Human Hematopoietic Stem Cell Adherence to Cytokines and Matrix Molecules

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Abstract

The hematopoietic microenvironment is a complex structure in which stem cells, progenitor cells, stromal cells, growth factors, and extracellular matrix (ECM) molecules each interact to direct the coordinate regulation of blood cell development. While much is known concerning the individual components of this microenvironment, little is understood of the interactions among these various components or, in particular, the nature of those interactions responsible for the regional localization of specific developmental signals. We hypothesized that cytokines act together with ECM molecules to anchor stem cells within the microenvironment, thus modulating their function. In order to analyze matrix-cytokine-stem cell interactions, we developed an ECM model system in which purified stem cell populations and plastic-immobilized individual proteins are used to assess the role of various matrix molecules and/or cytokines in human hematopoietic cell development. Analysis of these interactions revealed that a single ECM protein, thrombospondin, in conjunction with a single cytokine (e.g., c-kit ligand), constitutes a developmental signal that synergistically modulates hematopoietic stem cell function. (*J. Clin. Invest.* 1992. 90:251–255.) Key words: thrombospondin • progenitor cells • c-kit ligand • extracellular matrix

Introduction

Recent studies demonstrate that extracellular matrix (ECM)[1] molecules play a fundamental role in blood cell proliferation and/or differentiation. These molecules function to retain (or localize) hematopoietic cells within the marrow (1, 2), to augment and/or stimulate hematopoietic cell development (3, 4), and to localize hematopoietic growth factors (5, 6). However, the complexity of the matrix itself is a major obstacle to the functional analysis of ECM in hematopoiesis. While the extracellular matrix is comprised of various collagens, proteoglycans, and glycoproteins, its composition varies throughout the body (7). Bone marrow ECM contains fibronectin (Fn), thrombospondin (TSP), types III and IV collagen (8), all of which play a role in hematopoiesis, as do other marrow-specific components such as hemonectin (9) and tissue-specific proteoglycans (10). Indeed, the glycosaminoglycan side chains of proteoglycans, such as heparan sulfate, bind hematopoietic growth factors (5), suggesting that ECM growth factor complexes are important for blood cell development.

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1. *Abbreviations used in this paper:* BFU-E, erythroid burst-forming progenitor cells; BFU-MK, burst-forming megakaryocyte progenitor cells; CFC, colony-forming cells; CFU-GM, granulocyte-macrophage progenitor cells; CSF, colony-stimulating factor; ECM, extracellular matrix; EPO, erythropoietin; Fn, fibronectin; GEMM, pluripotent (mixed cellularity) progenitor cells which give rise to granulocyte, erythrocyte, macrophage, and megakaryocyte containing colonies; GM, granulocyte / / macrophage; HPP-CFC, high proliferative potential colony-forming cells; IL-3, interleukin-3; TSP, thrombospondin.

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Methods

Human bone marrow cells were obtained following informed consent and separated by three-color flow cytometry based on the expression of CD34, HLA-DR, and CD15 antigenic determinants as described elsewhere (11). Cytoadhesion assays were performed as follows: Target proteins (10–20 μg/ml, 0.1 ml/well) were immobilized onto plastic for 1 h at 37°C. The plates were rinsed with serum-free media and nonspecific binding blocked by incubating 30 min at 37°C with a solution of 10 mg/ml bovine skin gelatin (Sigma Chemical Co., St. Louis, MO). Cells were allowed to adhere to immobilized protein under serum-free conditions for 1 h at 37°C. Nonadherent cells were removed by two gentle washes with warm (37°C) serum-free medium. Adherent cells were then overlayered with semisolid media (methylcellulose or fibrinogen clot) containing, except where indicated, optimal concentrations of recombinant hematopoietic growth factors (1, 11). Controls consisted of analysis of cellular attachment to both gelatin-coated wells and wells coated with thyroglobulin (Sigma Chemical Co.) as an inappropriate protein. Gelatin and thyroglobulin controls demonstrated an average of 12±1% progenitor cell binding (mean±SE, $n = 7$). However, this attachment was not significantly different from zero ($P \leq 0.05$). Nonetheless, the attachment to inappropriate protein (thyroglobulin) was subtracted from the total progenitor cell attachment. Thus, specific percent attachment for each lineage is defined as: [the total number of adherent progenitor cells (i.e., colonies) minus the number bound to the inappropriate immobilized protein (thyroglobulin) divided by the total number of input progenitor cells] times 100. The recombinant target proteins used in this study were expressed in yeast. Thrombospondin was purified from human platelets (1) and is a glycosylated protein. Recombinant interleukin-3 (IL-3) and granulocyte-macrophage colony-stimulating factor (GM-CSF) were purchased from Genzyme Corp., Boston, MA, and erythropoietin (Epo) from Amgen Biologicals, Thousand Oaks, CA.

Results and Discussion

In our experiments, we dissected the numerous hematopoietic regulatory interactions by utilizing recombinant cytokines,

pure ECM molecules, and a purified population of human marrow cells that resembled hematopoietic stem cells. Such isolated marrow cell subpopulations are capable of not only multilineage differentiation, but also of self-renewal (12–16). Enriched populations of primitive human hematopoietic stem cells were isolated by three-color flow cytometry as described previously. Briefly, human bone marrow cells where fractionated by equilibrium density centrifugation, counterflow centrifugal elutriation, and fluorescent activated cell sorting (by expression of the CD34, HLA-DR, and CD15 antigenic determinants) (12, 13). The resultant CD34$^+$ DR$^-$ CD15$^-$ cell population represents a rare marrow subpopulation (0.04–0.06% of nucleated marrow cells), contains relatively few committed progenitor cells, and is highly enriched for several classes of hematopoietic progenitor cells: blast colony-forming cells, the primitive burst-forming megakaryocyte progenitor cells (BFU-MK), the high proliferative potential colony forming cells (HPP–CFC), and the long-term marrow culture initiating cells (12–14, 17).

A solid phase cytoadhesion assay was developed in which target proteins were immobilized onto plastic. Following immobilization of the target protein and subsequent blocking of nonspecific binding sites, CD34$^+$ DR$^-$ CD15$^-$ cells were allowed to adhere and quantitative total cell binding or human hematopoietic progenitor cell attachment were evaluated. We used this assay to assess cellular attachment to the ECM proteins TSP and Fn, as well as those recombinant human cytokines known to support proliferation of hematopoietic stem cells: IL-3, GM-CSF, and the c-kit ligand. In addition, EPO was examined to determine lineage-related binding differences.

The isolation of the CD34$^+$ DR$^-$ CD15$^-$ cell phenotype did not, in general, result in selection of a cell population with enhanced cytoadhesive capacity (data not shown). Rather, subpopulations of human CD34$^+$ DR$^-$ CD15$^-$ hematopoietic progenitor cells have specific attachment patterns to matrix molecules (Fig. 1 *B*) and cytokines (see below). Thus, 50–60% of the CD34$^+$ DR$^-$ CD15$^-$ progenitors (i.e., the erythroid burst-forming cells [BFU-E], as well as granulocyte, erythrocyte, macrophage, megakaryocyte colony-forming cells [CFU-GEMM], BFU-MK, HPP–CFC) adhere to TSP, whereas only 20% of the granulocytic progenitor cells (CFU-GM) bind to TSP (Fig. 1). This observation confirms and extends previous reports which show that some 15–30% of (unfractionated) human progenitor cells of all classes adhere to TSP (1, 2). Conversely, CD34$^+$ DR$^-$ CD15$^-$ progenitor cells show little or no ability to adhere to Fn. This lack of binding to Fn is consistent with in vivo studies of murine stem cells which also fail to bind to this molecule (18). However, more mature progenitor cells (~30% of BFU-E and 10% of granulocyte/macrophage colony-forming cells [CFU-GM] in unfractionated bone marrow) do bind to this molecule (1, 19), as do 10–40% of the progenitor cells that express HLA-DR (12). A number of groups have shown that the acquisition of the DR$^+$ phenotype is a relatively late differentiation event, and that DR is expressed on more committed progenitor cells (12, 14). Therefore, we conclude that the cytoadhesion characteristics of CD34$^+$ DR$^-$ CD15$^-$ progenitor cells define a subpopulation of more primitive progenitor cells that are characterized by enhanced binding to TSP and limited attachment to Fn.

Assessment of CD34$^+$ DR$^-$ CD15$^-$ progenitor cell adhesion to recombinant cytokines demonstrates that these growth factors, in addition to their known effects on proliferation, also

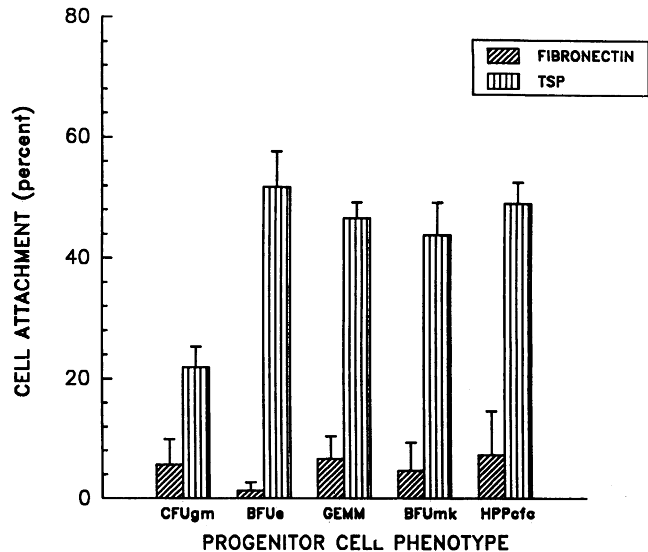

Figure 1. Adhesion of human CD34$^+$ HLA-DR$^-$ CD15$^-$ cell populations to extracellular matrix proteins. Human progenitor cell attachment to immobilized proteins was performed as described previously (1), and modified in Methods. Protein-adherent cells were left in situ and overlayered with semisolid media containing optimal concentrations of hematopoietic growth factors and cultivated for 14–28 days (1, 11). Values for all figures are mean±SE of 5–7 individual bone marrow samples cultured as 2–3 replicate cultures for each class of progenitor cells per attachment protein on separate occasions. The mean number of input CD34$^+$ DR$^-$ CD15$^-$ progenitor cells (per 2,500 total nucleated cells) were 93±10 CFU-GM, 72±19 BFU-E, 13±2 CFU-GEMM, 10±3 BFU-MK, 34±4 HPP–CFC (mean±SE; $n = 7$). Diagonal bars, progenitor cell attachment to fibronectin; vertical bars, attachment to TSP.

function as hematopoietic cytoadhesion molecules. Interestingly, lineage-related differences are observed in progenitor cell attachment to immobilized cytokines. For example, little attachment of CFU-GM, CFU-GEMM, and BFU-MK to erythropoietin is evident (Fig. 2 *A*). However, approximately four times as many erythroid precursors (BFU-E) adhere to erythropoietin than do CFU-GM. The HPP–CFC also adhere to erythropoietin, but to a lesser degree, perhaps indicating the multilineage potential of this cell (20). By contrast, GM-CSF binds few erythroid and pluripotent progenitors but noticeably binds ~15 times as many CFU-GM than it does BFU-E.

Both IL-3 and the c-kit ligand also bind CD34$^+$ DR$^-$ CD15$^-$ progenitor cells and, again, specific attachment patterns are seen. Less than 15% of erythroid and pluripotent CD34$^+$ DR$^-$ CD15$^-$ progenitor cells attach to IL-3, in contrast to a 40–60% adherence for CFU-GM, HPP–CFC, and BFU-MK (Fig. 2 *B*). Unlike IL-3, c-kit ligand binds relatively high numbers of CD34$^+$ DR$^-$ CD15$^-$ erythroid and GEMM progenitor cells, and even higher percentages of BFU-MK and HPP–CFC.

Analysis of CD34$^+$ DR$^-$ CD15$^-$ progenitor cell attachment data thus allows the definition of a relative cytoadhesion phenotype (Table I). It is interesting to note that the majority of primitive progenitors (HPP–CFC, BFU-MK, and CFU-GEMM) adhere to TSP, IL-3, and c-kit ligand, whereas the more committed progenitors (such as the CFU-GM) predominantly attach to IL-3 and GM-CSF. Most CD34$^+$ DR$^-$ CD15$^-$ erythroid progenitors adhere to TSP, with a lesser number also

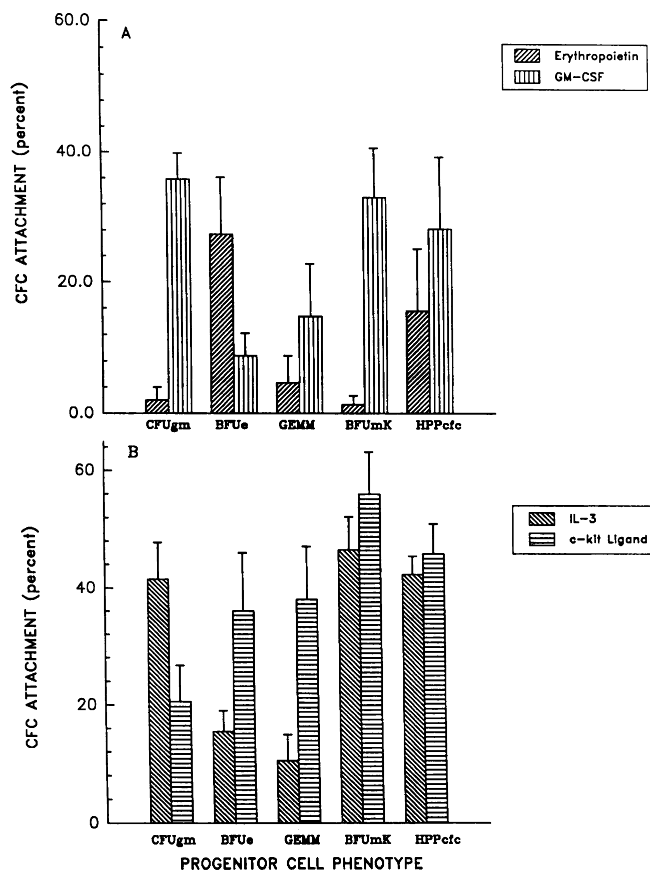

Figure 2. Attachment of CD34⁺ HLA-DR⁻ CD15⁻ hematopoietic progenitor cells to recombinant human cytokines. (*A*) Cytoadhesion to erythropoietin and GM-CSF. Diagonal bars, attachment to erythropoietin; vertical bars, attachment to GM-CSF. (*B*) Adhesion to IL-3 and c-kit ligand. Diagonal bars, progenitor cell attachment to IL-3; horizontal bars, attachment to c-kit ligand. Attachment procedure as in Methods.

having the capacity to bind to erythropoietin or c-kit ligand. The BFU-MK adhere to TSP, IL-3, and c-kit ligand, and also attach to GM-CSF, but to a lesser degree. Finally, the majority of the CFU-GEMM show a restricted cytoadhesive pattern, attaching to TSP or c-kit ligand.

Given the function of hematopoietic cytokines in mediating progenitor cell localization, we next examined whether the process of cytoadhesion was sufficient to initiate cellular proliferation (as quantified by colony formation). In order to perform these studies, $CD34^+$ DR^- $CD15^-$ cells were attached to immobilized proteins and subsequently cultivated without exogenous hematopoietic growth factors in the overlayer. (The maturation factor EPO was added to some cultures to permit development of BFU-E and CFU-GEMM colonies, see Fig. 1, legend). These studies demonstrate that hematopoietic progenitor cell attachment to extracellular matrix molecules (i.e., TSP and Fn), as well as to IL-3 and GM-CSF fails to stimulate proliferation (data not shown). Cytoadhesion of $CD34^+$ DR^- $CD15^-$ progenitor cells to c-kit ligand, however, is sufficient to stimulate colony formation (Fig. 3 *A*). Again, differential lineage effects are noted with few CFU-GM colonies, and no BFU-MK colonies developing under these conditions. Strikingly, equivalent numbers of BFU-E and HPP-CFU colonies develop when the sole stimulus is immobilized c-kit ligand.

Table I. Binding of $CD34^+$ HLA-Dr^- $CD15^-$ Progenitor Cell Populations to Recombinant Growth Factors and Matrix Molecules

Progenitor phenotype	Fn	TSP	EPO	GM-CSF	IL-3	KL
CFU-GM	+	+	+	+++	+++	+
HPP-CFU	+	+++	+	++	+++	+++
BFU-E	+/−	+++	++	+	+	++
BFU-MK	+	+++	+	++	+++	+++
GEMM	+	+++	+	++	+	+++

The relative attachment of progenitor cells to cytoadhesion molecules was summarized based on the frequency of $CD34^+$ DR^- $CD15^-$ cell attachment. Using data presented in Figs. 1 and 2, the relative attachment was determined as follows: + = 0–25% attachment, ++ = 26–50% attachment, +++ = 51–75% attachment, ++++ = 76–100% attachment.

Thus, soluble c-kit ligand, which alone lacks the ability to act as a human colony-stimulating factor (CSF) (21) functions as a CSF when immobilized. Megakaryocyte burst-forming cells are the sole exception to this type of c-kit ligand-mediated regulation. In the absence of additional (soluble) hematopoietic growth factors, no BFU-MK colonies occur, but colonies do develop when progenitors are allowed to adhere and are cultivated with additional factors in the overlayer (Fig. 3 *A*). This demonstrates that primitive megakaryocyte progenitor cells adhere to c-kit ligand, but the cytoadhesive event is insufficient (as a solitary stimulus) to initiate proliferation.

In order to reconstitute a simplified hematopoietic microenvironment, we examined the interaction of $CD34^+$ DR^- $CD15^-$ cells with a single hematopoietic growth factor in the presence of a single ECM molecule. When TSP and c-kit ligand were admixed in equimolar concentrations and coimmobilized onto plastic, the observed colony formation was much greater than that predicted by the binding capacity of either molecule alone (Fig. 3 *B*). This effect is particularly evident for CFU-GM and HPP–CFC, as it is to a lesser degree for CFU-GEMM and BFU-E. This suggests the existence of a subpopulation of $CD34^+$ DR^- $CD15^-$ cells that adhere to either c-kit ligand or TSP, but fails to proliferate (or survive) in the absence of binding to the second ligand, even though adequate soluble growth factor is present to promote progenitor cell proliferation. Alternatively, TSP and c-kit ligand may form a protein-protein complex which "reconstitutes" a new or better cytoadhesive complex, thus binding more cells. In either case, it appears that a subpopulation of $CD34^+$ DR^- $CD15^-$ progenitor cells exists that requires cooperative binding to both TSP and c-kit ligand to proliferate. Interestingly, primitive megakaryocyte precursors (BFU-MK) fail to respond to this simplified growth factor-ECM complex again, suggesting that other regulatory mechanisms may be operative in this lineage.

The observation of a synergistic cytoadhesive response to TSP and c-kit ligand, coupled with the actions of immobilized c-kit ligand as a hematopoietic growth factor, suggest that TSP and c-kit ligand together may constitute a growth-inductive signal. Neither TSP nor c-kit ligand function alone as CSFs for $CD34^+$ DR^- $CD15^-$ progenitor cells. However, equimolar TSP and soluble c-kit ligand added directly to the fluid-phase of cultures function as a colony-stimulating activity (Fig. 4 *A*).

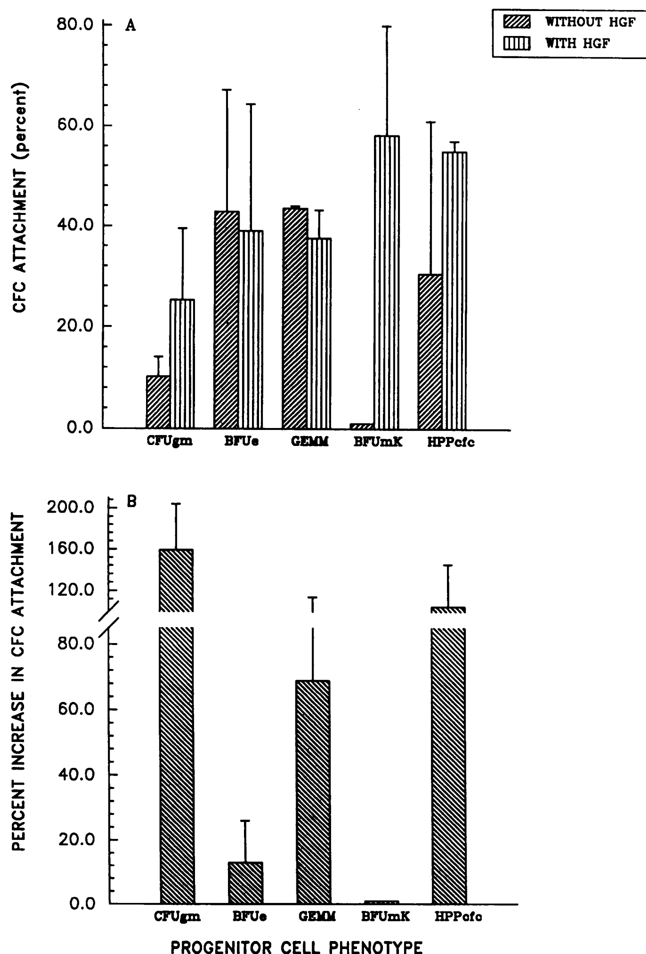

Figure 3. Attachment of CD34$^+$ DR$^-$ CD15$^-$ progenitor cells to c-kit ligand and/or TSP. (*A*) Cytoadhesion of progenitor cells to immobilized c-kit ligand in the presence or absence of additional growth factors. (*B*) Increase in progenitor cell attachment to an equimolar combination of immobilized TSP and c-kit ligand. Progenitor cells were attached as described in the Methods. For cultures without additional growth factors (*diagonal bars*), attached cells are overlayered with semisolid media only (except for BFU-E and GEMM colonies, which require 1 μm/ml of erythropoietin). Growth factor-replete cultures (*vertical bars*) were identical to those in Figs. 1 and 2. Equimolar amounts of TSP and KL were admixed in solution and immobilized onto plastic as described in Fig. 1. *HGF,* hematopoietic growth factors.

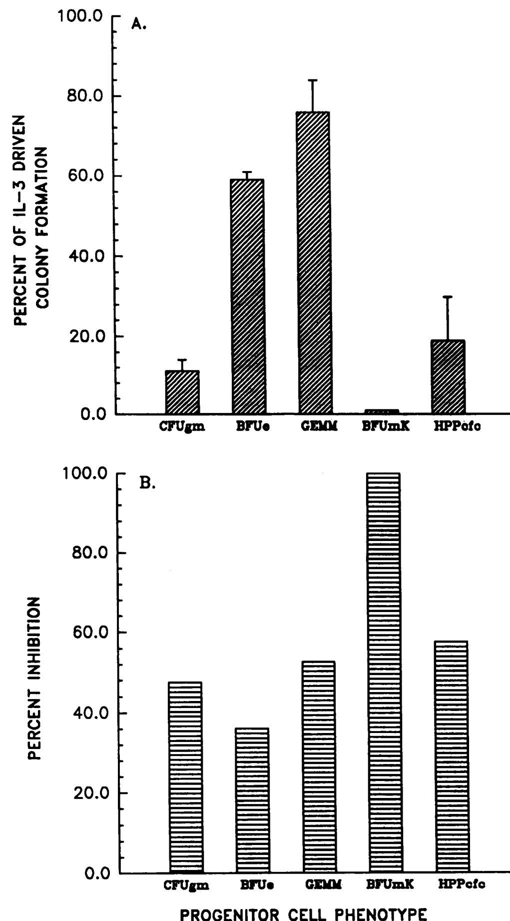

Figure 4. Responsiveness of CD34$^+$ DR$^-$ CD15$^-$ progenitor cells to co-cultivation of TSP and hematopoietic cytokines. (*A*) TSP and c-kit ligand function as a colony-stimulating factor when equimolar concentrations of TSP and KL are utilized as a stimulus for colony formation. Data are expressed as the percent of input, IL-3-promoted colony development (see text). (*B*) Inhibition of IL-3-driven colony formation by TSP. As in *A,* TSP was admixed with IL-3, and the results were expressed as the percent reduction in colony numbers. Results shown are a single representative experiment of two individual marrow samples, cultured in 2-3 replicates for each progenitor cell type for each sample.

Importantly, ~ 60% of the input BFU-E and > 80% of the input CFU-GEMM respond to this dual regulatory signal. TSP plus c-kit ligand also stimulates small numbers of CFU-GM and HPP-CFU. Again, the BFU-MK fail to respond to this combined matrix-cytokine signal, thus confirming the regulatory uniqueness of this lineage. In order to examine the specificity of the TSP-c-kit ligand interaction, TSP was cocultured with IL-3 (a powerful cytokine that drives the development of all lineages). In sharp contrast to the interaction between TSP and c-kit ligand, coculture of TSP with IL-3 results in a diminution of IL-3 responsiveness (Fig. 4 *B*). This inhibition suggests that in this context, TSP may down-modulate IL-3-promoted hematopoietic progenitor cell development. These data demonstrate both a role for TSP in modulating hematopoietic growth factor responsiveness and the uniqueness of the TSP-c-kit ligand interaction.

Recently, a number of studies have identified the c-kit ligand as an important hematopoietic microenvironmental regulator (22-24). The absence of or alterations in the c-kit ligand gene results in the microenvironmental defect associated with mutations at the steel locus. Furthermore, the c-kit ligand itself is a growth factor which augments primitive progenitor cell, mast cell, and committed progenitor cell development (22-24). The c-kit ligand exists in both membrane-bound and soluble forms (25). Functionally, its soluble form promotes progenitor cell development by synergistically modulating growth factor responsiveness, whereas the membrane-associated molecule directly stimulates cell proliferation and works as a ligand for cell-to-cell interactions (25, 26). Our data demonstrates that soluble c-kit ligand, when immobilized, functions as a colony-stimulating factor in the absence of other cytokines. Thus, changes occurring during the binding of c-kit ligand to plastic

are sufficient to allow this cytokine to function as a CSF, perhaps mimicking the membrane-bound form of this molecule.

Information concerning interactions between primitive progenitor cells, matrix molecules, and hematopoietic growth factors is limited. Recent studies demonstrate that progenitor cell proliferation (in the presence of stromal cells) is associated with glycosaminoglycan-bound GM-CSF (6), and that precise proteoglycanside chains (i.e., heparan sulfate) are responsible for binding both GM-CSF and IL-3 (5). These first reports of ECM growth factor interactions clearly demonstrate that matrix bound growth factor is presented in a biologically active form. Our observations demonstrated that another ECM growth factor "complex" (i.e., TSP and c-kit ligand) plays a role in hematopoietic stem cell development, and that the coimmobilization of these two proteins identifies a cell population of hematopoietic progenitor cells that requires adhesion to both ligands to undergo colony formation. Moreover, this ECM growth faction combination, when cocultured with $CD34^+$ DR^- $CD15^-$ progenitor cells, functions as a colony-stimulating activity for specific lineages of hematopoietic cells. These data thus represent the first report of a cytokine and ECM molecule functioning as a signal complex.

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