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The Integrin α9β1 Contributes to Granulopoiesis by Enhancing Granulocyte Colony Stimulating Factor Receptor Signaling

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Summary

The integrin α9β1 is widely expressed on neutrophils, smooth muscle, hepatocytes, endothelia and some epithelia. We now show that mice lacking this integrin have a dramatic defect in neutrophil development, with decreased numbers of granulocyte precursors in bone marrow, and impaired differentiation of bone marrow cells into granulocytes. In response to granulocyte colony stimulating factor (G-CSF), α9-deficient bone marrow cells or human bone marrow cells incubated with α9β1 blocking antibody demonstrated decreased phosphorylation of signal transducer and activator of transcription 3 and extracellular signal-regulated protein kinase. These effects depended on the α9 subunit cytoplasmic domain, which was required for formation of a physical complex between α9β1 and ligated G-CSF receptor. Integrin α9β1 was required for granulopoiesis and played a permissive role in the G-CSF signaling pathway, suggesting that this integrin could play an important role in disorders of granulocyte development and other conditions characterized by defective G-CSF signaling.

Introduction

Integrins are transmembrane heterodimers that participate in the translation of spatially fixed extracellular signals into a wide variety of changes in cell behavior (Hynes et al., 1999). Members of this family share common functions (e.g. cell attachment, spreading and survival) and utilize a number of common signaling intermediates (e.g the focal adhesion kinase and src family kinases) (Clark and Brugge, 1995; Hynes, 1992). However, the diverse and largely non-overlapping phenotypes of mice expressing null alleles of individual integrin subunit genes underscores the biological importance of integrin specificity(Hynes and Bader, 1997; Sheppard, 2000).

The phenotype of α9-deficient mice provided strong evidence that α9β1 plays a unique and non-redundant role in vivo. These mice survive embryonic development normally, but all die by 12 days of age from respiratory failure (Huang et al., 2000). The presence of bilateral chylothorax suggested a defect in lymphatic development in these animals.

The integrin α9β1 is widely expressed in airway epithelium; smooth, skeletal, and cardiac muscle cells, hepatocytes; and neutrophils (Palmer et al., 1993). Multiple ligands have been identified for this integrin, including the inducible endothelial counter-receptor, vascular cell adhesion molecule-1 (VCAM-1)(Taooka et al., 1999), the extracellular matrix proteins tenascin C (Yokosaki et al., 1994) and osteopontin (Smith et al., 1996; Yokosaki et al., 1999), some members of ADAMs family (a disintegrin and metalloproteases) (Bridges et al., 2004; Eto et al., 2002; Tomczuk et al., 2003), coagulation factor XIII and von Willebrand factor (Takahashi et al., 2000).

α9β1 is highly expressed on human neutrophils and is critical for neutrophil migration on VCAM-1 and tenascin-C (Taooka et al., 1999). However, the in vivo function of α9β1 on neutrophils is not clear. Because of the early post-natal mortality in α9-deficient mice, neither we nor others have previously characterized the effects of loss of this integrin on neutrophil function. By evaluating peripheral blood and bone marrow from juvenile α9-deficient mice, we now describe a dramatic defect in granulocyte development in these animals. Characterization of this defect led us to the identification of a pathway by which integrin α9β1 enhanced G-CSF receptor signaling and facilitated the effects of G-CSF on neutrophil development. These findings suggest a potentially important role for the α9β1 integrin in diseases characterized by defects in neutrophil development or other abnormalities in G-CSF signaling.

Results

Integrin α9-deficient mice have defective granulopoiesis

Peripheral blood from α9-deficient mice and littermate controls was stained for the presence of the neutrophil cell surface marker Gr-1 (Figure 1a). The percentage of neutrophils was dramatically decreased in *Itga9*$^{-/-}$ (Figure 1b). There were no major differences in total white blood cells, platelets, lymphocytes or eosinophils between *Itga9*$^{-/-}$, *Itga9*$^{+/-}$ and *Itga9*$^{+/+}$mice (Figure 1b). The percentage of relatively mature myeloid cells (metamyelocytes and granulocytes) and myelocytes in bone marrow was also decreased in *Itga9*$^{-/-}$mice. There were no differences in any other cell types (Figure 1c). Integrin α9β1 is expressed on granulocytes in human peripheral blood (Taooka et al., 1999). We confirmed (by immunoblotting) that α9β1 is also expressed on murine bone marrow cells (Figure 1d). Expression of α9β1 was also detected by flow cytometry on a subset of human bone marrow cells (Figure 1e). Integrin α9β1 is known contribute to cell adhesion and migration in vitro (Taooka et al., 1999; Young et al., 2001). It was thus possible that the decreased number of neutrophils could be due to redistribution. We therefore examined neutrophil and metamyelocyte numbers in the spleen. Neutrophils were 2% and metamyelocytes were 1% of total spleen cells. There was no difference between integrin α9 null mice and controls, suggesting the decrease in neutrophils in peripheral blood and bone marrow of α9 null mice is not due to redistribution.

Integrin α9-deficient mice have a defect in granulocyte differentiation

To determine whether the neutropenia in α9-deficient mice could be a consequence of cytokine signaling defects in hematopoietic progenitors, we tested the *in*

vitro responses of bone marrow to a range of hematopoietic growth factors. Responses to granulocyte macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-3, IL-6 and stem cell factor (SCF) were the same for bone marrow harvested from *Itga9*$^{-/-}$and *Itga9*$^{+/-}$mice. However, in response to granulocyte colony stimulating factor (G-CSF) there was a selective decrease in both the number of colonies formed (Figure 2a) and the number of cells in each colony in *Itga9*$^{-/-}$cells (Figure 2b). Integrin α9 null bone marrow cells had decreased number of colonies in response to range of concentration of G-CSF (Figure 2c). Both wild-type and integrin α9 null colonies contained 40%-50% mature neutrophils, suggesting that loss of α9 does not lead to maturation arrest (Figure 2d). This defect was partially rescued by restoring the expression of α9 to bone marrow from *Itga9*$^{-/-}$mice (Figure 2e).

To determine the significance of this pathway for human bone marrow cells, and to exclude the possibility that the abnormal response of α9 deficient mouse bone marrow to G-CSF was simply due to a decreased number of granulocyte precursors, we examined the effects of a blocking antibody to α9β1 on *in vitro* colony formation with normal human bone marrow. Responses to G-CSF were substantially attenuated in these cells by treatment with the α9β1-blocking antibody, Y9A2 (Figure 2f). Together, these results suggest that α9β1 contributes to neutrophil development by specifically facilitating neutrophil differentiation in response to G-CSF.

Integrin α9-deficient bone marrow cells exhibit attenuated signal transducer and activator of transcription 3 (STAT3) activation in response to G-CSF

To determine if the reduced responsiveness to G-CSF could be due to decreased expression of G-CSF receptor in bone marrow cells from integrin α9 null mice, we examined the surface expression of G-CSF receptor on mouse bone marrow cells by incubation with labeled G-CSF. There was no difference in receptor expression between integrin α9 null mice and controls (Figure 3a-b).

To determine whether the impaired responsiveness to G-CSF could be explained by interaction between α9β1 and the G-CSF signaling pathway, we first examined whether α9β1 and the G-CSF receptor were expressed in the same cells. Flow cytometry showed that the majority of human bone marrow cells that express the G-CSF receptor also express α9β1 (Figure 3c). To assess the ability of the α9 deficient bone marrow cells to produce the signals immediately downstream of the G-CSF receptor, we stimulated bone marrow with G-CSF, and evaluated activation of the proximal signaling intermediate, STAT3. As expected, STAT-3 phosphorylation was readily observed 5 min and 30 min after G-CSF stimulation of *Itga9*$^{-/-}$ bone marrow cells. However, this effect was markedly reduced in α9 deficient bone marrow cells (Figure 3d). Similarly, STAT3 phosphorylation was decreased in human bone marrow treated with integrin α9β1 blocking antibody (Figure 3e). These results suggest that expression of α9β1 does not affect G-CSF receptor expression, but enhances downstream STAT3 signaling in response to receptor ligation.

The cytoplasmic domain of integrin α9 is critical for enhancement of G-CSF signaling

To further elucidate the role of the integrin α9 subunit in G-CSF signaling, we stably expressed the G-CSF receptor and the integrin α9 subunit (and thus α9β1) in CHO cells. Co-transfected CHO cells were stimulated with G-CSF while plated on collagen (an adhesive substrate that is not a ligand for α9β1) or on both collagen and Tnfn3RAA (an integrin α9β1 specific ligand)(Yokosaki et al., 1998). G-CSF induced STAT3 phosphorylation was markedly reduced in cells plated on collagen alone, suggesting that ligation of integrin α9β1 is required for enhancement of G-CSF signaling (Fig. 4a). G-CSF-induced phosphorylation of STAT3 was also markedly inhibited by α9β1 blocking antibody (Y9A2), as we saw in human bone marrow cells (Figure 4a).

To determine whether the cytoplasmic domain of the α9 subunit specifically enhances G-CSF signaling, chimeric α subunits composed of the extracellular and transmembrane domain of α9 fused to the cytoplasmic domain of either the integrin α5 or α4 subunit were used (Young et al., 2001). CHO cell lines expressing equal amounts of each α9 construct were transfected to express the G-CSF receptor, and similar G-CSF receptor expression was confirmed by flow cytometry (Figure 4b). We also examined adhesion of these transfected cell lines on Tnfn3RAA and the irrelevant ligand, plasma fibronectin. As expected, integrin α9β1 expressing cells, but not mock transfectants, adhered to Tnfn3AA and all cell lines adhered equally well to fibronectin (Figure 4c). There were no differences in morphology or cell proliferation between these cell lines (data now shown). G-CSF-induced phosphorylation of STAT3 was dramatically decreased in cell lines expressing either chimeric α9 subunit (Figure 4d).

To identify the cytoplasmic sequences critical for enhancing G-CSF signaling, a series of α9 cytoplasmic domain deletion mutants were used (translation stop codon was

introduced into α9 cDNA to generate different lengths of mutated α9 protein: 984aa, 990aa and 1003aa; the full length of α9 is 1006aa). These deletion mutants were stably co-expressed with G-CSF receptor in CHO cells and the expression of G-CSF receptor was similar in each line (Figure 4b). These mutants were chosen, in part, to evaluate the potential significance of two cytoplasmic proteins, spermine spermidine acetyl transferase (SSAT) and paxillin that we have previously found to bind to the α9 cytoplasmic domain and influence α9β1-dependent effects on cell behavior (Chen et al., 2004; Liu et al., 2001; Young et al., 2001). Both of these functional effects are lost for α9(984X) but preserved for α9(990X), and interaction of both paxillin and SSAT is preserved for α9(990X). However, both of these mutants demonstrated markedly reduced G-CSF-induced STAT3 phosphorylation (Figure 4e). Only the C-terminal three amino acids of the α9 cytoplasmic domain could be deleted without affecting this response (Figure 4e). Thus, sequences within the α9 cytoplasmic domain are critical for enhancement of G-CSF signaling, but this response is not simply due to association with paxillin or SSAT. These results suggest that sequences within the α9 cytoplasmic domain are critical for enhancement of G-CSF signaling, but that this response is not simply due to association with paxillin or SSAT.

Integrin α9β1 enhances G-CSF signaling in response to naturally occurring ligand

Tnfn3RAA used in the above experiments is a recombinant form of the third fibronectin type III repeat of chicken tenascin-C containing an RGD to RAA mutation. To verify that a natural ligand for integrin α9β1 has the same function, we plated CHO cells on plates coated with collagen alone or collagen and human recombinant VCAM-1.

Phosphorylation of STAT3 was enhanced in CHO cells from VCAM-1 coated plates and was inhibited by antibody against integrin α9β1 (Figure 4f).

Integrin α9β1 enhances G-CSF signaling in hematopoietic cells

CHO cells expressing integrin α9β1 and the G-CSF receptor were convenient for examining the crosstalk between them in a simple system. However, CHO cells are non-hematopoietic cells. To confirm the relevance of our findings to hematopoietic cells, we used a pre-B cell line BaF3 expressing the G-CSF receptor. We could not detect expression of integrin α9 in BaF3 cells by reverse transcript PCR or immunoblot (data now shown). We thus infected BaF3 expressing the G-CSF receptor with a retrovirus expressing either integrin α9 or α9α5. Cells expressing both the wild type integrin α9β1 and the G-CSF receptor demonstrated increased phosphorylation of STAT3 in response to G-CSF, whereas cells expressing the α9α5 chimera were no different than cells expressing the G-CSF receptor alone (Figure 5a).

STAT3 has been reported as important for G-CSF induced proliferation and differentiation(McLemore et al., 2001), however observations in mice with a conditional knockout of STAT3 suggested that STAT3 could be a negative regulator of granulopoiesis(Lee et al., 2002). Some studies suggest that other signaling molecules might be more important in G-CSF induced proliferation and differentiation, such as ERK(extracellular signal-regulated protein kinase) (Kamezaki et al., 2005), Akt and STAT5(signal transducer and activator of transcription 5)(Dong et al., 1998; Ilaria et al., 1999; Zhu et al., 2004). We thus studied the activation of ERK, Akt and STAT5 in integrin α9β1 expressing BaF3 cells. Both the magnitude and duration of ERK

phosphorylation were increased in α9β1 expressing cells (Figure 5b). However, phosphorylation of Akt and STAT5 were unaffected by expression of α9β1. To determine the relevance of these findings to primary bone marrow cells, we performed similar experiments with bone marrow from wild type and α9 null mice. ERK phosphorylation was enhanced in cells expressing α9β1, whereas Akt and STAT5 phosphorylation was unaffected by the presence of the integrin (Figure 5c).

Suppressor of cytokine signaling 3(SOCS3) is an important negative regulator of G-CSF signaling. SOCS3 down-regulates signaling by binding the phosphorylated tyrosine 729 of the cytoplasmic domain of the G-CSF receptor(Zhuang et al., 2005). To determine if α9β1 affects G-CSF signaling by modulating interactions with SOCS3, we examined ERK activation in BaF3 cells expressing the integrin and a mutant G-CSF receptor in which tyrosine 729 was changed to phenylalanine (Y729F). This mutation has been shown to prevent the interaction of the G-CSF receptor with SOCS3(Hortner et al., 2002; Zhuang et al., 2005). As has been previously reported, the Y729F mutation enhanced G-CSF-induced ERK phosphorylation, but this effect was still further enhanced in cells expressing integrin α9β1 (Figure 5d). Thus, that the effects of α9β1 ligation of G-CSF receptor signaling are not likely due to inhibition of interactions with SOCS3.

In approximately 20% of patients suffering from severe congenital neutropenia(SCN), C-terminus truncated mutations are found in the G-CSF receptor(Dong et al., 1995; Dong et al., 1997). These patients have an increased risk of developing acute myeloid leukemia (AML)(Dong et al., 1997). G-CSF receptor truncated at 715 mutant (Δ715) shows a hyperproliferative response to G-CSF in vitro and increased phosphorylation of ERK(Ward et al., 1999; Zhu et al., 2004). To determine

whether α9β1 ligation could modulate signaling mediated by the membrane proximal region of the G-CSF receptor, we co-expressed the truncated Δ715 mutant and α9β1 in BaF3 cells and examined ERK phosphorylation and proliferation in response to G-CSF. As previously reported, cells expressing Δ715 had increases in G-CSF induced ERK phosphorylation (Figure 5e) and increased rates of proliferation (Fig. 5f), even in the absence of integrin α9β1. However, expression of the integrin did not further enhance ERK phosphorylation or G-CSF induced proliferation in these cells (Figure 5e-f). Expression of α9β1 increased G-CSF-induced proliferation in cells expressing either the wild type or Y729F mutant. Flow cytometry demonstrated similar amounts of expression of wild type, Y729F and Δ715 G-CSF receptors in both wild type and α9β1-expressing BaF3 cells (Figure 5g) and similar amount of α9β1 in all of the α9β1-expressing cell lines (data not shown). Taken together, these results suggest that α9β1 modulates signaling through sequences in the distal region of the G-CSF receptor cytoplasmic domain to enhance ERK and STAT3 activity without affected signals mediated by STAT5, SOCS3 or Akt.

In response to G-CSF, ligated integrin α9β1 physically interacts with the G-CSF receptor and enhances its phosphorylation

To determine whether α9β1 could physically interact with the G-CSF receptor, we plated CHO cells expressing the G-CSF receptor and wild type α9 or an α9α5 chimera on the α9β1 specific ligand Tn3fnRAA and immunoprecipitated α9-containing complexes with the non-blocking α9β1 antibody, A9A1. In cells treated with G-CSF, but not in untreated cells, the G-CSF receptor was detectable in immunoprecipitates of wild

type α9β1, but not in immunoprecipitates containing the chimeric α9α5 subunit, despite equal capture of both integrins as determined by immunoblot for the associated β1 subunit (Figure 6a). Formation of complexes containing the G-CSF receptor and wild type α9β1 clearly depended on ligation of the integrin, since complex formation was completely inhibited by the α9β1 blocking antibody, Y9A2.

Two integrin α9 cytoplasmic domain deletion mutation α9(984X) and α9(990X), which cannot enhance G-CSF signaling, were also evaluated for ability to physically associate with the G-CSF receptor by co-immunoprecipitation. Neither mutant appeared to associate with the G-CSF receptor (Figure 6b). In contrast, the one deletion mutant that preserved enhancement of G-CSF signaling (α9(1003X)) responded to G-CSF stimulation by associating with the G-CSF receptor to the same extent as seen with full-length α9. To confirm that physical association was an important determinant of enhanced signaling, we also performed co-immunoprecipitation experiments in lysates from untreated or G-CSF stimulated BaF3 cells expressing wild type G-CSF receptor, the Y729F mutant (for which α9β1 also enhances signaling) or the Δ715 mutant (for which α9β1 does not enhance signaling). As predicted, G-CSF treatment induced co-association of the integrin with both the wild type and Y729F mutants but not with the Δ715 mutant (Figure 6c).

Tyrosine phosphorylation of the G-CSF receptor is an important early step in G-CSF signaling. Phosphorylation of the G-CSF receptor was evaluated by immunoprecipitation of the receptor followed by immunoblotting with an phosphotyrosine antibody. In response to G-CSF, the G-CSF receptor was phosphorylated in cells expressing wild type, full-length α9 but not in cells expressing

the α9a5 chimera. Phosphorylation of the G-CSF receptor was also inhibited by blocking antibody to α9β1(Figure 6d). Thus, α9β1 is recruited to a protein complex containing the ligated G-CSF receptor and the presence of α9β1 in this complex enhances phosphorylation of the G-CSF receptor in response to G-CSF.

Discussion

In this report we describe a specific and dramatic defect on development of neutrophils in mice lacking the integrin α9 subunit. This phenotype appears to be explained by a role for the α9β1 integrin in enhancing signaling through the G-CSF receptor, an effect that we show depends on physical association of α9β1 with the G-CSF receptor. Both enhancement of signaling and receptor co-association depend on specific sequences within the α9 subunit cytoplasmic domain.

The observation that an integrin can physically associate with and specifically enhance signaling through another cell surface receptor is not novel. Several integrins have been shown to enhance signaling through growth factor receptors, including receptors for platelet derived growth factor (Baron et al., 2002; Schneller et al., 1997), vascular endothelial growth factor (Soldi et al., 1999) and epithelial growth factor (EGF) (Moro et al., 2002; Moro et al., 1998). A similar interaction has been described between a β1 integrin and the interleukin-3 receptor (Defilippi et al., 2005). In most of these cases, co-ligation of the integrin and the growth factor receptor leads to both enhancement in growth factor receptor signaling and to physical co-association of the two receptors. At least one example has been described where ligation of an integrin, α5β1, induces tyrosine phosphorylation of a growth factor receptor (the EGF receptor) that appears to

be independent of growth factor receptor ligation (Moro et al., 1998). Our data suggest that cooperative interaction between ligated G-CSF receptors and α9β1 occurs at a step quite proximal to the initiation of G-CSF signaling, since three early steps in this pathway, phosphorylation of STAT3, ERK and phosphorylation of the G-CSF receptor itself are clearly enhanced by α9β1. However, in this case it does not appear that α9β1 signaling is itself sufficient to initiate G-CSF signaling, since in the four cell systems we studied (human and murine bone marrow and transfected CHO and BaF3 cells) neither enhanced STAT3 phosphorylation nor G-CSF receptor phosphorylation was detected in the absence of exogenous G-CSF. Based on our findings that co-ligation of the integrin and the G-CSF receptor led to physical association of both receptors, and on our observations that each of four α9 subunit mutants that failed to promote co-association also failed to enhance G-CSF signaling, we speculate that physical co-association is critical for enhancement of signaling.

The precise molecular mechanism(s) by which physical co-association of integrins and other receptors promote enhanced signaling have not been determined. Since G-CSF receptor phosphorylation is required for association with STAT3 and its subsequent phosphorylation by activated Janus kinases (JAKs), it is likely that enhanced phosphorylation of the G-CSF receptor is a proximal step in enhancement of G-CSF signaling by α9β1. Because the amount of G-CSF receptor phosphorylation is thought to be determined by the balance of phosphorylation by activated JAKs and dephosphorylation by receptor-associated phosphatases we speculate that the activity or physical association of one or more of these kinases or phosphatases is regulated through α9β1.

Our data clearly demonstrate that the short α9 subunit cytoplasmic domain is critical for both enhanced G-CSF signaling and physical association between ligated G-CSF receptor and α9β1. Integrin cytoplasmic domains are highly divergent, but even the most closely related α4 subunit cytoplasmic domain cannot substitute for α9. These findings raise the possibility that one or more cytoplasmic proteins could bind to the α9 cytoplasmic domain and mediate receptor co-association and enhancement of G-CSF receptor signaling. We and others have previously identified two proteins that interact with the α9 cytoplasmic domain, SSAT(Chen et al., 2004) and paxillin(Liu et al., 2001; Young et al., 2001). Both interactions are biologically important, since binding to paxillin appears to be critical for prevention of cell spreading in cells expressing α9β1 (Young et al., 2001) and SSAT appears to be critical for α9β1-mediated enhancement of cell migration (Chen et al., 2004). One the deletion mutants examined in the current study, 990X, retains the ability to interact with both proteins and to mediate both inhibition of cell spreading and enhancement of cell migration. However, this mutant was unable to mediate either enhanced G-CSF signaling or receptor co-association, suggesting that interactions with paxillin and/or SSAT are not sufficient to explain these effects. While we cannot exclude participation of either or both of these proteins, it is clear that interactions with more C-terminal sequences within the α9 cytoplasmic domain are also required.

Previous studies have identified residues in the G-CSF receptor cytoplasmic domain involved in activation or repression of downstream signaling intermediates. The α9 cytoplasmic domain could modulate responses to G-CSF by direct or indirect interactions with any of these. For example, the membrane proximal region of the G-CSF

receptor (proximal to amino acid 715) has been shown to activate Akt and ERK and to enhance proliferation in response to G-CSF (Zhu et al., 2004). Our results, demonstrating no enhancement of Akt phosphorylation in cells expressing the wild type G-CSF receptor and no enhancement of ERK phosphorylation or proliferation by α9β1 in cells expressing the Δ715 truncation mutant G-CSF receptor, suggest that α9β1 ligation does not modulate responses mediated by the membrane proximal region of the receptor. Our data also suggest that α9β1 does not increase G-CSF receptor signaling by inhibiting interactions with SOCS3, since α9β1 still enhanced both ERK phosphorylation and G-CSF-induced proliferation in cells expressing the Y729F mutant, which cannot bind to SOCS3. Together, these results suggest that α9β1 most likely enhances STAT3 and ERK activation and neutrophil development through interactions with regions of the distal G-CSF receptor cytoplasmic domain other than the Y729 SOCS3 binding site.

G-CSF has been reported to inhibit neutrophil apoptosis and therefore enhance neutrophil survival (Colotta et al., 1992; Martin et al., 1995; Rex et al., 1995). One recent report suggests that ligation of α9β1 also prevents apoptosis and enhances survival(Ross et al., 2006). It is thus conceivable that the effects we describe could be explained by enhancement of neutrophil survival. However, we have been unable to demonstrate any effects of α9β1 on apoptosis or survival using bone marrow from α9-deficient mice or human bone marrow treated with α9β1 blocking antibody (data not shown). We therefore favor the hypothesis that α9β1 contributes to increased circulating neutrophil numbers and increased bone marrow colonies through enhancement of effects on G-CSF mediated proliferation, differentiation or both.

Our studies in CHO cells and human bone marrow suggest that the effects of α9β1 on enhancing G-CSF signaling might depend on integrin ligation. Multiple ligands α9β1 ligands have been identified, including the VCAM-1(Taooka et al., 1999), tenascin C (Yokosaki et al., 1994), osteopontin (Smith et al., 1996; Yokosaki et al., 1999), ADAMs proteases (Bridges et al., 2004; Eto et al., 2002; Tomczuk et al., 2003), coagulation factor XIII and von Willebrand factor (Takahashi et al., 2000). There are a number of extracellular matrix proteins expressed by bone marrow stem cells and stromal cells in bone marrow. VCAM-1 is expressed on stromal cells and mediates cell adhesion of hematopoietic progenitor cells(Simmons et al., 1992). Osteoponin and tenascin C are also expressed in bone marrow(Seiffert et al., 1998; Yamate et al., 1997). Osteoponin is a component of the hematopoietic stem cell(HSC) niche which plays an important role in HSC migration and proliferation(Nilsson et al., 2005). So far there are no reports that inactivating any of these α9β1 ligands lead to defects in granulopoiesis, which may be a consequence of redundancy given the large number of known ligands. In our studies, it appeared likely that bone marrow cells generate their own α9β1 ligands in vitro, since an antibody that interferes with ligand binding had the same effect as genetic absence or deletion of the integrin. Furthermore, addition of exogenous α9β1 ligands to cultured bone marrow cells did not further enhance the effects on G-CSF receptor signaling caused by expression of the integrin (data not shown).

Recent evidence suggests that G-CSF signals important effects on a number of cell types other than granulocytes. For example, the G-CSF receptor is expressed on endothelial cells, where expression of the adhesion receptors, E-selectin, vascular endothelial cell adhesion molecule-1 and intracelleular adhesion molecule-1 at the cell

surface are increased in response to G-CSF (Fuste et al., 2004). The G-CSF receptor is also expressed on neurons and G-CSF inhibits apoptosis of mature neurons. G-CSF has been reported to inhibit acute neuronal degeneration and contribute to long-term plasticity after cerebral ischemia (Schneider et al., 2005). Although we have not examined effects in other cell types, α9β1 is also widely expressed and we speculate that α9β1-mediated enhancement of G-CSF signaling could contribute to effects of G-CSF at one or more of these other sites.

In summary, we have identified a pathway by which the integrin α9β1 dramatically enhances the response of bone marrow precursors to G-CSF signaling and thereby contributes to normal granulocyte development. This pathway involves physical association of α9β1 with the ligated G-CSF receptor and enhancement of the G-CSF signaling pathway, beginning at a step proximal to receptor phosphorylation. Defects in this pathway could contribute to the development of congenital neutropenia and might also be important at other sites of G-CSF signaling.

Experimental procedures

Cytokines, Reagents and Antibodies

PE conjugated anti-Gr1 and anti-G-CSF receptor were from BD Bioscience (San Jose, CA). Antibody against phospho-STAT3 (Tyr705), STAT3, phospho-ERK (Thr202/Ser204), ERK, phospho-STAT5(Tyr694), STAT5, phospho-Akt(Ser473), Akt, HA, and phosphotyrosine mouse mAb were from Cell Signaling (Beverly, MA). The α9β1-specific ligand Tnfn3RAA (Prieto et al., 1993; Yokosaki et al., 1998; Yokosaki et al., 1994), α9 mouse monoclonal antibody Y9A2 (Wang et al., 1996), A9A1 (Vlahakis et

al., 2005) and rabbit polyclonal antibody 1057 (Palmer et al., 1993) were prepared in our laboratory. Purified normal IgG was from Jackson Immunoresearch (West Grove, PA). Purified mouse IgG1 and biotinylated mouse IgG1 control were from eBioscience (San Diego, CA). Collagen was from Sigma-Aldrich (St. Louis, MO). Goat polyclonal anti-G-CSF receptor was from R&D Systems (Minneapolis, MN). Human G-CSF and polyclonal antibody against integrin β1 was from Chemicon International (Temecula, CA). Mouse cytokines G-CSF, GM-CSF, IL-3, IL-6, and SCF were from Stem Cell Technologies (Vancouver, Canada).

Animal Husbandry

Mice maintained on a C57/BL6 background were housed at the animal care facility at the University of California San Francisco and all studies were approved by the institutional review board. Mice were. Integrin α9β1 null mice were used for analysis between 6 and 7 days after birth.

Murine bone marrow analysis

Marrow was removed from femurs and tibias by flushing with PBS buffer. The cell pellet was incubated with red blood cell lysis buffer, re-suspended in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum and captured on glass slides by cytospin. Slides were stained with Wright-Giemsa (Hema 3, Fisher Scientific, Tustin, CA) and 500 cells were counted per slide.

Flow cytometry

Murine bone marrow or peripheral blood cells were collected at autopsy and suspended in phosphate buffered saline (PBS). Human bone marrow cells were obtained from Allcells (Berkeley, CA). Cells were incubated with primary antibody for 30 min at 4°C,

followed by a secondary goat anti–mouse antibody conjugated with phycoerythrin. The expression of G-CSF receptor on mouse bone marrow cells surface was assayed by G-CSF phycoerythrin fluorokine kit from R&D Systems (Minneapolis, MN).

Hematopoietic progenitor cell assays

2×10^4 bone marrow cells were plated in either 1 ml of methylcellulose media MethoCult M3231 (Stem Cell Technologies, Vancouver, Canada) for mouse bone marrow cells or MethoCult M4230 for human bone marrow cells, supplemented with the indicated cytokines. Colonies were counted on days 7–8. Recombinant cytokines were used at the following concentrations: G-CSF, 20 ng/ml; GM-CSF, 10 ng/ml; IL-3, 10 ng/ml; IL-6, 500 ng/ml; and stem cell factor, 100 ng/ml. To examine cell morphology and cell number, entire methylcellulose cultures were harvested, washed extensively, counted and evaluated by Wright-Giemsa staining.

Retrovirus infection

Retroviruses (pBABEpuroα9(Young et al., 2001) or empty pBABEpuro) were generated by transfection into the Phoenix-E virus packaging cell line. Bone marrow cells were infected by incubation with supernatants from infected Phoenix E cells.

Immunoblot analysis and co-immunoprecipitation

For immunoblotting, bone marrow cells were pelleted by centrifugation, resuspended in PBS, and treated for 10 min at 0 °C with 2 mM diisopropyl fluorophosphate (DFP), a cell-permeable serine protease inhibitor, as described previously (Selsted et al., 1992). Cells were lysed in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM sodium ortho-vanadate, 50 mM NaF, 1% Triton X-100, lysates were separated by SDS-PAGE, transferred to immobilon, incubated with primary antibodies and detected by ECL

(Amersham Pharmacia Biotech, Piscataway, NJ). CHO cell lines were serum starved overnight, plated on Tnfn3RAA or collagen coated plates, stimulated with human G-CSF (20ng/ml) for 5 and 30 min and lysed as described above. BaF3 cells were grown in RPMI medium with 10% fetal calf serum and 2 ng/ml murine recombinant interleukin 3. Cells were serum starved overnight, plated on Tnfn3RAA and fibronectin coated plates and stimulated with human G-CSF (100ng/ml) for 5 and 30 min at 37 °C. Cells were lysed in 10mM Tris-HCl(pH7.5), 150mM NaCl, 1% Nonidet P-40, 2mM Na_3VO_4, 1 tablet per 10ml of solution of Complete Mini Protease Inhibitor Cocktail tablets (Roche).

For co-immunoprecipitation, CHO or BaF3 cell lines were lysed on ice for 30 min in an immunoprecipitation buffer: 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 10 mM EDTA, 1 mM sodium ortho-vanadate, 50 mM NaF, 1% CHAPS, 1 tablet per 10ml of solution of Complete Mini Protease Inhibitor Cocktail tablets (Roche). Lysates were clarified by centrifugation at 16,000 g and incubated with protein G-Sepharose coated with the α9β1 antibody, A9A1 at 4°C overnight. Beads were washed 5 times and precipitated polypeptides were extracted in Laemmli sample buffer, separated by SDS-PAGE under reducing conditions, probed with a G-CSF receptor monoclonal antibody (for CHO cells) or anti-HA (for BaF3 cells) and detected by ECL.

Generation of stable cell lines

Integrin α9-, α9α4, α9α5 or α9(984X), α9(990X) and α9(1003X) expressing CHO cells were transfected with human G-CSF receptor cDNA (IMAGE: 5217305 clone from ATCC) in pSPORT6 vector at a 5:1 ratio with pcDNA3.1.Hygro to induce hygromycin resistance. Stable clones were obtained by hygromycin selection and cell sorting by G-CSF receptor expression. BaF3 cells were infected by incubation with α9-expressing

retroviruses. Hemagglutinin (HA) tagged G-CSF receptor and mutated receptors were described previously(Zhu et al., 2004). Integrin α9 expressing BaF3 cell were transfected by electroporation, Stable cell lines were established by selection with 500 μg/mL of G-418.

Cell Proliferation in response to G-CSF

BaF3 cells proliferation was assayed as previously described(Zhu et al., 2004). Briefly, BaF3 cells were plated in Tnfn3RAA coated plates at a density of 1×10^5 cells/ml with 100ng/ml human G-CSF in 10% serum medium. Cell numbers were counted daily and assessed for viability by trypan blue exclusion.

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Figure 1. α9-deficient mice are neutropenic.

(a) Flow cytometry analysis of neutrophils in peripheral blood. Peripheral blood was collected from wild type, *Itga9*$^{+/-}$and *Itga9*$^{-/-}$ mice, stained with PE conjugated anti-Gr-1 and analyzed by flow cytometry. (b) Percentage of neutrophils in peripheral blood from wild type, *Itga9*$^{+/-}$and *Itga9*$^{-/-}$ mice (determined by Wright-Giemsa staining). Values are means from at least 6 mice from each group. *p=0.003 *Itga9*$^{+/-}$versus *Itga9*$^{-/-}$). (c) Bone marrow analysis. Cell counts and differential counts were performed on cells recovered from six sex-matched littermates of each genotype. *p=0.002, ** p=1.1×10^{-4} ($\alpha9^{+/+}$ versus $\alpha9^{-/-}$). (d) Expression of integrin α9β1 in mouse bone marrow cells. Lysates of bone marrow cells were blotted by α9 cytoplasmic domain antiserum 1057(Palmer et al., 1993). (e) Expression of integrin α9β1 in human bone marrow cells. Cells from human donor bone marrow were stained with anti-integrin α9β1, Y9A2. Open peaks represent fluorescence (FL) of mouse IgG1 control stained cells, and shaded peaks represent fluorescence of CHO cells stained with Y9A2. Error bars denote the mean ± SD.

Figure 2. **α9-deficient bone marrow is hypo-responsive to G-CSF**

(a) Bone marrow cells were plated in methylcellulose-containing media supplemented with the indicated cytokine. Hematopoietic colonies containing greater than 30 cells were scored after 7–8 days. N=7 for each genotype. *p=5.6×10^{-4}. (b) Cellular content of α9-deficient colonies relative to controls. Ten to eighty consecutive colonies from parallel cultures of bone marrow cells were picked, pooled, and counted. Mean ± SD of results from four to six mice per genotype. *p=1.5×10^{-3}. (c) Colony forming assay for mouse bone marrow cells stimulated by G-CSF (0 to 100ng/ml) (d) Differential counts of bone

marrow cells. Cells were collected from colony forming assay. Black bars: neutrophils and bands; gray bars: myelocytes and metamyelocytes; white bars: other cells. (e) Mouse bone marrow cells were infected with a retrovirus expressing full-length, mature α9 (α9) or with empty vector (vector). Colony forming assays were performed with 20ng/ml G-CSF. *$p=5\times10^{-3}$ (f) Colony forming assays with human bone marrow cells stimulated by a range of concentrations of G-CSF (0 to 20ng/ml) in the presence of integrin α9β1 blocking antibody Y9A2 (gray bars) or control normal mouse IgG (black bars). Error bars denote the mean ± SD.

Figure 3. G-CSF induced STAT3 phosphorylation is attenuated by loss or blockade of integrin α9β1.

(a) Flow cytometry of G-CSF receptor expression on mouse bone marrow cells. Cells were incubated with PE-conjugated G-CSF (shaded peak) or streptavidin-PE (open peak). (b) Mean fluorescence intensity for G-CSF receptor expression (n-=3 for each genotype). (c) Flow cytometry analysis of expression of G-CSF receptor and integrin α9 in human bone marrow mononuclear cells. Cells were incubated with PE-conjugated anti-G-CSF receptor and biotinylated anti-α9β1 (Y9A2) or biotin mouse IgG1 control followed by incubation with FITC-conjugated streptavidin. G-CSF receptor positive and negative cells were gated as light shaded and dark shaded peaks. (d) STAT3 phosphorylation in mouse bone marrow cells. Bone marrow cells from α9-deficient or wild type mice were treated with G-CSF for 5 or 10 minutes and analyzed by immunoblotting with anti-Phospho-STAT3 (Tyr705) or anti-total STAT3. (e) STAT3 phosphorylation of human bone marrow cells incubated with α9β1 blocking antibody (Y9A2) or control antibody for 30 minutes following by G-CSF treatment. Error bars denote the mean ± SD.

Figure 4. G-CSF induced STAT3 phosphorylation depends on specific sequences in the α9 cytoplasmic domain.

(a) Cells were treated with α9β1-blocking antibody, Y9A2 or control IgG and plated on either an irrelevant ligand (collagen) or on collagen and an α9β1 specific ligand, (Tnfn3RAA) and were either untreated or treated with G-CSF for 5 or 30 min. Lysates were evaluated by immunoblotting with anti-Phospho-STAT3 (Tyr705) or anti-total STAT3. (b) Flow cytometry of G-CSF receptor Expression on transfected CHO cells Open peaks represent fluorescence (FL) of unstained CHO cells, and shaded peaks represent fluorescence of CHO cells stained with the anti-G-CSF receptor. (c) CHO cells expressing either G-CSF receptor alone, integrin α9β1 alone, or both G-CSF receptor and integrin α9β1 were plated on either 5ug/ml Tnfn3RAA or fibronectin after incubation with or without α9β1 mAb, Y9A2. Adherent cells were stained with crystal violet and quantified by measurement of absorbance at 595nm(Young et al., 2001). (d) STAT3 phosphorylation in response to G-CSF in CHO cells expressing G-CSF receptor and wild type integrin α9 or α9 chimeras. (e) STAT3 phosphorylation in response to G-CSF in CHO cells expressing the G-CSF receptor and full-length integrin α9 or α9 deletion mutants. (f) STAT3 phosphorylation in response to G-CSF in CHO cells expressing G-CSF receptor and integrin α9. Cells were plated on collagen or collagen and human recombinant VCAM-1 coated dishes. Error bars denote the mean ± SD.

Figure 5. Integrin α9β1 enhances the activation of STAT3 and ERK in response to G-CSF in BaF3 cells.

(a) G-CSF-stimulated STAT3 phosphorylation of BaF3 cells expressing human G-CSF receptor and integrin α9β1. (b) G-CSF-stimulated ERK, STAT5 and Akt phosphorylation. (c) G-CSF-stimulated STAT3, EKR, STAT5 and Akt phosphorylation of mouse bone marrow cells from wild type and α9-deficient mice. (d) and (e) G-CSF stimulated ERK phosphorylation in BaF3 cells expressing G-CSF receptor mutants(Y729F and Δ715). (f) Proliferation assay of BaF3 cells expressing wild type or mutant G-CSF receptor. BaF3 cells were cultured in G-CSF (100ng/ml) containing medium. Viable cell number was determined by counting and trypan blue exclusion. (g) Flow cytometry of G-CSF receptor expression (shaded peaks) in BaF3 cells expressing wild type, Y729F or Δ715 G-CSF receptor. Error bars denote the mean ± SD.

Figure 6. G-CSF-induced G-CSF receptor phosphorylation and association with integrin α9β1

(a) CHO cells expressing G-CSF receptor and wild type α9 or an α9α5 chimera were treated with α9 blocking antibody (Y9A2) or control IgG for 30 min, plated on Tnfn3RAA and treated with G-CSF (20ng/ml). Lysates were immunoprecipitated with α9β1 antibody A9A1, and precipitated protein was detected with anti-G-CSF receptor or integrin β1 cytoplasmic domain antiserum (to monitor equal capture of α9β1). (b) CHO cells expressing G-CSF receptor and full-length integrin α9 or α9 cytoplasmic domain deletion mutants were incubated on Tnfn3RAA in the presence or absence of G-CSF and lysates were analyzed as above. (c) BaF3 cells expressing wild type, Y729F mutant or Δ715 mutant G-CSF receptor and full-length integrin α9 were incubated on Tnfn3RAA in the presence or absence of G-CSF. Lysates immunoprecipitated with α9β1 antibody

A9A1 and probed with anti-HA or anti-integrin α9 cytoplasmic domain (to monitor equal capture of α9β1). (d) CHO cells expressing G-CSF receptor and wild type integrin α9 or an α9α5 chimera were treated by anti-α9β1 Y9A2 or control (IgG) and G-CSF as described above. Cells were lysed and immunoprecipitated by G-CSF receptor antibody. Precipitated protein was probed with anti-phosphotyrosine or anti-G-CSF receptor (to monitor equal capture of G-CSF receptor).