Potentiation of hematopoietic cell migration with an IGF–interleukin-3 fusion protein

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Abstract A chimera of an N-terminally modified insulin growth factor (IGF)-II, NQPQMVHTY-hIGF-II(9–67) (BOMIGF), fused to interleukin-3 (IL-3) significantly improved the migration of CD34$^+$ human hematopoietic cells with respect to the effects observed during co-stimulation with BOMIGF and IL-3. A phosphatidylinositol-3 (PI-3) kinase inhibitor specifically inhibited migration in the presence of the chimera, while no significant difference in the inhibition of migration was observed in the presence of a Rho kinase inhibitor. These results suggest a key role of the PI-3 kinase pathway in the potentiation of migration caused by the linkage of BOMIGF and IL-3.

Key words: Mitogen-activated protein kinase; Phosphatidylinositol-3 kinase; Protein kinase C; Rho kinase; Fibronectin

1. Introduction

Our laboratory has been studying the synergistic hematopoietic effects associated with the delivery of factors as fusion proteins of insulin growth factors (IGFs) and cytokines. We are able to produce, using the baculovirus expression system, a biologically active and secreted form of a slightly modified IGF-II, NQPQMVHTY-hIGF-II(9–67) (BOMIGF), which contains the leader sequence and the first nine amino acids of bombyxin (an insect insulin like peptide) [1]. By joining the cDNA of a cytokine of choice to the sequence of BOMIGF within a baculovirus expression vector, we were able to generate high yields of chimeric proteins [2]. We have produced, by this system, a fusion protein of BOMIGF and interleukin-3 (IL-3) (BOMIGF–IL-3), which potentiated hematopoietic cell proliferation [3] and cell survival (DiFalco, Ali and Congote, submitted) as compared with the effects observed when the single components of the chimera were added simultaneously. Treatment with BOMIGF–IL-3 increases the number of hematopoietic precursor cells found in bone marrow and spleen tissues of myelosuppressed C57BL/6 mice [4]. In normal mice, the increase of hematopoietic stem cell precursors caused by BOMIGF–IL-3 was observed especially in the spleen [3]. This result may be attributed to a preferential ability by the chimera to mobilize stem cells from bone marrow and lead to increased accumulation of stem cells in the spleen. These observations prompted us to analyze the effect of the chimera on migration of the CD34$^+$ hematopoietic cell line TF-1 in vitro.

CD34$^+$ stem cells are present in small amounts in circulation during steady-state hematopoiesis as part of a normally occurring trafficking of hematopoietic stem cells between the bone marrow and other hematopoietic organs such as the spleen and liver. This process is particularly crucial in facilitating bone marrow recovery following hematopoietic stress such as cytotoxic chemotherapy. Although the mechanism for the mobilization of stem cells into peripheral blood is still poorly understood, it is thought to require increased proliferation of early progenitor cells followed by their migration and egress from the bone marrow cavities. Several hematopoietic growth factors, and in particular IL-3, as well as complex adhesive interactions between stem cell surface binding molecules and their ligands found in the extracellular matrix and/or on bone marrow stromal accessory cells play a key role in the mobilization process [5–7]. Although the role of IGFs on hematopoietic cell mobility has not been well characterized, there are many reports on the action of IGFs on migration of a variety of cells such as trophoblasts [8], smooth muscle cells [9,10] and heart fibroblasts [11]. In the case of IGFs, the chemotactic activities seem to be mediated by different signal transduction pathways, including phosphatidylinositol-3 (PI-3) kinase [12], protein kinase C (PKC) [13], Rho kinase [14] and phosphorylation of the mitogen-activated protein kinase (MAPK) [8,11]. MAPK activation seems to be dependent on upstream increased tyrosine phosphorylation of focal adhesion kinase (FAK) mediated by the IGF-I receptor. The requirement of FAK phosphorylation is still unsettled, probably due to variations in responses observed within the same cells having a different cytoskeletal architecture [15–17]. The same signal transduction pathways are involved in the mechanism of action of IL-3, albeit in studies based mainly on the effects of the cytokine on proliferation and survival [18–23].

The effects of IGFs and IL-3 on migration are the result of a complex cross-talk between the growth factors and integrins. $\alpha_4\beta_1$ integrin is important for the action of IL-3 in CD34$^+$ progenitor cells [5], whereas $\alpha_5\beta_1$, $\alpha_V\beta_5$, $\alpha_2\beta_1$ and $\alpha_V\beta_3$ are involved in the action of IGFs, depending on the interacting component of the extracellular matrix, such as fibronectin, vitronectin or collagen [24–26]. The action of IGFs on migration is also affected by IGF binding proteins [24]. In this communication we show how the linkage of BOMIGF with

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Abbreviations: BOMIGF, NQPQMVHTY-hIGF-II(9–67); IL-3, interleukin-3; MAPK, mitogen-activated protein kinase; PI-3, phosphatidylinositol-3; PKC, protein kinase C

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IL-3 results in an improved chemotactic activity for hematopoietic cells.

2. Materials and methods

2.1. Materials

Recombinant BOMIGF and the recombinant chimera of BOMIGF with human IL-3 were produced as previously described [3]. Human IL-3 was from R&D Systems. Fibronectin was from BD Biosciences. All kinase inhibitors were from Calbiochem, with the exception of Y-27632, obtained from Tocris. Exoenzyme C3 was from Upstate Biotechnology. The peptides RGDS and RGES were from Peninsula Laboratories. Anti-β1 integrin antibody (anti-CD29) was from Beckman–Coulter.

2.2. Cell cultures

The factor-dependent human hematopoietic cell line TF-1 (American Tissue Type Culture Collection) was maintained in culture as previously described [3]. Recently, we have replaced GM-CSF with 0.5 nmol/ml BOMIGF–IL-3 as a cytokine growth supplement. The serum-free medium used for migration experiments was RPMI 1640 supplemented with 300 μg/ml fatty acid-free, tissue culture-tested bovine serum albumin (BSA; Sigma) and 30 μg/ml bovine transferrin (ICN).

2.3. Cell migration assay

Migration experiments were performed using Transwell (Costar) chambers equipped with filter membranes with pore sizes of 5 or 8 μm in diameter [27], as indicated in the individual experiments. TF-1 cells were incubated in starvation medium (RPMI 1640 medium with 0.5% fetal calf serum and 300 μg/ml BSA) for 18 h. Subsequently, cells were washed, resuspended in the serum-free medium indicated above and 1.5×10^5 cells were dispensed into the upper chamber of Costar Transwells. Growth factors were added to the lower chamber at a final concentration of 0.1–25 mM. After a 16 h incubation at 37°C, cells in the bottom chamber were collected, mixed with trypan blue (vital stain) and counted with a hemocytometer under a light microscope. In experiments where the effects of fibronectin were studied, the Transwells were preincubated with 10 μg/ml fibronectin in RPMI 1640 medium alone for 3 h at 37°C prior to addition of cells. In experiments in the presence of inhibitors, the cells were incubated first for 20 min with the inhibitors. The cytokines were added subsequently and the incubation continued for 18 h. RGDS and RGES were added together with the cytokines. For the experiments with exoenzyme C3, the cells were electropermeabilized with the exoenzyme as described by Laudanna et al. [28], but without addition of NAD.

3. Results and discussion

Fig. 1A shows the importance of fibronectin for the migration of TF-1 cells in Costar Transwells, where the addition of this matrix protein considerably increases the number of cells going through to the lower cell chamber. Cell migration was measured by adding 25 nM BOMIGF, IL-3, the chimera and the corresponding equimolar mixture of BOMIGF and IL-3 to the lower chamber of the Transwells. When the results obtained after incubation of the cells with the cytokines are expressed as percent stimulation over control cell cultures, it was evident that the cytokines stimulated migration even in the absence of fibronectin (Fig. 1B). In fact, the number of cells migrating in the presence of the chimera was significantly higher than that observed with the co-addition of BOMIGF and IL-3. This is better appreciated in the dose–response curve shown in Fig. 2A. There was a significantly higher stimulation of cell migration in the presence of the chimera at concentrations of 10 and 25 nM as compared with BOMIGF and IL-3 added simultaneously ($P<0.05$ and 0.04, respectively, paired t-test). This difference was not significant in the presence of fibronectin (Fig. 2B). In fact, the stimulation of migration caused by the cytokines was lower than that observed in the absence of the cell matrix protein. These results indicate that, in the absence of fibronectin, the linkage of IGF with IL-3 improves the chemotactic activity of both compounds for TF-1 cells. This potentiation has also been observed for the stimulation of cell proliferation [3] and cell survival (DiFalco, Ali and Congote, submitted). Preliminary experiments using a murine hematopoietic cell line (32DI, grown in the presence of IGF-I and IL-3) confirmed the specific stimulation of the chimera on cell migration. However, in 32DI cells the chimera was more active than the equimolar mixture of BOMIGF and murine IL-3 both in the presence or absence of fibronectin (DiFalco and Congote, unpublished results).

Different signal pathways are known to be involved in the mechanism of action of IGFs and IL-3. In order to have a better idea of the relative importance of some of these pathways on the action of the chimera on migration, we first

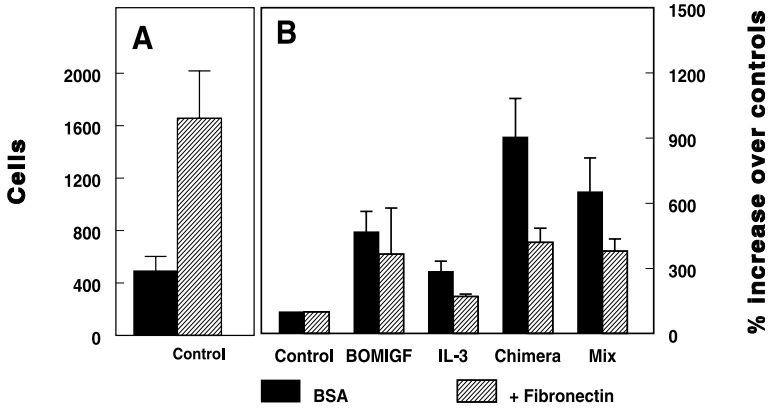

Fig. 1. Role of fibronectin and cytokines on TF-1 cell migration. A: Effect of fibronectin on migration. Mean ± S.E.M. $n=10$ and 11 for migration in control wells and wells previously coated with fibronectin, respectively. The difference between both groups was significant ($P<0.02$, Welch's test). B: Effect on migration of 25 nM BOMIGF, IL-3, BOMIGF–IL-3 (chimera) and an equimolar mixture of 25 nM each of BOMIGF and IL-3. The results have been expressed as percent stimulation over the number of cells migrating in the presence (+) or absence (BSA in the medium) of fibronectin without cytokines (control = 100). The chimera significantly stimulated migration in the absence of fibronectin as compared with the equimolar mixture using the paired, non-parametric Wilcoxon test ($P<0.04$, $n=8$). These experiments were done with Transwells of 5 μm diameter.

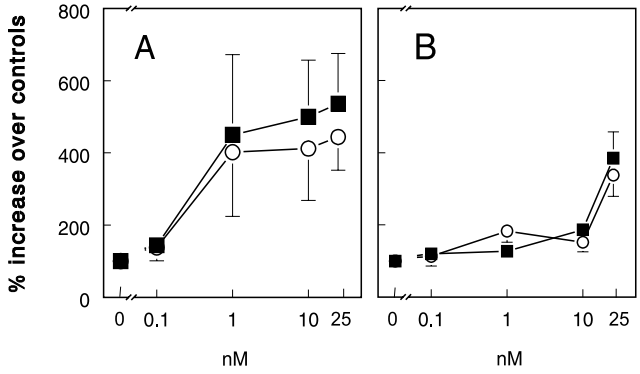

Fig. 2. Dose–response curves for cell migration. Cell migration was measured as indicated in Section 2 with increasing concentrations of the chimera (squares) or the equivalent equimolar mixture of BOMIGF and IL-3 (open circles) in the absence (A) or presence (B) of fibronectin. Cells migrating through 8 μm Transwells were counted and the results are expressed as percent increase over control cell cultures ± S.E.M. Although there was a wide variation of migration from one experiment to the other, the paired *t*-test indicated that the stimulation of cell migration at the concentrations of 10 and 25 nM in the absence of fibronectin was significantly higher than that observed in the presence of BOMIGF and IL-3 added simultaneously ($P < 0.05$ ($n = 8$) and $P < 0.04$ ($n = 9$), respectively). No significant differences were observed in the presence of fibronectin.

analyzed the effects of different inhibitors, including those targeting integrin interactions, on the mobility of TF-1 cells through fibronectin-coated wells. In these initial experiments we used inhibitor concentrations known to be effective in other hematopoietic cells. The PI-3 kinase inhibitor LY-294002 decreased migration by about 45% (Fig. 3A(I)). Although this kinase plays an important role in the actions of both IGFs and IL-3, it seems that it is not the unique mediator of cell migration. The PKC inhibitor stauroporine almost completely eliminates cell migration, suggesting a critical role of the enzyme on TF-1 migration. The MAPK kinase inhibitor PD98059, on the contrary, did not inhibit migration at concentrations known to be effective in other cell lines. As indicated in Section 1, FAK phosphorylation is important for MAPK activation. Although the chimera is capable of stimulating FAK phosphorylation, after extensive studies under a variety of conditions, we could not find a tangible significant difference on FAK phosphorylation displayed by the action of the chimera or that of the mixture of individual factors (DiFalco and Congote, unpublished results). Fig. 3A(II) shows

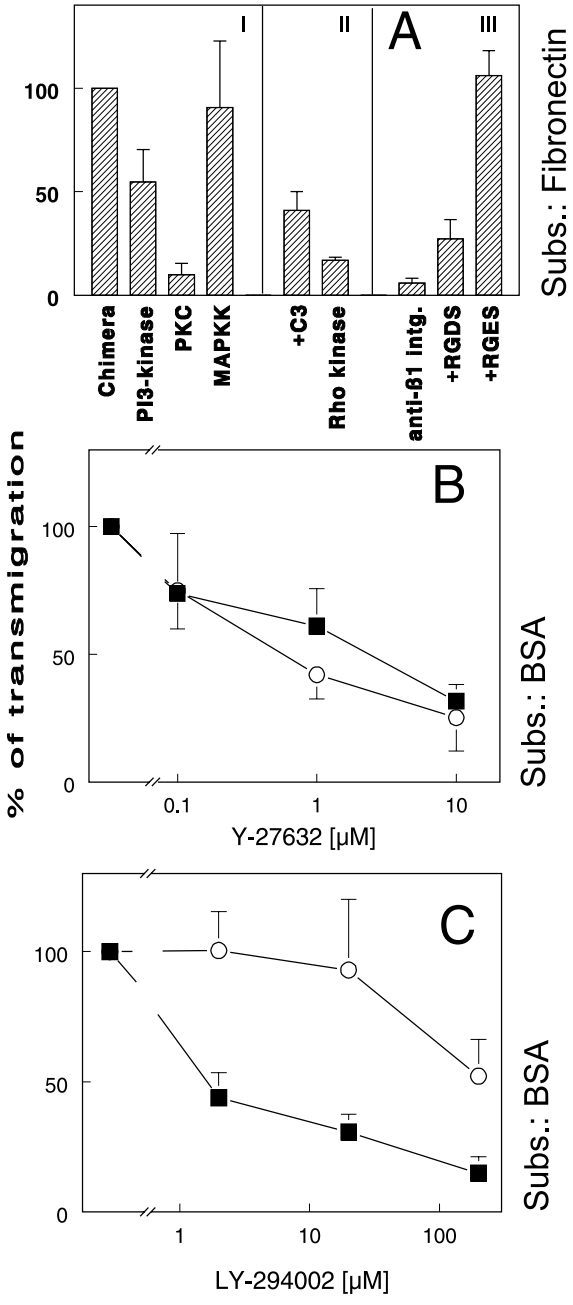

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Fig. 3. Effects of inhibitors of different signal transduction pathways on TF-1 cell migration. A: TF-1 migration assays were performed with Transwells (5 μm) preincubated with fibronectin in the presence of BOMIGF–IL-3. The results are expressed as percent of the values obtained with the chimera without inhibitors and the *P* values were calculated by analysis of variance and Student–Keuls multiple comparisons test. A comparison of the effects obtained with 20 μM LY-294002 (PI-3 kinase inhibitor, $P < 0.01$), 0.5 μM stauroporine (PKC inhibitor, $P < 0.01$) and 20 μM PD-98059 (MAPK kinase inhibitor, non-significant). (II) Effects of inhibitors of the Rho pathway. C3, effect of electropermeabilized exoenzyme C3 (ADP-ribosylation of Rho, $P < 0.01$); Rho kinase, cells incubated with 10 μM Y-27632 ($P < 0.001$). (III) Inhibitors related to integrin function. β1-integr., cells incubated with 50 μg/ml anti-CD29, an integrin β_1 subunit inhibitory antibody ($P < 0.001$). RGDS, cells incubated with 100 μM fibronectin competitor peptide RGDS ($P < 0.01$). RGES, cells incubated with 100 μM of the control peptide RGES (non-significant). B: Effect of increasing concentrations of Y-27632 on TF-1 cell migration in the presence of 10 mM chimera (squares) or 10 mM each of BOMIGF and IL-3 (open circles). The results are expressed as percent of the cell migration as compared with the migration obtained with 10 nM concentrations without inhibitor (100%). Mean ± S.E.M. ($n = 4$). C: Effect of increasing concentrations of LY-294002 on TF-1 cell migration in the presence of 10 mM chimera (squares) or 10 mM each of BOMIGF and IL-3 (open circles). The results are expressed as percent of cell migration as compared with the migration obtained with 10 nM concentrations without inhibitor (100%). Mean ± S.E.M. ($n = 4$). The higher sensitivity of TF-1 cells towards the PI-3 kinase inhibitor in the presence of the chimera was significant at the concentrations of 2 and 20 μM ($P < 0.05$ and $P < 0.01$, respectively, Student–Newman–Keuls multiple comparisons test). Subs., coating substrate. Experiments B and C were done with 8 μm Transwells in the absence of fibronectin but coated with the albumin (BSA) of the incubation medium.

the possible involvement of the Rho kinase pathway on the migration of TF-1 cells in the presence of the chimera. The exoenzyme C3, originally purified from *Clostridium botulinum*, is known to inactivate Rho through ADP-ribosylation [28]. C3, introduced into TF-1 cells by electroporation, was able to considerably reduce migration. The involvement of Rho was further confirmed by the inhibition of migration in the presence of Y-27632, an inhibitor of the Rho-associated protein kinase p160ROCK [29,30]. Fig. 3A(III) shows the possible involvement of integrins containing the β1 chain on migration. The antibody against this chain eliminated migration and the peptide RGDS, containing the integrin binding tripeptide RGD, substantially reduced migration. This inhibition was specific for the RGD sequence, because the control peptide RGES was unable to eliminate the chemotactic activity of the chimera.

Since the effects of the chimera are far more evident in the absence of fibronectin in TF-1 cells, we did not study any further the integrin-related inhibitors indicated in Fig. 3A(III). Instead, we decided to analyze in more detail the effect of two of the four inhibitors of intracellular signal transduction pathways of Fig. 3A(I,II), this time in the absence of fibronectin (Fig. 3B,C): Y-27632, because the Rho-associated protein kinase is known to play a key role in migration, and LY-294002, the inhibitor of PI-3 kinase. Although the effect of 20 μM LY-294002 was not particularly striking (Fig. 3A(I)), we have found that PI-3 kinase activity is increased in the presence of the chimera (DiFalco, Ali and Congote, submitted). Therefore, this enzyme is an attractive candidate as a mediator of the preferential action of the chimera in TF-1 cells. Cells were incubated in the presence of increasing concentrations of Y-27632 and after 20 min solutions of 10 nM chimera or the equivalent mixture of 10 nM BOMIGF and 10 nM IL-3 was added to the lower Transwell chamber and incubated for 18 h. The migrating cells were collected and counted. Fig. 3B shows the decrease of cell numbers with increasing concentrations of the inhibitor. This decrease was practically identical in the presence of the chimera or the equimolar mixture of BOMIGF and IL-3. Therefore, although the Rho kinase plays an important role in TF-1 cell migration, it is unlikely that the observed preferential increase of migration in the presence of the chimera could be attributed to a specific stimulation of the Rho kinase pathway. The same experiment was carried out using the PI-3 kinase inhibitor LY-294002 (Fig. 3C). Since the inhibition of migration with the inhibitor was not complete at a concentration of 20 μM (Fig. 3A), we extended the range of concentrations up to 200 μM. Fig. 3C shows that the PI-3 kinase inhibitor preferentially inhibits the chimera-mediated migration of TF-1 cells. This increased sensitivity towards LY-294002 was significant at concentrations of 2 and 20 μM ($P < 0.05$ and $P < 0.01$, respectively).

It can be concluded from these experiments that the linkage of IGF-II with IL-3 results in a synergistic effect on cell migration in TF-1 cells. Although the mechanisms involved in this preferential increase remain to be elucidated, it is clear that the PI-3 kinase is a key player in the action of the chimera. This can be deduced from the significant and specific elimination of cell migration in the presence of a PI-3 kinase inhibitor. These results are supported by our observation that the chimera increases the PI-3 kinase activity of TF-1 cells (DiFalco, Ali and Congote, submitted). They provide a likely explanation for the increased number of spleen colonies observed after injection of the chimera into mice [3] and suggest a possible use for IGF–IL-3 chimeras as a mobilizing agent in transplantation of bone marrow hematopoietic cells. An additional advantage associated with the administration of IGF–cytokine chimeras in vivo may be related with the presence in serum of numerous IGF binding proteins. The latter could potentially increase the half life of the IGF-linked cytokines by extending the protective effects they normally have for circulating IGFs. This possibility remains to be explored.

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