FROM THE CHICAGO MEETINGS

Pathophysiology of CML: Do defects in integrin function contribute to the premature circulation and massive expansion of the BCR/ABL positive clone?

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Hematopoiesis takes place in close contact with the marrow microenvironment. Normal progenitors adhere through a variety of receptors to stroma and extracellular matrix components, including fibronectin. Adhesion through $\beta 1$ -integrin receptors to fibronectin not only anchor progenitors to the stroma but also result in direct adhesion-mediated signaling that inhibits progenitor proliferation. In contrast to normal hematopoiesis, chronic myelogenous leukemia (CML) is characterized not only by abnormal, premature circulation of primitive progenitors in the blood but also by continuous progenitor proliferation. Although CML progenitors express the same integrin receptors as normal progenitors, they fail to adhere to stroma and fibronectin, suggesting structural or functional abnormalities of these receptors. Furthermore, CML cells present in contact with stroma or fibronectin continue to proliferate, suggesting that failure to adhere through integrin receptors may also underlie the abnormal proliferation of CML progenitors. The observation that integrin-mediated adhesion and proliferation-inhibitory signaling can be restored through treatment with interferon- α or an activating anti- $\beta 1$ -integrin antibody suggests a functional rather than structural defect that may be related to the presence of the BCR/ABL gene rearrangement in these cells. Insights into the role of integrins as adhesion molecules but also receptors that instruct hematopoietic progenitors to survive, proliferate, and possibly differentiate will not only further our understanding of the normal hematopoietic process but also provide insights into diseases characterized by deranged adhesion and proliferation that may lead to novel therapeutic approaches. (J Lab Clin Med 1997;129:584-91)

Abbreviations: CFC = colony-forming cell; CML = chronic myelogenous leukemia; FAK = focal adhesion kinase; IgG = immunoglobulin G; LTC-IC = long-term culture-initiating cell; MAPK = mitogen-activated protein kinase; PI3-K = phosphatidylinositol-3 kinase; STAT = signal transducer and activator of transcription

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Contact with stromal fibronectin inhibits normal CFC proliferation

Antibody mediated engagement of $\beta 1$ -integrins inhibits normal CFC proliferation

Fig. 1. Integrin-mediated proliferation inhibition of normal hematopoietic progenitors. Normal steady-state marrow-derived CD34⁺ cells were induced to proliferate by incubation in stroma-conditioned medium for 3 to 4 days. Cells were then cocultured with either bovine serum albumin, poly-L-lysine, stromal feeders, or glutaraldehyde-fixed stromal feeders in the presence or absence of anti-transforming growth factor- β -neutralizing antibodies or with the C-terminal $\alpha 4\beta 1$ -integrin-binding fragment (33/66 kd) of fibronectin for 4 to 6 hours. Alternatively, cells were incubated with blocking anti- $\alpha 4$, anti- $\alpha 5$, anti- $\beta 1$ -integrin antibodies or control mouse IgG for 30 minutes, followed by incubation with a goat anti-mouse IgG for 4 to 6 hours. Afterwards, cells were subjected to cold or tritiated thymidine before plating in methylcellulose progenitor assay to determine the fraction of cells in S phase.^{15,16}

Hematopoiesis is a complex process in which hematopoietic stem cells proliferate and differentiate into myeloid and lymphoid lineage committed progenitors. These committed progenitors undergo further multiplications before they terminally differentiate into mature blood elements. The process of myelopoiesis, B-lymphopoiesis, T-lymphopoiesis, and natural killer cell generation occurs in close proximity with a permissive microenvironment, which in adult life is provided in the bone marrow. The factors responsible and necessary for an orderly hematopoietic process are not yet understood. The bone marrow microenvironment contains "stromal" cells, such as macrophages, endothelial cells, fibroblasts and osteoblasts. These cells are responsible for the production and deposition of a complex extracellular matrix and local production and concentration of hematopoietic cytokines. Hematopoietic cells inter-

act through specific cell surface expressed receptors with either immobilized or secreted growth promoting/inhibitory cytokines and with adhesive ligands present on stromal cells or extracellular matrix components.¹ It is believed that the concerted action of all these interactions results in ordered, normal hematopoiesis.

INTEGRINS AND NORMAL HEMATOPOIESIS

That $\beta 1$ -integrins may play an important role in localizing progenitors in the marrow microenvironment follows from a number of in vivo and in vitro observations. First, engraftment of murine stem cells can be inhibited by anti- $\alpha 4\beta 1$ antibodies.^{2,3} Likewise, homing of human stem cells to a xenogeneic ovine marrow microenvironment can be inhibited by anti-human $\alpha 4$ antibodies.⁴ Intravenous administration of anti- $\alpha 4$ antibodies, but not

Fig. 2. Focal adhesions. Integrins, cell surface glycoproteins responsible for both cell-ECM adhesion and cell-cell adhesion events, consist of an α -subunit and β -subunit. The extracellular domain is responsible for ligand recognition and binding. The intracellular domain of the β 1-subunit colocalizes with cytoskeletal proteins in focal contacts. Engagement of integrins by their ligand results in the recruitment of FAK, c-Src, and other adaptor and signaling molecules that in turn activate a number of signal transduction cascades, such as the RAS/MAPK and the inositol and protein kinase C pathways, through which they can affect cell proliferation, differentiation, and survival (outside-in signaling). In addition, the interaction of the β 1-integrin intracellular domain with the cell cytoskeleton allows transfer of signals originating from cytokine stimulation or other adhesion receptors to the integrin to modify its affinity status (inside-out signaling).

anti- β 2-integrin antibodies, to baboons results in peripheralization of granulocyte/macrophage colony-forming cells and stem cells.⁵ Finally, β 1-defective hematopoietic stem cells can not successfully compete with wild-type stem cells in competitive engraftment experiments because of their inability to migrate.⁶ All these studies indicate that α 4 β 1-dependent interactions are important to localize progenitors in the marrow microenvironment and to allow their migration and adhesion to the microenvironment.

In vitro, it can be demonstrated that undifferentiated and lineage-committed progenitors from normal marrow adhere in a lineage-specific and differentiation stage-specific manner to several extracellular matrix components, including heparan sulfate,⁷ thrombospondin,⁸ and fibronectin.⁹ We and others have demonstrated that primitive progenitors, functionally defined as LTC-ICs, and more committed CFCs adhere to the vascular cell adhesion molecule and fibronectin present in stroma⁹⁻¹² through the α 4 β 1 and α 5 β 1 integrins and the cell surface proteoglycan CD44. In addition, progenitors express β 2-integrins whose role in progenitor-marrow microenvironment interaction is less clear¹² and selectins that may allow them to interact with endothelial cells.¹³

As has been described for other biologic systems,

integrin-mediated interactions between progenitors and fibronectin or other stromal components may also directly affect progenitor proliferation or differentiation (or both). In a series of experiments we have demonstrated that contact with stromal extracellular matrix, more specifically fibronectin, inhibits proliferation of CFCs and LTC-ICs. This is mediated in part through engagement of integrins, suggesting that integrin stimulation can directly affect progenitor proliferation. For instance, 2 to 3 times more CFCs are generated and 2 to 3 times more LTC-ICs are maintained when CD34⁺ cells are cultured for 5 to 8 weeks in stromal cultures but separated from stroma (noncontact culture) than when cultured in direct contact with the stromal cells (contact culture).¹⁴ Because the continued addition of peptides representing cell adhesion sites in the C-terminal domain of fibronectin to stroma-contact cultures increases the number of CFCs recovered after 5 weeks,¹⁵ similar to what we see in noncontact cultures, we hypothesized that direct contact between progenitors and stromal elements may inhibit proliferation of progenitors, leading to decreased production of CFCs and maintenance of LTC-ICs. We therefore examined the effect of coculture of CFCs with stromal ligands on their proliferation (Fig. 1).¹⁵ Coculture of proliferating CFCs with glutaraldehyde-fixed stroma inhibits CFC pro-

Fig. 3. Interferon- α restores integrin-dependent adhesion and integrin-mediated proliferation inhibition of CML hematopoietic progenitors. CML steady-state marrow-derived CD34⁺/HLA-DR⁺ cells were induced to proliferate by incubation in stroma-conditioned medium for 3 to 4 days. During the last 48 hours, 1000 U interferon- α was added to some of the samples. Cells were then cocultured with either bovine serum albumin or with the GRGDS containing α 5 β 1-integrin binding fragment (75 kd) or the C-terminal α 4 β 1-integrin binding fragment (33/66 kd) of fibronectin for 2 to 6 hours. Alternatively, cells were incubated with blocking anti- α 4-, anti- α 5-, or anti- β 1-integrin antibodies or control mouse IgG for 30 minutes, followed by incubation with a goat anti-mouse IgG for 4 to 6 hours. Afterwards, adherent and nonadherent cells were either replated directly in a methylcellulose assay to determine the fraction of committed progenitors that adhere to fibronectin or were subjected to cold or tritiated thymidine before plating in methylcellulose progenitor assay to determine the fraction of cells in S phase.

liferation that can be prevented by antibodies against α 4. Coculture of CFCs with α 5-binding or with α 4-binding domains of fibronectin inhibits CFC proliferation.¹⁶ Finally, incubation of CD34⁺ cells with blocking anti- α 4, α -5, or - β 1 antibodies and a secondary goat anti-mouse antibody induces receptor capping and cytoskeletal rearrangement, which leads to inhibition of CFC proliferation.¹⁶ These studies demonstrate thus that aside from anchoring progenitors in the microenvironment, β 1-integrins may also be responsible for transferring growth modulatory signals.

Integrins are a family of divalent cation-dependent cell surface glycoproteins responsible for both cell-extracellular matrix adhesion and cell-cell adhesion events.¹⁷ Integrins are heterodimeric as a result of the noncovalent association between an α -subunit and β -subunit. Integrins have a large, heterodimeric extracellular domain responsible for ligand recognition and binding. Ligand specificity is dictated

mostly by the α -chain. Integrins have a small membrane spanning domain and an intracellular domain. The intracellular domain of the β 1-subunit may have a dual role in that it mediates the initial steps in a signal transduction cascade as a result of binding of the integrin to its ligand (outside-in) and is responsible for affinity modulation of the integrin in response to signals provided to the cells through cytokines or other adhesion receptors (inside-out)¹⁷ (Fig. 2). Integrins colocalize with α -actinin, talin, vinculin, and F-actin in focal contacts.¹⁷⁻¹⁹ Members of the Rho family, including Rho and Rac, influence the formation and dynamic stability of actin-based cytoskeletal structures. Talin and the β 1-integrin itself bind to the FAK.²⁰ Thus engagement of integrins results in the recruitment of FAK to focal contacts and its autophosphorylation. Subsequent recruitment and activation of c-Src, paxillin, Crk, Grb-2, and the p85 subunit PI3K.¹⁸⁻²⁰ This can result in the activation of the Ras/MAPK pathway,²¹

The $\beta 1$ activating antibody, 8A2, restores adhesion and adhesion-mediated signaling in CML

Fig. 4. The activating anti- $\beta 1$ -integrin antibody, 8A2, restores integrin-dependent adhesion and integrin-mediated proliferation inhibition of CML hematopoietic progenitors. CML steady-state marrow-derived CD34⁺/HLA-DR⁺ cells were induced to proliferate by incubation in stroma-conditioned medium for 3 to 4 days. Before adhesion or adhesion-mediated proliferation inhibition assays, cells were incubated with the activating antibody 8A2 (a gift from Dr. Nick Kovach, University of Washington, Seattle) or control mouse IgG. Cells were then cocultured with either bovine serum albumin or fibronectin for 2 to 6 hours. Adherent and nonadherent cells were either replated directly in methylcellulose assay to determine the fraction of committed progenitors that adhere to fibronectin or were subjected to cold or tritiated thymidine before plating in methylcellulose progenitor assay to determine the fraction of cells in S phase.

changes in the expression of immediate-early inflammatory response genes,²² and alterations in the levels of cyclins²³ and cyclin-associated molecules,²⁴ all of which can affect cell proliferation, differentiation, and survival.

How these observations relate to the assembly of focal contacts that allow adhesion or integrin-dependent signaling that affects proliferation or differentiation (or both) of hematopoietic cells is still unknown. We have preliminary evidence that integrin-mediated alterations in progenitor proliferation depends on reorganization of the cytoskeleton¹⁶ and that integrin engagement on CD34⁺ cells results in the phosphorylation of FAK (unpublished observations). However, the nature of other downstream intracellular structural and signal molecules involved in focal contact formation leading to adhesion, migration, and growth inhibition observed in the hematopoietic system after engagement of integrins is still unknown.

DEFECTS IN INTEGRINS IN CML HEMATOPOIESIS

CML is a malignant disease of the pluripotent hematopoietic stem cell characterized by the Philadelphia chromosome (Ph)²⁵ and a rearrangement between the BCR gene (break-point cluster region) and the ABL gene.²⁶ Clinically, CML is characterized by a massive expansion of immature progenitors and precursors that leave the marrow microenvironment prematurely.²⁷ Although normal progenitors coexist with the malignant clone in CML, their growth seems to be inhibited, possibly as

a result of abnormalities inherent to the CML marrow microenvironment.²⁸

Why CML progenitors circulate prematurely in the blood is not completely understood. We and others have demonstrated that, compared with normal progenitors, Ph⁺ CML LTC-ICs and CFCs adhere significantly less to stroma^{29,30} or fibronectin³¹ even though Ph⁺ progenitors express normal numbers of $\alpha 4$, $\alpha 5$, and $\beta 1$ ^{29,31} adhesion receptors. This may explain, at least in part, their premature circulation. The mechanisms that underlie the massive expansion of Ph⁺ hematopoietic progenitors, precursors, and mature cells are also not understood. Chronic phase CML progenitors are not growth factor-independent nor are they hypersensitive to cytokines.³² There is evidence that CML progenitors are less subject to apoptosis when cultured under unfavorable conditions *ex vivo*,^{33,34} which may lead to accumulation of the malignant clone. In comparison with normal progenitors, more CML progenitors are proliferating, even though the cell cycle duration of CML progenitors may be longer than that of normal progenitors.³⁵ Because adhesion to the microenvironment in the normal hematopoietic process may be important not only in localizing progenitors in the marrow but also in regulating their proliferation, we examined whether defective integrin-mediated adhesion to stromal elements in CML may explain the abnormal circulation and also proliferation observed in this disease.

A number of studies have demonstrated that Ph⁺ CFCs and LTC-ICs are not subject to normal

Fig. 5. Possible interactions between p210^{BCR/ABL} and integrin-dependent signals. The BCR/ABL mRNA encodes for the P210^{BCR/ABL} tyrosine kinase. P210^{BCR/ABL} has increased tyrosine kinase activity, resulting in autophosphorylation of P210^{BCR/ABL} itself, FAK, Crkl, Cbl, and possibly STAT 1 and STAT 5. Phosphorylation of P210^{BCR/ABL} allows recruitment of Grb2, which then leads to complex formation with son of sevenless (Sos) and activation of Ras. Finally, compared with p145^{ABL}, P210^{BCR/ABL} binds significantly more to F-actin.

growth inhibitory signals when they are present in contact with stroma.^{36,37} Likewise, contact with the $\alpha 4\beta 1$ and $\alpha 5\beta 1$ binding domains of fibronectin or direct engagement of integrins with anti-integrin antibodies³⁷ does not inhibit Ph⁺ CFC proliferation. In contrast to normal progenitors, incubation of CML progenitors or cell lines that contain the BCR/ABL cDNA with blocking anti- $\beta 1$ antibodies does not result in receptor capping.³⁸ Interestingly, integrin-dependent adhesion and proliferation inhibitory signaling can be restored by preincubating CML cells with either interferon- α ^{29,37} (Fig. 3) anti-sense oligonucleotides against BCR/ABL³⁹ or with tyrosine kinase inhibitors specific for the BCR/ABL oncoprotein.⁴⁰ This indicates that integrins on CML progenitors are functionally rather than structurally abnormal. Integrin-dependent adhesion requires that the integrin binds to its ligand, which depends on the affinity state of the integrin and on postreceptor events that allow assembly of focal adhesions needed for firm adhesions and transfer of outside-in signals (to affect proliferation). This led us to hypothesize that the defect in CML may be that integrins are present in a low-affinity state or that the interaction of integrins with cytoskeletal elements is abnormal, resulting in failure to laterally associate, adhere, and transfer microenvironmental growth-inhibitory signals. Although affinity studies have not

yet been performed on primary CML progenitors, we have shown that treatment with the activating anti- $\beta 1$ antibody, 8A2,⁴¹ restores adhesion and subsequent adhesion-mediated proliferation inhibition of CML progenitors when in contact with fibronectin⁴² (Fig. 4). This provides at least indirect evidence that $\beta 1$ -integrins may be present in a low-affinity state on CML progenitors. It is well known that inside-out signals transferred from other cell surface adhesion receptors or cytokine receptors via the cytoskeleton to integrins can switch integrins to a functional high-affinity state. Our observation that receptor capping is defective³⁸ supports the hypothesis that decreased receptor affinity may be related to abnormal interactions between the integrins and cytoskeletal components, which do not allow inside-out activation of the integrin. Thus, abnormal interactions between integrins and one or more components of focal adhesions could then explain not only the poor integrin-mediated adhesion and defective growth-inhibitory outside-in signal transduction but also the low-affinity state of integrins on CML progenitors.

These studies suggest strongly that the BCR/ABL gene rearrangement may be responsible for the low-affinity state of $\beta 1$ -integrins on the majority of CML progenitors or the defective interactions between integrins and other components of focal adhesions in CML. However, it remains to be determined how

BCR/ABL causes these defects. The BCR/ABL mRNA encodes for the P210^{BCR/ABL} tyrosine kinase that is necessary and sufficient for malignant transformation^{43,44} (Fig. 5). p210^{BCR/ABL} has, in comparison with p145^{ABL}, increased tyrosine kinase activity, resulting in autophosphorylation of the oncoprotein.⁴⁵ This allows recruitment of the adaptor protein, Grb2, and activation of Ras. Activation of Ras may also be due to p210^{BCR/ABL}-dependent phosphorylation of additional adaptor proteins such as Crkl⁴⁶ and Cbl.⁴⁷ Because activation of Ras/MAPK can alter the type of focal contact assembled after engagement of integrins,⁴⁸ it is possible that activation of Ras by p210^{BCR/ABL} may affect both integrin-mediated adhesion and signaling in CML. Aside from activating the Ras pathway, p210^{BCR/ABL} phosphorylates and activates a number of other intracellular proteins including FAK,⁴⁹ paxillin,⁵⁰ Crkl,⁴⁶ and PI3-K,⁵¹ all of which are required for focal contact formation and for transfer of signal events related to integrin stimulation. For instance, because integrin-mediated signaling requires recruitment and activation of FAK and paxillin,¹⁷⁻²⁰ one might speculate that chronic phosphorylation of either of these molecules may result in their inability to participate in the integrin-mediated signaling cascade. Finally, increased binding of p210^{BCR/ABL} to F-actin⁵² and therefore indirectly to integrins may lead to the inability of integrins to laterally associate and form caps and to localize to focal adhesions where they both transfer outside-in and accept inside-out signals.

CONCLUSION

Additional studies will be needed to identify structural and signal molecules associated with $\beta 1$ -integrins in membrane-associated focal contact complexes that are required to induce firm adhesion and to induce migration or transfer growth regulatory signals in normal hematopoietic progenitors. Such studies will provide significant new insights into mechanisms involved not only in survival, proliferation, and differentiation of normal progenitors but also in mobilization and engraftment of normal hematopoietic progenitors. Such studies will also lead to a better understanding of abnormal hematopoietic processes characterized by increased or decreased circulation and expansion of progenitors, which may ultimately provide us with novel therapies for such disorders.

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