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Spatially Separated Distribution and Highly Flexible Expression of Adhesion Molecules Facilitates Dynamic Hematopoiesis

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In bone marrow niches, direct interactions of Hematopoetic Stem Cells (HSCs) with supportive stromal cells and the extracellular matrix are essential for regulation of hematopoiesis. These interactions are mediated by several sets of adhesion molecules. *In vivo*, different adhesion molecules were spatially separated and showed specific distributions for the different adhesion molecules. These patterns for VLA-2, VLA-3, ALCAM, VLA-1, VLA-4, VCAM-1 and ICAM-3, varied in size, ranging from a single cell to smaller or larger cell clusters. ICAM-1 and VLA-5 were expressed by virtually all cells. The results are similar in seven individual steady state bone marrows. *In vitro*, adhesion molecule expression changed for several stromal cell types by exposing them to different inductors as present in different animal sera. Besides the normal blood cell turn over recruitment of more blood cell developmental centers is needed during increased demands for hematopoietic cells. During infection far more granulocytes are demanded by the organism than during normal turnover. Such adaptation to changing conditions is easily accomplished by highly flexible spatio-temporal distribution of adhesion molecules since it provides bone marrow stromal cells with the capacity to deliver the specific lineages that the organism demands.

Key words: Adhesion molecules, human bone marrow, stromal cells, integrin

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INTRODUCTION

Bone marrow is a widespread organ throughout the body and highly organized tissue, in which proliferation and differentiation of hematopoietic stem cells (HSCs) into blood cells is tightly regulated. In bone marrow, hematopoiesis occurs in special microenvironments (Adams and Scadden, 2006; Kiel *et al*., 2005; Taichman, 2005; Tokoyoda *et al*., 2004; Zhang *et al*., 2003). Hematopoiesis is an intimate interplay between hematopoietic and stromal cells, which create complex but unique microenvironments by producing certain Extracellular Matrix (ECM) molecules, growth factors, cytokines and chemokines (Calvi *et al*., 2003; Fuchs *et al*., 2004; Taichman, 2005; Tokoyoda *et al*., 2004; Torok-Storb *et al*., 1999; Li and Xie, 2005). Hence, those microenvironments provide all the instructive signals required for the homing and controlled development of hematopoietic stem cells, HSCs (Yin and Li, 2006; Wilson and Trumpp, 2006). Direct physical interactions between HSCs and supportive stromal cells are essential in keeping stem cells in the niche and maintaining their stem cell character, that is the ability to self-renew and to differentiate along multiple lineages (Calvi *et al*., 2003; Fuchs *et al*., 2004; Taichman, 2005; Tokoyoda *et al*., 2004; Zhang *et al*., 2003). Stromal cells support normal hematopoiesis in several ways, including retention of HSCs in bone marrow niches (Whetton and Graham, 1999) and production of proliferation and differentiation inducing, as well as inhibiting growth factors and cytokines (Taichman, 2005; Zhu and Emerson, 2002).

In addition to their interaction with stromal cells, HSCs also adhere to ECM molecules. ECM, in particular glycosaminoglycans, contributes significantly to the formation of niches and regulates hematopoiesis, indirectly by immobilization of HSCs and sequestering growth factors and directly by induction of signal transduction pathways (Rosso *et al*., 2004).

Adhesion of HSCs to both stromal cells and ECM is mediated by a diversity of integrin and non-integrin receptors. Adhesion molecules play an important role in regulation of hematopoiesis. The β1 integrin was reported to be indispensable for self renewal (Gottschling *et al*., 2007). Which member of this adhesion molecule family of proteins is involved in self renewal remains elusive. Several reports describe expression of adhesion molecules by bone marrow-derived cells and the implications for adhesion, survival and proliferation of HSCs (De Ugarte *et al*., 2003; Fox and Kaushansky, 2005; Scott *et al*., 2003; Taichman, 2005; Teixido *et al*., 1992). In particular, the interaction between VLA-4 (integrin α4β1; very late antigen-4) on HSCs and VCAM-1 on stromal cells has been considered to play a major role in several steps of hematopoiesis (Fox and Kaushansky, 2005; Teixido *et al*., 1992). However, expression of a wealth of adhesion molecules and redundancy in their binding capacity for other adhesion molecules and ECM components indicate that multiple integrin-mediated pathways are utilized to retain HSCs within specialized niches and control proliferation and differentiation of HSCs (Prosper and Verfaillie, 2001; Teixido *et al*., 1992; Wilson and Trumpp, 2006). In most studies expression of adhesion molecules is measured by FACS analysis and the role of these molecules in hematopoiesis has been studied with the use of blocking antibodies and strategies that down- or upregulate their function (Gottschling *et al*., 2007). Although these *in vitro* studies are important in unravelling their binding capacities and mechanisms of action, little is known about the spatio-temporal distribution of adhesion molecules in bone marrow and changes in expression under changing circumstances. Several reports indicate that stromal cells like osteoblasts and fibroblasts have a fixed phenotype (Kuznetsov *et al*., 1997; van Beurden *et al*., 2003). Moreover, stromal cells when immortalized show also a fixed phenotype (Roecklein and Torok-Storb, 1995) coupled to a fixed function (Breems *et al*., 1994; Neben *et al*., 1993). However, hematopoiesis is a highly dynamic process, since it has to adapt to continuously altering needs (Honey, 2006). For instance, at high altitudes the organism is in need for erythrocytes and hematopoiesis has to switch to production of more red blood cells. Such an adaptive response is hardly accomplished with fixed stromal phenotypes.

Development of stem cells towards differentiated end cells involves several lineage-specific pathways, each comprising multiple steps. With the existence of not only lineage-specific, but even stage-specific cellular niches, as has been reported for B-lymphopoiesis (Tokoyoda *et al*., 2004), this implies the presence of a heterogeneity of cellular environments with varying properties. A few reported studies indicate that together with ECM and secreted growth factors, also expression and function of adhesion molecules change with the circumstances. LFA-1 has previously been shown to be dynamically regulated on $CD34^+$ cells (Torensma *et al*., 1996). During B-lymphocyte development in mouse bone marrow, adhesiveness of B-cell precursor cells to VCAM-1 changes when they move between subsequent stage-specific cellular niches (Tokoyoda *et al*., 2004).

To further explore the spatial organisation of adhesion molecules in human bone marrow, the expression of a panel of adhesion molecules was studied by immunohistochemistry. Furthermore, to gain more

insight in the dynamics of adhesion molecule expression, we investigated *in vitro* the expression of adhesion molecules by stromal cells when exposed to different inductors. Since it is known that only certain batches of animal sera can sustain stem cell growth, we argued that different animal sera (batches) contain different inductors that putatively alter adhesion molecule expression.

MATERIALS AND METHODS

This study was conducted at the department of tumorimmunology at Nijmegen during 2005 and 2006.

Materials: Monoclonal antibodies (mAbs) Rek-1 and AZN-IC3.1 against intercellular cell adhesion molecules (ICAM)-1 and -3, respectively, were generated in our laboratory as described previously (Binnerts *et al*., 1994; Bleijs *et al*., 2000). Anti-α4 (CD49d; VLA-4) mAb ALC1/1.3.1 and anti-integrin subunit αV (CD51) mAb were obtained from the Fourth Workshop on Leucocyte Differentiation Antigens (Vienna, 1989). Anti-CD31 mAb WM-59, anti-CD34 mAb 563, anti-α1 (CD49a; VLA-1) mAb SR84, anti-α2 (CD49b; VLA-2) mAb AK7, anti-α3 (CD49c; VLA-3) mAb C3 II.1, anti-VCAM-1 (CD106) mAb 51-10C9, mouse IgG1 pure and purified mouse IgG2b, κ were purchased from BD Pharmingen (San Diego, USA), while anti-myeloid peroxidase (MPO) polyclonal Ab and anti-CD68 mAb EBM11 were from Dako (Glostrup, Denmark). Anti-ALCAM (activated leucocyte cell adhesion molecule; CD166) mAb AZN-L50 was obtained from Sanbio (Uden, the Netherlands); FITC-conjugated anti-CD41 mAb P2 from Immunotech (Marseille, France), anti-α5 (CD49e; VLA-5) mAb SAM-1 from Serotec (Oxford, UK) and FITC-F(ab')$_2$ goat-anti-mouse IgG from Zymed (San Franscisco, USA). All mAbs were IgG1 Abs, except for anti-α5 mAb SAM-1, which was of subtype IgG2b. Biotinylated horse-anti-mouse IgG and goat-anti-rabbit IgG Abs, streptavidine-biotin-alkaline phosphatase complex (ABC-AP) and levamisol were from Vector Laboratories, Inc. (Burlingame, USA). Tissuetek (OCT Embedding Matrix) was purchased from Cell Path (Newtown, Wales, UK) and Fast Red TR from Sigma (St. Louis, USA). Aldefluor® was obtained from Stem Cell Technologies (Meylan, France). Alpha minimal essential medium (αMEM) and fetal calf serum (FCS) were obtained from Gibco (Grand Island, USA). Human serum (HuS; heat-inactivated) was purchased from Sigma (St. Louis, USA) and horse serum (HoS) from Integro (Zaandam, the Netherlands). Basic fibroblast growth factor (bFGF) was from R and D Systems (Minneapolis, USA).

Collection of bone marrow tissue for immunohistochemistry: Human bone marrow tissue was obtained after informed consent from patients undergoing open heart surgery. Small bone marrow clumps were taken from the sternum immediately after splitting the bone. The tissue was immediately embedded in Tissuetek, frozen in liquid nitrogen and stored at −80°C until further use. The morphology of the samples was assessed on hematoxylin-eosin stained 3 μm thick sections.

Immunohistochemistry: Sections (3 μm thick) from frozen tissue were mounted on poly-L-lysine coated slides, dried and fixed in acetone for 5 min. A specific staining was blocked by incubation for 10 min with 20% (v/v) normal horse serum or normal goat serum. Subsequently, tissue sections were incubated with the primary antibody for 1 h at room temperature. In case of staining for adhesion molecules, the following antibodies were used at a final concentration of 10 μg mL^{-1}: anti-ALCAM mAb AZN-L50, anti-ICAM-1 mAb Rek-1, anti-ICAM-3 mAb AZN-IC3.1, anti-α1 mAb SR84, anti-α2 mAb AK7, anti-α3 mAb C3 II.1, anti-α4 mAb ALC1/1.3.1, anti-α5 mAb SAM-1 and anti-VCAM-1 mAb 51-10C9. To identify specific cell populations, tissue sections were incubated with appropriate antibodies against various cell markers. Staining with the following antibodies is shown in the figures: Anti-MPO polyclonal Ab 1: 750, anti-CD41 mAb P2 1: 10, anti-CD31 mAb WM-59 5 μg mL^{-1}, anti-CD68 mAb EBM11 1: 100 and anti-CD34 mAb 563 20 μg mL^{-1}. The secondary Ab was biotinylated horse-anti-mouse IgG 1:200 diluted or biotinylated goat-anti-rabbit IgG 1: 200 in case the first Ab was a mouse monoclonal or a rabbit polyclonal Ab, respectively. Secondary Abs were diluted in PBS with 2% (v/v) human serum. Biotinylated secondary Abs were detected with ABC-alkaline phosphatase and bound Abs were visualized by incubation with FastRed. Slides were counterstained with hematoxylin and mounted. Negative control slides were stained with mouse IgG (1:20) of the appropriate subclass.

Primitive hematopoietic cells in bone marrow sections were identified by staining for the enzyme aldehyde dehydrogenase with Aldefluor® as follows. Freshly cut sections (10 μm thick) from frozen tissue biopsies were mounted on poly-L-lysine coated slides and kept on ice until use. The staining was performed by incubation of the sections for 45 min at 37°C in the dark in a solution of 100 μL assay buffer containing 0.5 μL Aldefluor®. Slides were rinsed with PBS and mounted. Fluorescence was analyzed on a fluorescence microscope.

Isolation and culture of bone marrow stromal cells: Human bone marrow tissue was obtained after informed consent from either healthy allogeneic bone marrow

donors (n = 5) or orthopedic patients undergoing hip replacement (n = 13). From normal bone marrow donors small clumps of bone marrow were collected by aspiration (Netelenbos *et al*., 2001). Particulate matter was removed from the bone marrow aspirate with 70 μm filters. Residual bone marrow present in the filters, consisting of cell aggregates, or hematons and small fragments of bone, was designated Filtrate. Bone material from the femur obtained from orthopedic patients was vortexed in order to separate most of the marrow from the bone. The resulting bone fraction was designated Bone. The marrow was centrifuged for 5 min at 400 g. From fractionated marrow the fat layer, containing fat and fat-associated cells, was collected (designated Fac) as well as the cell pellet (designated BM pellet). Dependent on the amount of fractionated material processed, each bone marrow fraction was divided over 1 to 4 flasks for culture under different conditions.

Bone marrow fractions were grown in α-MEM supplemented with 20% (v/v) serum and antibiotics. Sera used were human pooled serum (HuS), horse serum (HoS) or heat-inactivated Fetal Calf Serum (FCS). Where indicated, dexamethasone was added to culture media with HuS at a final concentration of 10^{-9} M. Basic-fibroblast growth factor (final concentration 2.5 ng mL^{-1}) was added to overcome slow growth (Banfi *et al*., 2000). After two days of culture non-adherent cells were removed. Attached cells were grown until confluency. Cells were not passaged *in vitro* to minimize changes in protein expression. From almost all seeded bone marrow samples a stromal cell culture was obtained, thus resulting in fifty stromal cell cultures derived from 18 donors. All cells were maintained under an atmosphere of 5% CO_2/95% air in a humidified incubator at 37°C.

Flow cytometry: Confluent layers of bone marrow stromal cells were trypsinized to detach cells. After washing with PBS/BSA, the cells were incubated for 45 min at 4°C with 5-10 μg mL^{-1} of each of the following primary antibodies: anti-ALCAM mAb AZN-L50, anti-α2 mAb AK7, anti-α3 mAb C3 II.1, anti-α5 mAb SAM-1 and anti-αV mAb. As a negative control mouse IgG of the appropriate subclass was used. Bound antibodies were detected by incubation with FITC-conjugated goat-anti-mouse $F(ab')_2$ IgG. Cell associated fluorescence was measured with a Beckton Dickinson FACScan flow cytometer. Staining results were analyzed using BD Cell Quest Pro software.

Due to shortage of cells it was not possible to measure expression of all adhesion molecules mentioned above by all stromal cell cultures, derived from different donors and bone marrow fractions, cultured under different conditions. Expression data in Fig. 3 are shown as the mean expression by stromal cell cultures of a certain bone marrow fraction of different donors grown in the same culture medium.

RESULTS

Different lineages of hematopoietic and stromal cells in bone marrow are spatially separated: Spatial separation of the various lineages that develop out of HSCs could already be distinguished just based on morphology (Fig. 1). Staining with hematoxylin-eosin revealed separate areas with cells of the erythroid or myeloid lineage (Fig. 1a).

The identity of different cells was further confirmed by immunohistochemistry with antibodies against lineage-specific cell surface markers. For example, staining with an antibody against myeloid peroxidase (MPO) clearly identified mature cells of the myeloid lineage, which resided in clusters between MPO-negative erythroid cells (Fig. 1b). In contrast to these lineages that develop in clusters, other cells were present as single cells dispersed through the bone marrow. This was the case for distinct hematopoietic cells, such as $CD41^+$ megakaryocytes (Fig. 1c) and hematopoietic stem- and lineage committed cells which could be detected with Aldefluor® (Fig. 1d).

Interspersed between the hematopoietic cells resided several types of stromal cells, such as $CD31^+$ endothelial cells and $CD68^+$ macrophages (Fig. 1e and f, respectively). Thus, whereas the non-homogeneous distribution of the various lineages indicated that they were spatially separated, the distribution patterns still allowed cells belonging to different lineages to be in close contact with each other.

Adhesion molecules are differentially expressed at distinct locations: Immunohistochemical staining of human bone marrow sections for a panel of adhesion molecules showed great diversity in their expression pattern (Fig. 2). For each adhesion molecule bone marrow sections of 7 donors were stained. Notwithstanding small variations in the intensity of staining, for each adhesion molecule the staining pattern was the same for all 7 donors. Although for most adhesion molecules positive cells were found throughout the whole bone marrow section, they were not randomly distributed, but they were expressed in an ordered manner.

Different adhesion molecules were expressed by morphologically distinct cell types. In many positive cells a punctate membranous staining was observed (Fig. 2 g), consistent with the expected localization of adhesion molecules on the cell membrane. The polarized staining might reflect expression of an adhesion molecule at sites where the positive cell interacted with another cell. In very large cells, such as megakaryocytes, also the cytoplasm usually stained positive (Fig. 2a). Nuclei were

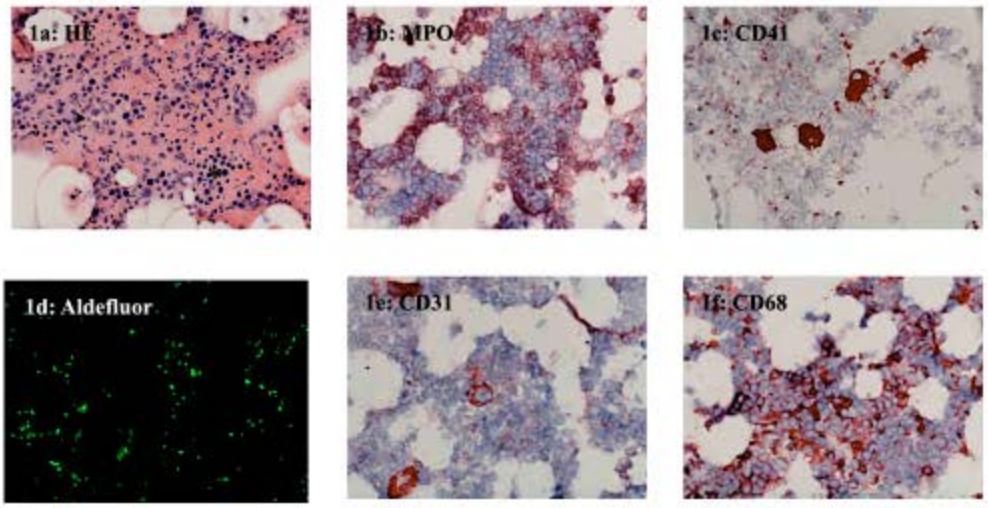

Fig. 1: Different lineages of bone marrow cells are spatially separated, Frozen sections of human bone marrow were stained with hematoxylin-eosin (a), anti-MPO polyclonal Ab (b), anti-CD41 mAb P2 (c), anti-CD31 mAb WM-59 (e) and anti-CD68 mAb EBM11 (f). The section in panel (d) was stained with Aldefluor® to detect the enzyme aldehyde dehydrogenase. Original magnification: x 400 for panels a-c, e, f; x 100 for panel (d) In panel a the asterisk denotes an erythroid cluster and the arrowhead a myeloid cluster

always negative. Whereas several cell types expressed more than one adhesion molecule, in most cases the overall staining pattern of the tested adhesion molecules differed. So, within each bone marrow section, different adhesion molecules were spatially separated.

Cells positive for integrin subunits α2 (VLA-2) or α3 (VLA-3) were distributed as single cells throughout the bone marrow. Despite this similarity, staining patterns of these adhesion molecules were nevertheless distinguishable because of differences in staining intensity and morphology of positive cells. The staining pattern of α2 was characterized by very intense staining of a high number of megakaryocytes (Fig. 2a). On the other hand, α3 was expressed by CD31-positive small blood vessels (Fig. 2b and 1e). Expression of ALCAM was found on a population of relatively small, round cells, dispersed through the bone marrow (Fig. 2c). Positive cells were present as single cells or in small clusters of two to five cells. Furthermore, ALCAM expression was also detected in megakaryocytes, albeit with much lower staining intensity.

Cells positive for integrin subunit α1 showed a stromal cell-like appearance and were also present as single cells or small clusters of a few cells (Fig. 2d). Furthermore, adipocytes stained positive for α1. Integrin subunit α4 (VLA-4) was expressed by larger clusters of cells lying adjacent to areas of negative cells (Fig. 2e). A comparable staining pattern of areas with positive and negative cells located next to each other was found for VCAM-1 (Fig. 2f) and ICAM-3 (Fig 2h). Yet, VLA-4, VCAM-1 and ICAM-3 were expressed by cells of different lineages. While the staining pattern of cells positive for VLA-4 mostly resembled that of the erythroid lineage based on morphological criteria, the distribution of ICAM-3-positive cells resembled that of the myeloid lineage. In case of VCAM-1, interstitial stromal cells as well as cells of the myeloid and erythroid lineages were found positive. ALCAM, VLA-4, VCAM-1 and ICAM-3 all showed a clear and often polarized staining of cell membranes (Fig. 2).

Expression of ICAM-1 and integrin subunit α5 (VLA-5) was not confined to small or somewhat larger groups of cells. In these cases, adhesion molecules exhibited widespread expression. Both ICAM-1 and VLA-5 were expressed by virtually all cells, except adipocytes, but in varying amounts. Within each bone marrow section, areas with high staining intensity were present next to areas with weaker intensity (Fig. 2i-j). In many positive cells and in particular in megakaryocytes, a granular cytoplasmic staining was observed.

Interestingly, VLA-4 and VLA-5 were expressed much more widely than markers for hematopoetic stem or progenitor cells, such as CD34 (Fig. 2k) or Aldefluor® (Fig. 1d). No staining was observed using isotype control antibodies (Fig. 2l).

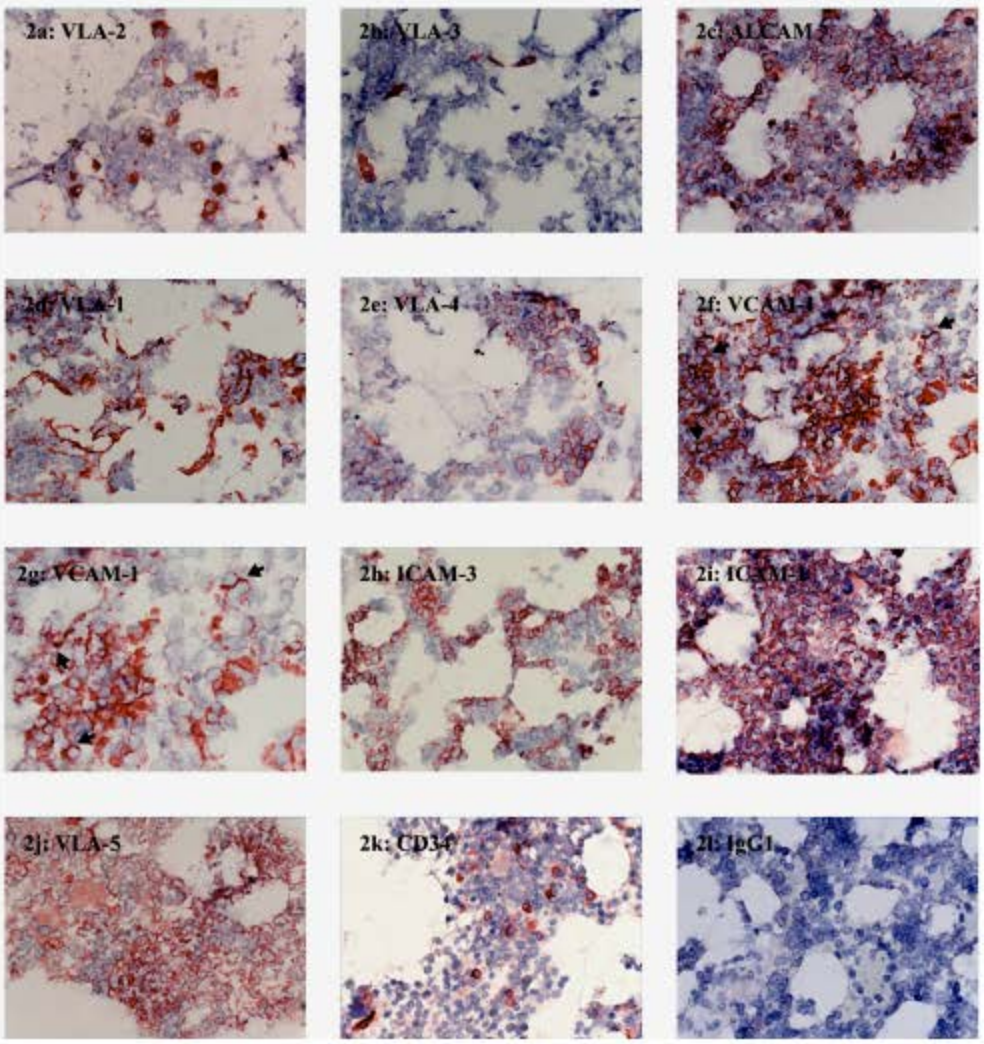

Fig. 2: Immunohistochemical detection of adhesion molecules in human bone marrow, Frozen sections of human bone marrow were stained with the following Abs: anti-α2 mAb AK7 (a), anti-α3 mAb C3 II.1 (b), anti-ALCAM mAb AZN-L50 (c), anti-α1 mAb SR84 (d), anti-α4 mAb ALC1/1.3.1 (e), anti-VCAM-1 mAb 51-10C9 (f, g), anti-ICAM-3 mAb AZN-IC 3.1 (h), anti-ICAM-1 mAb Rek-1 (i), anti-α5 mAb SAM-1 (j), anti-CD34 mAb 563 (k) and mouse IgG1 as negative control (l). Tissue sections were stained with the mAbs as described in Materials and Methods and counterstained with hematoxylin. Original magnification: x 200 (a); x 400 (b-f, h-l); x 630 (g). In panels (f) and (g) the arrows point to examples of polarized staining of the cell membrane

Expression of adhesion molecules by bone marrow stromal cells *in vitro* is dependent on both culture conditions and type of stromal cell: To investigate expression of adhesion molecules by human bone marrow cells under changing conditions *in vitro*, bone marrow samples were processed into four different fractions. Each sample was divided over four culture flasks and cultured under different conditions. Growing cells adhered to the culture disk and exhibited a stromal morphology (not shown). There was no difference in rate of proliferation when bone marrow cells were cultured in the presence of either FCS or Horse Serum (HoS). However, in the presence of Human Serum (HuS), slower proliferation was observed. Overall, expression patterns of adhesion molecules by bone marrow stromal cells of different isolates cultured in different media revealed large

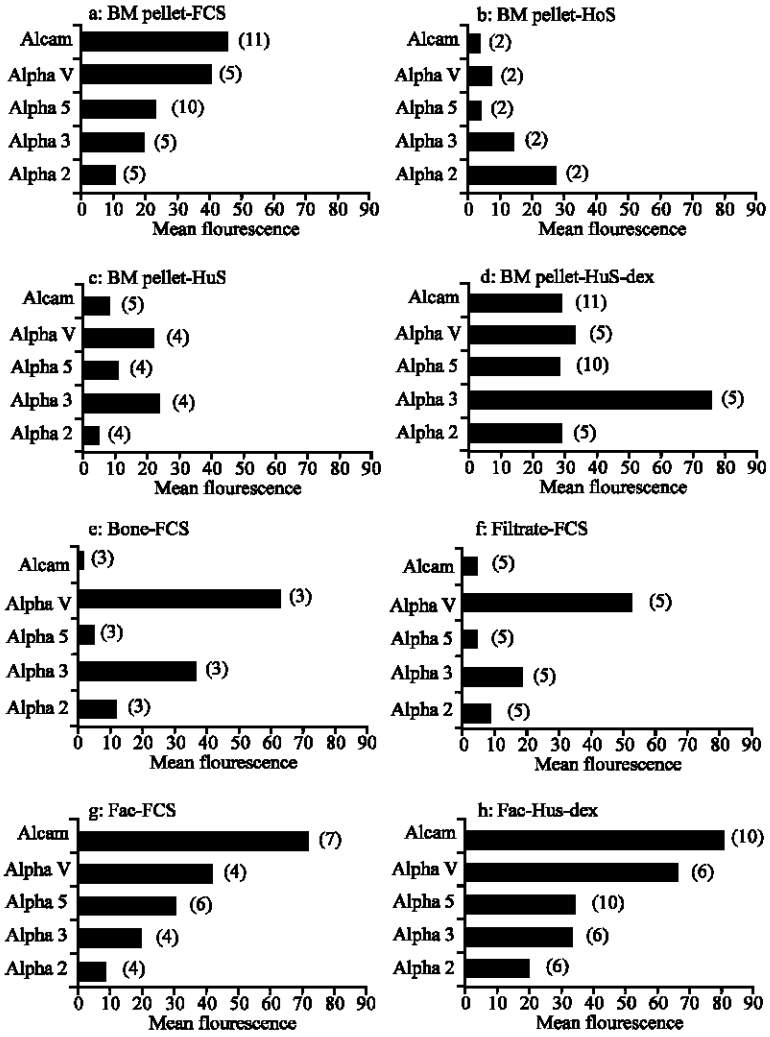

Fig. 3: *In vitro* expression profiles of adhesion molecules change with culture conditions and stromal cell type, Stromal cells derived from different bone marrow fractions were cultured in medium with FCS (a, e-g), horse serum (HoS) (b), human serum (HuS) without (c), or-with added dexamethasone (d, h) until confluency. Then, they were stained with mAbs against ALCAM and integrin subunits αV, α5, α3 and α2 and analyzed with flow cytometry. Stromal cells were derived from fractions Filtrate (f), Bone (e), BM pellet (a-d) and Fac (g, h) as described in Materials and Methods. The mean fluorescence was corrected for isotype control measurements. The data shown are the mean of the results obtained from stromal cell lines derived from n individual donors (n = the number shown in brackets behind each bar)

heterogeneity (Fig. 3). There was variation between donors in expression of individual adhesion molecules by stromal cells of a certain isolate and the effects of the culture medium on this expression. Nevertheless, when expression data derived from different donors were averaged for each adhesion molecule it became clear that its expression was dependent on both culture conditions and stromal cell type.

Culturing cells from a certain bone marrow fraction in different culture media yielded completely different adhesion molecule expression profiles. For example, in Fig. 3a it is shown, that culturing the pelleted cell-fraction in the presence of FCS yielded an expression profile in which ALCAM and αV were expressed more extensively than α5, α3 and α2. In contrast, when the same fraction was cultured in the presence of HoS, the pattern was the reverse, with α3 and α2 expressed to a higher extent than ALCAM and αV. In the presence of HuS the expression of αV and α3 was most pronounced. Addition of dexamethasone led to an increase in the expression of all

adhesion molecules and a change in the expression profile. The profile had been altered such that α3 had become the adhesion molecule that was mainly expressed, while expression of αV was now comparable to that of ALCAM, α5 and α2.

The effect of each culture medium on the adhesion molecule expression profile was not uniform for the stromal cells of all four different bone marrow fractions. For instance, the effect of FCS on the expression profiles on stromal cells in fractions Bone and Fac was completely different (Fig. 3e and g). In cells derived from fraction Fac, FCS in the medium induced relatively high levels of ALCAM expression, whereas in Bone-stromal cells hardly any ALCAM expression could be measured. In Bone-stromal cells, the adhesion molecules that were mainly induced by FCS were αV- and α3-integrins.

Under influence of FCS, expression profiles of BM pellet and Fac stromal cells were comparable and also between Bone and Filtrate similarities in expression profiles could be observed (Fig. 3a vs. g and 3e vs. f). However, these similarities in expression profiles between cells from different bone marrow fractions were not consistent for every culture condition. For example, in contrast to FCS, addition of HuS-dexamethasone to the medium resulted in totally different expression profiles by Fac and BM pellet stromal cells (compare Fig. 3a with g and 3d with h). These results reflect that expression of adhesion molecules by bone marrow stromal cells dynamically changed with culture conditions. This holds for all studied stromal cell types.

DISCUSSION

Immunohistochemistry for a panel of several adhesion molecules revealed that within human bone marrow several adhesion molecules are spatially separated. This is demonstrated by the unique overall expression pattern of each adhesion molecule. Different adhesion molecules are located on distinct hematopoietic and stromal cell types and show an ordered distribution in patches of varying size. The overall pattern was similar in bone marrow samples obtained from 7 individual donors. Hematopiesis in all donors can be considered as normal steady state hematopoisis. *In vitro*, expression of adhesion molecules by different subsets of bone marrow stromal cells is remarkably flexible as response on the different inductors present in different animal sera. Culturing cells from different bone marrow fractions in a given culture medium resulted in distinct expression profiles of adhesion molecules. So, a certain culture medium does not induce a certain phenotype, but this is also heavily dependent on the type of stromal cell. Hematopoiesis is highly adaptable due to changing conditions. When attending a Keystone meeting the bone marrow has to produce the normal blood cells needed for normal turnover but also more red blood cells to comply with the altitude in Keystone. Such adaptive behavior is not easily accomplished with fixed expression of adhesion molecules. The results indicate highly flexible expression of adhesion molecules by bone marrow stromal cells. Such dynamic expression triggered by changing factors endows stromal cells to rapidly adapt to new circumstances and contribute to dynamic niches in bone marrow.

Most studies on the role of adhesion molecules have been performed *in vitro* with isolated $CD34^+$ hematopoietic precursor cells adhering to a bone marrow stromal cell layer or purified proteins (Levesque *et al*., 1995; Simmons *et al*., 1992; Teixido *et al*., 1992). In particular, the interaction between VLA-4 and VLA-5 on $CD34^+$ hematopoietic cells and VCAM-1 and fibronectin on stromal cells has been studied quite extensively. Although these *in vitro* studies provide insight in the role of individual adhesion molecules in defined steps of the hematopoietic process, knowledge about the spatio-temporal distribution was still lacking and interactions between various adhesion molecules *in vivo* remain largely unknown. However, some of these issues may be solved by immunohistochemistry as performed in the present study, which provided a lot of additional information.

In conjunction with previously published *in vitro* studies, it was seen that β1 integrins, VLA-4 and VLA-5, are ubiquitously expressed. Interestingly, expression of α4 and α5 appears not to be restricted to $CD34^+$ cells (compare Fig. 2e and j with k), but is also evident in a substantial number of $CD34^-$ cells. These may be hematopoietic cells, but also stromal cells, since marrow mesenchymal progenitor cells have been reported to express several adhesion-related antigens including α4 and α5 (Conget and Minguell, 1999). Large megakaryocytes were positive for VLA-5, but not VLA-4, which is in agreement with *in vitro* studies in which VLA-5 was detected on mature polyploid megakaryocytes, while VLA-4 could be found only on immature megakaryocytes and myeloid cells (Molla *et al*., 1999; Mossuz *et al*., 1997). We also found very high levels of α2 expression in megakaryocytes, confirming published *in vitro* studies showing expression of VLA-2 by mature polyploid megakaryocytes (Molla *et al*., 1999). In addition to megakaryocytes, some smaller cells staining positive for α2 were present in bone marrow sections. These cells may be either immature human marrow stromal cells (Deschaseaux and Charbord, 2000) or short-term

reconstituting HSCs (Wagers and Weissman, 2006). Integrin subunit α1 has been indicated to be a marker for both stromal precursors and mature stromal cells (Deschaseaux and Charbord, 2000) in human bone marrow. In the present study we also found widespread expression of α1 by cells with a stromal appearance (Fig. 2d). In the bone marrow sections, ALCAM was detected on a cell population present as single cells or in small clusters. ALCAM has been reported to be expressed by the most primitive hematopoietic precursor and myeloid progenitor cells (Uchida *et al*., 1997) as well as by mesenchymal progenitor cells (Bruder *et al*., 1998; Cortes *et al*., 1999; Seshi *et al*., 2000). Upon fractionation of human bone marrow, highest levels of ALCAM were expressed by stromal cells in the Fac fraction. This suggests that most mesenchymal progenitor cells reside in the region closely associated with fat. On the contrary, negligible expression of ALCAM in fraction Bone indicates that stromal cells derived from bone pieces are differentiated and that mesenchymal stem cells in this fraction are absent or present in only very low numbers.

In addition to dependence on cell type, the *in vitro* study showed that adhesion molecule expression profiles also varied largely with culture conditions. Different animal sera or mixes of sera are used to culture LTBM-IC indicating that those animal sera contain inductors that allow HSC expansion. Expression of ALCAM and integrin subunits α2, α3, α5 and αV, as denoted in Fig. 3, is in agreement with previous studies, which also describe expression of these adhesion molecules in bone marrow stromal or mesenchymal progenitor cells. While cultures of human cells are usually performed in medium with FCS, bone marrow stromal cells are often maintained in media to which differentiation stimuli such as horse serum and glucocorticoids have been added. From the present study it is clear that each kind of serum as well as dexamethasone has its own effects on adhesion molecule expression and the effect is also dependent on the type of stromal cell. Bone marrow stromal cells have thus no fixed phenotype but are able to adapt to different inductors present in the different sera.

Such flexible expression pattern predicts that microenvironments acquire different expression profiles driven by changes in local factors. This is important in their function of supporting hematopoiesis, since that is a highly dynamic process, which has to comply to continuously altering needs.

The result of all these environment-induced changes in stromal cells is a multiplicity of cellular environments with varying functional properties, which support hematopoiesis not only lineage-specific, but even stage-specific (Tokoyoda *et al*., 2004). Although the mechanisms used are still not understood, some adhesion molecules have already been described to affect hematopoiesis, either directly by adhesion receptor-mediated signal transduction or indirectly by mediating the responses to cytokines and growth factors (Fox and Kaushansky, 2005; Kapur *et al*., 2001; Levesque *et al*., 1996; Prosper and Verfaillie, 2001; Schofield *et al*., 1998; Teixido *et al*., 1992). The (1 integrin is involved in self renewal (Gottschling *et al*., 2007) and changes in β_1 expression will thus directly affect this. Therefore, alterations in adhesion molecule expression, as found in the present study, may not merely reflect changes in stromal cell characteristics, but may be assumed to contribute directly to dynamic microenvironments. Considering this, ALCAM, ICAM-3, integrins VLA-1, VLA-2, VLA-3 and VLA-4 and VCAM-1 can be hypothesized to be better candidates to support certain steps in hematopoiesis than integral expressed integrin VLA-5 or ICAM-1. This does not exclude that these latter adhesion molecules do play a role in hematopoiesis, but in a general way, affecting cells of multiple lineages. Indeed, an important role for β1 and β2 integrins, such as VLA-4, VLA-5 and LFA-1 in hematopoiesis has been reported by Taichman (2005), Verfaillie (1998) and Kronenwett *et al*. (2000).

Overall, highly flexible, spatial distribution of adhesion molecules by bone marrow stromal and hematopoietic cells can be proposed to provide these cells with the ability to rapidly adapt to new and continuously changing circumstances and thus contribute to dynamic niches in bone marrow.

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