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CELLULAR INTERACTIONS IN HEMOPOIETIC PROGENITOR CELL HOMING: A REVIEW

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Abstract

Within the bone marrow microenvironment, dynamic cellular interactions are constantly occurring. These interactions involve hemopoietic stem cells, progenitor cells and maturing cells, physically interacting with other cells, some of which may function as accessory cells, and others which comprise the stromal elements; hemopoietic cells also interact with non-cellular elements, such as glycoproteins and fibrous proteins of the extracellular matrix (ECM). These interactions serve to regulate normal hemopoiesis by allowing the communication of regulatory information, migration and subsequent homing of stem cells within specific organs, and presentation of hemopoietic growth factors in a biologically relevant fashion. The goal of this review is to examine the specific cellular interactions that relate to the phenomenon of homing of intravenously transplanted stem cells to the bone marrow.

Key Words: Microenvironment, hemopoiesis, stromal cell, hemopoietic progenitor cell, bone marrow, proteoglycans, extracellular matrix.

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Introduction

Homing of hemopoietic progenitor cells (HPC) is the phenomenon which permits transplantation of bone marrow cell suspensions via the intravenous route (51). There is evidence that this homing is the initial event in hemopoiesis, and is necessary for sustained hemopoiesis (37). Once these intravenously transplanted cells have "homed" or selectively seeded lineage-specific stroma of the marrow, they may differentiate and mature into functional blood cells (51). It is generally accepted that this homing involves an intimate membrane interaction between HPC and stromal cells of the marrow (31, 51), in order for self-renewal, proliferation and differentiation to occur. In this review, we will examine the interaction between HPC and stromal cells which involves homing proteins as well as other cytoadhesive molecules.

Membrane interactions between HPC and stromal cells are quite complex and are known to involve a cellular component as well as polysaccharides and fibrous proteins of the extracellular matrix (ECM) (10, 14, 27, 30, 42, 54, 59). Some 15 years ago Allen and Dexter (5) developed an *in vitro* system of long-term bone marrow culture (LTBMC) in which one could dissect some of these cellular interactions that lead to adhesive events, and ultimately to the production of mature myeloid elements. It was within this system that Tavassoli and co-workers were first able to investigate the homing phenomenon, and to begin their characterization of homing protein, which accounts for the initial recognition event between HPC and stroma (1-4, 51).

LTBMC have enjoyed enormous successes in unraveling the complexities of hemopoiesis. In order to identify and isolate molecules that mediate cellular interactions this system has been made even more simple. Clonal precursor cell lines have been developed which bind to cloned stromal cell lines (6, 13, 16, 17, 33), reproducing in some ways HPC-stromal cell interactions, using defined cell populations.

Abbreviations Used

BFU-E	burst forming unit, erythroid
BSA	bovine serum albumin
CFU-E	colony forming unit, erythroid
CFU-GM	colony-forming unit, granulocyte, macrophage
CFU-S	colony-forming unit, spleen
CS	chondroitin sulfate
ECM	extracellular matrix
FN	fibronectin
GAG	glycosaminoglycan
GRGDS	gly-arg-gly-asp-ser
GRGES	gly-arg-gly-glu-ser
HPC	hemopoietic progenitor cell
IL-3	interleukin-3
LEC-CAM	lectin-binding domain, endothelial growth factor receptor domain, complement binding protein-cell adhesion molecule
LTBMC	long-term bone marrow culture
NAG	N-acetylglucosamine
PG	proteoglycan
RDG	arg-gly-asp
SEM	scanning electron microscopy
TEM	transmission electron microscopy

Methods

Electron microscopy

For transmission electron microscopy (TEM) studies, several round 12-mm coverslips were placed in 35-mm dishes before establishing co-cultures of HPC and stromal cells. After incubation the coverslips were removed and adherent cell layers were fixed with one-half strength Karnovsky's fixative buffered with 0.1 mol/liter cacodylate, pH 7.3, containing 0.2 % ruthenium red, for 1 hour at room temperature. They were then post-fixed in similarly buffered osmium tetroxide for 45 minutes at room temperature, and dehydrated through a graded series of ethanol. They were further dehydrated with a series of propylene oxide and infiltrated with graded concentrations of EPON 812 embedding medium. The coverslips were placed on top of filled BEEM® capsules and cured at 60 °C for 3 days. Coverslips were then removed in liquid nitrogen; sections were cut parallel to the coverslips so that the sections would pass the area of contact between progenitor and stromal cells. Sections were then stained and viewed in a JEOL 100CX Temscan transmission electron microscope operated at 60 kV.

For scanning electron microscopy (SEM) studies, co-cultures were established on coverslips as for TEM, removed, and adherent cell layers were similarly fixed in Karnovsky's fixative containing ruthenium red and postfixed in osmium tetroxide. They were then dehydrated in graded alcohol and embedded in EPON 812. Sections were obtained in an LKB microtome and examined as above.

HPC - stromal cell interactions

In our laboratory we have studied direct cell-cell interactions in which HPC adhere to stromal cells. For these studies we have used interleukin-3 (IL-3) dependent progenitor cell lines of the FDCP series, and B6Sut (13, 17), and stromal cell lines D2X and GBl/6 (6, 16) to serve as a model system. B6Sut and FDCP-mix are multipotential cells, while FDCP-1 is bipotential. In efforts to quantify this adherence, we have radiolabeled progenitor cells with ^{51}Cr and established a co-culture for 2 hours at 37 °C between these labeled cells and stromal cell monolayers grown in 24-well dishes. At the end of this time non-adherent progenitor cells are separated from progenitor cells that are bound to the stroma, radioactivity measured in both fractions, and % adherence determined. There is a significant difference in the ability of these HPC to bind stroma, presumably due to their cell surface membrane structures. FDCP-1 binds well (58.6 $\pm$ 3.8%), while B6Sut and FDCP-mix bind less well (26.3 $\pm$ 0.6%, 21.2 $\pm$ 1.8% respectively) and FDCP-2 displays very little binding (9.7 $\pm$ 2.8%) (22).

These interactions have been examined morphologically in detail in our laboratory with the following results. By scanning electron microscopy (Fig. 1C) HPC were covered in what appeared to be mostly short microvilli (M). In addition to these numerous microvilli, a previously undescribed morphological entity, known as "ruffle-like structures" was seen frequently on the surface of HPC (Fig. 1A-C; R), and sometimes appeared to interact with the stromal cell (S) at points of adherence (Fig. 1A, C, arrowheads). Microvilli on the stromal cell were also quite evident and sometimes lengthy (Fig. 1A, B). Ruffle-microvilli interactions could only be guessed at by SEM.

By transmission electron microscopy, finger-like outward projections (P) on the surface of HPC (Fig. 2A-C) could sometimes be seen to interact with stromal cells (S). While these extensions may represent microvilli on HPC, it is not possible to differentiate them from ruffles by TEM. Ruffles on HPC (Fig. 2A-C) could be seen interacting directly with stromal cell surface or with microvilli (M) of stromal origin.

Interestingly, these ruffles may also be present on the stromal cell membrane (Fig. 1B, far right arrowhead), although one can see only an "edge" of a ruffle, apparently originating from stroma, and involved in adherence of HPC. At this time it is uncertain that this "edge" seen in Fig. 1B represents the same morphological entity as the HPC ruffle.

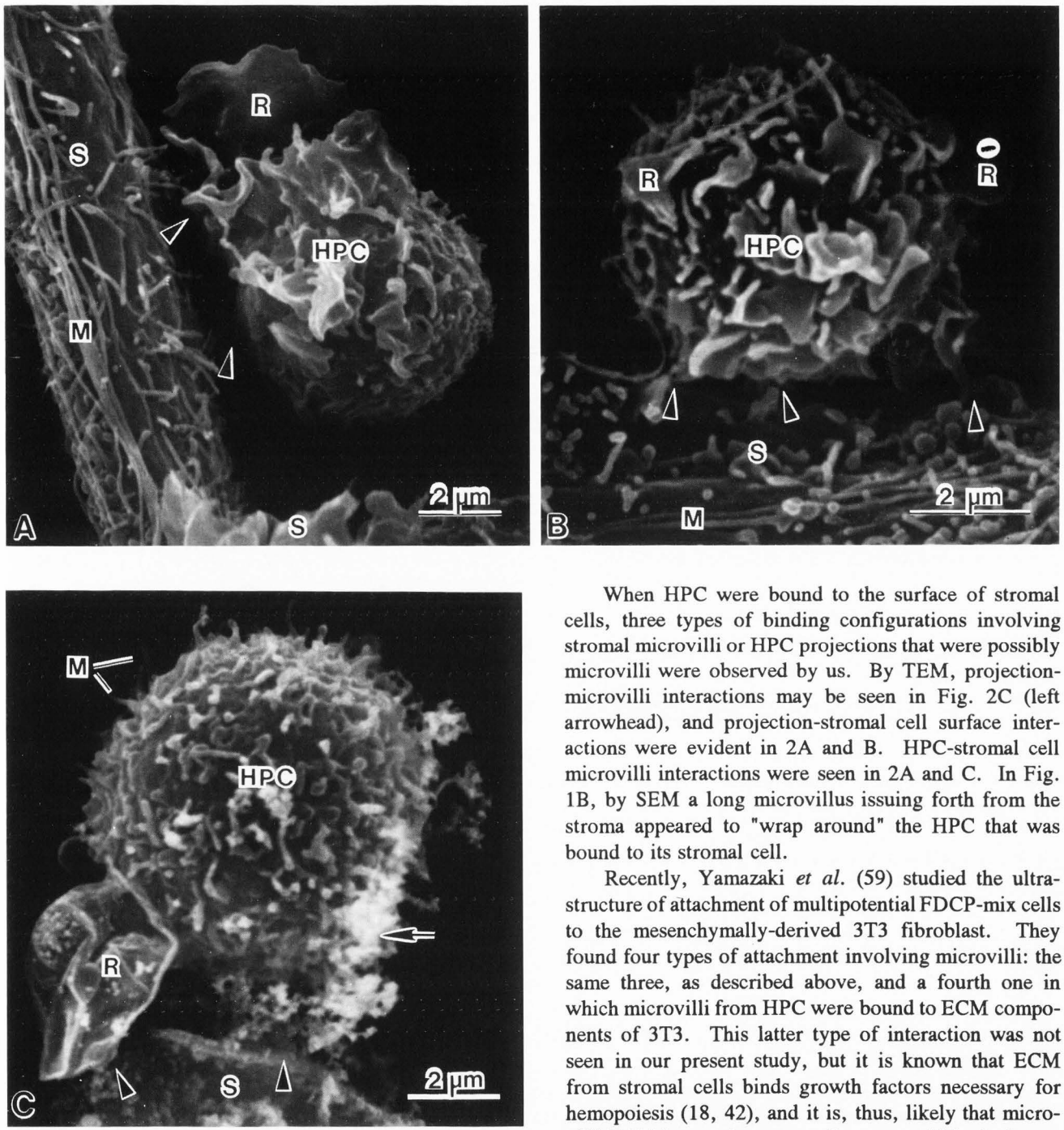

Figure 1. SEM of HPC B6Sut adhering to stromal (S) cell GBl/6. Microvilli (M) and "ruffle-like" structures (R) appear to participate in the cell-cell interaction. Arrowheads denote specific points of attachment. In panel C cells were stained with ruthenium red which stains proteoglycans. This stained material (arrow) appeared to be localized at some areas of contact between the cells.

When HPC were bound to the surface of stromal cells, three types of binding configurations involving stromal microvilli or HPC projections that were possibly microvilli were observed by us. By TEM, projection-microvilli interactions may be seen in Fig. 2C (left arrowhead), and projection-stromal cell surface interactions were evident in 2A and B. HPC-stromal cell microvilli interactions were seen in 2A and C. In Fig. 1B, by SEM a long microvillus issuing forth from the stroma appeared to "wrap around" the HPC that was bound to its stromal cell.

Recently, Yamazaki *et al.* (59) studied the ultrastructure of attachment of multipotential FDCP-mix cells to the mesenchymally-derived 3T3 fibroblast. They found four types of attachment involving microvilli: the same three, as described above, and a fourth one in which microvilli from HPC were bound to ECM components of 3T3. This latter type of interaction was not seen in our present study, but it is known that ECM from stromal cells binds growth factors necessary for hemopoiesis (18, 42), and it is, thus, likely that microvilli-ECM interactions generally occur within the hemopoietic compartment of the marrow. We have previously identified a chondroitin sulfate proteoglycan on the surface of FDCP-1 (36) with ruthenium red - positive material located on microvilli, making it also highly likely that ECM components from HPC may also be important in hemopoiesis, as will be discussed below.

One interesting observation of Yamazaki was the similarity to a state of emperipolesis in which an FDCP-mix cell was seen within a 3T3 cell. They observed this

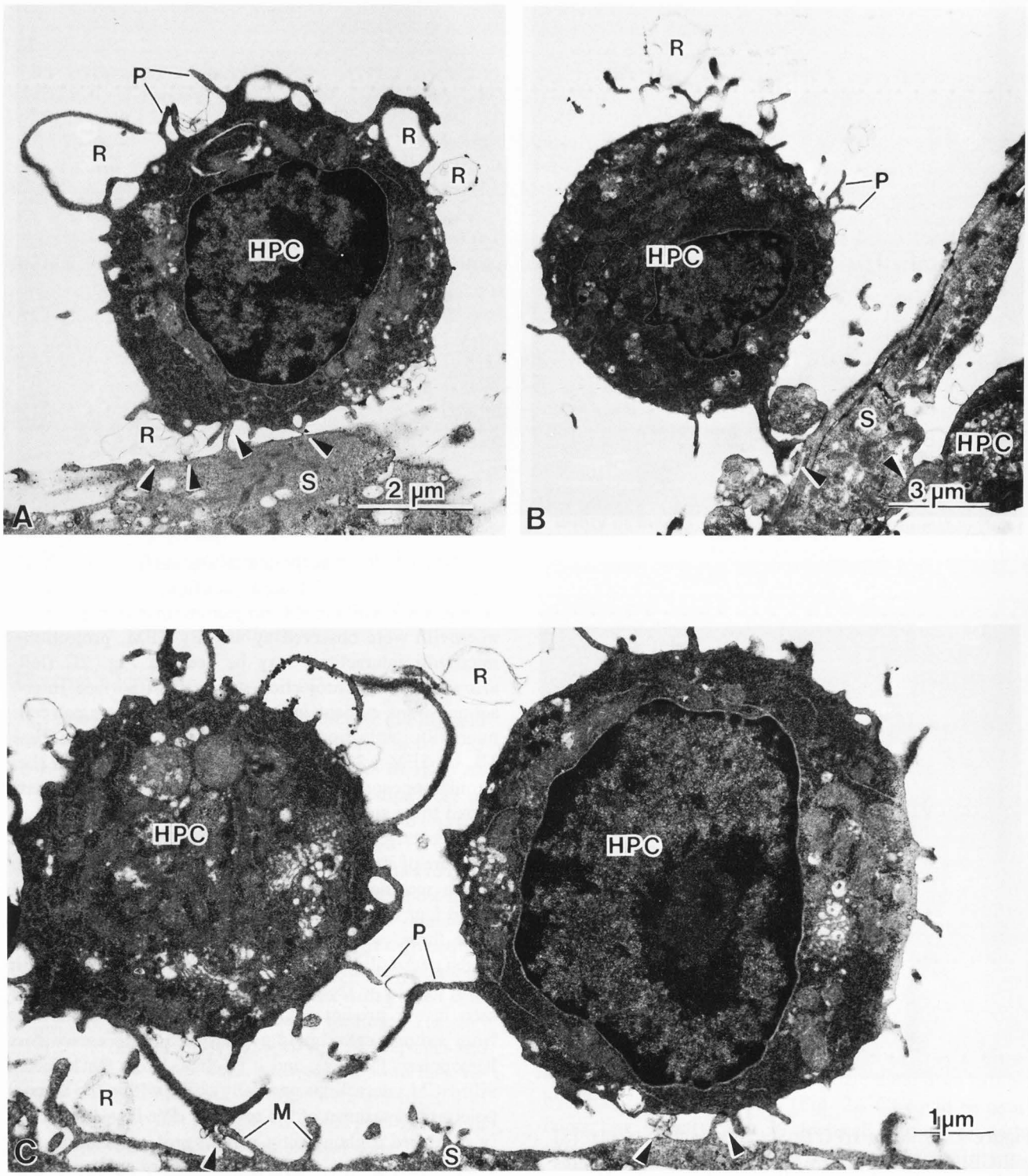

Figure 2. TEM of HPC B6Sut adhering to stromal (S) cell GBl/6. Finger-like projections (P) and "ruffle-like" structures (R) appear to participate in binding, specific events of which are denoted by arrowheads. Microvilli (M) emanating from the stroma appear to interact with projections from HPC. All three panels were made from cells which had been stained with ruthenium red.

occurrence only occasionally, while we have never observed it in our system, but do occasionally see HPC "enmeshed" within the membrane material of a stromal cell, almost appearing to have been "engulfed".

Involvement of homing protein in homing

A lectin called homing protein on the surface of progenitor cells with specificity for mannosyl and galactosyl residues recognizes and binds to a corresponding ligand on the surface of stromal cells (31). As the progenitor cell matures, it loses its homing protein, such that mature cells, therefore, do not possess it (31). Homing protein has been shown to be present on primitive HPC called CFU-S and on committed macrophage-granulocyte precursors CFU-GM; it is lacking on precursors of the erythroid series (29).

In these experiments synthetic probes galactosyl- and mannosyl-BSA but not fucosyl-BSA were able to selectively agglutinate CFU-S and CFU-GM from whole bone marrow (3). Erythroid progenitors BFU-E were selectively agglutinated by mannosyl-BSA only, whereas CFU-E were selectively agglutinated by fucosyl-BSA only. It is speculated that loss of this galactosyl-mannosyl-specific receptor allows release of mature blood cells into the circulation. The homing of erythroid progenitors might be dependent upon a molecular mechanism different from that of CFU-S and CFU-GM. Homing protein is absent on marrow stromal cells (21, 28).

Cloned HPC have allowed the purification of homing protein to a high degree, although the yield is low. Using carbohydrate affinity chromatography, homing protein was isolated and characterized as a single molecular species consisting of a heterodimer with Mr of 110,000 (32). The molecule is composed of two chains of Mr 87,000 and 23,000, with approximately 5% N-linked carbohydrate, as indicated from experiments with endoglycosidase F. Functionally, the molecule is a lectin, with a specificity for both mannosyl and galactosyl residues of a glycoconjugate (1-4). The ligand for this homing protein resides by definition on the surface of marrow stromal cells, and contains an as yet unknown configuration of membrane carbohydrate, probably the glycan moiety of a glycoprotein. It is most likely the combined configuration of the two sugars in the molecule that is being recognized by the lectin. Apparently, both mannosyl and galactosyl residues are necessary for binding, because competitive inhibition with one abolishes the binding altogether (2).

It has been determined that the Kd of binding of homing protein is 2.3×10^{-7} M and 1.0×10^{-7} M, respectively for galactosyl and mannosyl residues (26, 31), indicating rather low affinity binding. There are about one million sites per cell; interaction of this homing protein with its ligand does not lead to their internalization (31), which is expected since the ligand in its natural state is membrane bound and part of a glycoconjugate on the surface of the stromal cell. The distribution of homing protein is unknown, as ferritin-labeled neoglycoprotein probes lacked sensitivity to detect the lectin by electron microscopy (20). More sensitive ^{125}I-labeled neoglycoproteins were used for receptor kinetic studies.

Present knowledge of homing protein indicates that this molecule is consistent with a number of other membrane recognition molecules (24, 25, 40, 41, 50, 52, 53). Many of these receptors are calcium-dependent lectins, such as the peripheral lymph node lymphocyte homing receptor (9, 15, 49). It will be interesting to see if homing protein proves to be related to the LEC-CAM, or selectin family of cytoadhesion molecules (47, 48). The molecular structure of this family of receptors has been well studied, and the ligands for LEC-CAMS are glycoconjugates. At present one may only speculate that homing protein may be another member of this family.

Although the structure of the stromal glycoconjugate ligand for homing protein has not been revealed and must await purification, certain extrapolations concerning its molecular nature are permitted from other studies (51). The typical glycan structure seen on a cell membrane is most likely involved in binding homing protein, independent of its carrier. The glycan chain begins with two N-acetylglucosamine (NAG) residues of which the second one is connected to a mannosyl residue that branches to link to two other mannosyl residues. Each of these latter residues is linked to a NAG residue and, subsequently, to a galactosyl residues that may be exposed or covered by a sialyl residue. The only galactosyl available to homing protein for binding is in the penultimate position; the only mannosyl residues are at the branching site, either before or after a NAG residue.

Enzymatic treatment of stromal cell surface indicated that the removal of sialyl residue is necessary for binding. Therefore, the galactosyl residue can bind only when it is exposed, while this is not a requisite for mannosyl residue, which cannot be exposed at the branching site. Galactosyl residue, therefore, behaves very similarly to asialoglycoprotein binding to hepatocyte, as previously described by others (7, 44).

Most recently, Shiota and Tavassoli (23, 45, 46) have identified a stromal cell glycoprotein of Mr 37,000 selectively adsorbed by cloned HPC which appears to be involved in calcium-dependent adherence of HPC. Selective binding of the 37,000 protein present on GBI/6 stromal cell by FDCP-1, FDCP-2, B6Sut and FDCP-mix was observed. Treatment of stromal cells with endoglycosidase F/N-glyconase did not prevent the adsorption, suggesting that the binding does not occur via an N-glycan chain of the molecule. Studies are currently under-

way to determine whether this protein represents the homing ligand, and to assess the possible relationship between this adherent protein and other known proteins of similar molecular weight, including c-kit ligand. Because of the lack of N-glycosylation it is unlikely that the protein is c-kit ligand. Further, it has been found to be present in Sl/Sl^d stromal cell lines which are kit ligand deficient. However, this molecule appears to play a major role in selective adhesion of HPC to stroma, and its further characterization is anxiously awaited.

Several pieces of experimental evidence are paramount to the assignment of homing protein as the hemopoietic homing receptor. First, seeding of intravenously transplanted marrow cells is inhibited competitively by preincubation of cells with synthetic glycoproteins and simultaneous infusion of these glycoproteins (4). Only synthetic molecules of galactosyl and mannosyl, but not fucosyl specificity, inhibit the seeding. This same inhibition was observed in LTBMC (1, 2), and served as the molecular basis of much of the early work on hemopoietic homing.

Second, treatment of stromal cell surface with neuraminidase followed by galactosidase and mannosidase reduced or nearly abolished the homing of HPC to stromal cells (31). Treatment in the reverse order had no such effect. This indicated the presence, on the surface, of stromal cells of a glycoconjugate that can interact with homing protein.

Other Adhesive Interactions in Homing

Proteoglycans (PG) are a group of ECM molecules consisting of a core protein to which a repeating sequence of usually sulfated glycan structures called glycosaminoglycans (GAG) is attached (8, 19, 43, 54). Because of their composition PG have almost limitless potential for heterogeneity. Not only may their core protein content vary substantially but also their molecular size and number and types of GAG chains per molecule. More variation may be introduced within the GAG side chains with regard to length, composition and spatial arrangement of side chains along the disaccharide backbone.

This structural heterogeneity of PG gives some clues as to their potential for diverse functions within the extracellular spaces of tissues. Not only may they participate in formation of the "ground substance" of tissues, but PG may also bind various signaling molecules, such as growth factors. Recently, this function of selective extraction and binding of hemopoietic growth factors was shown (42). Other PG may be instrumental in selective binding of granulocyte-macrophage progenitors (10, 11, 39), while yet another class may be related to the developmental regulation of erythroid cells (38, 55-58). This latter class is preferentially associated with the cell membrane, rather than with the ECM.

That the adherence of HPC to marrow stroma is mediated by a receptor-ligand interaction of low affinity is understandable, since mature blood cells must be released into the circulation. However, because of this low affinity binding, attention has turned to the extracellular matrix, and in particular to PG, as an additional mechanism in homing. These PG may serve to strengthen the bond between HPC and stroma.

It was found in our laboratory that the HPC cell line FDCP-1 synthesizes a considerable amount of PG of one specific class, chondroitin sulfate (CS), that is associated with the membrane, but subsequently is released to the extracellular space (36). When FDCP-1 cells were cocultured with either hemopoietic stromal cells D2X or GBl/6, so that they "homed" to these cells, the stability of this PG on FDCP-1 was enhanced (36). These findings suggested that the synthesis of PG by HPC and its accumulation in the membrane may have a role in the interaction between HPC and stromal cells.

Recent work from our laboratory has indicated that this membrane-associated CS can also mediate binding of HPC to stromal cells (34, 35). In these studies enzymatic removal of CS abolished binding. At the molecular level this binding occurred via the interaction between the GAG part of CS and the heparin-binding domain of membrane-associated fibronectin (FN) on stromal cells. The presence of FN on stromal cell membrane has been well-documented (11, 12, 39, 43).

An additional binding event involving FN also occurred with these HPC, via the central cell-binding domain of FN which has as its essential structural feature a repeating sequence motif RGD (arg-gly-asp) (34, 35). Here integrins on the cell membrane of FDCP-1 are involved. Evidence for involvement of this particular domain was concluded from experiments in which inhibition of binding could be obtained by the synthetic pentapeptide GRGDS (gly-arg-gly-asp-ser), but not with the control peptide (GRGES, gly-arg-gly-glu-ser). This pentapeptide competitively inhibited the binding of HPC to the tripeptide sequence motif RGD (34, 35).

Thus, at least two domains of the FN molecule can interact with membrane associated molecules on the surface of HPC to participate in the homing phenomenon.

Conclusions

Homing protein is responsible for the initial recognition event between HPC and hemopoietic stroma within the hemopoietic compartment of the bone marrow. It is through this initial interaction that the high degree of specificity is provided that is necessary for HPC to identify and subsequently lodge within their requisite

"niche". But this interaction of HPC and its ligand is of relatively low affinity, and, thus, requires stabilization. Therefore, further strengthening of this bond is provided by various components of membrane-bound ECM, largely through interactions with FN-containing stroma. These secondary ECM-related interactions are of higher affinity, and when coupled with the initial homing protein-ligand interaction, serve to stabilize HPC within their environment. Using the techniques of molecular biology, one could confirm the role of homing protein by studying its induced expression in other types of cells. While such experiments would be cumbersome, they would allow more complete understanding of homing protein as a cytoadhesive molecule. The molecular basis of cytoadhesive interactions within the bone marrow are of intense current interest in experimental hematology, particularly as they relate to bone marrow transplantation therapy. Intriguing questions that remain to be more fully addressed include lineage distinctions between cytoadhesive interactions, and differentiation-induced alterations in cytoadhesion.

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Discussion with Reviewers

G.D. Roodman: Are the cell ligands which are required for stem cell homing located on the microvilli present on the stromal cells, and what is known of the distribution of the homing protein on the surface of the progenitor cells?

Authors: Concerning the location of ligand on stromal cell and the distribution of homing protein on progenitor cell surface, neither issue is completely resolved. Because there is at present no suitable probe for the ligand, its localization to stromal microvilli is only speculative. It has been shown that many receptors are preferentially distributed on microvilli, and this countereceptor may be no exception. Certainly, microvilli originating from both stem cell and stromal cell are involved in cytoadhesive interactions. Homing protein is known to be a surface membrane protein. Using ferritin-labeled neoglycoproteins we were unable to detect homing protein by electron microscopy (20); the sensitivity of this probe was not sufficiently high. However, using amide-modified latex minibeads covalently linked to the paraaminophenyl derivative of the appropriate sugars in pyranose form, specific binding of these probes for homing protein to a small fraction (less that one percent) of murine marrow cells was observed. By SEM, minibead probes were bound in patches to one or two areas of the cell, with binding occasionally recognized to be on microvilli. It is hoped that these particular probes may lend themselves to further studies on localization and purification of primitive cells within the bone marrow that carry homing receptors.

G.D. Roodman: Are there unique, lineage-specific homing proteins that differ in terms of their biochemical characteristics on different types of committed progenitors that differ from those on pluripotent stem cells?

Authors: It has been shown that synthesis of this galactosyl-mannosyl receptor does appear to be both lineage and developmentally regulated, in that it is present on both pluripotential and granulocyte-macrophage progenitor cells, but is absent on the surface of mature, circulating granulocytes (3). Differentiation in the erythroid series does not retain this protein in either BFUE, CFU-E or, as expected, mature erythrocytes. Instead, a homing protein with molecular specificity for fucosyl residues appears on erythroid lineage committed progenitors (29), but similarly to its myeloid counterpart, is lost upon terminal differentiation of erythroid cells. It is speculated that loss of these lectins may be instrumental in release of mature cells from the stroma into the circulation.

J.S. Greenberger: Do you think the ligand for homing receptor is possibly identical to c-kit ligand, or is it another gross factor that is membrane bound?

Authors: At this point, there is no data to strongly support c-kit as being the ligand for homing protein. A 37,000 stromal cell protein, described in this paper, is being studied by Dr. Tavassoli's laboratory as the possible ligand, and, although the molecular size of the two proteins is similar, their protein was found to be present in Sl/Sl^d marrow cell lines, which are deficient in c-kit ligand.

T.D. Allen: It is difficult to assume from TEM that profiles of cell extensions are necessarily those of microvilli. The longer they are, in fact, particularly when they have a curved profile, the more likely they are to be ruffles. A microvillus would be extremely unlikely to stay within the confines of the plane of a single thin section. It is far better to "size" microvilli in SEM, where the whole structure is visible. Do you concur?

Authors: Yes, we do. It would be helpful to understand the role of these "ruffles", because they are very dramatic structures by SEM. They may be a way in which the cell increases its surface area.