowledgments

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CHAPTER 8

***EVALUATION OF TWO BIODEGRADABLE POLYMERIC SYSTEMS AS
SUBSTRATES FOR BONE TISSUE ENGINEERING***

EVALUATION OF TWO BIODEGRADABLE POLYMERIC SYSTEMS AS SUBSTRATES FOR BONE TISSUE ENGINEERING

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Abstract

Rat bone marrow cells were seeded and cultured for one week on two biodegradable porous polymeric systems composed of poly(ethylene glycol)-terephthalate/poly(butylene terephthalate) (PEGT/PBT) and corn starch blended with poly(ϵ -caprolactone) (SPCL). Porous hydroxyapatite granules were used as controls. The ability of cells to proliferate and form extracellular matrix on these scaffolds was assessed using a DNA quantification assay and scanning electron microscopy examination, while their osteogenic differentiation was screened by the expression of alkaline phosphatase. In addition, the *in vivo* osteogenic potential of the engineered constructs was evaluated through ectopic implantation in a nude mice model. Results revealed that cells were able to proliferate, differentiate and form extracellular matrix on all materials tested. Moreover, despite the scaffold material used, all constructs induced abundant formation of bone and bone marrow after 4 weeks of implantation. The extent of osteogenesis (approx. 30% of void volume) was similar in all implants types. However, the amount of bone marrow and the degree of bone contact was higher on HA scaffolds, indicating that the polymers still need to be modulated for higher osteoconductive capacity. Nevertheless, the findings suggest that both PEGT/PBT and SPCL systems are excellent candidates to be used as scaffolds for a cell therapy approach in the treatment of bone defects.

Introduction

In several clinical situations, a large amount of bone tissue is required to regenerate osseous defects caused by trauma, tumour and abnormal skeletal development. The graft materials used to heal such problems depend on the type and size of the defect but essentially include autologous and allogeneic bone, as well as synthetic biomaterials such as metals, ceramics and polymers. Despite the wide range of available grafting materials, the development of novel and efficient therapies is required due to the serious limitations presented by the current bone grafts. Although autologous bone is seen as the golden standard to treat bone defects, since it is patient own and osteoinductive, it also implies a

very invasive surgical procedure that is associated with post-operative pain and donor site morbidity. In addition, there are limits to the amount of bone that can be collected and the harvested bone has to be manually shaped to fit the defect [1]. Allogeneic bone can solve some of these limitations, such as post-operative patient discomfort and availability of bone mass. Nevertheless, it also brings new drawbacks mainly due to the lack of reproducible osteoinduction and the possibility of immuneresponses and disease transmission [2]. With regard to the synthetic biomaterials, their success in reconstructing large bone defects is limited since they lack osteoinductive properties that are essential to induce a fast and complete regenerative process.

In recent years, the development of functional bone tissue equivalents has been widely investigated through bone tissue engineering strategies [3-9]. One of these approaches involves the use of patient own cultured osteogenic cells in combination with an appropriate biomaterial scaffold.

In 1988 Maniopoulos et al. [10] cultured bone marrow cells from the femora of rats, in the presence of the osteogenic differentiation factor dexamethasone and reported that these cells differentiated along the osteoblastic lineage and formed bone-like tissue in vitro. Since then, many investigators have described the culture expansion of bone marrow cells from human and several animal species [6, 11-28]. Those studies have demonstrated the ability of the bone marrow cell population to form a bone-like tissue in vitro and/or to induce the formation of bone when implanted ectopically in combination with a suitable biomaterial scaffold.

Bone formation by osteogenic cells is characterised by sequential events involving cell proliferation, expression of osteoblastic markers and synthesis, deposition and mineralisation of a collagenous matrix [29]. These events are, however, greatly affected by the type of scaffold material in which the cells were seeded and/or cultured [14-15, 30-33]. The scaffold material should, therefore allow attachment, growth and differentiation of osteoprogenitor cells. It should also have high porosity and interconnectivity between pores to facilitate the ingrowth of vascular tissue that will ensure the ultimate survival of the transplanted cells and/or tissue. Ideally, the scaffold material would be easily processed into the desired three dimensional shape and it would biodegrade after bone tissue formation, allowing to obtain a totally natural regenerated tissue. Depending on the type of bone defect (load bearing versus non load bearing) the material should also provide the mechanical support required.

Graft materials composed of synthetic biodegradable polymeric systems are excellent candidates as substrates for a cell therapy approach in the treatment of bone defects. These materials can be produced with high porosity in complex three dimensional shapes. Their

degradation and mechanical properties can be easily tailored by adjusting the composition and molecular weight of the polymers. To date, several synthetic biodegradable polymers have been evaluated as scaffolds for bone tissue engineering. The most widely investigated polymers are biodegradable poly(α - hydroxy esters), such as poly(L-lactic acid) (PLLA) [31, 34], poly(glycolic acid) (PGA) [35], poly(DL-lactic-co-glycolic acid) (PLGA) [16, 18, 33, 36-39]. Scaffolds made of PLGA with poly(ethylene glycol) [28] or with polycaprolactone [33, 40], as well as polycaprolactone alone [33], have also been subject of studies. Other polymeric systems that have been investigated include poly(propylene fumarate) [27] and polyurethanes [41].

With regard to the systems based in poly(α - hydroxy esters), reports have demonstrated that these materials support attachment, proliferation and differentiation of osteogenic cells [31, 36], as well as the deposition of a bone-like extracellular matrix and its mineralisation [18, 27-28, 33, 36]. Osteogenic cells cultured in these type of scaffolds were found to form bone tissue when implanted ectopically [16, 39]. Combinations of these polymeric systems with osteogenic cells were also reported to induce a higher degree of bone when implanted into osseous defects, as compared to materials alone or defects left empty [34-35, 38]. Polycaprolactone polymers without blending with PLGA were found to support bone marrow cell growth but not differentiation [33], while systems based in poly(propylene fumarate) were reported as suitable substrates with respect to attachment, proliferation and differentiation of these cells [27]. Despite the promising results obtained with the new polymeric systems, it is hard to find reports in which the results obtained are related to findings in calcium phosphates, since those materials are widely reported to allow bone marrow cell attachment, growth, differentiation and bone tissue formation [6, 11-15, 17, 19, 20-26].

The aim of the present study was to evaluate two biodegradable polymeric systems as substrates for osteoprogenitor cell attachment, growth, differentiation and bone tissue formation. One of the systems has already been approved for human clinical use [42] and it consists of a block copolymer composed by poly(ethylene glycol)-terephthalate and poly(butylene terephthalate) (PEGT/PBT), with bone bonding properties widely reported [43-45]. The second polymeric system evaluated is composed of corn starch blended with poly(ϵ -caprolactone) (SPCL). Cultured rat bone marrow cells were seeded on porous polymeric blocks of both materials, as well as on porous hydroxyapatite (HA) granules, and cultured for seven days prior to implantation. Osteoprogenitor cell growth and differentiation were evaluated during the culture period. At the end of seven days, the constructs were

subcutaneously implanted in nude mice for four weeks in order to evaluate their in vivo bone induction potential.

Materials and methods

PEGT/PBT copolymer

Poly(ethylene glycol)-terephthalate /poly(butylene terephthalate) (PEGT/PBT) was prepared at IsoTis NV (Bilthoven, The Netherlands). The copolymer had a PEGT/PBT weight ratio of 70:30 with a PEG molecular weight of 1000g/mol. Porous PEGT/PBT blocks were fabricated by salt leaching using NaCl as the leachable component. NaCl was sieved into particles ranging from 400 to 600µm in diameter and combined with 70:30 PEGT/PBT granules ground into powder. The mixture was compression moulded and, after cooling, the salt was dissolved in water. The porous blocks were then cut into 3x3x2mm samples. The intrinsic viscosity of the copolymer was between 0.65 and 0.89 dl/g and the porosity of the blocks, prior to testing and under dry conditions was 75% in volume. To improve cell attachment and proliferation on the material surfaces, a CO₂ plasma treatment was performed during 30 minutes, as described previously [46]. After treatment, the blocks were rinsed in water and sterilised in 70% ethanol for 2 hours followed by successive washes in PBS to remove ethanol residues.

SPCL blend

The material composed by corn starch (30%) blended with poly(ϵ -caprolactone) (70%) (SPCL) was obtained from Novamont Spa (Novara, Italy). The fibrous blocks were obtained by spinning, cutting and sintering of the polymeric blend. The material had a porosity of 70% in volume and the thickness of the fibres was approximately 125µm. Prior to testing the porous blocks were cut into 3x3x2mm samples.

HA granules

Porous granules of hydroxyapatite (HA, IsoTis NV, The Netherlands) were used as scaffold material. The processing route included the preparation of the HA slurry and mixing of the slurry with polymethylmethacrylate (PMMA) resin (volume ratio of HA/PMMA 1:1). After shaping in a mould and polymerisation, the mixture was subjected to drying, pyrolyzing (to remove all organic phases) and final sintering (1250 °C for 8h) in stages. The porosity of the material was approximately 50%, the interconnected pores had a median diameter of 440µm

and the size of the implanted particles was approximately 3x3x2mm. The granules were steam sterilised for 20 minutes at 121°C.

Isolation and culture of bone marrow cells

Bone marrow cells were obtained from the femora of young adult male Wistar rats (250-280g). The marrow cell preparation procedure was described in a previous report [6]. Briefly, femora were removed and washed in an antibiotic solution with a concentration 10 times higher than in culture medium. After the removal of the epiphyses, the bone marrow cells were flushed out with culture medium (see below). The bone marrow obtained from all the rats was pooled and plated in 80cm² flasks at a density equivalent to a femur per flask. The cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ and the culture medium during the entire experimental period consisted of minimum essential medium (α - MEM, Life Technologies, The Netherlands) containing 15% foetal bovine serum (FBS, Life Technologies, The Netherlands), antibiotics, 0.2mM L-ascorbic acid 2-phosphate (AsAP, Life Technologies, The Netherlands), 0.01M β -glycerophosphate (β GP, Sigma, The Netherlands) and 10⁻⁸ M dexamethasone (dex, Sigma, The Netherlands). The culture medium was refreshed after 24h and there after three times a week. At near confluence, the adherent cells were washed with phosphate buffered saline solution and enzymatically released by means of a 0.25% trypsin – EDTA solution (Sigma, The Netherlands).

Cell seeding and culture on the scaffolds

Prior to cell seeding all the materials were placed in α - MEM during 24 hours at 37°C to allow swelling. First passage cells were seeded on the three types of scaffold placed on bacteriological grade plates. Aliquots of 100 μ L of cell suspension were injected with a pipette tip into each block/granule at a density of 200,000 cells/scaffold. Cells were allowed to attach on the materials surface for 4 hours, after which time an additional 2mL of culture medium was added. Cells were grown up to 7 days in culture medium.

DNA assay

At 1, 3 and 7 days of culture, tissue engineered constructs (n= 4 per material and time period) were washed in PBS and digested with proteinase K solution (Sigma, The Netherlands), at 56°C for a minimum of 16 hours. After the digestion the samples were stored below -15°C until analysis using a Cyquant dye method. Heparin (Leo Pharm, The Netherlands) and Ribonuclease A solution (Sigma, The Netherlands) were added to the cell homogenate. The mixture was shaken and incubated at 37°C for 30 minutes. 100 μ l of each

sample were transferred to a 96 well plate and 100 μ l of 2x Cyquant GRDye (Molecular Probes, Portland) in PBS were added. The samples were incubated at room temperature and in the dark for 15 minutes. A standard curve was made using a stock solution of 100 μ g/ml DNA (Sigma, The Netherlands). To measure the samples, a LS-50B with fluorimeter (Perkin Helmer) was set with an excitation wavelength at 480 nm and emission wavelength at 520nm.

Scanning electron microscopy

After 1, 3 and 7 days of culture, samples were fixed, dehydrated, critical point dried (Balzers model CPD 030 Critical Point Drier), sputter coated with carbon (Balzers sputter coater model SCD 004) and examined in a Philips XL30 ESEM-FAG scanning electron microscope (n=3 per material and per culture period), at an accelerating voltage of 10-15kV.

Alkaline phosphatase staining

Expression of alkaline phosphatase (ALP) by bone marrow cells cultured on scaffolds was evaluated both after 1 and 7 days of culture (n=3 per material and time period), using an Azo-dye method. Briefly, the constructs were fixed for 2 hours in a mixture containing 4% paraformaldehyde in Sorensen buffer. After washing in demi water the samples were incubated for 15 minutes in a solution containing naphthol AS-BI phosphate (substrate) and Fast Blue R salt (Sigma, The Netherlands). Scaffolds without cells were also incubated in the same solution as controls.

In vivo implantation

Prior to implantation, tissue engineered samples as well as control scaffolds without cells were soaked in serum free medium and then washed in phosphate buffered solution pre-warmed to 37°C. Immunodeficient mice (HsdCpb:NMRI-nu) were anaesthetised, the surgical sites cleaned with ethanol and subcutaneous pockets were created, in which the samples were inserted. At the end of 4 weeks the samples (n = 8 per experimental condition) were removed and fixed in 1.5% glutaraldehyde in 0.14M cacodylic acid buffer, pH 7.3.

Histology

The fixed samples were dehydrated and embedded in methyl methacrylate. The sections were processed on a histological diamond saw (Leica SP1600, Leica, Germany) and stained with a 1% methylene blue solution and a 0.3% basic fuchsin solution in order to visualize bone formation.

Histomorphometry

In order to measure the materials porosity and the amount of bone and bone marrow present on the tissue engineered implants, as well as the degree of bone contact with the materials surface, quantitative analysis of the middle section of each implant (n=8 per material) was performed in a light microscope coupled to a computerised image analysis system (VIDAS). The measured parameters were defined as follows: Porosity: total pore area as compared to the total pore and material area; Bone formation: total bone area as compared to the total pore area; Bone marrow formation: total bone marrow area as compared to the total pore area; Bone contact: the length in which bone is in direct contact with the materials surface, without the interposition of a fibrous tissue layer detectable at light microscopic level, as compared to the total material length.

Statistics

Statistical evaluation was performed using single-factor analysis of variance (ANOVA) to assess statistical significance between the groups of scaffolds. In addition, two-tailed unpaired t student tests were used to evaluate statistical differences in between each two groups. Statistical significance was defined as $p < 0.05$.

Results

DNA assay

All scaffold materials, supported bone marrow cell attachment and proliferation during the 7 days of in vitro culture, as determined by DNA quantification over time (fig. 1). Statistical analysis revealed a significant increase on the amount of DNA present on each scaffold over time ($p=0.0003$ for HA, $p<0.0001$ for both PEGT/PBT and SPCL). At all measured time points, PEGT/PBT scaffolds contained a significantly higher cell number as compared to HA and SPCL, which can be due to a higher surface area of the PEGT/PBT blocks.

Scanning electron microscopy (SEM)

At day 1 of culture, scanning electron microscopy examination revealed the presence of isolated cells spread over the materials surfaces. In all evaluated scaffolds, the degree of cell-to-cell contact was low (fig. 2). After 3 days in culture, the amount of cells attached to the material surfaces increased, although, cells did not cover the entire surface of the scaffolds. On HA constructs, in the higher cell density areas, the first signs of extracellular matrix formation were detected, while on the polymeric samples cells had not yet visibly

started to produce matrix. At the end of 7 days, and regardless of the scaffold material, all constructs were covered with multilayers of cells and extracellular matrix (fig. 3). Abundant collagen-like fibres were detected (fig. 3c,f and i), indicating that prior to implantation the constructs consisted of scaffold material with cultured tissue.

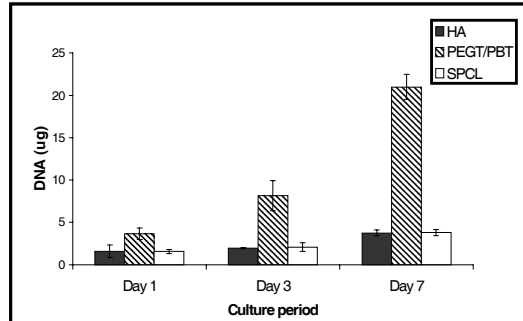
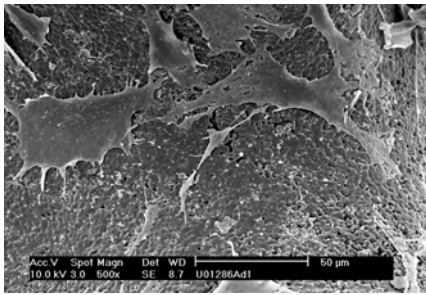
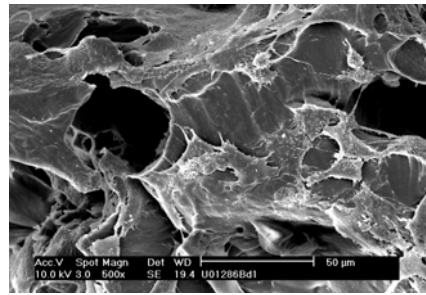


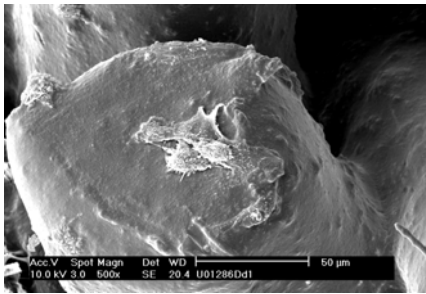
Figure 1 – DNA present on the scaffold constructs after 1,3 and 7 days of culture. Seeding density: 200,000 cells/scaffold. Scaffold apparent volume: 3x3x2mm.



(a)



(b)



(c)

Figure 2 – Scanning electron micrographs of rat bone marrow cells cultured for 1 day on the surface of porous (a) HA, (b) PEGT/PBT and (c) SPCL scaffolds. (500x).

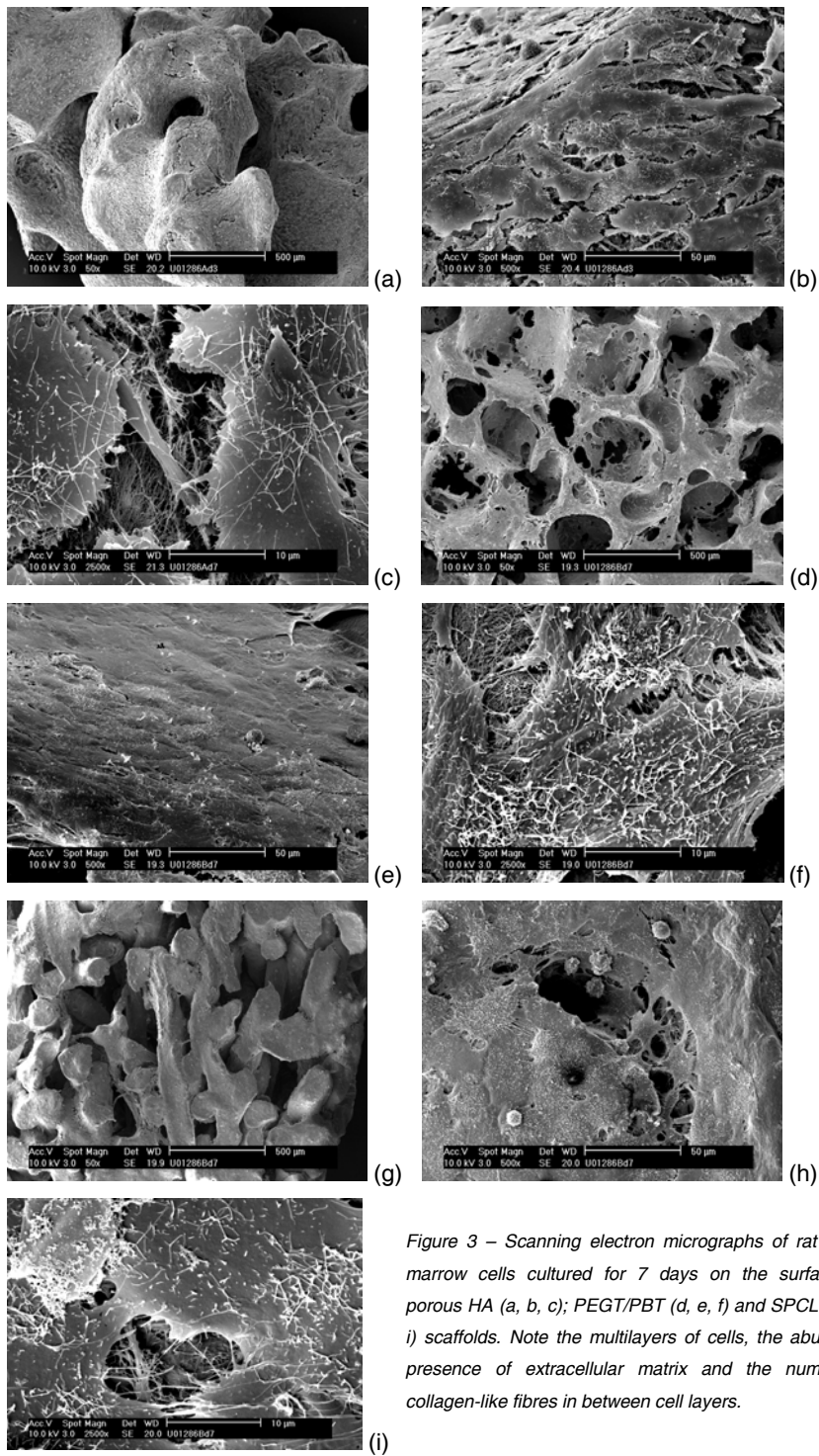


Figure 3 – Scanning electron micrographs of rat bone marrow cells cultured for 7 days on the surface of porous HA (a, b, c); PEGT/PBT (d, e, f) and SPCL (g, h, i) scaffolds. Note the multilayers of cells, the abundant presence of extracellular matrix and the numerous collagen-like fibres in between cell layers.

Alkaline phosphatase staining

The differentiated function of rat bone marrow cells grown on the various material scaffolds was evaluated by monitoring their alkaline phosphatase (ALP) activity both at day 1 and 7. After 1 day of culture, a substantial amount of cells present on both HA (fig. 4a) and PEGT/PBT (fig. 4b) constructs stained positive for ALP, as revealed by the blue coloration of the cells. On the SPCL surface, the number of cells positive for ALP was lower as compared to the other scaffolds (fig. 4c). With the increase of the culture period, the amount of cells expressing this osteoprogenitor cell marker, as well as the intensity of expression increased, despite of the scaffold type. At day 7, high ALP activity could be observed in all constructs and, at this time period, clear differences between cells cultured on the various scaffolds could not be detected (fig. 5). Control samples, without cultured cells, but also stained for ALP activity, did not exhibit any signs of blue coloration.

Histology

Regardless of the scaffold material, after 4 weeks of implantation, all implants without cultured cells, exhibited fibrovascular tissue invasion into the pore regions without any indication of in vivo osteogenesis. In contrast, in all implants in which bone marrow cells were cultured, consistent and abundant de novo formed bone with extensive areas of bone marrow could be observed (fig. 6). Bone was distributed over the pore area, penetrating along the entire volume of the scaffolds (3x3x2mm). Moreover, osteogenesis occurred not only on the materials pores but it was also found outside the scaffolds, encapsulating the implants in some areas (fig. 7). The newly formed bone exhibited a mineralised matrix with lacunae containing osteocytes and osteoblast layers lining the bone surfaces (fig. 8). In addition, abundant regenerated bone marrow tissue, which contained blood vessels and hematopoietic cells was detected in all implants (fig. 8). On the HA implants osteogenesis appeared to start in direct contact with the ceramic surface, without the interposition of a fibrous tissue layer (fig. 7a). On the polymeric samples, although areas of direct contact were detected, frequently the implants also exhibited islands of bone in the interior of the pores without a close contact with the material surfaces (fig. 7b and c). With regard to the presence of other tissue types, despite the intense bone formation observed, cartilaginous tissue was never observed.

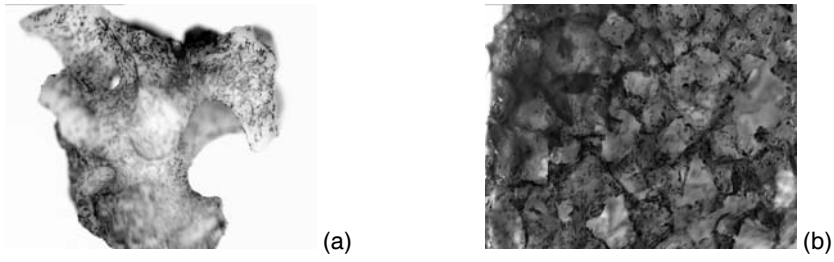


Figure 4 – Light micrographs representing the appearance of rat bone marrow cells stained for ALP activity after 1 day of culture on (a) HA, (b) PEGT/PBT and (c) SPCL scaffolds. (30x). In the Azo-dye method used, the dark coloration displayed by the cells represents ALP activity.

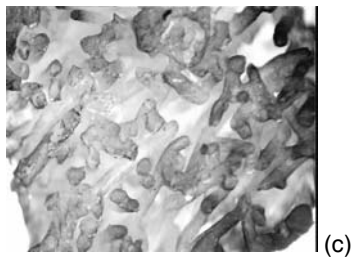
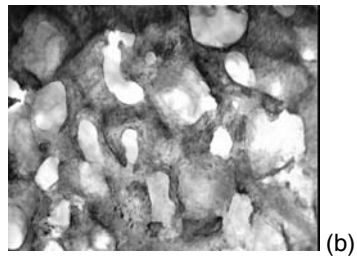
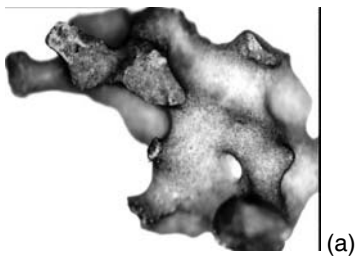


Figure 5 – Light micrographs representing the appearance of rat bone marrow cells stained for ALP activity after 7 days of culture on (a) HA, (b) PEGT/PBT and (c) SPCL scaffolds. (30x). In the Azo-dye method used, the dark coloration displayed by the cells represents ALP activity.

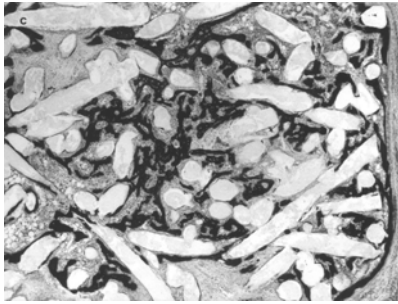
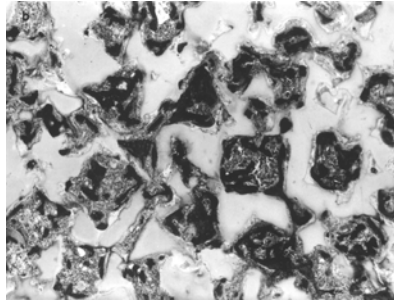
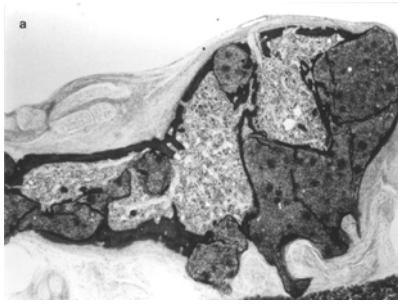


Figure 6 – Light micrographs of representative sections, illustrating osteogenesis in (a) HA, (b) PEGT/PBT and (c) SPCL scaffolds in which rat bone marrow cells were cultured for 7 days prior to implantation under the skin of nude mice for 4 weeks. (40x).

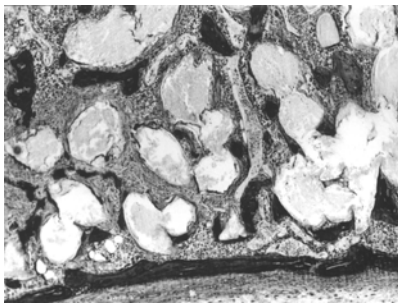
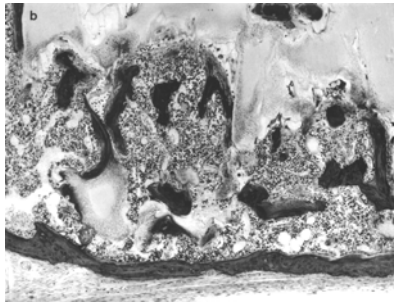
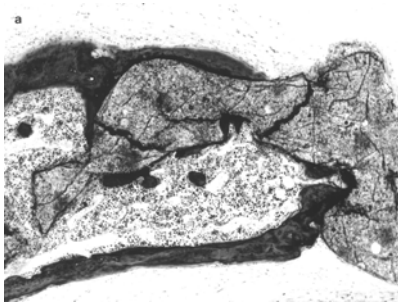


Figure 7 – Light micrographs illustrating areas in which bone was formed outside the pore area of (a) HA, (b) PEGT/PBT and (c) SPCL implants. (100x). Note bone and HA contact and also areas in which bone did not form in direct contact with the polymeric surfaces.

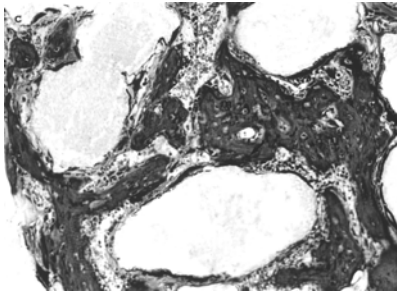
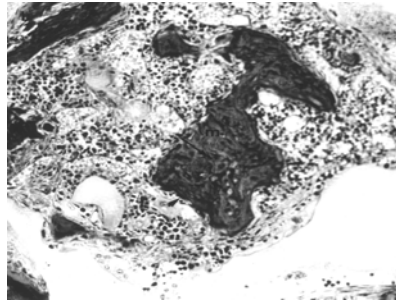
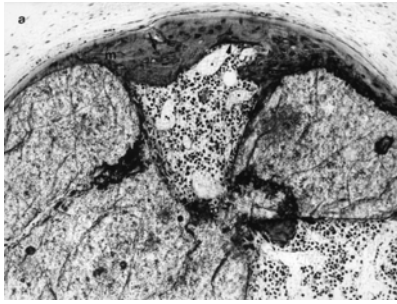


Figure 8 – Light micrographs illustrating the morphology of bone tissue formed on (a) HA, (b) PEGT/PBT and (c) SPCL implants. (200x). Note the mineralised bone matrix (m), with embedded osteocytes (arrow) and layers of osteoblasts (arrow head). Abundant bone marrow is also evident.

Histomorphometry

To evaluate the implants with regard to their porosity, extent of bone and bone marrow formation, as well as the degree of bone contact, histomorphometric analysis was performed on the samples harvested at the end of 4 weeks. With respect to porosity, results revealed no statistical differences between the three types of scaffold ($p=0.1025$). The HA presented a porosity level of $48.8\pm 11.3\%$, while the polymeric systems exhibited porosities of 56.6 ± 4.0 (PEGT/PBT) and 55.6 ± 3.4 (SPCL). For both PEGT/PBT and SPCL systems these results indicate a significant decrease in porosity after cell culture and implantation as compared to their porosity prior to testing (75 and 70%, respectively). The extent of newly formed bone present on the implants is represented in figure 9. The degree of osteogenesis, as compared to the available pore area, ranged from $27.7\pm 9.3\%$ on HA, 35.5 ± 10.3 on PEGT/PBT and 30.1 ± 2.9 on SPCL implants. At this 4 week survival period, a significant effect of the scaffold material on the extent of bone formation could not be detected ($p=0.2320$). Nevertheless, the occurrence of bone together with bone marrow was significantly higher on HA constructs as compared to PEGT/PBT and SPCL scaffolds ($p=0.0047$ and $p=0.0056$, respectively). In HA implants the total amount of bone and bone marrow occupied $89.0\pm 10.0\%$ of the available pore area, while it comprised $62.1\pm 14.7\%$ of

the void space in PEGT/PBT constructs and filled 62.4±14.3% of the pores on SPCL implants (fig. 10).

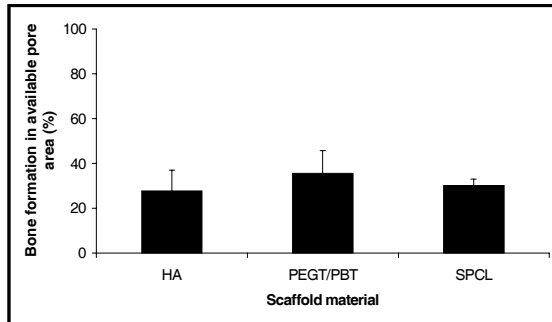


Figure 9 – Extent of bone formation on the tissue engineered constructs after 4 weeks of implantation. Effect of the material scaffold evaluated on the degree of bone formation was not significant ($p=0.2320$).

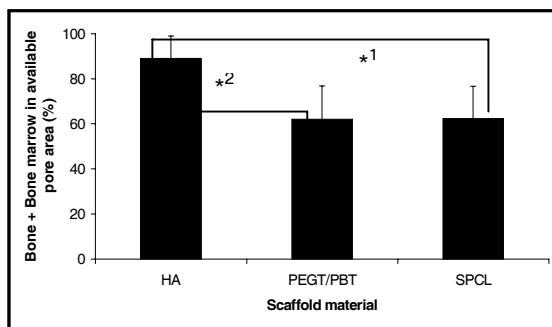


Figure 10 – Extent of bone and bone marrow present on the tissue engineered constructs after 4 weeks of implantation. * A statistical significant difference was found between HA and the polymeric scaffolds. $*^1 p=0.0047$ and $*^2 p= 0.0056$.

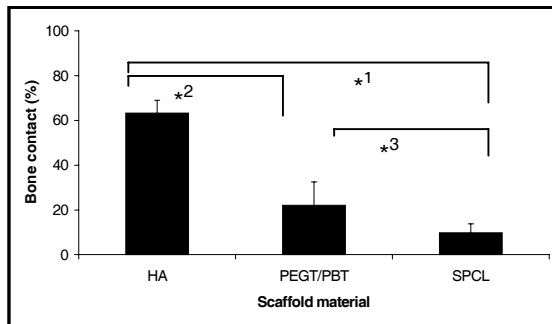


Figure 11 – Degree of contact between the newly formed and the implant materials at 4 weeks. * A statistical significant difference was found between HA and the polymeric scaffolds and between PEGT/PBT and SPCL implants. $*^{1,2} p<0.0001$ and $*^3 p= 0.0121$.

With respect to the degree of contact between the newly formed bone and the scaffold materials (fig. 11), the HA implants presented $63.3 \pm 5.7\%$ of its surface in direct contact with bone, while on polymeric substrates bone contact was substantially lower ($p < 0.0001$), ranging from $22.1 \pm 10.4\%$ on PEGT/PBT scaffolds to $9.9 \pm 3.9\%$ in SPCL implants. Moreover, the PEGT/PBT implants displayed a statistically higher degree of bone contact as compared to the SPCL constructs ($p = 0.0121$). In the PEGT/PBT samples direct microscopic contact with bone was related with the material ability to calcify during implantation. Bone contact on these implants was exclusively observed in areas where the material surface presented a calcification layer, while on SPCL samples surface calcification was never detected.

Discussion

The aim of the current investigation was to evaluate two biodegradable polymeric systems as substrates for bone tissue engineering. Although the study did not address the biodegradation behaviour of the polymers, previous findings by others [40, 46] have proven their degradation capability. In vitro degradation of PEGT/PBT systems is known to occur both by hydrolysis and oxidation [46], while systems based in polycaprolactone are known to degrade by hydrolysis [40].

In the present study, SEM and histological analysis indicated that all the scaffold materials tested possessed high degree of interconnectivity between the pores. The porosity of both polymeric systems decreased after cell culture and implantation, which is related to the hydrophilic character of the polymers that in contact with fluids will swell, reducing their void space. With regard to cell attachment and proliferation, DNA content and SEM analysis indicated that all scaffolds assessed allowed for bone marrow cell attachment, proliferation and production of extracellular matrix. With respect to the DNA analysis, PEGT/PBT scaffolds contained a substantially higher cell number as compared to HA and SPCL. Since after 7 days of culture all scaffolds were completely covered with cells, this appears to be related to a higher surface area of the PEGT/PBT samples, which allowed for additional cell growth.

As reported by other investigators [18, 25, 27, 33], the ability of the osteoprogenitor cells to differentiate along the osteoblastic lineage was assessed by their ALP activity. ALP is widely considered as a marker for the osteogenic phenotype [47]. After 7 days of culture, cells grown on both polymeric scaffolds and on HA stained intensively positive for ALP activity, suggesting that the engineering of hybrid (tissue and material) constructs with osteogenic potential was successful. Using a 2 dimensional culture system, Calvert et al. [33] reported

that poly(caprolactone) substrates, although allowing for bone marrow cell growth, inhibit osteogenic differentiation. In our study, the blend of corn starch and poly(ϵ -caprolactone) has shown to be a suitable substrate for bone marrow cell proliferation and differentiation. These divergent results can be attributed to both the presence of starch in our system and, more likely, to the different culture conditions used in the studies.

With regard to the *in vivo* osteogenic potential of the tissue engineered constructs, the results demonstrated that bone marrow cells cultured on all scaffolds, induced the formation of large quantities of bone tissue that supports hematopoiesis. Moreover, *de novo* formed bone and bone marrow were distributed over the entire scaffold volume, resulting in a penetration depth of bone tissue of at least 1.0mm. These findings are very relevant since in similar studies Ishaug et al. [16, 18] using rat bone marrow cells cultured on PLGA foams, reported a maximum penetration depth of bone tissue of approximately 0.25mm after 4 weeks of implantation and 0.15mm for the penetration depth of mineralised tissue after 4 weeks of *in vitro* culture. Furthermore, in the present study the amount of bone formation found on the polymeric constructs filled more than 30% of their available pore area, while the extent of bone and bone marrow occupied more than 62% of the pores. Although direct comparisons between these investigations and others using different biodegradable polymeric systems [16, 35, 38-41] are difficult due to the diverse study set ups, such as source and seeding density of osteogenic cells, polymers porosity and pore size and implantation models, it should be noted that to our knowledge, such high degree of bone tissue formation by cultured cells after 4 weeks has not yet been reported.

With respect to the histological characterisation of the implant, an interesting feature found both on HA and polymeric constructs was the formation of a bone tissue layer at the outside of the implants, which covered their outer surfaces and encapsulated the constructs in some areas. These observations are contradictory to those by Ohgushi et al. [11], who reported osteogenesis exclusively found in the material pores as a characteristic feature of ectopic bone formation induced by bone marrow cells in calcium phosphate ceramics. In our view, this discrepancy of results may be due to the fact that in the above mentioned work cells were seeded on the ceramic materials and directly implanted, while in our study cells were cultured on the scaffolds for one week prior to implantation. This procedure allowed cells to form a bone-like tissue layer not only in the inner but also on the outer surface of the implants. As a result, after implantation, fibrous tissue invasion from the host could be achieved through the centre of the pores but it could not completely invade the materials outer surface since an *in vitro* formed bone-like tissue was already present.

In this study, the process of bone formation comprised osteoprogenitor cell attachment, growth, differentiation and *in vivo* deposition of mineralised bone with subsequent

remodelling and bone marrow regeneration. The extent of bone present in HA constructs was similar to that observed on the polymeric implants. However, since the amount of bone marrow in HA samples was significantly higher as compared to the PEGT/PBT and SPCL implants, the process of osteogenesis seems to have occurred faster on HA constructs.

With regard to the degree of contact between the materials and the newly formed bone, in HA and as previously reported [11-12], bone formation appeared to start at the material surfaces, which resulted in a very high degree of bone contact. On the polymeric constructs this contact was substantially lower, for PEGT/PBT implants was approximately 22% while on SPCL was under 10%. The large difference observed between the two polymeric systems is justified by the formation of a calcification layer at the PEGT/PBT surfaces due to the uptake of fluid containing calcium ions, which confers to the material bone bonding properties [45]. In fact, in the present study direct contact between bone and PEGT/PBT implants was only observed in areas where the material surface was calcified. Comparisons of bone contact results obtained with PEGT/PBT and SPCL systems and other biodegradable polymers could not be performed due to the difficulty in finding reports that addressed the contact between bone induced by osteogenic cells on the implant and the material. Nevertheless, to obtain polymers with a degree of osteoconductivity similar to that of HA, further optimisation is required. For the PEGT/PBT samples precalcification of the material previous to implantation may substantially increase bone contact, while for the SPCL system a thin calcium phosphate coating on the materials surface may also increase bone contact.

Conclusions

Rat bone marrow cells seeded and cultured on porous biodegradable PEGT/PBT and SPCL blocks were able to differentiate, produce extracellular matrix and induce the abundant formation of bone and bone marrow tissue. In addition, at the implantation period assessed, the extent of newly formed bone on the polymeric constructs was similar to the degree of bone formation on HA implants. These findings indicate that the tested polymers are suitable scaffolds for a bone tissue engineering approach in the treatment of bone defects. Nevertheless, since the degree of bone contact was higher on HA scaffolds, the osteoconductive properties of the polymeric systems still need to be further modulated.

Acknowledgments

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CHAPTER 9

GENERAL DISCUSSION AND CONCLUDING REMARKS

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GENERAL DISCUSSION

The aim of this chapter is to review and discuss the characteristics of cultured bone marrow stromal cells and the parameters affecting their osteogenic character, in an attempt to define an optimal design for the construction of bone tissue engineered implants. The different studies described in this thesis (chapters 2 to 8) have demonstrated that bone marrow stromal cultures are composed of a heterogeneous cell population, in which the osteogenic lineage can develop. Using both in vitro and in vivo testing assays, the development of such lineage was found to be affected by growth and differentiation factors added to the culture medium. In addition, when using in vitro assays to characterise the osteogenic potential of human bone marrow stromal cells (HBMSCs), all tested cultures have shown to express markers and exhibit characteristics associated with the osteoblastic phenotype. Nevertheless, in vivo bone formation by HBMSCs was not consistently observed in all cases, indicating that cultures should possess a certain amount of cells with osteogenic potential, below of which in vivo bone formation could not be detected. As a consequence of these previous results, a method was developed to identify and quantify the subpopulation of cells most relevant for in vivo bone formation. Moreover, the presence of a bone-like extracellular matrix in the tissue engineered constructs was shown to be important for obtaining an implant with optimal properties regarding the period of time required to initiate bone formation and, thereby, implant stabilisation. Finally, the evaluation of different material scaffolds, which serve as delivery vehicles and as substrates for cell culture and differentiation, was addressed. In the following sections, the results obtained will be discussed and related to findings available from literature.

Heterogeneity of human bone marrow stromal cell cultures

The heterogeneous character of bone marrow is known to be reduced during culture due to the progressive loss of non adherent cells, macrophages, endothelial cells and cells with low proliferative capacity [1-5]. Nevertheless, our results (chapters 3 and 5) have shown that, even after extensive culture, the human bone marrow stromal cell (HBMSC) population still remains heterogeneous. Cultures were found to react with the monoclonal antibody Stro-1, however, this reactivity was restricted to a subset of cells and was not displayed by the entire population. During culture, the temporal pattern of stro-1 expression showed an increase during the preconfluent period followed by a progressive decline. The expression of this epitope on cultured HBMSCs was also found to decrease with the degree of

subcultivation. Since it is generally agreed that the osteoprogenitor cells reside exclusively in the Stro-1 reactive population [6-8], these findings demonstrate that other cell types than stromal precursors were present in the cultures. In addition, subsets of cells were found to possess epitopes for antibodies known to react with many different cell types (such as 1B10, CD34, CD146 and CD166 [6, 9-10]), which further demonstrated the heterogeneity of the cultured HBMSC population. Moreover, within the osteogenic population, heterogeneity was also observed with regard to cell differentiation stage. Both in chapters 3 and 5, data on gene expression confirmed that HBMSC cultures contained mRNA for both early (eg. parathyroid hormone receptor, osteopontin and alkaline phosphatase) and late osteogenic markers (osteocalcin). These findings are consistent with results from several other studies [11-14], in which HBMSC cultures were reported to coexpress bone cell related markers associated to different developmental stages. In addition, Stewart et al. [15] have demonstrated that dual labelling for Stro-1 and alkaline phosphatase allowed to identify osteogenic cells at different stages of differentiation on primary and first passage cultures and, in chapter 5, this was also shown for extensively expanded (4th passage) cultures, indicating that continuous subcultivation does not seem to reduce the heterogeneity of the osteogenic population with regard to the existence of cells in different developmental stages. Contradictory to these results are those from Pittinger et al. [16] who have claimed that a homogeneous population of stem cells can be isolated from bone marrow using standard density gradient procedures followed by in vitro culture. However, the characterisation of the cultures homogeneous character was based on their uniform reactivity with SH2 and SH3 antibodies, previously reported to recognise antigens for primitive cells of the osteoblastic phenotype [17], as well as on a lack of reactivity with antigens common on cells of the hematopoietic lineage. In our point of view, the homogeneous character attributed to the HBMSC cultures is at least controversial. Firstly, the characterisation of the so called 'pure' population did not include the Stro-1 antibody, which is widely reported to react with a distinct population of HBMSCs that contains all detectable colony forming units fibroblasts (CFU-Fs) and, therefore, all osteoprogenitor cells [6-8, 15, 18-19]. Moreover, results from the same study showed that individual colonies displayed varying degrees of multipotentiality, which further supports the existence of heterogeneity among cells. In fact, in chapter 3 of this thesis, HBMSC cultures from several donors were also found to uniformly (>93%) express SH2 antigen, independently of donor or culture period, while reactivity with Stro-1 (chapters 3 and 5) was restricted to a subpopulation of cells, which was dependent on the specific cell donor. These findings are indicative that SH2 binds to a broader cell population and not exclusively to stromal stem cells. Furthermore, studies reported in chapters 3 and 5, demonstrate that the differentiation stage of the cultured stromal cells is in

constant evolution since Stro-1 and ALP expression was shown to vary along the culture period. In summary, although immunoselection with specific antibodies has shown to reduce the heterogeneous character of HBMSC cultures, true homogeneous populations of stromal stem cells have not yet been identified and the required culture conditions to maintain either the stem cell character or a certain differentiation stage still need to be determined. Nevertheless, bone marrow stromal cell populations represent one of the most accessible sources of stem and/or progenitor cells, which make them excellent candidates for therapeutical use. The possibility to expand and direct the differentiation of these cells provides the opportunity to study events associated with osteogenic cell commitment and differentiation.

In vitro osteogenic potential of HBMSCs

The development of the osteoblastic lineage from bone marrow stromal precursors is characterised by a sequence of events involving cell proliferation, expression of bone related markers (cell differentiation) and synthesis and deposition of a collagenous extracellular matrix [20-21]. In the construction of bone tissue engineered implants, the optimisation of the culture conditions to better control these events is essential for the success of the technique. With respect to the cell proliferation step, in chapter 3 several growth factors were tested in an attempt to optimise cell proliferation rate, which will reduce the waiting period for the patient. Our results suggested that, although bFGF, EGF and TGF- β 1 actually participated in the proliferation mechanisms of these cells, bFGF and EGF were the most active in promoting cell growth and in maintaining the fibroblastic like morphology. These findings are in agreement with a report by Martin et al. [22], which demonstrated that bFGF and EGF are potent mitogens for HBMSCs. Additionally, in the same study, bFGF was reported to maintain cells in a more immature state during proliferation, inhibiting morphological changes from a fibroblastic morphology to a more flattened phenotype. Regarding the use of β ME to promote cell growth, our data indicated no stimulatory effect, contrary to the reported by Triffit et al. [23].

With respect to cell differentiation, the osteogenic potential of HBMSC cultures was characterised by the expression of bone matrix proteins, alkaline phosphatase and capacity to form a collagenous extracellular matrix. Several immunoreactivity and gene expression assays were used in these studies and results demonstrated that the cultures were immunoreactive and expressed mRNA for a wide range of markers associated with the osteoblast phenotype (chapters 3, 5 and 6). Moreover, the ability of HBMSCs to synthesise a collagenous extracellular matrix was established in chapter 3, in which both scanning

electron microscopy observations and immunostaining results revealed that the tissue engineered implants consisted of material covered with multilayers of cells embedded in an extracellular matrix rich in collagen type I.

The synthetic glucocorticoid dexamethasone (dex), has been extensively reported to stimulate osteogenic differentiation of HBMSC cultures [15, 24-27]. In our studies, signs of differentiation induced by dex included morphological changes from an elongated to a more polygonal cell shape (chapter 5) and an increase in the relative amount of cells expressing alkaline phosphatase (chapter 5 and 6). Additionally, in sub and near confluent cultures stimulation by dex increased the fraction of cells positive for Stro-1. These effects are in accordance with a model in which dex promotes the recruitment of cells into the osteogenic lineage and further stimulates their maturation [25].

In chapters 5 and 6, a strong donor dependency was observed, with regard to the fraction of cells expressing bone cell markers such as Stro-1, alkaline phosphatase, pro-collagen I and osteopontin. These findings are in agreement with several other studies, in which a large variability in the expression of osteogenic makers by HBMSC derived from different donors was reported [15, 24, 28]. Differences on the physiological status of the donor, as well as the aspiration site and procedure can account for these variations. With regard to the aspiration site, Phinney and coworkers [28] detected a large variation in the activity of alkaline phosphatase enzyme in HBMSC cultures from different donors despite the fact that all aspirates were obtained from the iliac crest. Furthermore, they observed clear differences in alkaline phosphatase activity of cultures established from the same donor over a 6 month period, which indicated that the method of bone marrow harvest plays a major role in producing cellular heterogeneity, pointing out the importance of developing standardised and optimised aspiration procedures. In fact, in order to produce an autologous artificial bone tissue, it is crucial that an appropriate bone marrow aspirate is collected from the patient. The cell content of the aspirate, as well as the proliferation and differentiation capacity of the cells are essential factors to be considered and will determine the final outcome of the technology.

In vivo osteogenic potential of HBMSCs

In a preliminary study described in chapter 2, stimulation of HBMSCs with rhBMP-2 was essential for their in vivo bone forming capacity. Nevertheless, further studies revealed that the presence of rhBMP-2 in culture was not required for in vivo bone formation by HBMSCs (chapters 3 to 6). These contradictory results are probably due to the use both of different cell sources and different proliferation conditions. In the study described in chapter 2, the bone marrow was obtained by flushing cells from a bone plug, while on subsequent studies

bone marrow aspirates were used. The cell population obtained by bone plug flushing most likely contained larger amounts of already differentiated cells with restricted proliferative capacity, as compared to bone marrow aspirates. In addition, in chapter 2, the culture medium used during the proliferation step was suboptimal as compared to the proliferation medium used in subsequent studies. Among others, it lacked the presence of bFGF, which besides of increasing cell growth, and therefore osteoprogenitor cell content, is also known to maintain the progenitors phenotype [22].

With regard to the effect of dex on the bone forming capacity of HBMSCs, results described in chapters 3 and 4 demonstrated that, in the majority of the assessed cultures, stimulation by dex was not required to obtain *in vivo* bone formation by HBMSCs. These findings are in agreement with those reported by Martin et al. [22] and suggest, as proposed by Kuznetsov et al. [5], that the HBMSC population contains subpopulations of both committed osteoprogenitors and undifferentiated cells. In the committed population, stimulation by dex, although not necessary, may stimulate further differentiation leading to an earlier start of bone formation. On the undifferentiated population, dex appears to recruit cells into the osteogenic lineage (chapter 4). The use of dex during the differentiation stage is, therefore, advisable to ensure that a sufficient number of HBMSCs will differentiate towards the osteoblastic lineage. In addition, dex appeared to contribute to a higher reproducibility in the degree of bone formation from donor to donor, increasing the extent of osteogenesis in samples with low bone forming ability.

In chapter 4, the effect of donor age on both growth rate and *in vivo* osteogenic potential of HBMSC cultures was assessed. With regard to the growth characteristics, an age related decrease was observed in the proliferation rate of cultures from donors older than 50 years as compared to younger donors. These findings agree with a recent study by Muschler and co-workers [29], in which an age related decrease in the number of nucleated cells per ml of bone marrow aspirate was observed. In a report by Phinney et al. [28], no age related effect could be detected on the growth rate of HBMSCs, nevertheless, the results from both studies do not conflict since the age range investigated by Phinney and coworkers ranged from 19 to 45 years, where we also did not detect differences in cell growth. In chapter 4, the decrease observed in the growth rate of HBMSC cultures from older donors is probably due to a reduction in the number of proliferative precursors (osteoprogenitors) present in bone marrow as age increases. This hypothesis is in conformity with findings reported by Bab et al. [30], in which the number of colony forming unit fibroblasts (CFU-F) from human bone marrow also exhibited an age related decrease. With regard to the effect of donor age on the *in vivo* bone forming capacity of HBMSCs, results indicated that cultures from several donors in all age groups possessed *in vivo* osteogenic potential. However, the increase of

age especially above 50 years resulted in a decrease in the frequency of cases in which in vivo bone formation was observed. These findings also point out a reduction in the amount of osteoprogenitor cells in bone marrow as age increases, and agree with reports from animal [31] and human [32-33] studies, in which the number of BMSCs colonies expressing alkaline phosphatase decreased during aging. Nevertheless, and as previously mentioned in this discussion, the bone marrow aspiration procedure may strongly affect the obtained cell population, therefore, in older patients, an optimisation of the aspiration procedure may increase the success rate of the approach. It is worth mentioning that recent investigations in our group demonstrated that the amount of nucleated cells per ml of bone marrow could be greatly expanded by collecting consecutive but small aspiration volumes in slightly different locations in the iliac crest. Another crucial factor to take into account when evaluating the in vivo osteogenic capacity of bone tissue engineered constructs (material with osteogenic cells and tissue) is vascularisation. After implantation, vascular supply must be rapidly established into the implantation region in order to bring nutrients and bioactive factors essential for cell survival and function.

Identification and quantification of the subpopulation of cells important for in vivo bone formation

As previously stated, in vivo bone formation by HBMSCs was not consistently observed in all cases (chapters 3 to 5). Therefore, in chapter 6 a method was developed to identify and quantify the subpopulation of cells important for in vivo osteogenesis. Since both preosteoblasts and osteoblasts possess a limited proliferative capacity and in our studies HBMSCs were extensively expanded prior to implantation, it seemed reasonable to assume that the highly proliferative cells (that is early progenitors) would be the most important population for the production of bone. Due to the lack of procedures to isolate these cells, we proposed an indirect quantification method based on the hypothesis that after dex stimulation, the increase on the proportion of cells expressing early osteogenic markers would provide a measurement for the amount of early (and therefore inducible) osteoprogenitor cells in culture. After calculating the degree of stimulation by dex displayed by each culture, with regard to ALP expression, the results were compared to their ability to form bone in an in vivo situation. The observations indicated that the degree of culture stimulation by dex was indeed related to the ability of the cultures to form bone tissue in vivo, suggesting that the ratio between the proportion of cells positive for ALP in the (+) dex and control conditions provides a simple method to assess the early osteoprogenitor cell content (that is, inducible osteogenic cells) of a given population. In summary, the method developed can be extremely relevant for the use of HBMSCs in bone reconstruction, since it

allows the detection of cultures with low osteogenic potential pointing out the need for a second bone marrow aspiration procedure or for the use of e.g. bone growth factors in the culture medium to enhance their osteogenicity.

The role of a bone-like extracellular matrix on the tissue engineered implants

In 1991, Davies et al. [34] described the process of in vitro bone formation by cultured rat BMSCs. Morphology, biochemical and gene expression analysis indicated that the in vitro formed bone closely resembled the natural bone in the early stages of in vivo bone formation [34-36]. In chapter 7, the osteogenic potential of implants containing a layer of cultured autologous bone-like tissue was compared to the osteogenicity of constructs that were implanted shortly after cell seeding and before extracellular matrix formation had started. Results demonstrated that bone-like matrix containing implants clearly induced faster bone formation as compared to the cell seeded scaffolds. The faster in vivo bone formation observed on the implants containing a bone-like tissue layer can be attributed to a combination of two factors. Firstly, the cultured cells were in a further stage in the process of osteogenic differentiation, since they had been in the presence of the differentiation factor dexamethasone for a longer period. Secondly, and as suggested by Yoshikawa et al. [37], the in vivo osteogenic potential of these implants can also be related to bone proteins and growth factors that are present in the formed extracellular matrix and contribute to enhance their osteogenicity. In fact, studies described in chapter 3 revealed that such constructs were composed of material covered with cells embedded within a collagenous extracellular matrix (rich in collagen type I) and the cells in question expressed mRNA for alkaline phosphatase, osteopontin, osteocalcin and receptor human bone morphogenetic protein 2. Furthermore, as reported in chapter 8, when cells were cultured on the scaffolds prior to implantation they formed a bone-like tissue layer not only in the inner but also on the outer surface of the implants. As a result, after implantation, a bone layer delineated the implants outer surfaces and encapsulated the constructs in some areas. These observations are contradictory to those by Ohgushi et al. [38], who reported that, in calcium phosphate scaffolds, in vivo bone formation was always restricted to the implant inner pores. In our view, this discrepancy of results may be due to the fact that in the above mentioned work cells were seeded on the material scaffolds and directly implanted. Therefore, fibrous tissue from the host could invade not only the implant pores but also directly contact the implants outer surface.

In summary, if the results obtained using an ectopic implantation model in a small animal (chapter 7) were extrapolated to a clinical situation, it is reasonable to assume that implant stability will be achieved earlier if bone-like tissue is present on the grafts at the time of implantation.

Evaluation of different materials as scaffolds for bone tissue engineering

In the development of cell therapy approaches for bone reconstruction, there is a need to engineer adequate materials that will serve as substrates for cell growth, differentiation and bone tissue formation, as well as delivery vehicles for cells and/or tissue at the implantation site. In addition, the scaffold also provides volume, reducing the amount of tissue required to fill the defects. The scaffold should, therefore, allow attachment, growth and differentiation of osteoprogenitor cells. It should also have high porosity to facilitate the ingrowth of vascular tissue that will ensure the survival of the transplanted cells and/or tissue. The selection of the specific material will depend on the site to be reconstructed. In load bearing sites high mechanical support will be required, while in non load bearing defects the mechanical requirements will be much lower. Ideally, the scaffold would also be easily processed into the desired three dimensional shape and it would degrade after bone tissue formation, allowing to obtain a totally natural regenerated tissue. In chapter 8, two biodegradable polymeric systems were evaluated as scaffolds for bone tissue engineering, aiming at non load bearing applications. One of the systems has already been approved for human clinical use [39] and it consists of a block copolymer composed by poly(ethylene glycol)-terephthalate and poly(butylene terephthalate) (PEGT/PBT). The second polymeric system evaluated is composed of corn starch blended with poly(ϵ -caprolactone) (SPCL). In vitro results demonstrated that both materials allowed for bone marrow cell attachment, growth, osteogenic differentiation and extracellular matrix formation. With regard to the in vivo osteogenic potential of the tissue engineered constructs, results have shown that bone marrow cells cultured on both polymeric systems induced the formation of large quantities of self maintained bone tissue, that supported hematopoiesis. In addition, histomorphometric measurements indicated the extent of de novo formed bone on both types of polymeric scaffolds was similar to that found in hydroxyapatite. Although direct comparisons between these studies and others using different biodegradable polymeric systems [40-43] are difficult due to the diverse study set ups and material characteristics, it should be noted that to our knowledge, such high degree of bone tissue formation after 4 weeks of implantation has not yet been reported by others.

Future applications and general considerations

The tissue engineering approach described in this report is a very powerful technology and the obtained results indicate that such approach would solve most of the drawbacks associated with the traditional bone replacement therapies. This technology can be applied to a wide variety of clinical situations such as spinal fusions, augmentation of bone in the jaw region, reconstruction of bone defects due to the excision of tumours and deformities and

replacement of low quality bone in hip arthroplasty revisions. Nevertheless, the period of time required to produce the tissue engineered bone, 4 to 5 weeks depending on the defect size, is a limiting factor since it excludes its application in acute trauma situations.

Results from current clinical trials do not envision problems with regard to the schedule of operations with weeks in advance and the time required to produce the tissue engineered bone. Nevertheless, the present technology can become more flexible if the entire procedure is divided into two steps. Cells can be expanded in culture and then cryopreserved prior to seeding and final culture on the biomaterial scaffold. This will provide the health care institution with more freedom to schedule operations and to later adjust this schedule. Another approach could be the storage of the tissue engineered bone prior to implantation, which then could be used in an off the shelf manner. Nevertheless, the optimal storage conditions, as well as the maximum period of storage without loss of cell viability and osteogenic potential needs to be investigated in future.

Current research is already directed in reducing handling during the period of in vitro culture in order to prevent any kind of contamination. The design of bioreactors in which cells can be expanded directly in the biomaterial scaffold from the beginning to the end of the procedure, will not only reduce risks of contamination but also make the approach more cost effective. Another field of interest that is currently under investigation in our group is the development of biomaterial particulates with very small diameter, which allow producing injectable bone fillers. The biomaterial with the cells can be injected into the jaws or vertebrae to fill defects in which low mechanical performance is required. This kind of approach possesses a major advantage for both patients and clinicians since it only requires a minimal invasive surgery procedure to reconstruct the defect. First results in this area indicated the feasibility of the technique in an ectopic implantation model. Additionally, the development of adequate biomaterial scaffolds is extremely relevant for the technology. Recent studies in our group demonstrated that materials with approximately the same chemical composition but different structures can originate quite different responses with regard to in vivo bone formation.

The studies described in this report mainly concern the investigation of a tissue engineering approach in the treatment of isolated bone defects. However, in cases in which all bones are affected, such as osteoporosis and osteogenesis imperfecta, it is not feasible to consider the treatment of all bones by replacement with tissue engineered bone. In the case of age related bone loss (osteoporosis), it can be envisioned that expansion of early osteoprogenitor cells in culture, followed by their systemic administration into the patient, may cure and/or diminish the severity of the disease. With regard to the treatment of diseases involving genetic mutations, molecular engineering of cells is an area that may

lead to the development of techniques, which will allow correcting several bone deficiencies. During *in vitro* expansion, HBMSCs may be genetically manipulated to produce a desired cellular product and then systemically distributed to establish a normal bone marrow microenvironment.

CONCLUDING REMARKS

The studies described in this thesis contributed to further characterise the osteogenic potential of cultured bone marrow stromal cells, as well to identify the cell subpopulation mainly responsible for this osteogenic character. In addition, some of the *in vitro* manipulations required for their extensive subcultivation and *in vivo* bone formation were defined. Finally, new and adequate scaffold materials were presented. The obtained results demonstrate the potential of the bone tissue engineering technology and indicate that the use of such cell therapy approach to treat bone defects may improve the quality of life for many patients. In fact, due to such promising results feasibility clinical trials are currently ongoing. To successfully and reproducibly regenerate bone using a tissue engineering strategy the technology, however, still needs fine tuning. Standardised and optimised bone marrow aspiration procedures have to be defined in order to obtain cell populations with optimal progenitor cell content. In addition, the development of antibodies that will allow to isolate homogeneous populations of undifferentiated cells and the definition of the culture conditions required to maintain either an undifferentiated cell character or a certain differentiation stage still need to be established.

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Summary

In the present thesis, a tissue engineering approach to treat bone defects was investigated. Such strategy was based on the use of patient own cultured bone marrow stromal cells (BMSCs) in association with biomaterials to produce autologous living bone equivalents. When engineering such implants, three main factors had to be taken into account: (i) the cells, (ii) the culture technology and (iii) the biomaterial scaffolds.

The capacity of BMSCs to proliferate, differentiate along the osteogenic lineage and form a bone like tissue was demonstrated in various in vitro assays making use of biochemical, immunological, microscopic and gene expression techniques. The ability of the cells to produce bone in vivo was established using an ectopic (extra osseous) implantation model. Results indicated that BMSC cultures were composed of a heterogeneous population containing a subpopulation of cells with high proliferative capacity and with potential to differentiate into bone forming cells. Both the growth and the differentiation pattern of these cells could be manipulated, to a certain degree, through the use of bioactive factors during culture. After implantation, the bone forming capacity of the cultures proved to be related to the amount of early osteoprogenitors and precursors cells that could be induced into starting the osteogenic differentiation process. In bone marrow aspirates, this subpopulation appeared to decrease with donor age and to be strongly dependent on the donor, indicating that the aspiration procedure plays an important role in the obtained bone marrow cell population. In order to evaluate the in vivo bone formation capacity of BMSC cultures prior to implantation, an experimental method was developed in which the amount of early osteoprogenitors and precursors cells could be quantified.

With regard to the technology design, data indicated that the culture of cells on the biomaterial scaffolds prior to implantation resulted in implants with faster in vivo bone forming ability as compared to scaffolds implanted after cell seeding. In addition, two biodegradable polymeric systems were proposed as scaffolds to be used in the described bone engineering approach after evaluating their ability to support bone marrow cell growth, differentiation and in vivo bone formation.

In summary, although the complete knowledge of the factors controlling BMSC growth and osteogenic differentiation still needs to be further expanded, the obtained results suggest that the bone tissue engineering approach described in this thesis presents a great potential for the repair of bone defects and will become an advantageous alternative to the traditional autologous bone grafting.

Samenvatting

In dit proefschrift wordt de techniek van bot tissue-engineering onderzocht met als doel het behandelen van botdefecten. Een dergelijke strategie werd gebaseerd op het gebruik van patient eigen materiaal, gekweekte stromale beenmerg cellen (BMSCs), in combinatie met een biomateriaal om een levend autoloog botequivalent te produceren. Wanneer men dergelijke implantaten produceert zijn er drie belangrijke factoren die in overweging genomen moeten worden: (i) de cellen, (ii) het kweekproces en (iii) het biomateriaal. De capaciteit van BMSCs om te kunnen prolifereren en tot botcellen te differentiëren, waarna een op bot gelijkend weefsel wordt gevormd, wordt middels diverse *in vitro* analyse technieken aangetoond zoals biochemische, immunologische, microscopische en gen-expressie technieken. De potentie van de cellen om bot te produceren wordt aangetoond in een *in vivo* model in een ectopische (niet botrijke) omgeving.

De resultaten laten zien dat de BMSC kweken uit een heterogene populatie van cellen bestaan met een fractie aan cellen die een hoge proliferatie capaciteit bevatten en die de potentie hebben om te differentiëren tot botvormende cellen. Zowel de groei als de differentiatie van deze cellen zouden, tot op zekere hoogte, door het gebruik van bioactieve factoren gedurende de kweek kunnen worden beïnvloed. Na implantatie bleek dat de botvormende capaciteit van de kweken gerelateerd was aan de hoeveelheid vroege osteoprogenitor- en voorlopercellen die aangezet kon worden tot osteogene differentiatie. In het beenmerg aspiraats lijkt deze populatie cellen af te nemen met toenemende leeftijd van de donoren en sterk te variëren tussen de donoren. Dit wijst erop dat de aspiratieprocedure een belangrijke rol zou kunnen spelen in het verkrijgen van de juiste populatie beenmergcellen. Om de *in vivo* potentie van de BMSC kweken voorafgaand aan implantatie te evalueren is een experimentele methode ontwikkeld die de hoeveelheid vroege osteoprogenitor cellen kan kwantificeren.

Met betrekking tot de beschreven techniek wijzen de resultaten erop dat het kweken van cellen op het biomateriaal voorafgaand aan de implantatie een snellere *in vivo* botvorming tot gevolg heeft ten opzichte van het direct implanteren van het materiaal na het zaaien van de cellen. Daarnaast worden twee biodegradeerbare polymeren voorgesteld als dragermateriaal voor de tissue engineering techniek nadat deze materialen zijn geëvalueerd op celgroei, celdifferentiatie en *in vivo* botvormende eigenschappen.

Samenvattend, hoewel de volledige kennis van factoren die de groei van BMSCs en de osteogene differentiatie beïnvloeden verder moet worden uitgebreid, suggereren de verkregen resultaten dat de bot tissue-engineering zoals beschreven in dit proefschrift een grote potentie heeft om als goed alternatief te dienen voor het gebruik van autoloog bot.

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Curriculum Vitae

Sandra C. Mendes was born on the 16th of September 1972 in Porto, Portugal.

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In September 1997 she started as PhD student on the Department of Bone Tissue Engineering at biotechnology company IsoTis NV, in cooperation with Twente University. The promotor and assistant promotor of the dissertation are Prof. Clemens van Blitterswijk and Dr. Joost de Bruijn, respectively. The subject of the research regarded the use of bone marrow stromal cells in combination with biomaterials for bone defects reconstruction.