Generation of Biologically Active Angiostatin Kringle 1–3 by Activated Human Neutrophils[1]

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The contribution of polymorphonuclear neutrophils (PMN) to host defense and natural immunity extends well beyond their traditional role as professional phagocytes. In this study, we demonstrate that upon stimulation with proinflammatory stimuli, human PMN release enzymatic activities that, in vitro, generate bioactive angiostatin fragments from purified plasminogen. We also provide evidence that these angiostatin-like fragments, comprising kringle domain 1 to kringle domain 3 (kringle 1–3) of plasminogen, are generated as a byproduct of the selective proteolytic activity of neutrophil-secreted elastase. Remarkably, affinity-purified angiostatin kringle 1–3 fragments generated by neutrophils inhibited basic fibroblast growth factor plus vascular endothelial growth factor-induced endothelial cell proliferation in vitro, and both vascular endothelial growth factor-induced angiogenesis in the matrigel plug assay and fibroblast growth factor-induced angiogenesis in the chick embryo chorioallantoic membrane assay, in vivo. These results represent the first demonstration that biologically active angiostatin-like fragments can be generated by inflammatory human neutrophils. Because angiostatin is a potent inhibitor of angiogenesis, tumor growth, and metastasis, the data suggest that activated PMN not only act as potent effectors of inflammation, but might also play a critical role in the inhibition of angiogenesis in inflammatory diseases and tumors, by generation of a potent anti-angiogenic molecule. ***The Journal of Immunology,*** **2002, 168: 5798–5804.**

The formation of new capillaries from preexisting blood vessels, termed angiogenesis or neovascularization, is a process that in adults is tightly controlled in physiological conditions and limited to processes such as reproduction, wound healing, and hair growth. However, dysregulated angiogenesis can be observed in many pathological conditions such as cardiovascular diseases, rheumatoid arthritis, psoriasis, diabetic retinopathy, and tumors (1), presumably as a consequence of the local imbalance between proangiogenic and anti-angiogenic factors. In this regard, a number of endogenous inhibitors targeting the vasculature have been identified, but only some of them, including angiostatin, endostatin, and anti-thrombin, seem to selectively act on the proliferating endothelial cell compartment of the newly formed blood vessels (2). Angiostatin, the first of these angiogenic inhibitors to be discovered, has been initially isolated from the urine of tumor-bearing mice, and then characterized as an internal fragment of plasminogen composed of the first four kringle domains of its precursor (angiostatin K1–4) (3). Several proteolytic mechanisms whereby plasminogen is cleaved to form angiostatin-related proteins have been subsequently identified, with the resulting cleavage products known to contain different NH_2 and COOH termini (4). In addition to the K1–4 species (generated by some metalloproteinases, cathepsin D, urokinase, tissue plasminogen activator, and plasmin autoproteolysis in the presence of free sulfidryl donors; Refs. 5–8), angiostatin K1–5 (9, 10) and angiostatin K1–3 have also been identified, the latter being a proteolytic byproduct of porcine pancreatic elastase activity (11). All of these angiostatin-related proteins exert similar endothelial cell inhibitory activities including apoptosis induction and migration inhibition, as well as tumor growth inhibition in experimental neoplasia, with various degrees of effectiveness (4). To our knowledge, only macrophages and tumor cells have been reported to generate angiostatin, mainly as angiostatin K1–4 and/or K1–5, depending on the enzymatic activities released by these cells (10, 12–14).

Polymorphonuclear neutrophils (PMN)[3] are the most abundant circulating blood leukocytes that, typically provide the first-line defense against infections. However, in recent years it has become clear that the contribution of neutrophils to host defense and natural immunity extends well beyond their traditional role as professional phagocytes. Indeed, neutrophils can be induced to express a number of genes whose products lie at the core of inflammatory and immune responses, engaging them in a complex cross-talk with immune cells that bridges innate and adaptive immunity (15). Interestingly, activated neutrophils are also able to produce a number of molecules that appear to either positively or negatively modulate the angiogenic process (15). In addition, PMN store a number of proteinases in their granules, including gelatinase B (matrix metalloproteinase (MMP)-9) and urokinase (16), which, at least in vitro, are known to generate angiostatin K1–4 (5, 7). However, despite of this body

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[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophils; NE, neutrophil elastase; VEGF, vascular endothelial growth factor; FGF2, basic fibroblast growth factor; CAM, chorioallantoic membrane; SFCM, serum-free culture medium; CB, cytochalasin B; GROα, growth-related gene product-α; HPg, human Glu-plasminogen; PAI, plasminogen activator inhibitor-1; CG, cathepsin G; MMP, matrix metalloproteinase; IE, *N*-methoxysuccinil-Ala-Ala-Pro-Val chlormethyl ketone.

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of information, the role of PMN in angiogenesis has remained poorly investigated.

In the present study, we addressed the question of whether neutrophils could directly generate biologically active angiostatin from plasminogen. We show that activated neutrophils are indeed capable of generating anti-angiogenic peptides comprising kringle domains 1–3 of plasminogen. We also provide evidence that neutrophil elastase (NE), directly secreted by stimulated PMN, is the enzyme responsible for the generation of angiostatin K1–3. Finally, we show that the affinity-purified angiostatin peptides generated by neutrophils can inhibit basic fibroblast growth factor (FGF2) plus vascular endothelial growth factor (VEGF)-induced endothelial cell proliferation in vitro, and both VEGF-induced angiogenesis in the matrigel assay as well as FGF2-induced angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay in vivo.

Materials and Methods

Cell purification and culture

Highly purified granulocytes (>99%) and Percoll-purified monocytes were isolated under endotoxin-free conditions from buffy coats of healthy donors (17). After purification, leukocytes were suspended at 10^7/ml in RPMI 1640 medium (Biowhittaker, Walkersville, MD) supplemented with 10% low-endotoxin FBS (<0.009 ng/ml; Biochrom, Berlin, Germany), and incubated for 3 h in polypropylene tubes at 37°C, 5% CO_2 atmosphere, before stimulation. In selected experiments, preincubation was conducted in the presence or absence of either 100 U/ml IFN-γ (Hoffman-La Roche, Basel, Switzerland) or 1000 U/ml IFN-α (Roferon-A; Hoffmann-La Roche; Ref. 18). Cells were then washed once with PBS, suspended in serum-free culture medium (SFCM), and routinely stimulated for 15 min with 100 nM fMLP (Sigma-Aldrich, St. Louis, MO) after a previous 5-min preincubation with 5 μg/ml cytochalasin B (CB) (19). In selected experiments, neutrophils were stimulated either for 15 min with IL-8 (provided by Dr. M. Ceska, Sandoz, Vienna, Austria), growth-related gene product-α (GROα), or for 1 h with TNF-α, GM-CSF (Peprotech, Rocky Hill, NJ), and LPS (from *Escherichia coli*, serotype 026:B6, Sigma-Aldrich). After stimulation, SFCM was harvested and stored at −20°C. The human pancreatic carcinoma cell line ASPC1 (from American Type Culture Collection, Manassas, VA) was maintained in RPMI 1640 supplemented with 10% FBS and used to generate SFCM, as described (20). All reagents used were of the highest available grade and were dissolved in pyrogen-free water for clinical use.

Angiostatin generation

A total of 100 μg/ml of human Glu-plasminogen (HPg; purchased from Enzyme Research Laboratories, South Bend, IN) were added to the SCFM harvested from the leukocyte cultures and then incubated at 37°C under continuous shaking for 14 h. Small aliquots of the latter mixtures were then withdrawn and assessed for angiostatin generation by Western blot analysis. In some experiments, HPg cleavage was also conducted in the presence of the following proteinase inhibitors (added 1 h before HPg): 5 mg/ml α1-antitrypsin, 1 mM *N*-methoxysuccinil-Ala-Ala-Pro-Val chlormethyl ketone (IE), 0.3 μM aprotinin, 25 mM EDTA (Merck, Frankfurt, Germany), 100 μg/ml plasminogen activator inhibitor-1 (PAI) (Calbiochem, San Diego, CA). Purified NE, cathepsin G (CG) (ICN Biochemicals, Aurora, OH), and MMP-9 (Roche Molecular Biochemicals, Mannheim, Germany) were also incubated with HPg in the absence or the presence of the specific inhibitors as controls. Samples (100 ng/lane) were electrophoresed under nonreducing conditions on 10% polyacrylamide gels in Tris-glycine running buffer and subsequently transferred to nitrocellulose membranes by electroblotting. Membranes were first blocked for 1 h at room T in TBS/T (20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20) containing 5% BSA, and then incubated overnight at 4°C in the presence of a 2–2.5 μg/ml of mAbs directed against kringles 1–3 (K1–3) of HPg (from American Diagnostica, Greenwich, CT, or clone 10-V-1 from Calbiochem). Ab binding was detected by using HRP-conjugated anti-mouse IgG (1/3000 dilution in TBS/T containing 3% BSA) and revealed using the chemiluminescence system (ECL; Amersham Pharmacia Biotech, Piscataway, NJ).

Lysine-Sepharose purification of angiostatin

To obtain sufficient amounts of PMN-derived angiostatin to be used for the various biological assays (see *Results*), 1.5 mg HPg was incubated for 14 h at 37°C with 10 ml of SFCM harvested from CB plus fMLP-stimulated PMN. The reaction products were then loaded to a column containing 5 ml of lysine Sepharose 4B (Amersham Pharmacia Biotech), previously equilibrated with binding buffer (50 mM phosphate buffer, pH 7.5). The column was washed with 10 vol of binding buffer, 5 vol of 0.5 M NaCl dissolved in binding buffer to remove nonspecifically bound proteins, and finally eluted with 0.2 M 6-aminocaproic acid to recover angiostatin, according to the protocol described by O'Reilly et al. (21). Fractions (0.5 ml) were collected, pooled, and 6-aminocaproic acid removed by gel filtration on a PD-10 desalting column (Amersham Pharmacia Biotech) previously equilibrated with either serum-free RPMI or distilled water. The resulting products were quantified by measuring their absorbance at 280 nm, filter-sterilized, divided into working aliquots, and stored at −80°C. Angiostatin preparations were subjected to Western blot analysis, and purity evaluated to be >95% by silver staining of polyacrylamide gels (7).

Measurement of NE activity

The amount of bioactive NE released from stimulated PMN was estimated by measuring the rate of hydrolysis of the NE specific chromogenic substrate, *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide (Sigma-Aldrich) (22). Briefly, 100 μl of either purified NE or PMN-derived SFCM were dispensed into 96-well plates and incubated for 5 min at 37°C. A total of 100 μl of substrate solution (1 mM *N*-methoxysuccinyl-Ala-Ala-Pro-Val *p*-nitroanilide diluted in 100 mM Tris-HCl buffer, pH 8) were then added, and substrate hydrolysis was monitored for up to 1 h in an automated microplate spectrophotometric reader (Spectra Count, BS1000; Packard Instrument, Meriden, CT) for the release of *p*-nitroanilide. Each sample was run in duplicate. The amount of bioactive elastase detected in SFCM was calculated according to a calibrated standard curve.

Endothelial cell proliferation assay

HUVEC were isolated from the umbilical vein as described (23) and grown in M199 medium (Sigma-Aldrich) supplemented with 20% FBS, antibiotics, 5 U/ml sodium heparin (Bristol-Myers Squibb, Latina, Italy), and 30 μg/ml endothelial growth supplement (Sigma-Aldrich). For the cell proliferation assay, low passage (p2-p4) HUVEC were harvested by trypsinization and seeded at 6,250 cells/cm^2 in 96-well plates in complete medium in the absence of endothelial growth supplement. After 24 h, cells were added with fresh medium containing FGF2 and VEGF (both at 30 ng/ml) in 5% FBS in the absence or in the presence of affinity-purified neutrophil-generated angiostatin (PMN-Angio K1–3) or commercial angiostatin K1–3 (Calbiochem). After a further 72 h of incubation, cells were trypsinized and counted.

In vivo angiogenesis

The Matrigel model of angiogenesis in vivo (24) as modified by Albini et al. (25) was used. A potent angiogenic mixture of VEGF (100 ng/ml) and TNF-α (2 ng/ml) was added to liquid Matrigel containing 26 U/ml of heparin (VEGF plus TNF plus heparin) at 4°C with or without 0.5 μg/ml commercial angiostatin or PMN-Angio K1–3, to a final volume of 600 μl. The Matrigel suspension was slowly injected s.c. into the flanks of C57 mice using a cold syringe, where the gel rapidly polymerizes to form solid implants. After 4 days, gels were collected, weighed, and subjected to analysis of the hemoglobin content as an estimate of vascularization as previously described (25). Portions of some samples were paraffin embedded and stained with H&E for histological analysis or with anti-Factor VIII mAb for histochemical identification of vessels.

Chick embryo CAM assay

Fertilized white leghorn chick eggs were incubated under conditions of constant humidity at 37°C. On the third day of incubation, a square window was opened in the egg shell after removal of 2–3 ml of albumen so as to detach the developing CAM from the shell. The window was sealed with a glass of the same size, and the eggs were returned to the incubator. At day 8, 1-mm^3 sterilized gelatin sponges (Gelfoam; Upjohn Company, Kalamazoo, MI) adsorbed with rFGF2 (500 ng/embryo) dissolved in 2 μl of PBS were implanted on the top of growing CAMs (26). Immediately after implantation, sponges were treated with 1 μg of PMN-Angio K1–3 preparations. Angiostatin treatment was repeated daily until day 10. Sponges containing vehicle or FGF2 alone were used as negative and positive controls, respectively. CAMs were examined under a stereomicroscope and blood vessels around the sponges were counted at day 11.

Statistical analysis

Data are expressed as means ± SD. Statistical evaluation was performed by the Student's *t* test, and considered significant if p values were <0.05.

Results

Conditioned medium from activated neutrophils results in proteolysis of plasminogen

In initial experiments, we analyzed human PMN for their in vitro capacity to generate enzymatic activities able to excise angiostatin from HPg. For this purpose, SFCM harvested from PMN stimulated under conditions known to induce optimal degranulation (CB plus 100 nM fMLP for 15 min) was incubated with 100 μg/ml HPg at 37°C for 14 h. Reaction products were then analyzed under nonreducing conditions by Western blot, using mAbs recognizing the kringle 1–3 (K1–3) domains of HPg (Fig. 1*A*). Only a prominent band at 88 kDa corresponding to HPg (Fig. 1*A*, *lane 1*) was detected in the digestions performed with SCFM derived from either resting (Fig. 1*A*, *lane 2*) or CB-treated neutrophils (data not shown). In contrast, SFCM recovered from CB plus fMLP-stimulated neutrophils completely degraded HPg, generating three major protein bands with molecular masses at ~32 kDa (Fig. 1*A*, *lane 3*), which corresponded to the molecular masses of angiostatin K1–3 (Fig. 1*A*, *lane 7*), but not of angiostatin K1–4 (Fig. 1*A*, *lane 6*). Under these experimental conditions, end-generation of angiostatin K1–3 peptides consisted of multiple steps. At earlier times of incubation, several transient bands of low intensity in the plasmin H chain and angiostatin K1–4 molecular mass range (66 and 45 kDa, respectively) were observed (data not shown). Importantly, formation of angiostatin K1–3 fragments was also induced by SFCM obtained from PMN stimulated with fMLP in the absence of CB (Fig. 1*B*, *lane 3*), IL-8 (Fig. 1*C*, *lanes 2* and *3*), GROα (Fig. 1*C*, *lanes 5* and *6*), and GM-CSF (Fig. 1*D*); though the extent of HPg degradation and angiostatin K1–3 formation depended on the concentration of the stimulus used. In contrast, other inflammatory compounds such as IFN-α (Fig. 1*B*, *lane 4*), IFN-γ (Fig. 1*B*, *lane 6*), LPS, or TNF-α (Fig. 1*D*) did not induce HPg degradation. However, under the same experimental conditions, either LPS or TNF-α triggered IL-8 release (data not shown) as expected (15), whereas both IFN-γ and IFNα enhanced agonist-stimulated NE secretion (see below). Consistent with the evidence that PMN generate angiostatin K1–3, incubation of HPg with SFCM harvested from autologous monocytes proved ineffective in degrading HPg (Fig. 1*A*, *lanes 4* and *5*).

Identification of neutrophil-secreted elastase as the angiostatin K1–3-generating enzyme

In addition to MMP-9 and urokinase that are known to generate angiostatin (5, 7), PMN also contain other protineases, including CG and NE, able to degrade HPg into proteolytic fragments (27). To identify the type of enzymes present in the PMN-derived supernatants that are responsible for the generation of angiostatin peptides, HPg and either SFCM collected from stimulated neutrophils or as controls, purified enzymes were mixed with selective

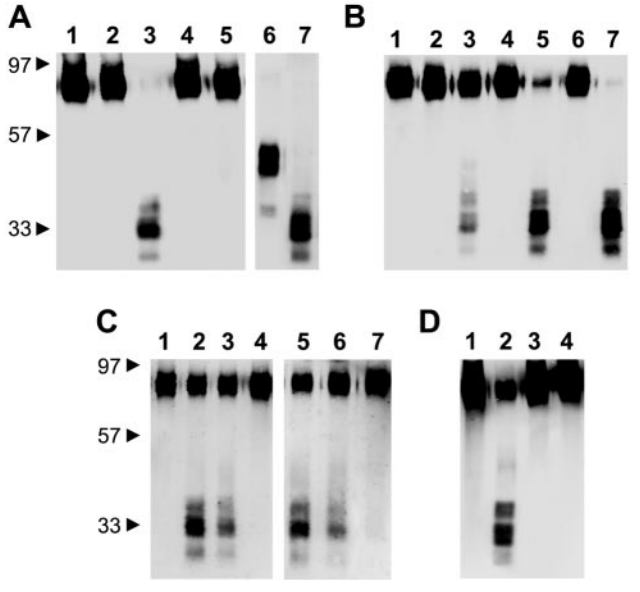

FIGURE 1. Generation of angiostatin K1–3 by SFCM harvested from activated human neutrophils. HPg (100 μg/ml) was incubated at 37° for 14 h with shaking in the presence of SFCM obtained from leukocytes activated under various conditions. Samples (100 ng/lane) were electrophoresed and analyzed by Western blot with a mAb raised against the K1–3 portion of HPg. *A*, Angiostatin K1–4 (*lane 6*) and angiostatin K1–3 (*lane 7*) from commercial sources were run in parallel as controls. *A*, HPg incubated with medium (*lane 1*) or SFCM from: resting PMN (*lane 2*); CB/fMLP-stimulated PMN (*lane 3*); resting monocytes (*lane 4*); CB/fMLP-stimulated monocytes (*lane 5*). *B*, HPg incubated with medium (*lane 1*) or SFCM from: resting PMN (*lane 2*), 100 nM fMLP-stimulated PMN (*lane 3*), PMN treated with IFN-α, alone (*lane 4*) or in combination with fMLP (*lane 5*), PMN treated with IFN-γ, alone (*lane 6*) or in combination with 100 fMLP (*lane 7*). *C*, HPg incubated with SFCM from: resting PMN (*lane 1*), PMN stimulated with 500 ng/ml (*lane 2*), 100 ng/ml (*lane 3*), and 10 ng/ml IL-8 (*lane 4*); PMN stimulated with 500 ng/ml (*lane 5*), 100 ng/ml (*lane 6*), and 10 ng/ml GROα (*lane 7*). *D*, HPg incubated with SFCM from PMN treated with medium (*lane 1*), 10 ng/ml GM-CSF (*lane 2*), 100 ng/ml LPS (*lane 3*), and 5 ng/ml TNF-α (*lane 4*).

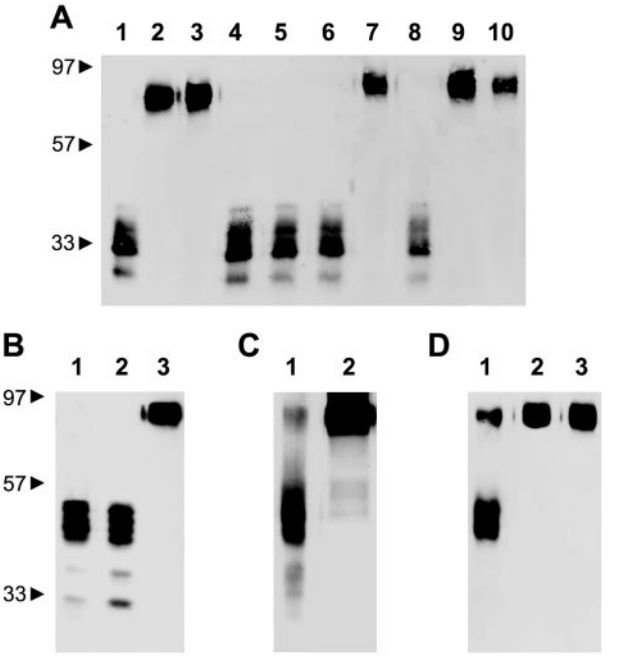

FIGURE 2. Effect of different types of proteinase inhibitors on the proteolysis of plasminogen mediated by neutrophil-derived SFCM. Selective proteinase inhibitors were added to either SFCM harvested from CB/fMLP-stimulated PMN or purified enzymes from commercial sources, 1 h before the incubation with HPg for 14 h. Samples were then analyzed for kringle-containing HPg fragments by Western blot. *A*, SFCM harvested from CB/fMLP-stimulated PMN incubated with HPg alone (*lane 1*) or with the following proteinase inhibitors: αl-anti-trypsin (*lane 2*), IE (*lane 3*), EDTA (*lane 4*), PAI (*lane 5*), aprotinin (*lane 6*). HPg incubated in medium alone (*lane 7*), or with purified NE in the absence of inhibitors (*lane 8*), or in the presence of αl-anti-trypsin (*lane 9*), or IE (*lane 10*), is also shown. *B*, HPg incubated with purified CG in the absence of inhibitors (*lane 1*), or with IE (*lane 2*), and αl-anti-trypsin (*lane 3*). *C*, HPg incubated with purified MMP-9 in the absence (*lane 1*) or in the presence of EDTA (*lane 2*). *D*, HPg incubated with ASPC1-conditioned SFCM without (*lane 1*) or with PAI (*lane 2*) or aprotinin (*lane 3*).

proteinase inhibitors before a 14-h incubation and Western blot analysis. Fig. 2 shows that, among many others, only α1-antitrypsin (Fig. 2*A*, *lane 2*), a serine proteinase inhibitor blocking the enzymatic activities of both elastase (Fig. 2*A*, *lane 9*) and CG (Fig. 2*B*, *lane 3*), as well as IE (Fig. 2*A*, *lane 3*), a specific elastase inhibitor (Fig. 2*A*, *lane 10*) which does not affect CG activity (Fig. 2*B*, *lane 2*), completely inhibited the conversion of HPg to angiostatin exerted by SFCM derived from stimulated PMN. Similar to neutrophil-derived SCFM (Fig. 2*A*, *lane 1*), purified NE generated angiostatin K1–3 fragments (Fig. 2*A*, *lane 8*), whereas purified CG incubated with HPg only yielded angiostatin K1–4 fragments (Fig. 2*B*, *lane 1*). Based on these findings and on the specific effect of IE, we conclude that NE is the enzyme responsible for the generation of angiostatin K1–3 by neutrophil-derived SFCM. This conclusion was consistent with the effects of the other inhibitors tested. For example, while EDTA, a metalloprotease inhibitor, was unable to inhibit the angiostatin-generating capacity of neutrophil-derived SFCM (Fig. 2*A*, *lane 4*), it effectively blocked the cleavage of HPg determined by recombinant exogenous MMP-9 (Fig. 2*C*, *lane 2*). Similarly, PAI and aprotinin did not affect the generation of angiostatin by PMN-derived SFCM (Fig. 2*A*, *lanes 5* and *6*), but effectively blocked the capacity of SFCM from ASPCI cells to generate angiostatin (Fig. 2*D*, *lanes 2* and *3*) that is mediated by urokinase (20). Finally, measurement of NE activity (Table I) revealed that the amount of bioactive elastase released by PMN under the various stimulatory conditions was in line with previous findings (18, 28–32) and correlated with the capacity of SCFM to convert HPg into angiostatin K1–3 (Fig. 1). In general, we observed that concentrations of NE >3 μg/ml were required to obtain a complete degradation of 100 μg/ml HPg in a 14-h incubation time. Moreover, both IFN-α and IFN-γ significantly primed PMN for an enhanced NE secretion in response to 100 nM fMLP, 100 ng/ml IL-8, and GROα (Table I), and as a consequence, rendered the related SCFM much more efficient in degrading HPg and generating angiostatin K1–3 (Fig. 1*B*, *lanes 5* and *7* for fMLP, and data not shown for IL-8 and GRO).

Neutrophil-derived angiostatin K1–3 inhibits in vitro FGF2 plus VEGF-induced endothelial cell proliferation and angiogenesis in vivo

We subsequently determined whether angiostatin K1–3 generated by PMN-derived SFCM exerted anti-angiogenic activities. Large preparations of SFCM harvested from PMN stimulated with CB plus fMLP and incubated with HPg were affinity-purified (21) (Fig. 3, *insert*, *lane 3*). Initially, we tested the effect of PMN-Angio K1–3 on the proliferation of HUVEC cells induced by FGF2 plus VEGF. As shown in Fig. 3, PMN-Angio K1–3 suppressed the FGF2 plus VEGF-induced HUVEC proliferation in a dose-dependent manner, being at 20 μg/ml even more effective than equivalent amounts of commercial angiostatin K1–3 in the same assay (data not shown). Subsequently, we examined whether PMN-Angio K1–3 possessed the ability to inhibit VEGF-induced angiogenesis in vivo, using the Matrigel implant model (25). VEGF and TNF-α (VTH) produced a potent angiogenic response in vivo that was strongly inhibited by the addition of 0.5 μg/ml PMN-Angio K1–3 (Fig. 4). Histological examination of VTH-containing implants demonstrated the formation of dilated, hemorrhagic vessels and a massive cellular infiltrate through the Matrigel (Fig. 4*A*). Gels containing commercial angiostatin (Fig. 4*C*), as well as implants containing neutrophil-derived angiostatin (Fig. 4*D*) showed reduced infiltrating cells with the formation of a few small blood

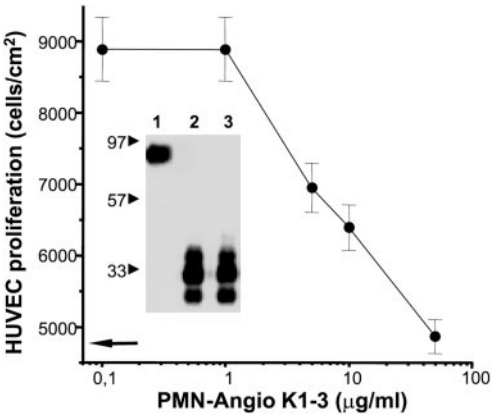

FIGURE 3. Inhibition of HUVEC proliferation in vitro by PMN-Angio K1–3. Proliferation of HUVEC mediated by FGF2 plus VEGF (both at 30 ng/ml) was conducted in the presence of increasing concentrations of PMN-Angio K1–3. Data represent the mean ± SD of three determinations. Arrow indicates the basal proliferation in the absence of any mitogen. *Inset*, Western blot analysis of a typical PMN-Angio K1–3 preparation (*lane 3*), as compared with commercial Angio K1–3 (*lane 2*).

Table I. *Release of bioactive elastase (nanograms per milliliter) by stimulated neutrophils*[a]

		+ IFN-α	+ IFN-γ
Medium	237 ± 71 (n = 8)	200 ± 25 (n = 4)	248 ± 97 (n = 4)
5 ng/ml TNF-α	373 ± 21 (n = 3)		
100 ng/ml LPS	240 ± 50 (n = 2)		
5 μg/ml CB	256 ± 96 (n = 4)		
100 nM fMLP	1172 ± 344 (n = 8)	2261 ± 673 (n = 4)*	3437 ± 645 (n = 4)*
100 nM fMLP[b]	1050 ± 215 (n = 3)	2233 ± 156 (n = 3)*	
CB + fMLP	7200 ± 986 (n = 8)		
10 ng/ml GROα	222 ± 50 (n = 2)		
100 ng/ml GROα	699 ± 283 (n = 4)		1816 ± 217 (n = 4)*
500 ng/ml GROα	1458 ± 200 (n = 2)		
10 ng/ml IL-8	270 ± 68 (n = 2)		
100 ng/ml IL-8	960 ± 103 (n = 4)	2063 ± 543 (n = 4)*	2291 ± 958 (n = 4)*
500 ng/ml IL-8	1963 ± 250 (n = 4)		
10 ng/ml GM-CSF	860 ± 221 (n = 3)		

[a] PMN (10^7/ml) were incubated for 3 h with or without 1000 U/ml IFN-α or 100 U/ml IFN-γ before stimulation. SFCM were collected after stimulation with fMLP, IL-8, and GROα for 15 min, or after stimulation with LPS, TNF-α, and GM-CSF for 1 h. The amount of bioactive NE released by PMN was estimated as indicated in *Materials and Methods*. Values indicate the means ± SD of the number of experiments indicated by the parentheses. Statistical evaluation was performed between agonist and medium, in the absence or the presence of IFN-α/IFN-γ.

[b] In these experiments, neutrophils were stimulated with fMLP added at the same time as IFN-α.

*, $p < 0.05$.

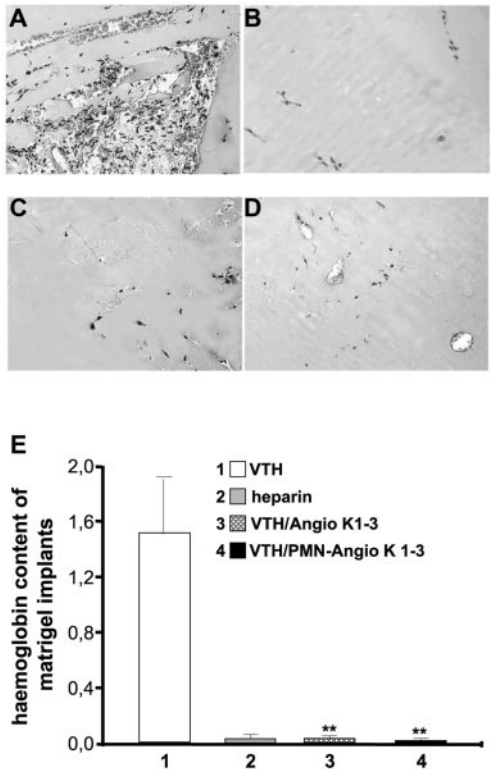

FIGURE 4. Inhibition of in vivo angiogenesis by PMN-Angio K1–3. *A–D*, Histology of Matrigel pellets implanted in vivo, stained with H&E. *A*, VEGF and TNF-α (VTH) induced a massive infiltrate with large, hemorraghic vessels in the matrix. In the presence of (0.5 μg/ml) commercial angiostatin (*C*) or PMN-Angio K1–3 (*D*) few cells penetrate into the matrix and only occasional small vessels are observed. *B*, Matrigel pellets containing only heparin. *E*, Hemoglobin content of the Matrigel sponges indicates the extent of blood content; and therefore, an estimate of the extent of vascularization. VTH induced a strong angiogenic response, whereas inclusion of PMN-Angio K1–3 significantly ($p < 0.03$ using Student's *t* test) reduced this response to background levels.

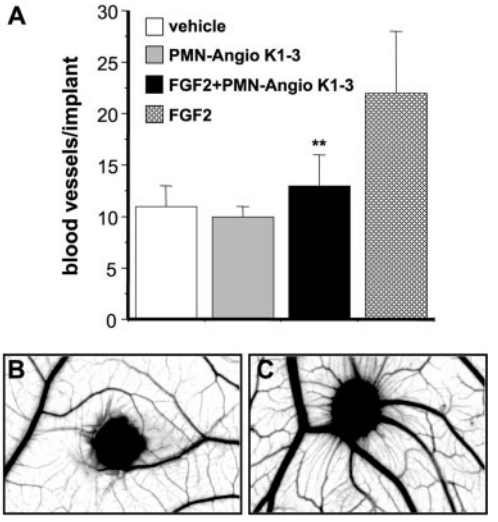

FIGURE 5. Effect of neutrophil-generated angiostatin on CAM neovascularization. Chicken embryo CAMs were implanted with gelatin sponges treated with 500 ng FGF2 or vehicle at day 8 of development. When indicated, 1 μg of PMN-Angio K1–3 were added daily to the sponges until day 10. The day after, macroscopic blood vessels infiltrating the sponge were counted under a stereomicroscope (*A*) and photographed (*B* and *C*). Note the absence of newly formed blood vessels converging toward the sponge in PMN-Angio K1–3-treated implants (*B*) when compared with implants treated with FGF2 alone (*C*). Data are the mean ± SD of four determinations. **, Statistically different from FGF2 alone ($p < 0.03$, Student's *t* test).

vessels. Furthermore, measurement of the hemoglobin content as an indicator of the angiogenic response showed that the mean hemoglobin level was significantly lower in VTH plus angiostatin-containing pellets ($p < 0.03$, Student's *t* test) than in gels with VTH alone (Fig. 4*E*). Finally, PMN-Angio K1–3 was also found to inhibit angiogenesis induced by rFGF2 in the chick embryo CAM assay (Fig. 5). Indeed, daily administration of PMN-Angio K1–3 prevented neovascularization triggered by FGF2 without affecting the physiological blood vessel formation observed in FGF2-untreated CAMs.

Discussion

Numerous studies have established that activated PMN express and secrete several inflammatory and immunoregulatory proteins, both in vitro and in vivo (15). Noteworthy is that many of these mediators could possibly influence, be it directly or indirectly, the neovascularization process. Neutrophil-derived proangiogenic factors include VEGF (15), heparin-binding epidermal growth factor (33), TNF-α (15), IL-8, GROα (15), and FGF2 (34); whereas the neutrophil-derived factors recognized to be anti-angiogenic molecules are IL-12 (15), inducible protein 10, monokine induced by IFN-γ (18), and cationic proteins, such as bactericidal/permeability-increasing protein (16). Similarly, PMN represent a particularly rich source of proteinases (16), which are known to exert contrasting functions in the context of the angiogenic process. On one hand, these enzymes degrade extracellular matrix molecules, modulate mechanical structures, and liberate extracellular matrix molecules-bound growth factors, which are all activities fundamental for the initiation of the angiogenic process. In contrast, proteinases are involved in the generation of several endogenous angiogenic inhibitors from large precursor proteins (2). However, despite this body of information, it is not clear yet what the actual role of PMN in the regulation of angiogenesis might be (35, 36), other than their ability to induce angiogenesis in vitro (37, 38), and to indirectly mediate the proangiogenic effects of Fas, laminin, and Tat in vivo (39–41).

In this study, we provide evidence that supernatants from human neutrophils activated with proinflammatory agonists including fMLP, a bacterial derived-peptide, chemokines such as IL-8 and GROα, and growth factors such as GM-CSF, contain proteolytic activities that mediate the conversion of purified plasminogen into angiostatin-like fragments corresponding to the known angiostatin K1–3. The latter has been originally identified as a product of the in vitro digestion of HPg with porcine pancreatic elastase (11), and has been subsequently demonstrated to be the portion of angiostatin K1–4 retaining the strongest inhibitory activity on endothelial cell growth, as well as the strongest tumor-suppressing activity (11, 42). To our knowledge, these findings are the first identification of a nonneoplastic human cell type able to generate angiostatin K1–3. Through the use of specific protease inhibitors and several other criteria, we identified NE as the major enzyme responsible for the angiostatin-generating capacity of neutrophil-derived supernatants. Furthermore, cytokines such as IFN-γ and IFN-α that enhanced the NE-secretory capacity of stimulated neutrophils also potentiated the ability of PMN to generate angiostatin K1–3. Moreover, supporting the specific role of NE, freshly isolated monocytes, stimulated as neutrophils, did not show any angiostatin-forming capacity. Although we cannot exclude that other more selective agonists might induce monocytes to generate plasminogen degrading activities, it is worth remarking that monocytes

only express small amounts of NE (43) that are completely lost during differentiation into macrophages and replaced with the macrophage metalloelastase (MMP-12) (43, 44).

The angiostatin K1–3 generated by neutrophils was then enriched by affinity purification and also shown to be biologically active on the basis of its ability to inhibit endothelial cell proliferation in vitro, and to inhibit angiogenesis induced by VEGF as well as by FGF2 in the chick embryo assay in vivo. The ability of human neutrophils to produce biologically active angiostatin-like fragments extends the previous findings on the production of angiostatin by other phagocytes, namely murine macrophages in a model of Lewis Lung Carcinoma (12), after thioglycollate-elicited peritoneal inflammation (13), and after TGF-β_1-activation (13). Interestingly, angiostatin generation by macrophages was shown to be dependent on either MMP-12 activity (12), or as a consequence of the proteolytic regulation of membrane bound plasmin (13), and different from neutrophils, they mainly generated angiostatin K1–4.

Even though the physiological role of PMN-generated angiostatin remains elusive, it potentially has broad implications. Most of the physiological events and pathological inflammatory disorders where angiogenesis plays a key role, such as wound healing, rheumatoid arthritis, cardiovascular diseases, psoriasis, and diabetic retinopathy (1) are processes in which neutrophils represent a prominent cellular component (45). The data shown in this study together with previous observations (15) suggest that the contribution of neutrophils to angiogenesis regulation most likely depends upon a fine balance of proangiogenic and anti-angiogenic factors. It is plausible that PMN, once recruited into an inflammatory site, may secrete either pro- or anti-angiogenic molecules depending on the environmental stimuli. For instance, previous in vitro findings demonstrated that if neutrophils sense LPS or TNF-α, they are induced to produce mainly proangiogenic factors, such as VEGF or IL-8 (15). In contrast, whether PMN encounter IFN-γ or IFN-α simultaneously with LPS or TNF-α, then they are triggered to additionally and specifically release anti-angiogenic molecules, including IL-12, inducible protein 10, monokine induced by IFN-γ, and IFN-inducible T cell α-chemoattractant (15, 18). Our present data are in complete agreement with these observations, given that LPS or TNF-α did not provoke any release of HPg-degrading activity, whereas both IFN-γ or IFN-α dramatically enhanced the release of NE by neutrophils stimulated with fMLP, IL-8, and GROα; therefore, strongly enhancing the angiostatin formation. Taken together, these data not only suggest that generation of angiostatin by neutrophils might play a negative feedback role in the neovascularization processes induced by proangiogenic peptides such as IL-8 and GRO (46), but also indicate that PMN may contribute to the anti-angiogenic activities of both IFN-γ and IFN-α in vivo (47–49).

In addition to inflammatory processes, neutrophils may also contribute to the generation of angiostatin in cancer. Although PMN are usually but a marginal component of both human and animal tumors, recent studies using cytokine gene transfer strategies have suggested that they are active in immunosurveillance against several tumor types (50). It has been shown that many cytokines and chemokines released by engineered tumor cells, for instance IL-1, IL-2, IL-12, IFN-α, IFN-β, IFN-γ, G-CSF, IL-8, and GROα (50) quickly recruit a massive local reaction that leads to the rejection of engineered tumor cells and the establishment of a significant immunity against the wild-type parental tumor. PMN play a key role in all of these cytokine-induced tumor rejections, often in cooperation with $CD8^+$ T lymphocytes. Although recruited PMN may produce several cytotoxic mediators, including reactive oxygen species, proteases, membrane-perforating agents, and soluble mediators of cell killing such as TNF-α, the formation of angiostatin K1–3 mediated by the release of NE might additionally contribute to the anti-tumor activities observed.

In conclusion, our results attribute a novel and unsuspected role of angiogenesis inhibition to neutrophils. Their ability to release large amounts of elastase and other proteinases, classically associated with defense purposes (45) or tissue injury (51, 52), might actually have an additional biological function. Based on the findings presented here, proteinase release by PMN might in fact contribute to generation of endogenous inhibitors of angiogenesis in vivo from large precursors having distinct functions, as has already been observed for degradation of collagen XVIII and serpin antithrombin in vitro (53, 54). Whether NE or other enzymatic activities released by activated neutrophils participate to the formation of anti-angiogenic factors (such as fragments of fibronectin, Kringle 5 of plasminogen, restin) has not yet been established and is currently under investigation in our laboratories.

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