Angiostatin inhibits monocyte/macrophage migration *via* disruption of actin cytoskeleton

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ABSTRACT **In light of the involvement of tumor-associated macrophages (TAM) in the promotion of tumor growth and metastasis, strategies to prevent TAM recruitment within the tumor microenvironment are currently under investigation. The recent observation that angiostatin reduces macrophage infiltration in an atherosclerosis model prompted our laboratory to further explore the use of human plasminogen angiostatin (hK1–3) protein as a macrophage modulatory agent. We demonstrate that hK1–3 blocks migration of murine peritoneal macrophages (91% decrease, $P<0.00005$) and human monocytes (85% decrease, $P<0.05$) *in vitro*. Cell viability of hK1–3-treated cells is not affected, as determined by fluorochrome-labeled inhibitors of caspase-propidium iodide (FLICA/PI) flow cytometry analysis. Furthermore, confocal microscopy of phalloidin-stained cells reveals that hK1–3 leads to disruption of actin filopodia/lamellipodia in human monocytes and induces distinct podosome accumulation in mature differentiated macrophages. Paradoxically, we observed a 3.5-fold increase in secretion and a 3- to 5.5-fold increase in gelatinolytic activity of macrophage-produced matrix metalloproteinase-9, which we suggest is a cellular response to compensate for the dominant static effect of hK1–3 on actin. We also demonstrate that hK1–3 induces the phosphorylation of extracellular signal-regulated kinase (ERK1/2) in human monocytes. hK1–3-mediated macrophage immobilization has the potential to be exploited therapeutically in pathological conditions associated with cellular hypoxia, such as cancer and atherosclerosis.—Perri, S.R., Annabi, B, Galipeau, J. Angiostatin inhibits monocyte/macrophage migration *via* disruption of actin cytoskeleton. *FASEB J.* 21, 3928–3936 (2007)**

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It is well established that tumor-associated macrophages (TAMs) are involved in tumor progression and metastasis (1–4). TAMs are skewed toward the M2 phenotype (polarized type II macrophages) (5), which is oriented toward promoting angiogenesis, tissue remodeling, and repair (5). TAMs are typified by low cytotoxic potential against tumor cells owing to reduced production of nitric oxide (NO) and proinflammatory cytokines (6). TAMs may possess decreased inflammatory cytokine production as a consequence of their exposure to tumor-derived anti-inflammatory molecules such as interleukin (IL) -4, IL-10, transforming growth factor-β1, and prostaglandin E_2 (7, 8). Type II macrophages promote tumor cell proliferation and metastasis by producing an array of proangiogenic factors and metalloproteinases (MMP) and by their involvement in signaling pathways that regulate the function of fibroblasts in the tumor stroma (5, 6, 9, 10). Several reports have demonstrated a correlation between infiltration of TAMs and prognosis in cancer patients whereby increased macrophage infiltration resulted in worse prognosis (9, 11–13). TAMs have also been associated with immune suppression and tolerance (14) as well as inhibition of T cell responses by up-regulating NO, prostaglandins, tumor necrosis factor α, and arginase activity, which leads to apoptosis of activated T cells (15, 16).

TAMs are derived from circulating monocytes and are recruited to the tumor microenvironment by tumor-produced monocyte chemotactic factors (17), some of which include CCL2/monocyte chemoattractant protein (MCP)-1, produced by gliomas, sarcomas, lung, breast, cervix, ovary, melanoma, and pancreatic cancer (18–21); CXCL8/IL-8, produced by breast and melanoma (22, 23); and CCL5/RANTES (regulated on activation, normal T cell expressed and secreted), produced by breast and melanoma (24, 25). High expression of monocyte chemotactic factors is associated with increased macrophage infiltration (26). Upon TAM infiltration, cancer cells sustain TAM survival, which subsequently induces TAMs to produce growth factors and matrix degradation enzymes that lead to tumor proliferation, angiogenesis, and invasion (7, 27–29).

Thus, it would be desirable to further explore strategies to reduce TAM infiltration in the tumor microenvironment. We previously demonstrated that the

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human plasminogen (hPlg) kringle 5 (K5) domain acts as a potent antiangiogenic agent, blocks migration of human monocyte-derived macrophages *in vitro*, and substantially reduces $CD45^+$ $Mac\text{-}3^+Gr\text{-}1^-$ macrophage recruitment to the tumor microenvironment *in vivo* (30). It was reported by Moulton *et al.* (31) that angiostatin, another internal proteolytic fragment of plasminogen that encompasses the first four kringle domains, was also observed to reduce *in vivo* infiltration of $Mac3^+$ macrophages in the plaque and surrounding vasa vasorum in an atherosclerosis model (31). This finding suggests that angiostatin may possess immune modulatory properties that can be exploited to suppress tumor-associated macrophages.

MATERIALS AND METHODS

Macrophage isolation

Murine peritoneal macrophages were isolated from C57Bl/6 mice (Charles River, Laprairie, Quebec, Canada) by lavage of the abdominal cavity with RPMI solution. Harvested peritoneal macrophages were resuspended in RPMI that contained 10% fetal bovine serum [FBS] and 1% penicillin-streptomycin (Wisent Technologies, St. Bruno, Quebec, Canada) and plated on untreated tissue culture dishes overnight. Nonadherent cells were removed, and adherent fraction was trypsinized and used for further experiments. Fresh human peripheral blood mononuclear cells (PBMCs) were obtained from normal volunteers and maintained in RPMI (Wisent Technologies) supplemented with 20% human serum (Cambrex, Walkersville, MD, USA) and 1% penicillin-streptomycin. To induce differentiation of monocytes into macrophages, 500 U/ml granulocyte/macrophage colony-stimulating factor (GM-CSF; Immunex, Thousand Oaks, CA, USA) was added.

Macrophage migration assay

Murine peritoneal macrophages (2.5×10^5) or human monocytes (1.5×10^5) were plated onto 0.15% gelatin/phosphate buffering solution (PBS)-coated 5 μm pore chemotaxis membranes (Corning, Acton, MA, USA) within Boyden chamber inserts. Murine macrophages were exposed to 1.0 and 5.0 μM recombinant human plasminogen angiostatin (hK1–3) (Cell Sciences Inc., Canton, MA, USA) protein for 24 h. Human monocytes were exposed to 10 μM hK1–3 for 24 h. Species-specific recombinant GM-CSF (murine (m)-GM-CSF [R&D Systems Inc., Minneapolis, MN, USA]) was used as a chemoattractant and diluted in serum-containing RPMI media. Each sample was tested in triplicate, and nonmigrating cells were removed by gently wiping the upper surface of the filter. The average number of migrating murine macrophages per field was assessed by counting three random, high-power fields per filter and then plotted. The total number of migrating human monocytes per filter was also counted and plotted.

Apoptosis assay

Murine peritoneal macrophages were plated on 60 mm dishes for 3 h in RPMI media that contained 10% FBS and 1% penicillin/streptomycin. Nonadherent cells were removed by washing three times in PBS. Adherent macrophages were either exposed to media alone or to increasing concentrations of recombinant hK1–3 protein (0.1, 0.5, 1.0, and 2.0 μM) that were diluted in media for 16 h under normal culture conditions. Whole-cell staining was performed with the polycaspase fluorochrome-labeled inhibitors of a caspase-propidium iodide (FLICA-PI) apoptosis kit (Immunochemistry Technologies, Bloomington, MN, USA) and staining was quantified by flow cytometry analysis.

Actin staining

Human monocytes were plated and allowed to adhere on round glass coverslips (VWR International, Mississauga, Ontario, Canada) within 24-well tissue culture plates for 1.5 h. The monocytes were washed with PBS and exposed to either serum-free media (SFM), 2 μM hPlg diluted in SFM, 2 μM and 5 μM hK1–3 diluted in SFM, or 1 μg cytochalasin D (Sigma, Oakville, Ontario, Canada) diluted in SFM for 24 h under normal culture conditions. hGM-CSF-differentiated macrophages were exposed to SFM, 2 μM hPlg diluted in SFM, or 2 μM hK1–3 diluted in SFM for 24 h under normal culture conditions. Cells were washed, fixed with 3.7% formaldehyde/PBS, permeabilized with 0.1% TritonX-100/PBS, and stained with phalloidin Alexa Fluor 488 (Cambrex) and 4′,6-diamidino-2-phenylindole (DAPI, Molecular Probes, Burlington, Ontario, Canada) per the manufacturer's instructions. Stained cells were mounted with Immunomount (Vector Laboratories, Burlingame, CA, USA) and stored in the dark at 4°C. Confocal microscopy images were acquired with the Zeiss LSM510 Meta confocal microscope.

Gelatin zymography

Monocytes/macrophages were exposed to either SFM, 2 μM hPlg in SFM, or 2 μM hK1–3 in SFM for 24 h, then conditioned media were collected and concentrated 4-fold. Gelatinolytic activity in conditioned media samples was assessed by gelatin zymography. An aliquot (20 μl) of the culture medium was subjected to sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS/PAGE) with a 7.5% (w/v) polyacrylamide gel that contained 0.1 mg/ml gelatin. The gels were then incubated for 30 min at room temperature twice in 2.5% (v/v) Triton X-100 to remove SDS, then rinsed five times in doubly distilled water. The gels were incubated at 37°C for 20 h in 20 mM NaCl/5 mM $CaCl_2$/0.02% (v/v) Brij-35/50 mM Tris/HCl buffer (pH 7.6), then stained with 0.1% Coomassie Brilliant Blue R-250, followed by destaining in 10% (v/v) acetic acid/30% (v/v) methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblot analysis

Monocytes/macrophages were exposed to either SFM, 2 μM hPlg in SFM, or 2 μM hK1–3 in SFM for 24 h and conditioned media were collected. Cell lysis buffer (Sigma) was used according to the manufacturer's instructions to prepare cell lysates. Protein samples were separated on 4–20% gradient SDS-PAGE gels (Invitrogen, Burlington, Ontario, Canada), transferred to PVDF membranes, and immunoblotted with anti-MMP-9 (R&D Systems Inc.), antiphospho-ERK1/2, anti-ERK (Cell Signaling Technology, Danvers, MA, USA), anti-HuR (Jackson ImmunoResearch Laboratories, West Grove, PE), antitubulin, and anti-GAPDH antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies.

RESULTS

Characterization of isolated monocytes/macrophages

We isolated both murine and human macrophages to test the effect of recombinant K1–3 exposure. **Figure 1*A*** depicts a representative Giemsa stain performed on a cytospin sample prepared immediately after isolation of murine peritoneal macrophages. Flow cytometry analysis demonstrates that the isolated murine macrophage population is strongly positive for CD11b/Mac-1 and CD14 when cultured in serum-free, serum-containing, or serum/murine recombinant GM-CSF-containing media for 24 h (Fig. 1*B*). The Mac-1 antibody detects a 170 kDa α chain of Mac-1 (CD11b/CD18, $\alpha_M\beta_2$ integrin), also known as complement receptor 3, which is expressed on macrophages. The CD14 antibody detects the lipopolysaccharide receptor on peritoneal resident macrophages but not on blood monocytes. Human monocyte preparations were isolated from PBMCs of healthy donors by a standard leukaphoresis protocol. The monocyte fraction was capable of differentiating into macrophages upon exposure to recombinant human GM-CSF for 7 days in normal culture conditions and stains positive for CD206, which represents the mannose receptor present on the plasma membrane of macrophages (data not shown).

Effect of hK1–3 on macrophage migration

In light of our previous observation that the soluble hPlg K5 domain inhibited *in vitro* migration of human PBMC-derived macrophages (30), we tested the ability of recombinant hK1–3 protein to affect macrophage migration. Murine peritoneal macrophages were exposed to SFM, serum-containing media (SM), serum- and mGM-CSF-containing media (SGM), hPlg in SFM, hK1–3 in SFM, or PBS in SFM for 45 min at 37°C and subsequently plated on the upper chamber of gelatin-coated filters within modified Boyden chambers. Media in lower chambers contained either serum, serum and mGM-CSF (chemoattractant), or no serum (basal). hK1–3 is capable of blocking basal as well as mGM-CSF-stimulated macrophage migration (up to a 91% decrease) in a dose-dependent fashion (basal: 2 μM, $P>0.05$, 10 μM, $P<0.05$, and 17 μM, $P<0.005$; stimulated: 2 μM, 10 μM, and 17 μM, $P<0.00005$; **Fig. 2**). hPlg appears to induce slight inhibition of macrophage migration under chemoattractant conditions (1 μM, $P<0.0005$) compared with control, although its effect is not at all comparable to the potent inhibitory effect induced by hK1–3. However, it is worth noting that hPlg does not inhibit macrophage migration beyond that of the background inhibition observed with the PBS control sample, as do all tested hK1–3 doses. Therefore, its effect should be considered insignificant. Consistent with these results, we also observed an hK1–3-induced suppression of migration (85% decrease) when human PBMC-derived monocytes were utilized in a similar migration assay ($P<0.05$, Fig. 2*C*). These observations suggest that direct exposure of monocytes/macrophages to hK1–3 protein substantially decreases their migratory capacity.

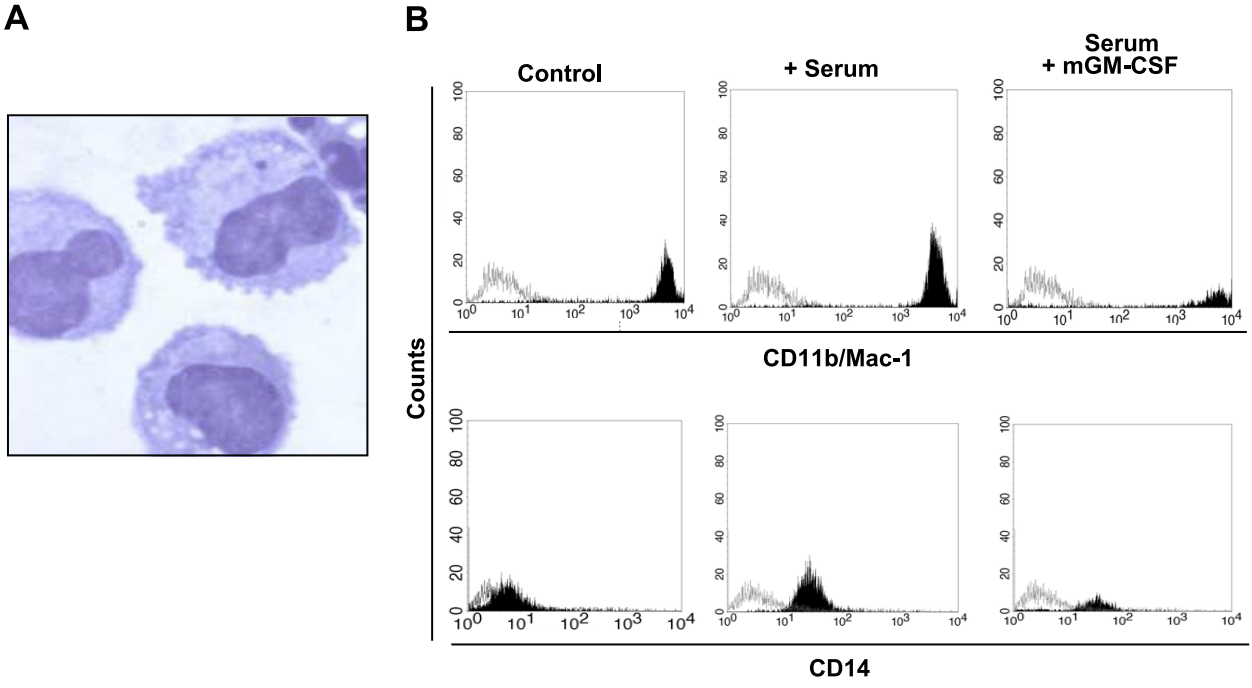

Figure 1. Macrophage characterization. *A)* Representative Giemsa stain performed on a cytospin preparation of freshly isolated murine peritoneal macrophages (100×magnification). *B)* Representative flow cytometry histograms of murine macrophages stained with anti-CD11b/Mac-1 and anti-CD14 antibody cultured in serum-free, serum-containing, and serum/mGM-CSF-containing media.

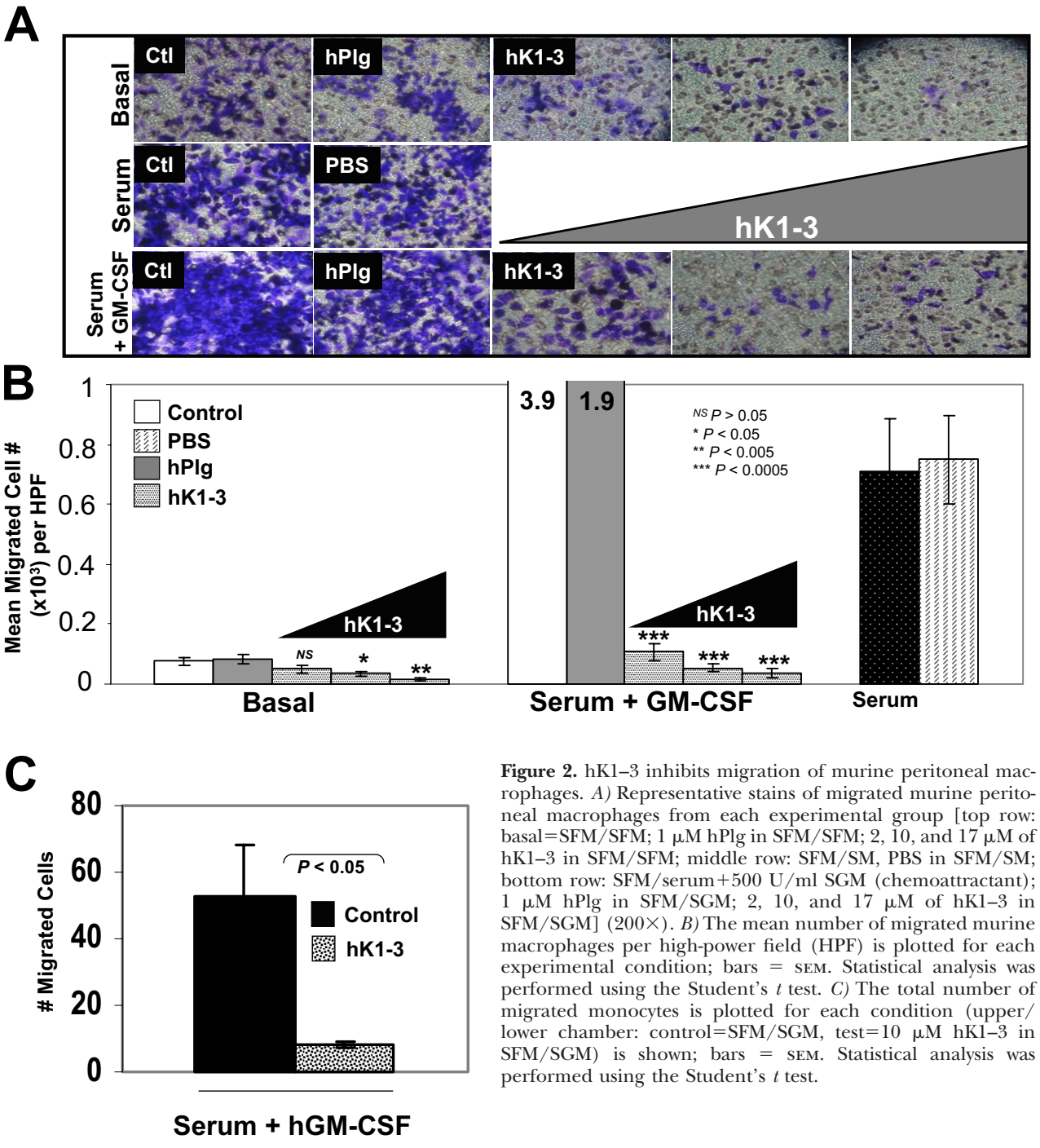

Figure 2. hK1–3 inhibits migration of murine peritoneal macrophages. *A)* Representative stains of migrated murine peritoneal macrophages from each experimental group [top row: basal=SFM/SFM; 1 μM hPlg in SFM/SFM; 2, 10, and 17 μM of hK1–3 in SFM/SFM; middle row: SFM/SM, PBS in SFM/SM; bottom row: SFM/serum+500 U/ml SGM (chemoattractant); 1 μM hPlg in SFM/SGM; 2, 10, and 17 μM of hK1–3 in SFM/SGM] (200×). *B)* The mean number of migrated murine macrophages per high-power field (HPF) is plotted for each experimental condition; bars = SEM. Statistical analysis was performed using the Student's *t* test. *C)* The total number of migrated monocytes is plotted for each condition (upper/lower chamber: control=SFM/SGM, test=10 μM hK1–3 in SFM/SGM) is shown; bars = SEM. Statistical analysis was performed using the Student's *t* test.

Effect of hK1–3 on murine macrophage viability

To determine whether the decrease in migration was attributable to an effect of hK1–3 on cell viability, we investigated whether macrophages exposed to hK1–3 led to apoptotic cell death. Murine peritoneal macrophages were exposed to increasing doses of hK1–3 diluted in SM or SM alone for 16 h under normal culture conditions. Cells were harvested and subsequently stained with a commercially available fluorescence-based FLICA/PI kit, which selectively stains cells that contain active caspases. Results reveal that hK1–3 was unable to induce cell death at the doses tested, as evidenced by negligible $FLICA^+$ staining as well as similar $FLICA^+PI^+$ staining as control-treated cells (Supplemental Fig. 1). The data suggest that exposure of macrophages to hK1–3 does not lead to cell death.

Effect of hK1–3 on actin cytoskeleton of monocytes and hGM-CSF-differentiated macrophages

In an attempt to explain the mechanism by which hK1–3 induces inhibition of macrophage migration, we assessed the ability of hK1–3 to affect actin polymerization. Human monocytes were plated on coverslips and

exposed to the following conditions: SFM, 2 μM hPlg diluted in SFM, 2 μM and 5 μM hK1–3 diluted in SFM, or 1 μg cytochalasin D diluted in SFM for 24 h in normal culture conditions. Cytochalasin D was used as a control since it binds to the ends of filamentous (F) actin filaments and induces potent inhibition of actin polymerization/depolymerization leading to the formation of distinct actin aggregates. Confocal microscopy images of phalloidin/DAPI-stained monocytes demonstrate that hK1–3 is capable of destroying actin filaments and decreases cell polarity in a dose-dependent manner compared with control-treated cells (**Fig. 3**). Control and Plg-treated cells possessed well-developed filopodia (arrowheads) with a clear, elongated migration front (arrows) containing actin distributed through the long axis of the cell and stress fibers, filopodia, and lamellipodia at the leading migration edge. The rear monocyte end is typified by a round shape with a less organized actin filament network. In contrast, disruption of actin polymerization in hK1–3-treated cells led to actin redistribution, which resulted in the formation of cluster-like actin aggregates (depolymerized actin), especially along the periphery of the cell. hK1–3-treated monocytes are clearly more rounded, with loose actin filaments that lack protrusions and migrating fronts, similar to the effect induced by cytochalasin D treatment. The ability of hK1–3 to disrupt monocyte actin polymerization may be partly responsible for the observed hK1–3-induced migration blockade.

Human monocytes were induced to differentiate into macrophages *via* exposure to 500 U/ml of hGM-CSF for 5 days, plated on coverslips, and exposed to SFM, 2 μM hPlg diluted in SFM, or 2 μM hK1–3 diluted in SFM for 24 h under normal culture conditions. Phalloidin/DAPI-stained confocal images of hK1–3-treated macrophages clearly depict the dominant presence of punctiform pericytoplasmic actin staining (dotted arrows) as well as extensive filopodia (arrowheads) at the migration front compared with control-treated cells (**Fig. 4**). This distinct phalloidin staining in hGM-CSF-differentiated macrophages may represent podosomes, punctate dot-like, F-actin-rich core structures surrounded by a ring of monomeric G-actin generally constrained to the cell periphery (32).

Effect of hK1–3 on macrophage-derived MMP-9 expression and activity

Our observation that hK1–3 induces strong inhibition of migration led us to test whether the activity of key proteases implicated in cell chemotaxis/motility such as MMP-2 (gelatinase-A) and -9 (gelatinase-B) were affected by hK1–3 exposure. Murine peritoneal macrophages and human monocytes were exposed to SFM, 2 μM hPlg in SFM, 2 μM hK1–3 in SFM, or 5 μg cytochalasin D in SFM for 24 h under normal culture conditions. Conditioned media were collected, concentrated 4-fold, and subjected to gelatin zymography analysis. We observed a 3.5-fold increase in secretion of MMP-9 in hK1–3-treated murine peritoneal macrophages as confirmed by anti-MMP-9 immunoblot analysis (**Fig. 5*A***). Furthermore, exposure of macrophages to hK1–3 led to an increase in the ability of macrophage-derived soluble MMP-9, but not MMP-2, to lyse gelatin compared with control-treated cells (Fig. 5*B*). More specifically, murine macrophages exposed to hK1–3 possessed a 3- to 5.5-fold increase in pro-MMP-9 (92 kDa) and active MMP-9 (83 kDa) activity (Fig. 5*B*, left panel). Increased active MMP-9 was also observed in hK1–3-treated human monocytes (Fig. 5*B*, right panel).

Effect of hK1–3 on downstream signaling

We assessed the ability of hK1–3 to modulate the protein expression level of signaling mediators reported to be associated with MMP-9 expression (33). We used immunoblot analysis to test extracellular signal-regulated kinase (ERK) and nuclear factor human antigen R (HuR), which has been reported to bind to AU-rich elements present in the 3′-untranslated region of MMP-9 mRNA (34) and prevent mRNA degradation (35–37). At 24 h, the phosphorylated (P)-ERK1/2 expression level remained similar in hK1–3 and control-treated murine macrophages *vs.* total ERK expression (Fig. 5*C*). In contrast, hK1–3-treated human monocytes increased expression of P-ERK1/2 compared with control-treated cells. hPlg-treated human monocytes exhibited the highest P-ERK1/2 expression. There was no detectable difference in the expression of

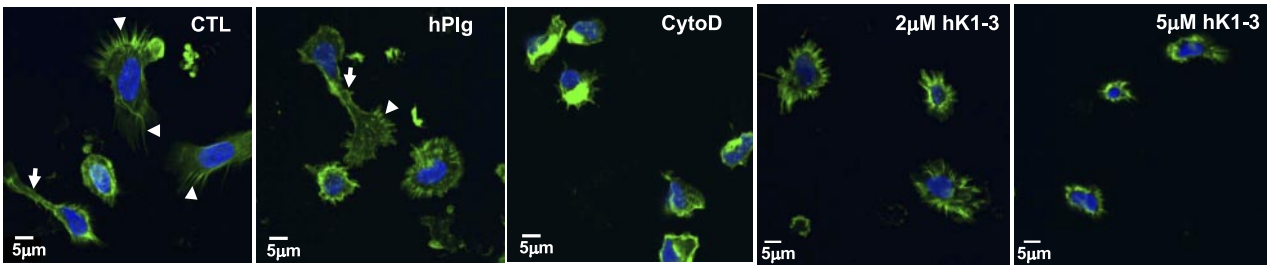

Figure 3. hK1–3 disrupts actin polymerization in human monocytes. Representative confocal microscopy images of human monocytes exposed to each experimental condition: SFM (control, ctl), 2 μM hPlg in SFM, 2 μM, and 5 μM hK1–3 in SFM, or 1 μg cytochalasin D (CytoD). F-actin revealed with phalloidin Alexa Fluor 488 (green) and nucleus with DAPI (blue). Arrowheads indicate filopodia and arrows depict the migrating front of the cell. Scale bars = 5 μm.

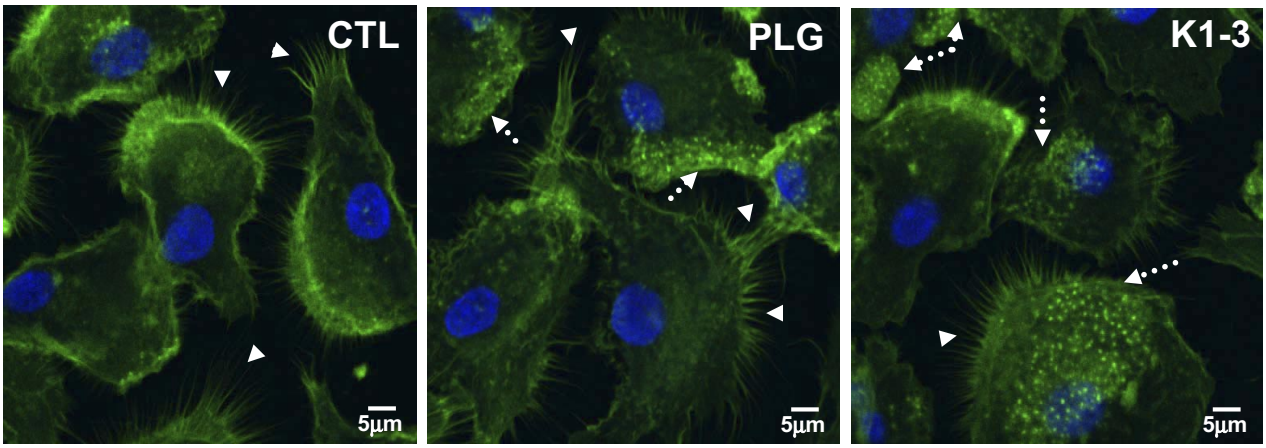

Figure 4. hK1–3 induces podosome accumulation in hGM-CSF-differentiated macrophages. Representative confocal microscopy images of human monocyte-derived macrophages exposed to each experimental condition: SFM (control, ctl), 2 μM hPlg in SFM, or 2 μM hK1–3 in SFM. F-actin revealed with phalloidin Alexa Fluor 488 (green) and nucleus with DAPI (blue). Arrowheads indicate filopodia and dotted arrows depict podosome accumulation preferentially localized at the migrating front of the cell. Scale bars = 5 μm.

HuR in human monocytes among experimental treatment conditions (Fig. 5*C*).

DISCUSSION

Our findings demonstrate that angiostatin (hK1–3), best known for its antiangiogenic properties (38), possesses additional immune modulatory properties *via* its effect on macrophage mobilization. It is worthy to note that other well-characterized endogenous antiangiogenic agents have also been reported to possess a more broadened cell specificity than was initially believed. For instance, Moulton *et al.* (31) reported that soluble VEGF receptor 1 (39) is capable of inhibiting plaque-associated endothelial cell sprouting as well as suppressing VEGF-induced migration of human blood monocytes. In an experimental model of bone metastasis

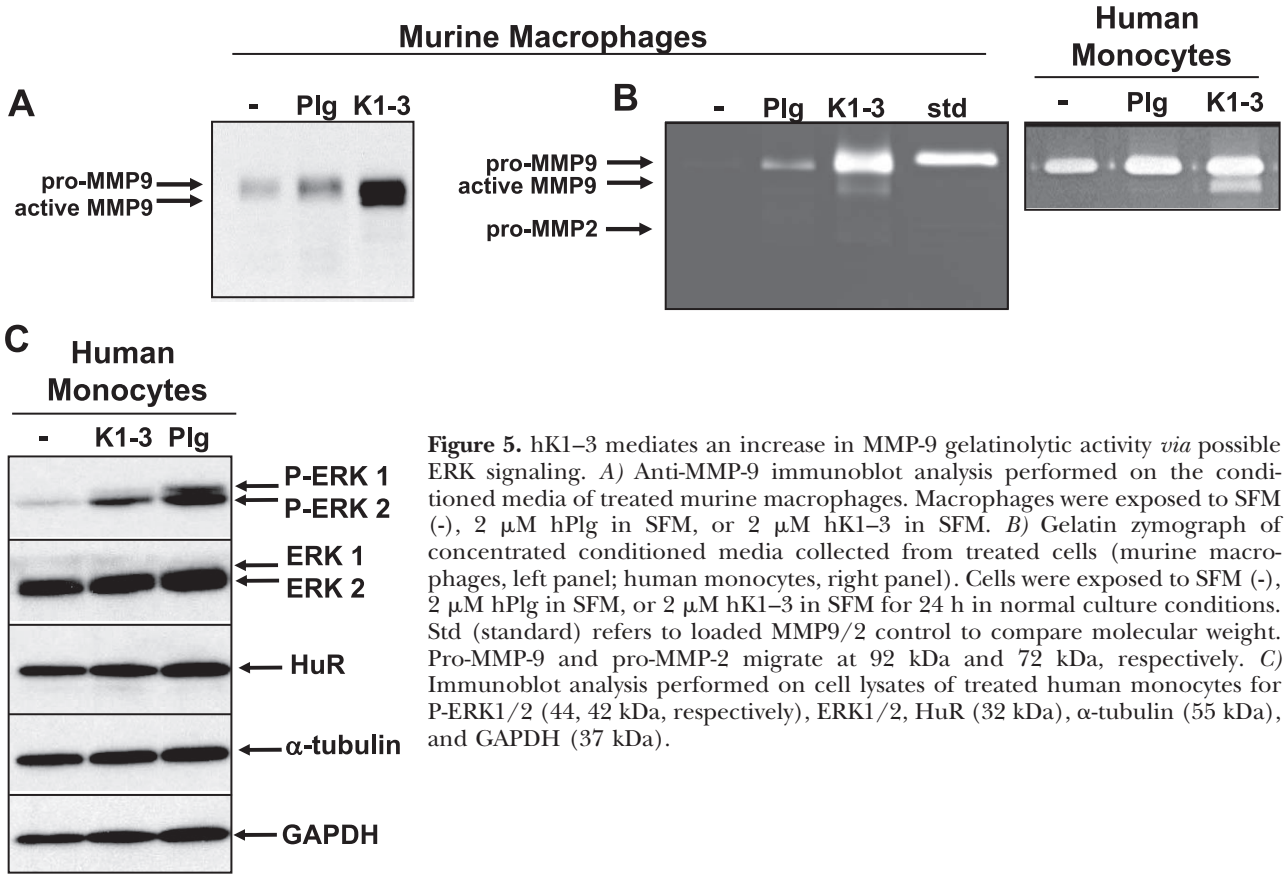

Figure 5. hK1–3 mediates an increase in MMP-9 gelatinolytic activity *via* possible ERK signaling. *A)* Anti-MMP-9 immunoblot analysis performed on the conditioned media of treated murine macrophages. Macrophages were exposed to SFM (-), 2 μM hPlg in SFM, or 2 μM hK1–3 in SFM. *B)* Gelatin zymograph of concentrated conditioned media collected from treated cells (murine macrophages, left panel; human monocytes, right panel). Cells were exposed to SFM (-), 2 μM hPlg in SFM, or 2 μM hK1–3 in SFM for 24 h in normal culture conditions. Std (standard) refers to loaded MMP9/2 control to compare molecular weight. Pro-MMP-9 and pro-MMP-2 migrate at 92 kDa and 72 kDa, respectively. *C)* Immunoblot analysis performed on cell lysates of treated human monocytes for P-ERK1/2 (44, 42 kDa, respectively), ERK1/2, HuR (32 kDa), α-tubulin (55 kDa), and GAPDH (37 kDa).

induced by breast cancer cells that mimic metastasis observed in naturally occurring human breast cancer (40), it was shown that angiostatin acts as an effective inhibitor of bone resorption by directly inhibiting cancer-induced bone destruction *via* direct inhibition of osteoclast activity and generation. More recently, Noonan *et al.* (41) reported that angiostatin loses its antiangiogenic potential in animal genes targeted for the IL-12 receptor as well as in animals with the IL-12 p40 subunit deleted. Because endothelial cells do not express the IL-12 receptor or respond to IL-12 stimulation, angiostatin-induces IL-12 production by either neutrophils, macrophages, or dendritic cells in order to exert its effect (41). Our laboratory confirmed that human K5 protein, another internal cleavage product of plasminogen, acts as a potent endothelial cell inhibitor (30), as others have observed (42). Moreover, we demonstrate that K5 directly inhibits *in vitro* migration of human monocyte-derived $CD206^+$ macrophages and leads to a significant reduction in $CD45^+$ $Mac\text{-}3^+Gr\text{-}1^-$ macrophage infiltration within tumor implants (30). We also show that K5 protein is chemotactic for $CD11b^+$ neutrophils in an *in vitro* chemotaxis assay and that K5-containing tumor implants possess a cellular infiltrate predominantly composed of neutrophils (43). Similarly, angiostatin was reported to inhibit IL-8- and macrophage inflammatory protein 2-induced neutrophil migration (44).

In this study we show that recombinant hK1–3 protein blocks *in vitro* migration of murine peritoneal macrophages (Fig. 2*A, B*) and human monocytes (Fig. 2*C*). This is consistent with observations by Benelli *et al.* (44), who report that angiostatin is capable of inhibiting MCP-1-induced monocyte migration. We show that, at a similar dose reported to cause endothelial cell death (45), hK1–3 does not inhibit migration by affecting macrophage viability (Supplemental Fig. 1). This result implies that hK1–3 induces macrophage immobilization *via* an alternative mechanism of action. It is possible that hK1–3 interacts with a different cell surface receptor on macrophages, which may lead to downstream signaling distinct from that described for its interaction with endothelial cells, which then results in apoptosis. Our confocal images of phalloidin-stained monocytes/macrophages reveal that hK1–3 is capable of disrupting actin filopodia/lamellipodia and promotes podosome accumulation in mature, differentiated macrophages. To the best of our knowledge, no study so far has correlated the ability of angiostatin to impair macrophage motility with actin cytoskeletal disruption. Clusters of podosomes have been observed at the leading edge of macrophages assembling from a larger precursor structure that undergoes fission (46). Microtubules have been shown to be persistently associated with podosomes at the leading macrophage edge (46, 47). It has been reported that microtubule destabilization in primary human macrophages led to podosome disassembly, which resulted in enhanced motility (48). Consistent with these findings, our results demonstrate that control and hPlg-treated human macrophages possess fewer podosomes (*i.e.*, more dynamic) and display increased migratory capacity. In contrast, hK1–3-treated macrophages appear to possess a distinct accumulation of less dynamic podosomes with an increased life span. The typical podosome life span at the leading edge of macrophages generally is a few minutes (46, 47). Podosome accumulation in hK1–3-treated macrophages may cause reduction in cell detachment, which may lead to decreased cell motility, as we observed in the migration assay. It has also been reported that microtubule destabilization in a murine peritoneal macrophage cell line significantly reduced the life span of podosomes—without leading to a complete loss of podosomes—and led to inhibition of motility, even though transient protrusions at the leading macrophage edge remained present (46). Similarly, we observed that hK1–3-treated macrophages maintained clear filopodia protrusions at the cell periphery.

Wang *et al.* reported that intact kringle 5 is required for plasminogen to bind to cell surface β-actin on prostate cancer cells and demonstrated that angiostatin 4.5, which consists of kringles 1 to 4 and 85% of kringle 5, cannot bind β-actin (49). However, Dudani *et al.* utilized a different angiostatin isoform (K1–4 produced by limited enzymatic proteolysis of Plg) and claimed that both Plg and angiostatin can bind actin on the endothelial cell surface (50). These findings suggest that hK1–3 protein most likely does not bind β-actin on the monocyte/macrophage cell surface in order to disrupt actin since it does not contain the K5 domain. It is possible that hK1–3 interacts with cell surface-expressed angiomotin, which has been identified as an endothelial cell surface receptor for angiostatin (51), binds to F-actin, and has been associated with endothelial cell motility and actin fiber formation (52–54). Benelli *et al.* demonstrate that angiomotin is also present on polymorphonuclear cells (44); thus, it is highly probable that monocytes/macrophages express cell surface angiomotin as well, which may promote hK1–3 binding leading to actin disruption. More recently, Dudani *et al.* (55) reported that angiostatin binds to surface-associated heat shock protein (HSP) -27 and, to a lesser extent, HSP-70 on endothelial cells. It is possible that HSPs may also be implicated in hK1–3 interaction with macrophages.

Paradoxically, we observed an increase in the gelatinolytic activity of macrophage-produced MMP-9 in hK1–3-treated cells (Fig. 6*B*). MMP-9 is linked with leukocyte transmigration, as recently observed by Kolaczkowska *et al.* (56) in a murine model of induced peritonitis whereby gelatinase B-deficient ($MMP\text{-}9^{-/-}$) mice possessed impaired leukocyte infiltration compared with controls. MMP-9 has also been reported to participate in lung-specific metastasis (57). Macrophages adopt an innate motile phenotype in response to chemotactic stimuli *via* secretion of proteolytic enzymes to detach and degrade the extracellular matrix. In our study, up-regulation of MMP-9 activity may arise as a positive feedback mechanism owing to the strong inhibitory action of hK1–3 on actin, which exerts a

dominant negative effect on macrophage migration. This phenomenon has also been described by Tomasek *et al.* (58), who report that disorganization of the actin cytoskeleton increases expression, secretion, and activation of MMP-2 in human fibroblasts, possibly as a mechanism to remodel the links between the matrix and the cytoskeleton.

Lu *et al.* (59) reported that stimulation of monocytes with lipopolysaccharide leads to the production of monocyte-derived MMP-9 *via* the phosphatidylinositol-3 kinase/Akt/inhibitor of κB kinase-α/nuclear factor-κB pathway. We observed that hK1–3-treated monocytes increased expression of P-ERK1/2 compared with control-treated cells. This finding is consistent with the data reported by Genersch *et al.*, which demonstrated that sustained ERK phosphorylation is associated with MMP-9 expression in endothelial cells (33). That there were no detectable differences in HuR expression in monocytes among experimental conditions suggests that MMP-9 expression may be regulated posttranslationally.

Overall, this study confirms previous observations that angiostatin impedes macrophage migration, and demonstrates for the first time that actin disruption induced by angiostatin may serve as a novel mechanism that leads to macrophage immobilization. We speculate that angiostatin's ability to induce a dominant static phenotype in macrophages, in addition to its previously reported endothelial cell-inhibitory properties and its direct cytotoxicity on tumor cells, could markedly enhance its effectiveness as an anticancer agent. These findings can be exploited therapeutically in the context of pathologies associated with high leukocyte infiltration, such as chronic inflammation observed with malignancies and atherosclerosis. Moreover, in light of angiostatin's novel macrophage modulatory property, its utility could also be extended to target M2-phenotypic macrophages implicated in immune regulation and tissue remodeling (60). FJ

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