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Cytokine and Chemokine Networks Influencing Stem Cell Proliferation, Differentiation, and Marrow Homing

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Abstract The hematopoietic stem cell (HSC) is an attractive target for gene therapy of genetic diseases of the immune and hematopoietic system, and for drug-resistance strategies in which genes conferring resistance to a variety of chemotherapeutic agents can be transduced. Stem cells are relatively easy to obtain; e.g., by marrow aspiration or G-CSF mobilization into the peripheral blood, and can be enriched e.g., by the use of anti-CD34 + monoclonal antibody. For conventional retroviral transduction, normally quiescent HSC must be activated into the cell cycle by priming with appropriate cytokines, and it has been critical to identify cytokine combinations that preserve the self-renewal capacity of long-term repopulating HSC. It has become apparent that strategies designed to optimize HSC cycling and proviral integration can compromise the capacity of transduced HSC to compete in vivo against endogenous HSC or HSC that have not been activated into cell cycle. Lentiviral vectors can integrate genes into non-cycling cells but there is an increased efficiency of transduction if Go HSC are activated into G1-phase of the cell cycle. This reduced efficiency of long-term engraftment of ex vivo cultured HSC may be due to impaired self-renewal capacity or reduced marrow homing efficiency. The latter may be attributed to down modulation of chemokine receptors necessary for chemotactic homing to the marrow. Alternatively, or in addition, there may be down modulation of (1) HSC adhesion molecules necessary for endothelial adhesion and egress from the circulation: (2) metalloproteinases secreted by HSC that facilitate their migration through extracellular matrix and promote release of critical soluble regulatory factors in the marrow microenvironment. A more controversial view is that cell death pathways, for example those involving FasR (CD95) may be activated in cycling HSC, resulting in their selective destruction upon transplantation and localization to sites rich in Fas ligand such as the liver. J. Cell. Biochem. Suppl. 38: 29–38, 2002.

Key words: cytokine; chemokine; hematopoietic stem cell; metalloproteinase; transplantation

The vast majority of clinical trials and basic research studies have used vectors based upon murine leukemia viruses, and in optimization of conditions for gene transfer into human HSC consideration must be given to vector design to ensure that transgenes are not silenced in the stem cell or its progeny. In addition, retroviral envelopes must be selected based upon the nature and extent of specific cell surface receptor expression by appropriately primed HSC. Retroviral gene transfer is enhanced by co-localizing HSC and virus in the presence of fibronectin or fibronectin fragments ("retronectin"), and by addition of polycations such as polybrene and protamine (Fig. 1). [reviewed Moore and MacKenzie, 1999]. In early studies, the cytokine combinations used to activate HSC into cell-cycle were either effective only against more committed progenitors, or favored differentiation at the expense of self-renewal. With the discovery of the importance of ligands for the receptors c-kit, Flt3, and c-mpl that are expressed on the most primitive HSC, it became possible to achieve a high efficiency of transduction of HSC as measured by in vitro marrow stromal co-culture assays (the long-term culture-initiating cell-LTC-IC, and the cobblestone area-forming cell–CAFC, assays; Fig. 1). However, with development of the NOD/SCID and SCID-Hu models for supporting long-term human hematopoiesis (Fig. 1), it became apparent that in vivo engraftment could be compromised. The negative impact of this loss of engraftment following in vitro manipulation

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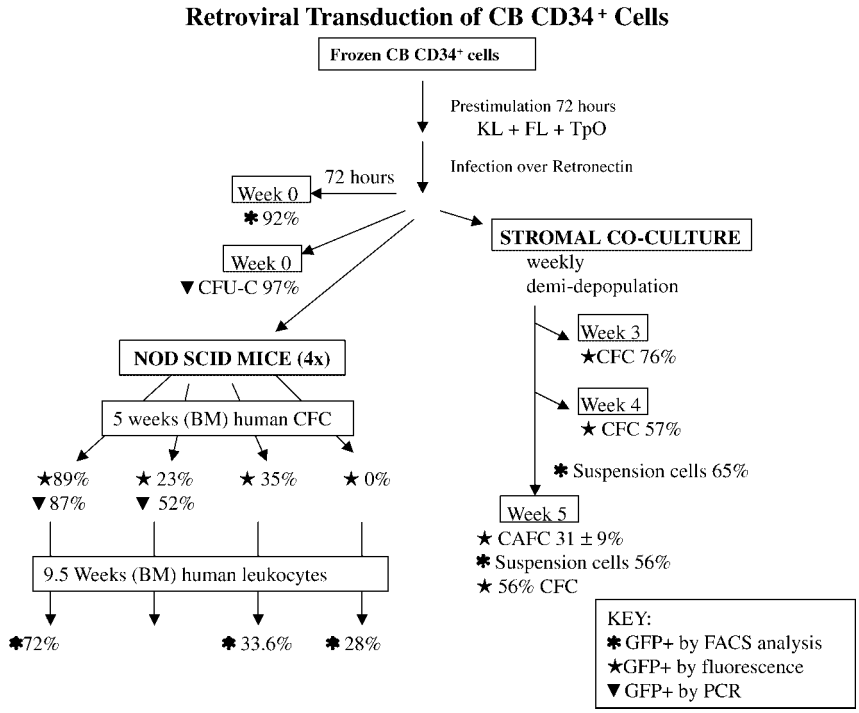

Fig. 1. Retroviral transduction of human hematopoietic stem cells and progenitors. In order to optimize retroviral transduction of cells with stem cell properties a PG13/SFG^{MPSV}-eGFP vector was used with the myeloproliferative virus LTR that provides for strong gene expression in primitive hematopoietic cells without evidence of silencing of gene expression in long-term culture. The enhanced green fluorescent protein (eGFP) signal allows direct visualization under fluorescence microscopy of transduced cells, forming hematopoietic colonies or cobblestone areas, as well as determination of transduction efficiency in different populations by FACS analysis. The virus was pseudotyped with the gibbon ape leukemia virus envelope and used at a titer of 5×10^6–10^7 pfu. CD34+ cells were isolated from umbilical cord blood (CC) using Miltenyi immunomagnetic bead separation. Cells were prestimulated for 72 h with Tpo, KL, FL (100 ng/ml), and IL-6 (20 ng/ml). The retroviral transduction was performed over 48 h in retronectin-coated plates with five changes of virus in the presence of the above cytokines. Following a further 48 h for optimal gene expression (total culture time 7 days), transduction efficiency was determined by FACS, by primary colony assay (CFU-GM/BFU-E) with direct fluorescent measurement, by PCR analysis and by LTC-IC and CAFC assay at 2- and 5-weeks, on MS-5 stroma. Transduced cells (equivalent to an input of 1×10^5 CD34+ cells) were also injected into 3.5 G γ-irradiated NOD/SCID mice.

and cell cycle activation of HSC was seen in the first clinical trial of *MDR* gene therapy [Hesdorffer et al., 1998], where only minimal MDR transduction of engrafted marrow cells was observed. The patients had received unmanipulated fresh cells as well as transduced cells, and this competition between fresh and cultured cells may have decreased the engraftment of the manipulated MDR-transduced and cycling HSC. To test this possibility, Qin et al. [1999] used a mouse model to study the competition between untransduced, unmanipulated fresh female marrow cells and cytokine-treated *MDR*-gene transduced male cells that were mixed and given to irradiated female mice. The transduced manipulated cells were at a significant disadvantage for engraftment as compared to fresh cells, when both were given together. The extent of the unfavorable competition depended on the relative numbers of transduced and untransduced cells administered.

SOURCES OF STEM CELLS FOR TRANSDUCTION

Gene transfer has been undertaken with stem cells derived from fetal liver (FL), umbilical cord blood (CB), adult bone marrow (BM), and apheresed peripheral blood (mPB) obtained in the regenerative phase following chemotherapy with or without hematopoietic growth factor (G-CSF, GM-CSF) mobilization, or with CSF mobilization alone. Stem cells obtained from fetal or neonatal sources are numerically fewer but possess greater self-renewal potential, longer telomeres and are more easily activated into cell cycle. Direct comparison of transduc-

tion efficiency of primitive hematopoietic cells has shown that CB > mPB > BM. While whole mononuclear cells from BM, PB, or CB can be used for gene transfer following cytokine priming, more efficient stem cell cycling and transduction is observed with isolated CD34 + populations or with stem cell enriched fractions (CD34 + CD38−; CD34 + Thy-1 +; CD34 + HLA-DRlo).

EVALUATION OF CELL CYCLE STATUS AND STUDY OF THE BIOLOGICAL BASIS FOR THE DISCREPANCY BETWEEN IN VITRO AND IN VIVO MEASURES OF STEM CELL FUNCTION FOLLOWING EX VIVO CULTURE OF CD34 + CELLS WITH CYTOKINES

A variety of combinations and concentrations of cytokines have been used to achieve stem cell cycling and stem cell expansion. The combination of Flt3 Ligand (FL), cKit Ligand (KL-also termed stem cell factor-SCF) and thrombopoietin (Tpo) has proved the most efficacious in activating HSC into cell cycle with retention of their self-renewal capacity (Figs. 1 and 2). The membrane growth factor KL is encoded by the Steel (*Sl*) locus and two alternatively spliced KL transcripts encode two stromal cell-associated KL proteins, KL-1 and KL-2. The KL-2 protein lacks the major proteolytic cleavage site for generation of soluble KL, thus representing a more stable cell-associated form of the ligand. Important insight about the respective roles in vivo of membrane-associated KL and soluble KL have come from studies of *Sl* mutant mice, since mice carrying *Sl* mutations have defects in hematopoiesis, melanogenesis and gametogenesis. The Sl^d allele encodes for a biologically active secreted soluble KL protein, and not membrane forms of the ligand as a result of an intragenic deletion that includes the transmembrane domain and the C terminus. Because the phenotype of Sl^d/Sl^d mice involves a severe hematopoietic deficiency it can be concluded that the membrane form of KL is critical for normal hematopoietic function. In mice generated to exclusively express the SL^{KL2} allele, steady-state hematopoiesis was normal but the mutant animals displayed increased sensitivity to sublethal doses of γ-irradiation with absence of the rapid elevation of soluble KL in the serum that is normally associated with myelosuppressive insult [Tajima et al., 1998]. The marrow microenvironment, or its in vitro substitute,

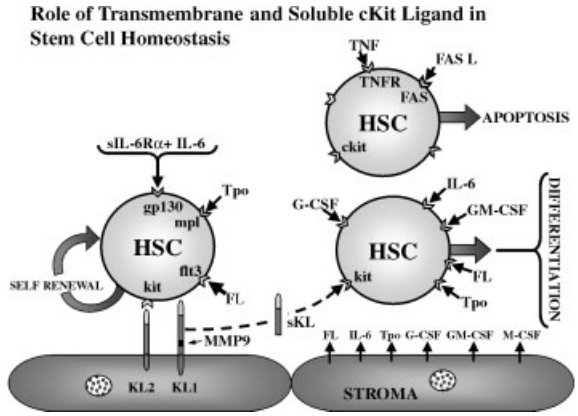

Fig. 2. The role of marrow stromal-derived hematopoietic growth factors in regulation of stem cell (HSC) self-renewal and differentiation. Spliced forms of ckit ligand, KL1, and KL2, interact with ckit receptors expressed on HSC, promoting adhesion and survival of the cells. In synergy with Tpo and FL signaling through the mpl and Flt3 receptors, and gp130 signaling following binding of a soluble IL-6 Receptor + IL-6 complex, HSC proliferation occurs with both self-renewal and differentiation. Metalloproteinases such as MMP9 are released within the marrow microenvironment by primitive hematopoietic cells including HSC, and by macrophages, following activation by SDF-1. Proteolytic cleavage of KL1 releases a soluble form of KL that can bind to ckit on HSC and on progenitors that are no longer adherent to stroma, preventing their apoptosis. In synergy with various stromal-derived growth factors sKL supports rapid differentiation of HSC and facilitates short term hematopoietic recovery. In the absence of ckit activation, particularly if associated with reduced flt3 and mpl signaling, HSC may undergo apoptosis, possibly associated with upregulation of TNFR and Fas.

the stromal cells in co-culture assay, produce a number of hematopoietic growth factors, either constitutively, or following inductive signals associated with stem cell adhesion or following stimulation by hematopoietic cell-derived cytokine such as IL-1 or TNF (Fig. 2). Interaction with membrane-anchored KL2 facilitates binding of HSCs expressing c-kit and may facilitate their survival in a quiescent state. Additional signals provided via ligand interactions with mpl, flt3, and gp130 provide cell-cycle activation and facilitate self-renewal of HSC. We postulate that the combination of cytokines will still provide a proliferative stimulus, once an HSC detaches from the stroma if the kit signal is provided by soluble KL, however, in this scenario differentiation may predominate over self-renewal. In the absence of kit signaling, particularly in the absence of mpl and Flt activation, stem cells may undergo apoptosis, possibly associated with expression of Fas and TNFR. The membrane-anchored signal is

necessary for preservation of the pool of long-term repopulating stem cells, while the soluble KL is required for optimal short-term hematopoietic recovery where rapid HSC mobilization, proliferation and differentiation into progenitor cells is required. The metalloproteinase MMP-9, rapidly produced by HSC and macrophages within the marrow following stimulation by the chemokine SDF-1, plays an important role in proteolytic cleavage of transmembrane cytokines including KL2, TNF, and possibly IL-1 and M-CSF, with release of soluble ligands with receptor agonistic action [Heissig et al., 2001] (Fig. 2). Flt-3 ligand is efficacious in prompting stem cell cycling with retention of self renewal, particularly when combined with Tpo and KL [Petzer et al., 1996]. In a primate study, the addition of FL and stromal support to a 4-day cytokine priming with IL-3, IL-6, and KL alone, increased the degree of subsequent long-term engraftment of retrovirally transduced cells from 0.01% to a clinically relevant 10–20% [Heim and Dunbar, 2000; Dunbar et al., 2001]. However, even in the presence of FL, prolonging in vitro cytokine priming actually compromised engraftment in a competitive study with short-term primed cells, despite a significant expansion of cells and CFC following long term culture. Interleukin-3 was used in a number of earlier cytokine combinations for HSC priming, and while capable of inducing maximal CFC and total cell expansion, it may be detrimental to HSC self-renewal. Reduced LTC-IC amplification in the absence of an effect on CFC amplification was seen, when FL and KL levels were decreased but IL-3 levels were high [Zandstra et al., 1997]. IL-3 may be directly influencing HSC self-renewal probability and favoring differentiation. In addition, IL-3 has an inhibitory effect on stem cell CXCR4 signaling, reducing HSC chemotactic response to SDF-1 [Jo et al., 2000]. Purified murine ckit+, Sca-1+, Lin− HSC are efficiently induced to proliferate by FL and KL, but retention of HSC activity requires additional exposure to a ligand that will activate gp130. IL-11 acts through gp130 and is a potent co-factor with FL and KL in stimulating murine HSC. Stem cells do not express IL-6Rα, but express abundant gp130 which can be activated by a soluble form of IL-6R which has bound IL-6. A fusion protein comprising IL-6 joined to sIL-6R by a flexible linker (HyperIL-6) can substitute for IL-11 in murine HSC cultures [Auder et al., 2001]. Ueda et al. [2000] reported that a combination of KL + FL + TPO plus IL6/sIL-6R was superior to other combinations in expanding CB cells with NOD/SCID engraftment potential. Enhanced signaling through gp130 on stem cells may be advantageous in two ways-favoring self-renewal and upregulating CXCR4, allowing efficient marrow homing [Moore, 2000]. In cultures of both murine and human stem cells, an initial dramatic loss of repopulating activity has been found to precede execution of a first cell division [Peters et al., 1996; Gothot et al., 1998; Habibian et al., 1998; Glimm et al., 2000; Wiesmann et al., 2000]. Ex vivo culture of bone marrow exhibits this engraftment defect concomitantly with progression of HSC through S-phase, suggesting that the cell cycle transit impairs their ability to engraft [Kittler et al., 1997]. In vitro cytokine stimulation of murine bone marrow cells induces cell cycle transit of primitive stem cells, taking 40 h for progression from G0 to mitosis and 12 h for subsequent doublings. Studies performed by Peters et al. [1996] have shown that murine marrow cells cultured with IL-3, IL-6, IL-11, and SCF for 48 h resulted in impaired engraftment. Further studies have shown that long term engraftment, (2 and 6 months), of male BALB/c marrow cells exposed to the same cytokine combination over 24–48 h and then transplanted into female recipients, resulted in engraftment nadirs in late S-and early G2-phases of the cell cycle [Habibian et al., 1998]. Based on this data, it can be concluded that position in the cell cycle will determine whether long term engraftment will occur and that certain phases of the cell cycle are incompatible with the multistep pathway of homing and proliferation of self-renewing HSC. In serum-free cultures of cord blood CD34 cells exposed to Tpo overnight and then cultured for a further 4 days with IL-3, IL-6, KL, FL, G-CSF, the first division of the CD34+ CD38−ve cells did not occur until Day 3, and yet by Day 6 more than 60% of the HSC had completed at least 3 divisions. On the 5^{th} day, NOD/SCID transplantable stem cell activity was restricted to the G1 fraction even though committed progenitors (CFC) and LTC-IC were evenly distributed between G0/G1 and S/G2/M, and in the former fraction all LTC-IC were in G1 [Glimm et al., 2000]. The observed engraftment defect of proliferating human HSC during their passage through S/G2/M phases of the cell cycle and their failure to re-enter, G0 may have a consi-

derable negative impact on the clinical utility of current ex vivo expansion or retroviral marking protocols that depend on maximal induction of stem cell cycling. The use of lentivectors for transduction obviates, the need for stem cell cycling in order to achieve vector integration. However, there is clear evidence that cells show higher transduction with lentivectors, when activated out of G0 and into G1. In the case of hematopoietic stem cells, this can be achieved by 18–36 h of activation in vitro, usually with a single cytokine such as Tpo [Glimm et al., 2000] or a combination of Tpo + KL + FL [Gothot et al., 1998]. In freshly harvested populations of human mPB CD34 +cells, NOD/SCID repopulating cells are in G0 rather than G1 (16-fold more engraftment with equal numbers of G0 vs. G1). Upon culture for 36 h with IL-3, KL and FL, cells move from G0 to G1 but not into cell cycle, however their repopulating capacity markedly diminished as HSC transited into G1 [Gothot et al., 1998]. Thus, both G0–G1 progression and entry into S/G2/M in vitro are associated with a decrease in HSC engraftment capacity.

HSC Homing to the Marrow

An explanation for the discrepancy between the increased stem cell content following ex vivo expansion as measured by in vitro CAFC/LTC-IC assay and impaired in vivo engraftment is that HSCs acquire a homing defect. Homing is a process by which stem cells specifically engraft in the bone marrow and not in other organs. The homing of HSC following their intravenous infusion is remarkably efficient, with 10–20% of long term repopulating HSC localizing in the marrow within 3–24 h. Homing of stem cells, and their retention within the marrow, depends upon adhesion molecule interactions between stem cells, sinusoidal endothelium, stromal cells, and bone marrow matrix (Fig. 3). Adhesive interactions profoundly influence the localization, proliferation and differentiation of HSC. Cytokine exposure may affect the expression

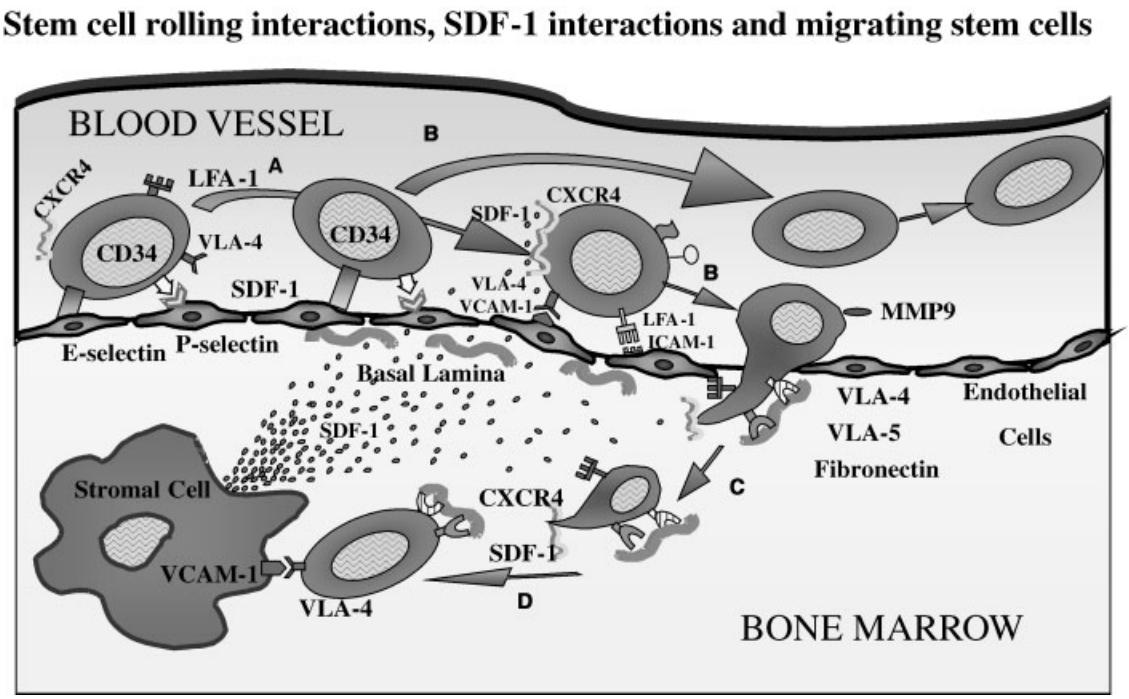

Fig. 3. Stem cell rolling interactions, SDF-1 interactions, and HSC transendothelial migration. **A**: HSC rolling interactions on marrow sinusoidal endothelium constitutively expressing E and P selectins. Following rolling, CXCR4 + stem cells are activated by SDF-1, which is secreted from bone marrow endothelial cells and triggers LFA-1/ICAM-1 and VLA-4/VCAM-1 interactions promoting firm adhesion to endothelial cells. **B**: Cells that do not express sufficient levels of CXCR4 will detach from the endothelial layer and return to the blood stream. **C**: The arrested human CXCR4 + HSC, in response to SDF-1, will extravasate and migrate through the underlying basal lamina ECM using VLA-4 and VLA-5 integrin receptors to fibronectin (FN), and facilitated by secretion of metalloproteinases (MMP). **D**: Migrating stem cells will eventually reach the ''stem cell niches,'' which consist of stromal cells that present the proper set of adhesion molecules (e.g., VCAM-1 and FN), SDF-1, and growth stimulatory factors [Modified after Peled et al., 2000].

and function of adhesion molecules that are important for engraftment. CD34 + cell expression of LFA-1, CD44, VLA-4, VLA-5, and CD62L (L-selectin) has been correlated with more rapid engraftment following clinical transplantation. Subsetting by flow cytometry of primitive murine Sca-1 +Lin − cells into adhesion molecule low or high populations has pointed to a requirement for expression of CD43 and VLA-5, but not LFA-1, VLA-4, or CD62L in engraftment of syngeneic mice [Orschell-Traycoff et al., 2000]. Becker et al. [1999] have demonstrated that when murine bone marrow cells are exposed to IL-3, IL-6 IL-11, and cKit L for 24–48 h, the expression of alpha-4 (VLA-4) and β-1 decreases, and α-1, α-5, α-6, and PECAM increases. Concomitantly, the adhesion of these cells to fibronectin declines. In summary, the changes of adhesion molecules expression on hematopoietic stem cells decreases the localization of these cells to the bone marrow, and may confer a propensity to migrate to non-hematopoietic organs where clearance and destruction of these cells occurs.

The role of chemotaxis in this homing has been revealed in a number of studies on the role of the chemokine Stromal-Derived Factor-1 (SDF-1/CXCRL12) produced by marrow stromal cells, and its interaction with cells expressing its receptor, CXCR4. CXCR-4 is expressed on CD34 +/CD38− and CD34 +/Thy-1 + stem and progenitor cell subsets [Mohle et al., 1998; Fig. 3]. Mice with a knockout of either the chemokine gene or its receptor die perinatally with failure of hematopoietic development in the marrow, probably reflecting impaired stem cell migration from the liver [Nagasawa et al., 1996; Zou et al., 1998]. In vitro chemotactic assays have shown that an SDF-1 gradient produces a rapid transmembrane and transendothelial migration of human HSC as detected by long term stromal co-culture assay, and this could be inhibited by blocking antibodies to CXCR4 [Jo et al., 2000]. In the NOD/SCID xenograft model, in vivo engraftment of human stem cells in the mouse marrow was blocked by antibodies to human CXCR4 indicating the in vivo significance of this chemotactic mechanism [Peled et al., 1999, 2000]. The addition of IL-3 to the KL, FL, and Tpo cytokine combination used to activate CD34 + cells into cycle and to promote HSC expansion caused a down-modulation of CXCR-4 signaling, with the chemotactic response to SDF-1 in vitro being reduced by 20–50% [Jo et al., 2000]. In vivo data has also shown that preincubation of stem cells with IL-3 reduces short and long term engraftment potential [van der Loo et al., 1995]. The integrins VLA-4, VLA-5 and LFA-1, interacting with VCAM-1 and ICAM-1 on marrow stroma and endothelium play a crucial role in SDF-1 mediated homing (Fig. 3). SDF-1 induces a rapid but transient activation of these integrins on stem cells, facilitating the binding of these cells to endothelium and their egress from the circulation [Peled et al., 2000; Fig. 3]. Co-injection of BM cells and antibodies to VLA-4 or VCAM-1 blocked progenitor homing in mouse transplant models [Frenette et al., 1998; Vermeulen et al., 1998]. Preincubation with antibodies to VLA-4 and VLA-5 prevented, and antibodies to LFA-1 reduced, engraftment of human CB CD34 + cells in NOD/SCID mice [Peled et al., 2000]. SDF-1 also rapidly upregulates expression of matrix metalloproteinase-9 (MMP-9) on primitive hematopoietic cells facilitating transit of these cells across endothelium and basement membrane [Fig. 3; Heissig et al., 2001]. Overexpression of SDF-1 in the circulation by intravenous administration of an adenovector expressing the *chemokine* gene leads to a reversal of the normal SDF-1 gradient with egress of cells from the marrow and elevation of circulating numbers of CXCR4-expressing lymphocytes, monocytes, platelets, and hematopoietic stem and progenitor cells [Hattori et al., 2001]. This SDF-1-mediated HSC mobilization is dependent upon MMP-9 activation, since it was completely blocked in MMP9-deficient mice [Heissig et al., 2001].

SDF-1 AND TNFα SIGNALING IN STEM CELLS

Interactions between T-lymphocytes and macrophages that have been exposed to SDF-1 have been shown to lead to T-cell death. SDF-1 up-regulated surface expression of TNFα on monocytes and macrophages, and TNF-R on CD8 T-cells and the subsequent interaction between these cells triggered rapid (24 h) T-cell apoptosis [Herbein et al., 1998]. It is possible that a similar interaction between HSC and macrophages occurs in vivo and in CD34 cell cultures. We have shown that SDF-1 upregulates TNF-RI (which is the main mediator of cellular TNF response including TNFα-mediated cytotoxicity), and TNF-RII (which is more tightly regulated but can mediated cytotoxicity)

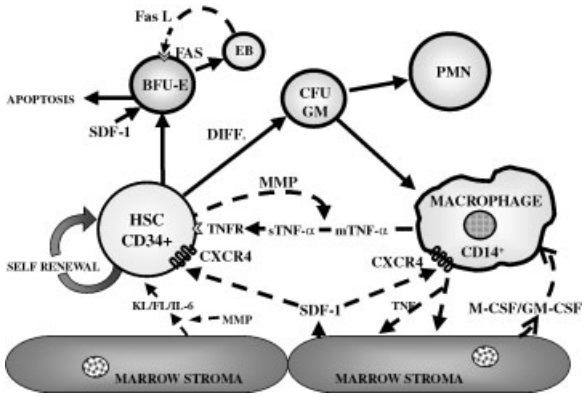

Fig. 4. The role of SDF-1 produced by bone marrow stromal elements. Chemoattraction of hematopoietic stem cells (HSC) and CD14+ macrophages is mediated by SDF-1 interacting with CXCR4 expressed on these cells. Both cell types show rapid adhesion to stroma following CXCR4 signaling leading to increased avidity/affinity of VLA4. SDF-1 upregulates the TNFα receptors (TNFRI and RII) on HSC and membrane-associated TNFα on macrophages. SDF-1 activation also induces release of the metalloproteinase MMP-9 by CD34+ cells, which cleaves both mTNF on macrophages and the KL1 variant of the stromal cell membrane-associated ckit ligand with release of soluble cytokines (sTNFα and sKL). Soluble KL prevents, and sTNFα may promote, apoptosis of HSC and myeloid progenitors (CFU-GM). TNFα released by macrophages can induced increased stromal cell production of a number of hematopoietic growth factors (G-CSF, GM-CSF, IL-1, IL-6, M-CSF, and possibly Tpo). SDF-1 may also upregulate Fas (CD95) on erythroid progenitors (BFU-E), which may then undergo apoptosis in the presence of Fas ligand produced by differentiating erythroid cells.

on CD34+ cells and sensitizes them to TNF-mediated apoptosis (Fig. 4). Tumor necrosis factor has been shown to elicit a variety of responses, inhibitory and stimulatory, on primitive human or murine hematopoietic progenitors depending on cell population exposed and growth factors present. Some effects are due to the ability of TNFα to induce production and release of other cytokines, however, direct effects on primitive hematopoietic cells have been observed. Low doses (0.1 ng/ml) of TNFα in presence of FL, KL, IL-3, IL-6, and G-CSF markedly decreased (>10-fold below input) the output of LTC-IC in short-term culture of adult BM CD34+CD38− cells under conditions that did not affect CFC production. The TNFα effect is quite rapid, occurring within 3 days of exposure, and at a dose of TNFα was not cytotoxic nor inhibitory to cytokine-induced proliferation in single cell assay [Maguer-Satta et al., 2000]. Dybedal et al. [2001] reported that NOD-SCID engraftment of LTC-IC in the marrow was profoundly depressed (>95%) if mice were treated with TNF 24, 64, and 96 h after transplantation of CD34+ cells. Production of intracellular ceramide is a well described consequence of TNF stimulation and the apoptosis-inducing action of TNFα on hematopoietic cells is mimicked by exposure to C2-ceramide, a diffusible analog of ceramide that enters cells passively [Maguer-Satta et al., 2000]. At lower doses that do not induce apoptosis, C2- and C6-ceramide and D-erythro-MAPP (an inhibitor of the alkaline ceramidase that breaks down ceramide to sphingosine) reduced or eliminated LTC-IC within 24 h of culture [Maguer-Satta et al., 2000]. TNFα at low concentrations appears to affect stem cell fate decisions, reducing self-renewal at the expense of differentiation.

FAS RECEPTOR EXPRESSION

Fas (CD95) is a 45 kDa type I integral membrane protein, which is identical to Apo-1 antigen and belongs to the nerve growth receptor/TNF superfamily [Itoh et al., 1991]. This protein is expressed on hematopoietic cells such as fetal liver CD34+ cells [Barcena et al., 1999], neutrophils and monocytes [Iwai et al., 1994]. Stem cells and hematopoietic progenitors have a low [Sato et al., 1997], to negative [Nagafuji et al., 1996; Niho and Asano, 1998], level of expression of Fas receptor protein (CD95) on their cell membranes. In vitro stimulation results in a marked increase on Fas antigen (CD95) expression on these cells [Maciejewski et al., 1995; Nagafuji et al., 1996; Sato et al., 1997]. In vitro, functional expression of CD95 can be induced by in vitro culture with negative hematopoietic regulators (TNFα and IFNγ), and these cytokines induce Fas-mediated growth suppression and apoptosis of murine progenitor cells [Dybedal et al., 2001]. They also suppress human colony formation by myeloid, erythroid, and multipotential progenitors and decrease long-term culture initiating cells in bone marrow cultures [Selleri et al., 1995; Moreau et al., 2001]. Takenaka et al. [1996] reported that human CD34+ cells isolated from bone marrow do not express CD95 and approximately half of them express Bcl-2. When CD34+ cells are cultured with SCF, IL-3, IL-6, G-CSF, and Epo, they gradually become positive for CD95 and rapidly lose Bcl-2 expression. Dybedal et al. [2001] showed that when murine lineage-depleted bone marrow progenitor cells are cultured with SCF alone, or with a

combination of TNF and CSF, GM-CSF, IL-3, IL-6, and SCF, upregulation of Fas expression (CD95) occurred and an almost complete block of clonogenic growth was obtained when Fas was activated by Anti-Fas agonistic antibody. Increased expression of CD95 has been reported for human BM cells cultured in KL, IL-3, IL-6, GM-CSF, G-CSF, or Epo [Takenaka et al., 1996; Stahnke et al., 1998]. In human fetal liver cultures, endogenous Fas ligand has an inhibitory effect on fetal HSC which could be blocked by soluble FasL and agonistic anti-CD95 monoclonal antibody [Barcena et al., 1999]. Similar findings were reported using adult human CD34 + CD38-BM cells, with reduced apoptosis in cells cultured for 48 h with sFasL [Josefsen et al., 1999]. It may be concluded that interaction between endogenous FasL and FasR can lead to increased stem/progenitor apoptosis in culture, and inhibiting this interaction leads to improved stem cell expansion. We believe that inhibition of this pathway may also improve in vivo engraftment of cultured cells. In support of this concept is a study that showed that G-CSF mobilized CD34 cells showed significant loss of NOD/SCID engraftment potential even when cultured with a cytokine combination optimal for preservation of in vitro stem cell capacity (Tpo + KL + FL) [Young et al., 2001]. Cultured CD34 + cells did not show impaired adhesion to VCAM-1 coated plates under both static and flow conditions, and chemotaxis towards SDF-1 was not impaired, suggesting normal function of CXCR4 and VLA4. CD34 +Thy-1 + cells expressed increasing levels of Fas receptor beginning at 20hrs of culture and peaking after 3 days. This was associated with altered homing of 3 day cultured cells, with increased numbers lodging or being sequestered in the liver 24 h after transplantation, where they may interact with endogenous Fas ligand and undergo apoptosis. In support of this model, the addition of caspase inhibitors to the CD34 cell cultures improved subsequent NOD/SCID engraftment 2–3 fold [Young et al., 2001].

CONCLUSIONS

Strategies are required to improve engraftment of cell-cycle activated, cultured HSC. Attempts have been made to reverse the cell-cycle activation by the use of agents that inhibit HSC proliferation. Transforming growth factor β1(TGF-β1) arrests proliferating repopulating HSC, and can block the S-phase entry of high proliferative potential CFCs and can inhibit the proliferation of primitive HSC [Cashman et al., 1999; Dao et al., 1998]. However, when HSC isolated from adult mouse bone marrow are cultured with TGF-β1, this cytokine is unable to overcome the failure of quiescent cells (Rhodamine low) to engraft after transplantation in murine competitive repopulating assays [Wiesmann et al., 2000]. Similar results were reported with human cord blood [Glimm et al., 2000]. Additional studies using a stem cell inhibitor (*N*-Acetyl-Ser-Asp-Lys-Pro) that prevents the entry of colony forming units into S-phase have failed to demonstrate a beneficial effect in recovery kinetics in short term hematopoietic reconstitution [Szilvassy et al., 2000]. In another strategy, rhesus monkey CD34 + cells that had been activated into cycle by a 4 day culture with a combination of cytokines were then transferred to culture with fibronectin fragment and KL alone for a further 48 h [Takatoku et al., 2001]. Stable in vivo engraftment levels of up to 29% were observed following infusion of the cells maintained for 2 days under these non-stimulatory conditions. The intriguing results with caspase inhibitors suggests that cell cycling per se is not the cause of poor engraftment, but may be linked to upregulation of Fas and activation of proapoptoctic pathways [Young et al., 2001]. A number of inhibitors of Fas and its downstream pathway of caspase activation are available for evaluation. It is also possible that retroviral transduction of proliferating HSC performed in marrow stromal or endothelial co-culture may better preserve the engraftment and self-renewal capacity of HSC, with adherent cells being less likely to upregulate Fas.

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