Expression of granulocyte colony-stimulating factor is induced in injured rat carotid arteries and mediates vascular smooth muscle cell migration

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Chen, Xing, Sheri E. Kelemen, and Michael V. Autieri. Expression of granulocyte colony-stimulating factor is induced in injured rat carotid arteries and mediates vascular smooth muscle cell migration. Am J Physiol Cell Physiol 288: C81–C88, 2005. First published September 22, 2004; doi:10.1152/ajpcell.00322.2004.—Granulocyte colony-stimulating factor (G-CSF) is a lineage-restricted hematopoietic growth factor that stimulates proliferation and maturation of hematopoietic progenitors and is a known powerful mobilizer of bone marrow-derived stem cells. Very little has been reported on G-CSF expression and modulation of vascular smooth muscle cell (VSMC) activation. The purpose of this study was to characterize the expression and effects of G-CSF on primary human VSMC and balloon angioplasty-injured rat carotid arteries. In cultured human VSMC, G-CSF mRNA and protein expression are induced by several cytokines, with the most potent being fetal calf serum and T-lymphocyte-conditioned media. G-CSF is not expressed in naive rat carotid arteries but is induced in neointimal SMC in carotid arteries subject to balloon angioplasty. G-CSF is chemotactic for human VSMC. There is a significant difference between unstimulated cells and those treated with G-CSF at 100 and 1,000 pg/ml (P < 0.01 and 0.05 for 3 experiments). G-CSF also activates the GTPase Rac1, a regulator of cellular migration in VSMC. Inhibition of Rac1 inhibits G-CSF-driven VSMC migration. Important signal transduction protein kinases, including p44/42 MAPK, Akt, and S6 kinase, are also activated in response to G-CSF. This is the first report describing the expression of G-CSF in injured arteries and the multiple effects of G-CSF on VSMC activation. Together, our data suggest that G-CSF is an important mediator of inflammatory cell-VSMC communication and VSMC autocrine activation and may be an important mediator of the VSMC response to injury.

THE INITIATION AND PROGRESSION of many vascular pathologies, including atherosclerosis, restenosis, and allograft injury, are caused by a localized inflammatory reaction mediated by infiltrating inflammatory cells. These immune cells secrete cytokines that both activate and injure normally quiescent vascular endothelial cells and vascular smooth muscle cells (VSMC). Activated vascular cells in turn secrete growth and chemotactic factors that further recruit inflammatory cells, a cycle that is responsible for the progressive nature of these diseases (28, 37, 39). As part of the VSMC response to injury (25), VSMC migrate from the lumen into the intima of the vessel, where they proliferate and synthesize cytokines, contributing to a loss of lumen diameter and vascular contractility. VSMC migration and hypertrophy are responsible for most of the obliterative arterial intimal thickening present in mechanically induced restenosis as well as in cardiac allograft vasculopathy (12, 30). VSMC are plastic in that, in response to cytokine stimulation, they transform from a differentiated, contractile state to a dedifferentiated, synthetic cell. Regulation of VSMC dedifferentiation remains to be completely understood. It has been suggested that the cytokine-induced activation of VSMC is one of the most critical cellular events in neointimal development. Accordingly, VSMC migration represents an important point of therapeutic intervention to attenuate many vascular diseases.

Immune products, including granulocyte-monocyte colony-stimulating factor (GM-CSF), IFNγ, IL-1, IL-6, and TNFβ, influence the VSMC phenotype and mediate the vascular response to injury (27, 38). Although it has been shown that cytokine-activated but not quiescent VSMC secrete both GM-CSF and M-CSF (8), little has been reported on the expression of granulocyte colony-stimulating factor (G-CSF) cytokine in injured arteries or activated VSMC or on the effect of G-CSF on VSMC pathophysiology. G-CSF is considered to be a lineage-restricted hematopoietic growth factor which stimulates terminal mitotic divisions and the final cellular maturation of hematopoietic progenitors, particularly granulocytes (18). G-CSF has multiple effects on its target cells, including promotion of cell survival, proliferation, chemotaxis, and maturation (20). All of these pathophysiological responses are involved in VSMC activation and development of intimal hyperplasia. Indeed, recently it was observed that infusion of G-CSF into patients undergoing coronary interventional procedures unexpectedly increased the rate of in-stent restenosis (13).

Using cDNA microarray analysis to investigate the VSMC transcriptional response to inflammatory stimuli, we observed an unexpected expression of G-CSF mRNA. Very little has been reported regarding the expression and effects of G-CSF in VSMC. In this report, we describe how we verified the expression of G-CSF by examining its expression in cytokine-stimulated VSMC and balloon angioplasty-injured rat carotid arteries. We also show that G-CSF is chemotactic to human VSMC and activates the GTPase Rac1, which is responsible for regulation of migration. Inhibition of Rac1 by dominant-negative or pharmacological inhibition abrogates G-CSF-initiated migration. G-CSF also initiates signal transduction pathways in VSMC by activating several cellular protein kinases, including Akt, p44/42 MAPK, and pS6. Together, this suggests an important, previously unrecognized role for this cytokine in vascular immune cell communication.
MATERIALS AND METHODS

Cells and culture. Primary human coronary VSMC were obtained as cryopreserved secondary culture from Cascade (Portland, OR) and subcultured as described (3). Cells from passages 3–6 were used in the described studies. G-CSF was purchased from Sigma (St. Louis, MO). For microarray analysis, 1 × 10⁶ VSMC were rinsed with PBS and incubated in 0.5% fetal calf serum (FCS) for 48 h. One group of VSMC treated this way was subjected to microarray analysis, while a second group was stimulated with 50% T-cell-conditioned medium (Fisher Biotechnology, St. Louis, MO) for 48 h and then subjected to microarray analysis.

Rat left common carotid artery balloon angioplasty. Left common carotid artery balloon angioplasty was performed in 350-g male Sprague-Dawley rats (Charles River Breeding Laboratory, Wilmington, MA) that were under pentobarbital sodium anesthesia (65 mg/kg delivered by intraperitoneal injection; Steris Laboratories, Phoenix, AZ) as described previously (1). Briefly, the left external carotid artery was cleared of adherent tissue, allowing the insertion of a 2-French Fogarty arterial embolectomy catheter (model 12 060 2F; Baxter Healthcare, Santa Ana, CA). The catheter was guided a fixed distance down the common carotid artery to the aortic arch, inflated with a fixed volume of fluid, and withdrawn back to the site of insertion a total of three times. Once this step was completed, the catheter was removed and the wound was closed (9-mm Autoclips; Clay Adams, Parsippany, NJ) and swabbed with Povidine surgical scrub (7.5% povidone-iodine; Chastion, Danville, CT). Animals were housed in Plexiglas cages, maintained under a 12:12-h light-dark cycle, and allowed access to standard laboratory chow and drinking water ad libitum until tissue collection. All surgical procedures were performed in accordance with the guidelines of the Animal Care and Use Committee of Temple University and the American Association for Laboratory Animal Care.

Tissue processing and immunohistochemistry. Primary antibodies to G-CSF (Santa Cruz Biotechnology, Santa Cruz, CA) and proliferating cell nuclear antigen (PCNA; Transduction Labs, Lexington, KY) were used at 2 μg/ml in 1% BSA-PBS and applied for 1 h, followed by biotinylated secondary antibody (1:200 dilution) and then avidin-biotin-peroxidase complex, each for 30 min. Nonspecific isotype antibodies were used as negative controls. Staining was visualized with the substrate diaminobenzidine (Vector Laboratories, Burlingame, CA), producing a reddish brown color, and then counterstaining with hematoxylin was performed as described previously (2).

RNA isolation and cDNA microarray analysis. The complete detailed protocol for microarray analysis can be found on the Gene Logic web site (http://www.genelogic.com/docs/pdfs/backgrounder_DataGeneration.pdf). Briefly, total cellular RNA was extracted using TriReagent (Molecular Research Center, Cincinnati, OH), which allowed simultaneous extraction of RNA and protein as described previously (2). cDNA was synthesized using T7-oligo(dT)24 and SuperScript II reverse transcriptase and labeled using biotinylated CTP in an in vitro transcription reaction. cRNA (~10 μg) was fragmented and analyzed using the Affymetrix U-133 chip (Affymetrix, Santa Clara, CA) and hybridized and washed according to the manufacturer’s protocol. GeneChips were scanned and data were normalized to several housekeeping genes as well as spike-in cDNA to construct a scatter plot using the Affymetrix MAS 5.0 algorithm. Electronic Northern blotting based on relative change values (E Northern) were computed from the percentile and median values of expression from the test sample over the control sample. Relative change and E Northern blot analysis were performed using the GeneExpress algorithm.

Western blotting. Human VSMC extracts were prepared as described elsewhere (2). Membranes were incubated with a 1:2,000 dilution of primary antibody and a 1:2,000 dilution of secondary antibody. G-CSF antibody was obtained from Santa Cruz Biotechnology. Equal protein concentrations of cell extracts were determined using a Bradford assay, and equal loading on gels was verified by performing Ponceau red S staining of the membrane. Reactive proteins were visualized using enhanced chemiluminescence. Multiplex phospho-p90 ribosomal S6 kinase (p90RSK), Akt, p44/42, and S6 were purchased from Cell Signaling (Beverly, MA) and used according to the manufacturer’s instructions. Statistics based on densitometric analysis of at least three independent experiments were obtained using a paired sample t-test.

Rac1 activation. Rac1 activation was determined using the p21-activated kinase 1 protein (PAK) pull-down assay as described previously (3). Human VSMC were serum starved in 0.5% FCS overnight, challenged with 200 pg/ml G-CSF for various time periods, and lysed in sample buffer (25 mM HEPES, 150 mM NaCl, 5 mM MgCl₂, 0.5 mM EGTA, 20 mM β-glycerophosphate, 0.5% Triton X-100, 50 mM glycerol, 10 mM NaF, and 2 mM Na-vanadate, plus protease inhibitors). The volume of lysate was adjusted to normalize for equal concentrations of proteins. Cells suspensions were incubated with glutathione S-transferase (GST)-PAK Sepharose (Cytoskeleton, Denver, CO) for 1 h at 4°C. Only activated Rac1 binds the PAK protein (9). Beads were washed three times, bound proteins were detected using Western blotting with Rac1 antibody, and bands were quantitated by performing densitometry.

Migration and chemotaxis. Transwell Boyden chamber plates (6.5-mm diameter; Costar, Cambridge, MA) with 8-μm polycarbonate membrane pore size were seeded with 40,000 VSMC/membrane in medium containing 0.5% FCS as previously described (3). G-CSF at 1,000, 100, or 10 pg/ml were placed in the lower chamber, and cells were incubated for varying times as indicated at 37°C, and then cells were fixed and stained in Dif-Quick cell stain (American Hospital Supply, Deerfield, IL). The upper layer was scraped free of cells. VSMC that had migrated to the lower surface of the membrane were quantitated by counting four high-powered fields in each membrane.

RESULTS

G-CSF is expressed in stimulated human VSMC. To determine the VSMC transcriptional response to inflammatory stimuli, primary human coronary arterial VSMC were serum starved in 0.5% FCS for 72 h and then challenged with T-lymphocyte-conditioned medium. After 30 h, RNA was extracted for cDNA microarray analysis. Analysis of the relative change of unstimulated vs. stimulated VSMC indicated that inflammation-induced transcripts could be clustered into several categories on the basis of function, including cytokine, cell cycle, cytoskeletal, and remodeling proteins. One transcript that was increased >4-fold in stimulated cells was G-CSF, which was unexpected because G-CSF is considered to be a lineage-restricted hematopoietic growth factor (Fig. 1A). Very little has been reported to date regarding G-CSF expression and function in VSMC, so we chose this cytokine for further study.

To verify G-CSF expression in VSMC, cultured human VSMC were serum starved into quiescence in 0.5% FCS for 72 h and then stimulated with a serum or a variety of cytokines for 40 h. The results presented in Fig. 1B demonstrate a basal
level of G-CSF protein expression in unstimulated VSMC but an increase in expression in response to serum and every cytokine tested, including 15% FCS, T-cell-conditioned medium, IFN-γ, PDGF, TGF-β, TNF-α, and G-CSF. The strongest stimuli are FCS, which induces an average 920% increase in G-CSF expression, and T-cell-conditioned medium, which increases G-CSF 690% above unstimulated levels, as quantitated using scanning densitometry (data not shown). IFN-γ, PDGF, TGF-β, TNF-α, and G-CSF were also increased G-CSF expression between 200 and 80%, respectively. Taken together, these results suggest that G-CSF expression can be induced in human coronary arteries by serum and cytokine stimulation.

G-CSF is expressed in injured, not naive, rat carotid artery. Cytokine-activated VSMC are the most abundant neointimal cell type in the injured artery, and it was important to determine whether G-CSF was expressed by VSMC in response to a pathophysiological process. Rat carotid arteries were injured by performing balloon angioplasty, and after 3 and 15 days, serial sections were examined for G-CSF and PCNA expression using immunohistochemistry. Figure 2 shows that G-CSF protein was not detectable in uninjured arteries but was detectable in medial VSMC 3 days postinjury and in neointimal cells 15 days postinjury. At both time points, antibody recognition was particularly concentrated in an area closest to the lumen of the vessel. This finding is in contrast to that for PCNA, which was predominately expressed in adventitial cells 3 days postinjury and was undetectable 15 days postinjury.

G-CSF is chemotactic for human VSMC. G-CSF is chemotactic for hematopoietic cells (35), and we hypothesized that it would have the same effect on VSMC. Human VSMC in 0.5% FCS were seeded into the upper chamber of a Boyden chamber. After attachment, G-CSF in concentrations ranging from 10 to 1,000 pg/ml was added to serum-reduced medium in the lower chamber, and differences in chemotaxis between G-CSF and unstimulated VSMC were quantitated by counting migrated cells after 3 h. Figure 3 shows a significant difference between unstimulated cells and those treated with G-CSF at 100 and 1,000 pg/ml (P < 0.01 and P < 0.05, respectively, for 3 experiments). G-CSF at 10 pg/ml was not significantly different in all experiments. There was no difference between 100 and 1,000 pg/ml G-CSF with regard to the migration of VSMC. The kinetics of migration also was investigated. VSMC were seeded as described, and 100 pg/ml G-CSF were added to the bottom chamber for 2, 4, and 6 h. Figure 4 shows a significant difference between 4- and 6-h migration vs. 2 h and no G-CSF (P < 0.05 for 3 experiments). There were no differences between 4 and 6 h and between 2 h and no G-CSF added. Taken together, these data indicate that G-CSF had a chemotactic effect on human VSMC.

G-CSF activates Rac1. Cellular movement in response to extracellular signals is regulated by the coordinated activation of small GTPase proteins, including Rac1 (9). To test the hypothesis that the chemotactic effects of G-CSF are mediated by Rac1, the effects of G-CSF on Rac1 activity were examined using the GST-PAK Sepharose pull-down assay. Figure 5 shows that the addition of G-CSF to human VSMC activated Rac1. This demonstrates activation of Rac1 GTPase activity, not increased expression, because absolute levels of Rac1 as measured using Western blotting of cellular extracts were similar. No change in Cdc42, a similar GTPase protein also involved in motility activation, was observed (data not shown). This suggests that G-CSF activation of Rac1 was likely responsible for G-CSF-induced VSMC migration.

Rac1 activity is essential for G-CSF-induced VSMC migration. It was important to determine whether Rac1 activation was requisite for G-CSF-induced VSMC migration. Human VSMC were infected with 30 MOI of AdRac1N17 for 48 h before being subjected to the migration assay or the addition of the Rac1 pathway inhibitor Sch-51344 (10 μM) directly to the lower chamber. G-CSF at 100 pg/ml was added to the bottom chamber, and VSMC were allowed to migrate for 4 h. Figure 6 demonstrates that elimination of Rac1 activity resulted in significant reduction of G-CSF-induced VSMC migration for Rac1N17 and Sch-51344 (P < 0.001 and P < 0.01, respectively). It is important to note that inhibition of Rac1 activity was necessary for basal levels of migration because Rac1 inhibited VSMC migrated more slowly than normal VSMC, even in the absence of G-CSF (P < 0.05). Consequently, whereas these data indicate that Rac1 activity is necessary for G-CSF-driven VSMC migration, it also suggests that the Rac1 pathway is not exclusive for G-CSF.

G-CSF activates multiple intracellular signaling proteins. In granulocytes, engagement of the G-CSF receptor is linked to activation of multiple intracellular signaling pathways (26). The effect of G-CSF on signaling proteins in VSMC was investigated by Western blot analysis using a cocktail of
Fig. 2. G-CSF expression in balloon angioplasty-injured rat carotid arteries. Immunohistochemical analysis of G-CSF and proliferating cell nuclear antigen (PCNA) expression in an uninjured artery (A–C) and a carotid artery that was injured by balloon angioplasty 3 days earlier (D–F) or 15 days earlier (G–I). E: arrows indicate PCNA-positive VSMC. Reddish brown staining indicates antibody recognition, and sections were counterstained with hematoxylin. Magnification, ×400.

Fig. 3. G-CSF-stimulated VSMC chemotaxis is dose dependent. Human VSMC in 0.5% FCS were seeded into the upper chamber of a Boyden chamber. After attachment, G-CSF in concentrations ranging from 10 to 1,000 pg/ml was added to serum-reduced (0.5% FCS) medium in the lower chamber, and differences in chemotaxis between G-CSF and unstimulated VSMC were quantitated by counting migrated cells after 3 h. Values are means from 3 experiments performed in triplicate from 3 independent groups of VSMC. Errors are reported as SD. *P < 0.05. **P < 0.01.

Fig. 4. G-CSF-stimulated VSMC chemotaxis is time dependent. Human VSMC in 0.5% FCS were seeded into the upper chamber of a Boyden chamber. After attachment, G-CSF at 100 pg/ml was added to serum-reduced (0.5% FCS) medium in the lower chamber, and differences in chemotaxis between groups were quantitated by counting migrated cells after 2, 4, and 6 h. Values are means from 3 experiments performed in triplicate from 3 independent groups of VSMC. Errors are reported as SD. *P < 0.05.
activation-specific phosphokinase antibodies. In these experiments, primary human VSMC were serum starved in 0.5% FCS for 48 h and then stimulated with 200 pg/ml G-CSF for the times indicated. Equal amounts of protein extracts were incubated with glutathione S-transferase (GST)-p21-activated kinase (PAK) Sepharose beads, and activated, PAK-bound Rac1 was identified by performing Western analysis and quantitated using densitometry of the corresponding bands. One-tenth the amount of extract was also blotted with Rac1. Gel shown is representative of 3 experiments performed on 3 independently derived groups of VSMC.

Fig. 5. G-CSF stimulates Rac1 activity in human VSMC. A: human VSMC were serum starved for 48 h and then stimulated with 100 pg/ml G-CSF for the times indicated. Figure 7 illustrates that several protein kinases involved in signal transduction were activated above basal levels by G-CSF. In three experiments, p90 reached a peak of 372 ± 147% above basal levels at 10 min and declined to basal levels by 30 min poststimulation. Akt was rapidly activated 99.6 ± 14.7% above background levels by 10 min poststimulation. S6 kinase was activated 314 ± 179% above basal levels, peaking between 3 and 60 min poststimulation, and p44/42 MAPK peaked 223 ± 77% above background levels 15 min poststimulation. Significant differences vs. unstimulated cells were noted for S6 kinase at 30 min, for p44/42 MAPK at 10 –30 min, for Akt at all times poststimulation, and for p90RSK at 10 min poststimulation (∗P < 0.05 for all samples in all experiments). Close to 100% of p44/42 MAPK and S6 kinase were phosphorylated at 30 min poststimulation. Although a significant difference in the phosphorylation of p90RSK was observed at 10 min poststimulation, no significant differences were observed for Akt at any time poststimulation. Rac1 was activated in response to G-CSF stimulation, with a peak observed at 10 min poststimulation

Fig. 6. Effect of Rac1 on G-CSF-induced VSMC migration. Human VSMC in 0.5% FCS were seeded into the upper chamber of a Boyden chamber. After attachment, G-CSF at 100 pg/ml was added to serum-reduced 0.5% FCS in the lower chamber, and differences in chemotaxis between groups were quantitated by counting migrated cells after 4 h. Values are means from 3 experiments performed in triplicate from 3 independent groups of VSMC. Errors are reported as SD. *P < 0.05. **P < 0.01. ***P < 0.001. Some VSMC were infected with AdRac1N17 (dominant-negative Rac1) 48 h earlier; other samples had Sch-51344 (10 μM) added to the lower chamber at the time of seeding.

Fig. 7. G-CSF stimulates signaling pathways in human VSMC. Human VSMC were serum starved for 48 h and then stimulated with 100 pg/ml G-CSF for the times indicated. A: equal amounts of protein extracts were separated using SDS-PAGE, transferred to nitrocellulose, and incubated with an antiserum cocktail containing anti-phospho-p90RSK, Akt, p44/42 MAPK, and S6 kinase. The blot was stripped and incubated with anti-GAPDH antibody to indicate equal protein loading. Representative results of 3 experiments performed with identical results are shown. B: scanning densitometric quantitation of G-CSF-stimulated protein kinase activation in human VSMC showing the means of 3 independent experiments. ∗P < 0.05. C: ratio of total to phosphorylated protein. Relative amounts of protein were determined by performing scanning densitometry.
p90RSK was noted at 10 min, the majority of the protein remained unphosphorylated. Together, these data indicate that G-CSF activated multiple protein kinases that can mediate signaling pathways in human VSMC.

DISCUSSION

Large numbers of inflammatory cells are found in lesions of atherosclerosis, restenosis, and chronic allograft vasculopathy (11). These immune cells secrete cytokines that have profound and largely undercharacterized phenotypic effects on endothelial cells and VSMC. G-CSF is a lineage-restricted hematopoietic growth factor that stimulates terminal mitotic divisions and the final cellular maturation of hematopoietic progenitors, particularly granulocytes (18). While both GM-CSF and M-CSF expression have been noted, only one report has documented G-CSF expression in VSMC, in response to the inflammatory cytokine IL-1β (8, 34). Because very little has been reported regarding G-CSF expression in VSMC, the detection of G-CSF mRNA by performing cDNA microarray in stimulated human VSMC was unexpected. This expression was verified at the protein level by performing Western blot analysis of VSMC challenged with several growth factors and cytokines. Although G-CSF expression has not been characterized in VSMC, the G-CSF promoter contains elements of transcription factors activated by cytokines, including stimulating protein 1 (SP1), NF-κB, cAMP-response element-binding protein (CREB), nuclear factor of activated T cells (NFAT), yin yang 1 (YY1), and c-Fos, among others (23). Not surprisingly, the most complex factors, FCS and T-cell-conditioned medium, both of which contain multiple inflammatory and growth factors, elicited the most robust expression of G-CSF. There appeared to be no difference between inflammatory cytokines such as IFN-γ and strictly proliferative cytokines such as PDGF in terms of G-CSF expression. Nonetheless, every factor examined was capable of increasing G-CSF expression above basal levels.

Because activated VSMC constitute the major cell type in restenotic lesions, we investigated the expression of G-CSF in balloon angioplasty-injured rat carotid arteries using immunohistochemistry. No detectable G-CSF was observed in naive, uninjured arteries, but G-CSF was identified in medial VSMC in injured arteries after 3 days or in neointimal cells in injured arteries after 15 days. This is not entirely unexpected, because the restenotic injury is a complex lesion consisting of a multiple-cytokine milieu (28). This corroborates the Western blot analysis data indicating cytokine-dependent induction of G-CSF expression, and this is the first report of G-CSF expression in response to mechanical arterial injury. Also important is the contrast to PCNA staining in terms of both expression and location, suggesting that G-CSF-expressing cells, while activated, do not necessarily proliferate. Although no studies have reported G-CSF expression in injured arteries, G-CSF expression could represent an important prestenotic event in the initiation and development of the restenotic lesion by several means. First, G-CSF is a potent leukocyte chemoattractant (24), and its expression in the arterial wall could act as a homing mechanism to promote leukocyte infiltration. Second, G-CSF is proliferative for hematopoietic cells, and the ED50 for mouse myeloblastic NFS-60 cell proliferation is 100 pg/ml (32). In this way, once present in the restenotic lesion, immune cells are stimulated to divide and mature. Third, we recently showed that G-CSF is proliferative to human VSMC (6). Consequently, expression of G-CSF by activated VSMC could act as an autocrine regulator of VSMC proliferation.

G-CSF is chemotactic for granulocytes (24). An early report also shows that G-CSF is chemotactic for endothelial cells (5). In a myeloid cell line, G-CSF induced Egr-1 upregulation through interaction of serum response element-binding proteins (19). Because Egr-1 plays a key role in vascular cell pathophysiology (14), we hypothesized that G-CSF would also promote VSMC migration as well. Indeed, we noted a dose- and time-dependent effect of G-CSF on VSMC migration. The present study is the first to establish a chemotactic effect of G-CSF on VSMC. G-CSF exerts its proliferative effects through activation of the Ras family of GTPases, which are intimately involved in the regulation of migration as well as proliferation (25, 26). These proteins act as molecular switches and cycle between inactive GDP-bound and active GTP-bound molecules to modify upstream signals to downstream effectors as required. Rac1 is a Ras family member that also plays an obligate role in cell migration. The Rac proteins in particular play important roles in VSMC pathophysiology, including the regulation of oxidative processes, proliferation, and migration (22). The experiments presented in Figs. 5 and 6 are the first to determine that G-CSF activates Rac1 in VSMC. Elimination of Rac1 activity results in significant reduction of G-CSF-induced VSMC migration. Accordingly, Rac1 activation is an obligatory molecular event that mediates G-CSF-induced migration of VSMC.

Activated Rac1 can transduce signals from cell surface receptors to corresponding cytoplasmic targets (10, 22). Although the G-CSF receptor does not contain a cytoplasmic kinase domain, binding of G-CSF to its receptor results in a rapid tyrosine autophosphorylation of its receptor as well as the phosphorylation and activation of numerous intracellular protein tyrosine kinases (7). These include the Ras-MAPK, JNK/SAPK, and p38 pathway activation (4, 24, 25). In our experiments, we show that G-CSF induces rapid activation of p90RSK and p44/42 MAPK, and Akt and more gradual, less robust activation of pS6 kinase.

Several reports have demonstrated a MAPK- and NF-κB-mediated autocrine growth loop for G-CSF in nonhematopoietic tumor cells in which serum deprivation stimulated constitutive production of G-CSF (21, 36). Although known for some time to be proliferative for granulocytes and human endothelial cells (5, 26), there is only one other report in the literature describing expression of G-CSF in human VSMC (34). In this report, G-CSF mRNA was expressed in response to the inflammatory cytokine IL-1β. The authors of that study suggested that because G-CSF is proliferative for granulocytes, it might be involved in the progression of vascular inflammatory diseases. Our study extends that hypothesis and is the first to report that G-CSF has activating effects on human VSMC and confirms that this cytokine may exert pleiotropic effects outside the hematopoietic system.

Numerous recent studies have indicated that bone marrow-derived and circulating vascular cell precursors, or stem cells, contribute to the cellular component of the neointimal lesion in both mechanical and allograft-injured arteries by homing to the site of the lesion (29, 31, 33). These reports suggested that migration and transdifferentiation of multipotent cells can be
an important source of neointimal occlusion in several vascular pathologies. G-CSF is a known powerful chemoattractant and mobilizer of bone marrow-derived stem cells (15, 24). It has been demonstrated that bone marrow stem cell transplantation improves myocardial perfusion but is limited by the invasive-ness of stem cell collection. A recent report (13) described the MAGIC cell randomized clinical trial, in which endogenous stem cells were mobilized by infusion of G-CSF in patients with coronary stents. In this study, an unexpectedly high rate of in-stent restenosis was noted in patients who were administered G-CSF, so much so that enrollment in that arm of the study was terminated. It was suggested that the stem cells contributed to arterial occlusion within the lesion. Consequently, G-CSF secretion by activated arterial VSMC may contribute to recruitment and homing of vascular precursor cells to the site of the arterial lesion.

There are several novel points in this study. First, G-CSF can be expressed by human VSMC, and this expression can be increased by several cytokines. Second, G-CSF is not detectable in normal rat carotid arteries but can be induced in medial and neointimal VSMC by injury. Third, G-CSF is chemotactic for VSMC, likely through Rac1 activation. Fourth, G-CSF can increase activation of signal transduction pathways in VSMC. Taken together, these points indicate that G-CSF expression by activated VSMC has important implications for vascular inflammatory cell communication, resulting in the progressive nature of several vascular pathologies.

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REFERENCES


