Pro-angiogenic role of neutrophil-like inflammatory heterophils during neovascularization induced by growth factors and human tumor cells

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Abstract

A quantitative in vivo angiogenesis model employing collagen onplants placed on the chick embryo chorioallantoic membrane (CAM) has been used in this study to assess the spatial and temporal associations between neutrophil-like inflammatory cells, namely chicken heterophils, and the development of new blood vessels. Previously we have demonstrated that monocytes/macrophages infiltrating the onplants were associated with extracellular matrix remodeling and angiogenesis, in particular by delivering MMP-13 collagenase. By introducing chicken gelatinase B (chMMP-9) as a specific marker for heterophils, we now show that the onset and extent of angiogenesis induced by purified growth factors or by human HT-1080 fibrosarcoma cells correlated with the initial influx of chMMP-9-positive heterophils. This early heterophil arrival was followed by the infiltration of monocytes/macrophages and appeared to sustain further blood vessel formation. The disruption of inflammatory cell influx by two mechanistically distinct anti-inflammatory drugs cortisone and ibuprofen significantly inhibited angiogenesis, indicating a functional involvement of these inflammatory cells in new blood vessel development. A direct addition of isolated heterophils or purified chMMP-9 into the HT-1080-onplants engrafted into cortisone- or ibuprofen-treated embryos reversed the anti-angiogenic effects of the drugs. The exogenously-added heterophils induced in vivo a further infiltration of endogenous heterophils and monocytes and dramatically rescued the impaired angiogenesis, highlighting the importance of early inflammatory leukocytes in tumor-induced angiogenesis. Moreover, purified heterophils incorporated into onplants lacking growth factors or tumor cells, induced angiogenesis in non-treated embryos, further indicating a direct pro-angiogenic role for neutrophil-like leukocytes.
Introduction

Tumor-induced angiogenesis occurs in a complex microenvironment, in which a balance between anti-angiogenic and pro-angiogenic factors has been shifted in favor of mediators promoting the expansion of pre-existing blood vessels\textsuperscript{1,2}. One of the possible mechanisms underlying the angiogenic switch is a production by tumor cells of inflammatory mediators responsible for leukocyte recruitment. In turn, infiltrated leukocytes release an array of highly active mediators such as cytokines, chemokines and growth factors, which further contribute to tumor progression by inducing tumor cells to produce additional angiogenic factors. Correspondingly, chronic inflammation and infiltration by leukocytes has been linked to neovascularization at the sites of human tumor development. Additional data suggests that inflammation can even be a prerequisite for the development of certain malignancies\textsuperscript{3-13}. Inhibition of tumor development and tumor angiogenesis by anti-inflammatory drugs, including those targeting cyclooxygenase (COX)-2, further suggests an active role of infiltrating leukocytes in neovascularization\textsuperscript{2,14-21}.

Monocytes/macrophages, mast cells, neutrophils and T-lymphocytes represent the major types of infiltrating leukocytes found around and within solid tumors\textsuperscript{3,5,7,8,10,11}. During inflammatory responses, neutrophils are usually the first recruited effectors that produce potent chemotactic factors likely guiding further influx of other inflammatory leukocytes such as monocytes/macrophages and T-lymphocytes\textsuperscript{8,11,22-25}. Altogether, inflammatory conditions strongly contribute to a microenvironment favoring tumor growth and neovascularization.

In addition to cytokines and growth factors, inflammatory leukocytes also release a number of proteases, including matrix metalloproteinases (MMPs)\textsuperscript{4,7,11,26-28}. MMPs are critical
for remodeling of the tissue extracellular matrix (ECM) and are involved in many steps of tumor progression, including tumor angiogenesis\textsuperscript{29,30}. Recent evidence points to the critical and specific functions of MMPs derived from activated stromal cells and inflammatory leukocytes at sites of tumor development\textsuperscript{7,8,11,28,31-36}. Although there is no strict specificity, distinct subsets of tumor-associated leukocytes are often linked to production of characteristic MMPs\textsuperscript{28}. In this regard, infiltrating neutrophils constitute a particular source of MMP-9 (gelatinase B), because it is pre-synthesized and stored in specialized granules and thus is ready to be released rapidly at the site of inflammation\textsuperscript{26,37-42}. Other types of inflammatory leukocytes such as monocytes/macrophages and mast cells produce a number of MMPs, including MMP-9, which functionally contribute to angiogenesis triggered by inflammation\textsuperscript{25,43,44} and tumor growth\textsuperscript{1,7,34,35,45}.

To investigate the role of specific inflammatory MMP-carrying leukocytes in growth factor- and tumor-induced angiogenesis, we employed the chick embryo system involving collagen onplants placed on the top of the chorioallantoic membrane (CAM)\textsuperscript{46,47}. In this model, pre-existing blood vessels of the underlying CAM are induced by exogenously-added growth factors or tumor cells to sprout and grow upward into a three-dimensional collagen graft. These new blood vessels anastomose, generating a complete, blood-circulating angiogenic network. Using the CAM/collagen onplant model we have previously shown that the onset of angiogenesis is critically dependent on a stromal collagenase, chicken MMP-13 (\textit{ch}MMP-13), imported by hematopoietic cells of monocyte-macrophage lineage\textsuperscript{48}.

In this study we analyzed the role of host heterophils (the avian analogue of mammalian neutrophils\textsuperscript{49}) in angiogenesis induced by purified angiogenic growth factors (bFGF and VEGF) or, alternatively, by human tumor cells (HT-1080 fibrosarcoma) incorporated into the collagen onplant. Detailed morphological and immunohistochemical examination indicated that
heterophils rapidly enter the collagen/CAM tissue and that levels of angiogenesis in the onplants correlated positively with the heterophil influx. These inflammatory granulocytes were the main, if not the only cell type positive for chicken MMP-9 in the embryo, indicating that chMMP-9 can be used as a marker for heterophils and suggesting that heterophils import chMMP-9 protein to the sites of angiogenesis. Influx of heterophils into collagen onplants was followed by appearance of chMMP-13-positive monocyte/macrophages. The disruption of inflammatory cell influx by cortisone and the non-steroidal inhibitor ibuprofen, two mechanistically distinct anti-inflammatory drugs, resulted in significant inhibition of tumor-induced angiogenesis, indicating that the inflammatory leukocytes are required to sustain new blood vessel formation. A direct addition of isolated heterophils or purified chMMP-9 into onplants placed atop the CAM of ibuprofen-treated embryos completely reversed the anti-inflammatory effects of the drug and rescued angiogenesis, highlighting the importance of the influx of cytokine- and MMP-bearing inflammatory cells during growth factor- and tumor-induced angiogenesis.
Materials and methods

CAM angiogenesis assay

CAM angiogenesis assays were performed using shell-free 10 day-old chick embryos as described\textsuperscript{46,48}. Fertilized COFAL-negative White Leghorn chicken eggs (Charles River Labs, North Franklin, CT) were incubated in a rotating incubator at 38°C and 85% humidity. At day 3, the contents of the eggs were transferred to sterile plastic weigh boats, covered with square Petri dishes and kept in a stationary incubator at 38°C with 85% humidity.

Collagen onplants were prepared as described\textsuperscript{46,48}. Briefly, 8 volumes of 3.0 mg/ml type I rat tail collagen (BD Biosciences, Franklin Lakes, NJ) were neutralized with 1 volume of 10x PBS and 1 volume of 0.15 N NaOH/0.25 M HEPES buffer (pH 7.4). Two volumes of neutralized collagen were combined with one volume of PBS containing 1 mg/ml BSA (control onplants). To induce angiogenesis, angiogenic growth factors bFGF and VEGF were added at a final concentration of 16.7 μg/ml and 5 μg/ml, respectively (PeproTech, Rocky Hill, NJ). Alternatively, HT-1080 fibrosarcoma cells (ATCC, Rockville, MD) were used to induce angiogenesis (5x10\textsuperscript{4} cells per onplant). In the tumor cell-induced angiogenesis assays, neutralized collagen was prepared with 10x DMEM, and two volumes of neutralized collagen were combined with 1 volume of HT-1080 cell suspension in DMEM with 1 mg/ml BSA.

To assemble onplants, 30 μl of the final collagen mixture were placed atop two layers of nylon mesh (Tetko, Inc., Kansas City, MO) and allowed to polymerize at 37.5°C for 90 min. Solidified onplants were grafted on top of the CAM of day 10 chick embryos incubated ex ovo. Where indicated, the onplant-bearing animals were treated systemically with cortisone (0.5 mg/embryo; Sigma, St. Louis, MO) or ibuprofen (0.75 mg/embryo; Cayman Chemical, AnnArbor, MI). Both anti-inflammatory drugs were prepared in DMEM supplemented with 1%
methylcellulose and injected at 0.1 ml per embryo under the CAM twice: immediately prior to placing the onplants on day 10 and a second time on day 12. Hydroxamate MMP inhibitor GM6001 (Calbiochem, La Jolla, CA) was either added directly over the onplants (5 μl of 25 μM solution per onplant) or injected under the CAM for systemic treatment (10 μl of 1.25 mM solution) at the time of grafting and 48 h later. Recombinant chicken tissue inhibitor of metalloproteinase-2 (TIMP-2) was incorporated into the collagen onplant mixture to a final concentration of 2.85 μM. chMMP-9 was purified from serum-free conditioned medium from HD11 mononuclear cells as described and added at 100 ng per onplant. Angiogenesis was scored at 66-72 h using a stereomicroscope (Olympus, Melville, NY), as described. Blood vessels, visualized within the grids of the top mesh, i.e. above the lower mesh, were regarded as angiogenic. Data are presented as percentage of angiogenic grids (number of grids containing blood vessels over the total number of grids scored).

**Isolation of heterophils from peripheral blood**

Heterophils were isolated from the peripheral blood of 17 day-old chick embryos essentially as described. Blood was collected from the allantoic vein into 50 mM EDTA in PBS, layered over a discontinuous 1.077/1.119 Ficoll-Hypaque gradient, and centrifuged at 250xg for 45 min at 22°C. Erythrocytes were collected from the cell pellet and resuspended in PBS supplemented with 1.0% chicken serum (PBS/CS). Heterophils were collected from the 1.077/1.119 interface and the 1.119 band, placed into a siliconized tube and washed with PBS/CS. Heterophils comprised 85-95 % of the total leukocytes in the isolated preparations. Following centrifugation at 250xg for 7 min, erythrocytes and heterophils were resuspended at 2x10⁷ cells/ml in PBS/20 mM HEPES and kept on ice until use.
**Immunofluorescent staining and immunohistochemistry**

Collagen onplants with the underlying CAM and samples of normal CAM from the embryos containing no onplants, were snap-frozen on dry ice in the O.C.T. compound (Sakura Finetek, Torrance, CA) or fixed with Zn-formalin and embedded in paraffin.

For immunofluorescent analyses, 20 μm cryosections were placed on poly-L-lysine coated slides, fixed in 4% paraformaldehyde, and blocked in PBS with 10%NGS (NGS/PBS). Staining was performed using 0.3 μg/ml rabbit anti-chMMP-9 antibody53 or mouse monoclonal antibody (mAb) CVI-ChNL-68.1 (ID-DLO, Lelystad, The Netherlands) against chicken macrophages (1:10,000). After washing, sections were incubated with the secondary Alexa546-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (1:1000 in NGS/PBS) (Molecular Probes, Eugene, OR). Detection of the mouse mAb was performed using the Renaissance Tyramide Signal Amplification Kit (NEN, Boston, MA). The slides were incubated with 2 mg/ml RNAse (Labscientific, Livingstone, NJ) for 2 h and then mounted in ProLong antifade reagent containing the green nucleic acid dye YO-PRO-1 iodide (1:400). Images were captured using a Zeiss Axioskop (Carl Zeiss, Inc., Thornwood, NY) microscope fitted with a cooled CCD CAMera (Diagnostic Instruments, Santa Barbara, CA), and processed using Adobe Photoshop 6.0 software.

For immunohistochemical analysis of chMMP-9 expression, cryosections were fixed with cold methanol. Detection of chMMP-13 was performed on paraffin-embedded sections treated with 10 mM citrate buffer for antigen retrieval. Endogenous peroxidase was blocked with 0.3% hydrogen peroxidase. Non-specific binding was blocked with PBS/2% BSA/5% NGS. Slides were incubated overnight at 4°C with 2 μg/ml rabbit anti-chMMP-9 or anti-chMMP-13
antibodies\textsuperscript{48} or murine mAb 29-7 reacting with a yet unidentified surface antigen of human cells. After washing, the slides were incubated for 1 h with secondary biotinylated goat anti-rabbit or anti-mouse IgG (1:1000), followed by incubation with HRP-NeutrAvidin conjugate for 30 min (Pierce, Rockford, IL) and then with a DAB chromogenic substrate. Sections were counterstained with Mayer’s hematoxylin. Digital images were captured under low (4x and 10x) and higher (20x and 40x) magnifications using the Olympus BX60 microscope equipped with a digital DVC video camera. The quantitation of \textit{ch}MMP-9-positive heterophils or \textit{ch}MMP-13-positive monocytes in individual onplants or normal CAM was performed on 20x images overlaid with a 9x7 square grid. Positively stained cells were scored in the grids overlapped with tissue. Data are presented as the number of \textit{ch}MMP-9-positive heterophils or \textit{ch}MMP-13-positive monocytes per grid (mean±SEM).

**Protein extraction from the onplant tissue**

Samples of normal CAM and collagen onplants were harvested at the indicated time points. A total of 4-6 onplants from 3-5 embryos were combined, frozen on dry ice and kept at -80°C. The samples were thawed on ice, cut into pieces and homogenized at 4°C in 0.6 ml extract buffer (0.1 M Tris, 1.0% SDS, 10 mM EDTA, 10 μg/ml each of aprotinin, pepstatin and leupeptin, 1 mM PMSF, pH 8.0). Extracts were cleared by centrifugation at 14,000 rpm for 20 min at 4°C. Protein content was determined using the BCA kit (Pierce).

**Western blotting**

Twenty five μg of extracted protein were separated by SDS-PAGE on 4-20\% or 8\% gels. Resolved proteins were transferred to the nitrocellulose or PVDF membranes. After blocking
with 5% non-fat dry milk, the membranes were incubated overnight at 4°C with 1 μg/ml anti-chMMP-9 antibody, washed and incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat or donkey anti-rabbit IgG, and developed with Super Signal® West Pico Chemiluminescent Substrate (Pierce).

Zymography

A total of 5 μg of extracted protein was mixed with 10xSDS sample buffer and ran on 8% SDS-polyacrylamide substrate gels containing 0.8% gelatin. Following PAGE, gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature and incubated overnight at 37°C in 50 mM Tris, 150 mM NaCl, 5 mM CaCl₂, pH 7.4. Gels were stained with Coomassie Blue R-250. Bands of gelatinolytic activity correspond to the areas devoid of blue staining.

Statistical analysis

The statistical differences between data sets were analyzed by Student’s t-test using the Prizm program (GraphPad Software, San Diego, CA). The data are presented as scattered plots or bar graphs with the mean ± SEM. Data were considered statistically significant when p < 0.05.
Results

Influx of chMMP-9-positive heterophils into the CAM during angiogenesis

In preliminary immunohistological analysis of leukocyte-enriched fractions from peripheral blood of chick embryos, heterophils were identified as the only cells positively stained with a chMMP-9-specific antibody. In normal quiescent CAM tissue, heterophils were rarely found extravascularly and no chMMP-9 protein was associated with non-cellular components (data not shown). However, a rapid influx of chMMP-9-positive heterophils was induced in the collagen CAM onplants (Figure 1). Twenty four h after grafting both control and growth factor-containing onplants, chMMP-9-positive cells were readily identified in the underlying CAM and in the tissue overlaying the collagen onplant, i.e. 24-48 h before earliest appearance of new blood vessels (Figure 1A,B). Heterophil morphology of chMMP-9-positive cells was confirmed at higher magnification by their lobulated nuclei and granular chMMP-9-specific staining in the cytoplasm (Figure 1A,B, insets). The levels of heterophil infiltration were higher in the onplants supplemented with angiogenic growth factors, where heterophils were frequently found around blood vessels (Figure 1B, arrows). By 48 h, more chMMP-9-positive heterophils were identified in the upper regions of the CAM, suggesting further infiltration of the collagen onplant tissue (Figure 1C,D). Levels of chMMP-9 staining did not appear to change substantially between 48 and 66 h (Figure 1E,F).

In addition to distinct cellular staining, chMMP-9 was also associated with ECM fibrils, especially in the growth factor-containing onplants (Figure 1F, asterisks). Non-cellular chMMP-9 staining was particularly strong at the air/collagen interface visualized as ribbon-like structures (indicated by # in Figure 1A). Both the cellular and ECM immunostaining were completely abrogated by pre-absorbing the chMMP-9 antibody with purified chMMP-9 protein (Figure 1G),
indicating that both types of staining were specifically associated with *ch*MMP-9 protein. Angiogenesis, scored at 66 h, confirmed that the levels of angiogenesis were 4-5-fold higher in growth factor-supplemented collagen onplants (Figure 1, scatter graph). Thus, heterophil infiltration preceded and paralleled the development of a new vascular network, suggesting a functional role of infiltrating *ch*MMP-9-positive heterophils in CAM angiogenesis.

The kinetics of *ch*MMP-9 protein expression in collagen CAM onplants during angiogenesis was examined by Western blotting (Figure 1, H). *ch*MMP-9 protein was essentially undetectable during the first day after placing the onplants onto the CAM. By 26 h, *ch*MMP-9 proenzyme was readily identified in both types of onplants, but was 2 to 3-fold higher in growth factor-containing onplants compared to controls as judged by densitometry analysis of blots. This differential was also observed at 50 h. Incubation up to 66 h, when angiogenesis was scored, did not cause any substantial changes in *ch*MMP-9 levels and the zymogen form continued to be the major species detected. These changes in *ch*MMP-9 protein levels were consistent with and corresponded to the kinetics of heterophil influx, suggesting that heterophils constitute a major cellular source of *ch*MMP-9 in the collagen CAM onplants.

**Kinetics of monocytes/macrophage infiltration**

Cells of monocyte/macrophage lineage represent another major inflammatory cell type associated with the angiogenic onplant tissue. Therefore, we analyzed whether monocyte/macrophage influx overlapped or coincided with the heterophil population during infiltration of collagen CAM onplants. Immunostaining with an antibody specific for chicken monocytes/macrophages showed that at 24 h, fewer monocytes/macrophages were present within the onplant tissue compared to heterophils and were identified only in the underlying CAM
(Figure 2A,B). At 48 h, some monocytes/macrophages could be detected around the grids of the lower mesh, i.e. at the collagen/CAM interface (Figure 2C,D, arrowheads), but in general their numbers remained quite sparse. In both control and growth factor-supplemented onplants, the number of monocytes/macrophages significantly increased by 66 h (Figure 2E,F). This was in contrast to the influx kinetics of heterophils, which after rapid influx leveled off between 48 and 66 h. Thus, monocytes/macrophages and heterophils represent two distinct inflammatory leukocyte populations infiltrating with different kinetics the collagen CAM tissue during angiogenesis.

**Quantitative analysis of heterophil influx**

Heterophil infiltration was quantitatively assessed on cryosections of CAM collagen onplants immunohistochemically stained with anti-\(ch\)MMP-9 as our newly-established heterophil marker. Multiple digital images were captured at 4-20x magnification and then analyzed quantitatively by scoring for the frequency and density of \(ch\)MMP-9-positive heterophils (Figure 3A). The examination of these cells at 40x magnification, confirmed that all the scored cells were indeed heterophils, characterized by multi-lobed nuclei (counterstained to appear blue) and granular cytoplasm containing the brownish-stained antigen (Figure 3B).

During the entire 72 h-incubation, \(ch\)MMP-9-positive heterophils were absent or rare in normal CAM from embryos containing no onplants (Figure 3C). In contrast, heterophils were identified in the underlying CAM of control onplants at 14 h and steadily increased until 48 h and then slightly declined at 72 h. Remarkably, in the presence of angiogenic growth factors, \(ch\)MMP-9-positive heterophils were first identified as early as 2 h after placing onplants on the CAM, i.e. when no heterophils were observed in the control onplants. Within the next 12 h, the
number of heterophils in growth factor-containing onplants increased so rapidly that their frequency exceeded 3-fold that observed in control onplants.

**Tumor-induced angiogenesis in collagen CAM onplants**

Our studies indicate that the varying levels of neovascularization induced by angiogenic growth factors correlated well with the levels of leukocyte influx, including heterophils and monocytes. We next verified whether such influx of inflammatory cells would be reproduced during angiogenesis induced by malignant tumor cells. Therefore, the purified growth factors were replaced in the collagen onplants with $5 \times 10^4$ human fibrosarcoma HT-1080 cells. The diagram in Figure 4A schematically represents the tumor-angiogenic system introduced in this study and depicts the tissue and cellular components of a collagen CAM onplant after three days of angiogenic stimulation.

Levels of angiogenesis induced by HT-1080 cells were on average 4-5 fold higher than those induced in control collagen onplants (Figure 4B) and were comparable with the maximum levels of growth factor-induced angiogenesis (Figure 1, scatter graph). Importantly, similar to growth factor-induced angiogenesis, angiogenesis induced by HT-1080 cells was sensitive to inhibitors of MMPs, using both natural (chicken TIMP-2) and synthetic (GM6001) inhibitor compounds delivered either topically or systemically (Figure 4B). The wide variation and range in angiogenic scoring of individual onplants depicted in the scatter graph (Figure 4B) is typical for measurements in highly complex in vivo systems. Nevertheless, the $p$ values (less than 0.005) clearly indicate significant difference between the experimental variables.

To visualize tumor cells within the complex vascular/stromal tissue of the collagen onplants, we stained cryosections with mAb 29-7 that reacts with a surface antigen of human
cells (Figure 4C, left panel). Based on the 29-7 antigen distribution, three days after placing collagen onplants on the CAM, HT-1080 cells spread beneath the air/collagen interface among the infiltrating stromal cells and between the grids of the nylon meshes (circles in Figure 4C, left panel), while the underlying CAM was generally free of human tumor cells. Newly-formed blood vessels induced by the tumor cells were present throughout the underlying CAM (arrows in Figure 4C, left panel).

If the HT-1080-onplants were incubated on the CAM of chick embryos for 3 additional days after angiogenic scoring (i.e., 6 days after onplant grafting), large primary tumors formed on the top of the onplant (Figure 4C, right panel). These tumors were highly vascularized and yielded secondary micrometastatic foci in the liver and lungs (data not shown). Thus, incorporation of 5x10^4 HT-1080 cells into a collagen onplant resulted in a pronounced, MMP-dependent, angiogenic response in 3 days (Figure 4B) and the development of invasive, vascularized malignant tumors in 3-6 days (Figure 4C), demonstrating that the modified CAM/onplant assay indeed functions as a quantitative tumor angiogenesis model.

Influx of chMMP-9-positive heterophils during tumor-induced angiogenesis

We next verified whether tumor-induced angiogenesis in CAM collagen onplants would be accompanied by infiltration of specific leukocytes. Since heterophils were the first to influx CAM collagen onplants, kinetics of chMMP-9-positive cells in HT-1080 onplants was analyzed and quantified between 2-72 h (Figure 4D). In contrast to the growth factor-supplemented onplants, chMMP-9-positive heterophils were very rare in HT-1080 cell-containing onplants 2 h following grafting. However, heterophils were readily identified at 12 h, indicating that a few hours were required to accumulate effective levels of angiogenic factors and heterophil
attractants within the HT-1080-onplants. Nevertheless, a significant increase in heterophils occurred in the onplants containing HT-1080 cells prior to and during the neovascular growth period (Figure 4D). In control onplants, the frequency of chMMP-9-positive heterophils at 72 h was significantly lower than that of HT-1080-onplants (Figure 4D), which closely correlated with the different levels of angiogenesis achieved at this time point (Figure 4B).

**Depletion of heterophil influx during tumor cell-induced angiogenesis by cortisone**

To analyze whether angiogenesis induced by tumor cells depended on the influx of inflammatory leukocytes, infiltration of leukocytes into HT-1080 onplants was prevented by treatment with the anti-inflammatory drug cortisone. HT-1080 cells in cortisone-treated and non-treated onplants were visualized with the human-specific mAb 29-7. Cortisone treatment did not appear to affect the growth and spread of tumor cells in the onplants although caused a generalized thinning of the CAM tissue (Figure 5A, upper panels). However, immunohistochemical staining of cortisone-treated and non-treated onplants with the chMMP-9 specific antibody (Figure 5A, lower panels) and quantitation of the resulting images (Figure 5B, upper panel) confirmed that the frequency of chMMP-9-positive heterophils in cortisone-treated onplants were decreased approximately 3-fold as compared to non-treated controls.

Western blot and zymography analyses confirmed that chMMP-9 content was correspondingly lower in collagen onplants treated with cortisone as determined by the diminished lysis zone typical for the chMMP-9 region in a zymograph and, more specifically, by the near absence of a chMMP-9-specific band in the Western blot (Figure 5C, upper and lower panels, respectively). Densitometry of the zymograph and Western blot images demonstrated a corresponding 60.2±7.9% and 81.6±3.9% decrease in chMMP-9 protein in cortisone-treated
onplants. In agreement with the suggestion that the presence of tumor-associated leukocytes is critical for proper development of the angiogenic network, cortisone caused a severe suppression of angiogenesis in collagen onplants containing HT-1080 cells (Figure 5B, lower panel). Levels of angiogenesis were diminished by 80% in cortisone-treated animals, establishing a strong correlation between inflammatory cell influx and neovascularization.

Modulation of inflammatory cell influx by a COX inhibitor ibuprofen

To extend and corroborate our findings with cortisone-treated onplants, we used a non-steroidal anti-inflammatory agent ibuprofen, a potent pan-COX inhibitor. HT-1080 onplants from control embryos and ibuprofen-treated embryos were analyzed for levels of angiogenesis and degree of infiltration by chMMP-9-positive heterophils and chMMP-13-positive monocytes (Figure 6). At a dose of 0.75 mg/embryo, which calculates to approximately 50 μM, ibuprofen caused a 50-70% reduction of angiogenesis. The gross morphology of onplants and the distribution of HT-1080 cells within the onplants from ibuprofen-treated embryos appeared similar to that of untreated animals (Figure 6A, upper panels). This finding indicated that the anti-angiogenic effect of ibuprofen was not associated with a direct inhibition of HT-1080 cell proliferation. This indication was confirmed by the similar size of tumors developed on top of the onplants 6-7 days after grafting on the CAM (data not shown). In further agreement, ibuprofen did not cause significant effects on HT-1080 cell proliferation in vitro at a dose range of 10-100 μM (data not shown). Moreover, when growth factor-supplemented onplants were placed on the CAM of ibuprofen-treated embryos, levels of angiogenesis were diminished almost to control levels (data not shown), further demonstrating the effects of ibuprofen on host tissues.
Immunohistochemical analysis of the HT-1080 onplant tissue from embryos treated with ibuprofen demonstrated a substantial reduction of infiltration by *ch*MMP-9-positive heterophils and *ch*MMP-13-positive monocytes/macrophages (Figure 6A, middle and lower panels, respectively). Quantification of digital images demonstrated 53.9±9.4% inhibition of heterophil influx by ibuprofen in four independent experiments. Concomitantly, influx of *ch*MMP-13-positive monocytes/macrophages was reduced by 55.2±9.7% (n=3). The diminished infiltration of both types of inflammatory leukocytes correlated well with overall inhibition (64.9±6.8%, n=9) of the angiogenic response in ibuprofen-treated embryos (Figure 6B).

**Rescue of anti-angiogenic effects of ibuprofen and cortisone**

Since heterophils were the first leukocytes infiltrating the onplant tissue and their infiltration was prevented by anti-inflammatory agents, it suggested that the inhibitory effects of cortisone and ibuprofen might be overcome by the incorporation of purified heterophils into the onplants. Incorporation of as few as 5x10^4 purified heterophils indeed completely reversed the anti-inflammatory effects of ibuprofen on HT-1080 cell-containing onplants, rescuing both the angiogenic response and the influx of endogenous inflammatory leukocytes (Figure 6B). In five independent experiments, levels of angiogenesis decreased by ibuprofen to 44.9±8.0% of non-treated control were brought back to 96.4±14.8% by exogenous heterophils. Angiogenic rescue was accompanied by a complete restoration of infiltration by *ch*MMP-9-positive heterophils and *ch*MMP-13-positive monocytes (100.2±15.9%, n=4 and 113.2±26.4%, n=3 of untreated levels, respectively) (Figure 6A, right panels, and corresponding bar graphs in Figure 6B). Importantly, the density of heterophils, identified in the rescued onplant tissue 3 days after incubation on the CAM, exceeded approximately 10-20-fold the initial density of the exogenously-added purified
heterophils. This suggests an induction of endogenous heterophil influx, likely in an autocrine manner. In contrast, addition of $5 \times 10^4$ purified erythrocytes per onplant did not overcome the inhibitory effects of ibuprofen on angiogenesis and infiltration by inflammatory cells (Figure 6B), indicating that the addition of nucleated, but non-inflammatory cells cannot bring about the rescue. Similarly to ibuprofen-treated animals, the addition of purified heterophils to HT-1080 cell-containing onplants grafted in embryos treated with cortisone also restored angiogenesis and infiltration by chMMP-9-positive heterophils (data not shown).

Given that heterophils have been the major cell type associated with and delivering chMMP-9 in the angiogenic onplant tissue (Figures 1 and 3), we next verified whether a direct addition of purified chMMP-9 to the onplants would overcome the anti-angiogenic effects of ibuprofen on HT-1080 cell-induced angiogenesis. In two independent experiments, angiogenesis in ibuprofen-treated animals was inhibited by 36.5% (Figure 7). Although not as acute as the inhibition in the experiments presented in Figure 6, this inhibition of angiogenesis was statistically significant ($p=0.009$) and was fully overcome by the addition of 100 ng of purified chMMP-9 ($p=0.0001$). This finding indicates that purified chMMP-9, possibly in conjunction with other angiogenic factors, provides a means to restore angiogenesis diminished by the anti-inflammatory drugs such as ibuprofen. The results of the rescue by chMMP-9 suggested that the enzyme itself might be able to directly elicit angiogenic responses. However, no pro-angiogenic effects of purified chMMP-9 were demonstrated when 100 ng of the enzyme were incorporated directly into control collagen onplants which were not supplemented with any growth factors or tumor cells (Figure 7). Therefore, chMMP-9 alone apparently is not sufficient to induce angiogenesis in this experimental system, but rather requires cooperation with other factors.
provided possibly by tumor cells in a setting where inflammatory cell influx has been compromised.

To directly assess the pro-angiogenic capacity of inflammatory heterophils, purified heterophil preparations were incorporated into control onplants containing no growth factors or HT-1080 cells. The addition of only $5 \times 10^4$ heterophils per onplant substantially induced angiogenesis, resulting in approximately a 3-fold increase in levels of angiogenesis in three independent experiments (upper panels in Figure 8A,B). Correspondingly, this induced angiogenic response was accompanied by increased levels of infiltration by endogenous chMMP-9-positive heterophils and chMMP-13-positive monocytes (2.9-fold and 2.5-fold, respectively, as illustrated in the middle and lower panels of Figure 8A,B). The addition of isolated erythrocytes to control onplants failed to significantly induce angiogenesis and leukocyte infiltration (data not shown). These experiments demonstrate that relatively low numbers of exogenously-added heterophils not only rescue drug-inhibited tumor angiogenesis, but also exhibit an inherent capacity to induce a substantial pro-angiogenic response in vivo in the absence of added growth factors and/or tumor cells.
Discussion

A wealth of compelling evidence spatially and temporarily links cancer progression and tumor angiogenesis with the infiltration of tumor sites by inflammatory leukocytes. The critical role of persistent inflammation in tumor development has been convincingly demonstrated by the anti-cancer effects of long-term use of anti-inflammatory drugs in humans. In agreement, specific functions of tumor-associated leukocytes were experimentally confirmed in several animal models, clearly suggesting a pro-angiogenic role of inflammatory leukocytes. However, the actual scenario and kinetics of early leukocyte influx as well as interrelationships between specific inflammatory cell types during the initial stages of tumor-induced angiogenesis is still not fully understood.

To experimentally address some of the essential questions regarding the putative pro-angiogenic role of specific inflammatory leukocytes, we have modified a previously described growth factor-induced in vivo angiogenesis model employing collagen CAM onplants. In the modified assay, sprouting of angiogenic vessels from the pre-existing CAM vasculature is induced by human tumor cells. This model provides an accurate and reproducible measure of new vasculature formation and also allows for analysis of host infiltrating leukocytes in the tumor-induced angiogenic process. Not only is the observed angiogenesis dependent on MMP-mediated collagen remodeling, but it also coincides with a rapid influx of MMP-bearing leukocytes: first, chMMP-9-positive heterophils, followed by chMMP-13-positive monocytes/macrophages.

Early heterophil arrival into collagen onplants is a part of a broader infiltration scenario comprising influx of a variety of host cells, including myofibroblasts, angiogenic capillaries, and blood vessels into the original onplant space. The early infiltrating chicken heterophils were
shown to be a major source of leukocyte-derived $ch$MMP-9 protein. In the absence of exogenous growth factors, low levels of $ch$MMP-9-positive heterophil infiltration were associated with the low angiogenic response in the control onplants. More importantly, increased levels of heterophil infiltration correlated closely with high levels of angiogenesis induced in the collagen onplants by growth factors or tumor cells, while inhibition of heterophil influx by anti-inflammatory agents directly associated with diminished the levels of angiogenesis.

During angiogenesis in collagen CAM onplants, cleavage, remodeling and degradation of collagen occur coordinately$^{46,48}$. Although heterophils are the earliest leukocytes to appear in the onplant tissue, substantial influx of monocytes/macrophages in the underlying CAM tissue was detected approximately 24 h later after the initial wave of infiltrating heterophils. That these events were temporally linked was indicated by the observation that the addition of purified heterophils to control onplants was followed shortly by an induced influx of monocytes. Functional distinction between the two leukocyte populations is also indicated by different MMPs mediating ECM remodeling at the angiogenic site: $ch$MMP-13 collagenase, critical for the cleavage of fibrillar collagen is delivered by monocytes/macrophages$^{48}$, while $ch$MMP-9 gelatinase, likely degrading the denatured collagen and possibly cleaving other polypeptides, is imported specifically by the heterophils.

In mammals, inhibition of neutrophil influx into various inflamed tissues correspondingly decreased both MMP-9 levels and neovascularization$^{25,41,43}$. Similarly, diminished macrophage infiltration and lack of MMP-9 in MMP-9$^{-/-}$ mice were associated with a decrease in angiogenesis, specifically in microvessel density and capillary branching$^{44}$. In the present study, evidence for a critical role of $ch$MMP-9-positive heterophils in tumor-induced angiogenesis was provided by experiments where leukocyte influx into HT-1080 cell-containing onplants was
abrogated by two potent, mechanistically distinct, anti-inflammatory agents, cortisone or ibuprofen\textsuperscript{15,50,59}. In cortisone-treated animals, an inhibited development of angiogenic capillaries and blood vessels was associated with significant decrease in the levels of heterophil influx as well as \(ch\)MMP-9 protein, providing an experimental link between infiltrating heterophils, \(ch\)MMP-9 delivery and tumor-induced angiogenesis.

The effects of the non-steroidal anti-inflammatory drug, ibuprofen, on HT-1080 cell-induced angiogenesis corroborate the cortisone effects and expand our data on coordinated inhibition of angiogenesis and heterophil infiltration. Ibuprofen, known to target both tumor and inflammatory cells\textsuperscript{59}, was used at concentrations which did not affect HT-1080 cell growth and distribution, but very effectively inhibited influx of the inflammatory leukocytes, \(ch\)MMP-9-positive heterophils and \(ch\)MMP-13-positive monocytes. Concomitantly, this inhibition of inflammatory cell influx was accompanied by a corresponding decrease in angiogenic response, indicating a pro-angiogenic role of inflammatory leukocytes in tumor-induced angiogenesis. Surprisingly, only limited quantitative data are available in the literature to support a causal relationship between coordinated inhibition of tumor angiogenesis and leukocyte infiltration by anti-inflammatory drugs, including COX inhibitors\textsuperscript{60,61}. Therefore, the present study provides a strong experimental link for a direct correlation between the tripartite effects of anti-inflammatory drugs, inhibition of leukocyte influx and inhibition of tumor-induced angiogenesis.

The present study also provides direct evidence that specific inflammatory leukocytes, i.e. neutrophil-like heterophils, could themselves be highly pro-angiogenic in vivo. The addition of only \(5 \times 10^4\) heterophils directly into collagen onplants, not supplemented with any exogenous growth factors or tumor cells, induced a strong stimulation of new blood vessel formation. Further support for a direct causal effect of inflammatory cells on malignant progression is
illustrated by the complete rescue of ibuprofen-inhibited angiogenesis in tumor cell-containing onplants by the addition of purified heterophils. Remarkably, exogenously-added heterophils not only restored the levels of accompanying leukocyte infiltration of onplants, but also the levels of tumor-induced neovascularization. To our knowledge, this is the first demonstration that these inflammatory leukocytes are directly pro-angiogenic. In a mammalian system, stimulatory effects of a different inflammatory leukocyte type on tumor growth and angiogenesis were recently demonstrated when purified mast cells were mixed with plasmacytoma cells before implantation into the mice\textsuperscript{58}.

It is not clear what factors are responsible for the pro-angiogenic role of inflammatory heterophils. It is unlikely that the simple physical presence of exogenous live cells elicited the observed effects, since nucleated chicken erythrocytes clearly failed to stimulate or rescue angiogenesis. It is more likely that the released products of heterophils are involved in their pro-angiogenic functions. Like their mammalian counterparts, heterophils are terminally differentiated leukocytes that do not synthesize their products in response to specific stimuli, but store pre-synthesized mediators, including MMP-9, in granules for rapid release at sites of inflammation or angiogenesis.

There is a great deal of evidence linking mammalian MMP-9 to neovascular tissue function as an angiogenic switch, as a mediator of tissue remodeling and/or as a catalytic provider of bioactive cytokines, regulators and inhibitory peptides\textsuperscript{32-36,40,45,62}. Thus, \textit{ch}MMP-9 found in the earliest arriving cell type that infiltrate the newly-stimulated CAM vascular tissue, could be considered as a prime catalytic effector molecule. Unlike the interstitial collagenase, \textit{ch}MMP-13, which stimulates angiogenesis when added to the collagen onplants\textsuperscript{48}, purified \textit{ch}MMP-9 did not by itself stimulate angiogenesis in the CAM assay. However, purified
chMMP-9 was capable of rescuing angiogenesis compromised by anti-inflammatory effects of ibuprofen. Therefore, other intrinsic factors released by heterophils, possibly acting in tandem with catalytic chMMP-9, are likely involved in the pro-angiogenic-action of these inflammatory leukocytes. It would be premature at this point to speculate on the molecular identity of released products of the avian heterophils that might be functionally active in CAM vascular tissue or CAM tumor tissue. However, the facile yet quantitative model described herein for both growth factor- and tumor-induced angiogenesis, might in the near future allow for screening isolated secreted products of heterophils for their ability to stimulate angiogenesis and/or to induce the further influx of specific inflammatory cells.
Figure Legends

Figure 1. Angiogenesis in the collagen CAM onplants is accompanied by infiltration of chMMP-9-positive heterophils and accumulation of chMMP-9 protein. Collagen onplants were supplemented with buffer alone (control onplants: A, C, and E) or angiogenic growth factors (bFGF/VEGF: B, D, F and G). Collagen onplants with the underlying CAM were harvested at 24 h (A and B), 48 h (C and D), or 66 h (E, F, and G), embedded in OCT compound and frozen. Cryosections were immunostained for chMMP-9 with an affinity-purified rabbit polyclonal antibody (red). Tissue sections were counterstained with YO-PRO-1 iodide, resulting in green-stained cell nuclei. The blank circular areas represent areas previously occupied by the two-tiered nylon meshes, which are frequently displaced during tissue processing. At low magnification, scattered chMMP-9 staining appears to be associated with individual cells or small clusters of cells (arrowheads). The insets in panels A and B illustrate at higher magnification heterophils with distinctively shaped nuclei (green) and cytoplasmic chMMP-9-positive granules (red). Specific staining of the extracellular fibrils for chMMP-9 is seen in F (asterisks). The lumens of large blood vessels in panels B, C, and E are denoted with “lu”. The bright ribbon-like staining at the upper border of the collagen-air interface is indicated by # (panel A). In panel G, a 66 h growth factor-containing onplant is chMMP-9-negative after the chMMP-9 antibody was immunodepleted by pre-incubation with purified chMMP-9, confirming the specificity of the staining in the other panels. Bar represents 200 μm. Scatter graph in the upper right illustrates the levels of angiogenesis in control and growth factor-containing (bFGF/VEGF) onplants scored at 66 h and presented as a fraction of grids with newly developed blood vessels (mean ± SEM). Statistical significance (*) is indicated for p<0.0001). (H) Expression of chMMP-9 protein in the collagen CAM onplants. At 3 h (lanes 2-3), 17 h (lanes 4-
5), 26 h (lanes 6-7), 50 h (lanes 8-9), and 66 h (lanes 10-11), the collagen onplants with (+) or without (-) angiogenic growth factors (bFGF/VEGF) were excised from the CAM, pooled, extracted, and analyzed by SDS-PAGE and Western blotting with chMMP-9 antibody. As a positive control, the chMMP-9 proenzyme produced by PMA-treated chicken monocytic cells (HD11) was run in lane 1 (2 μl of cell lysate). The position of 75 kDa chMMP-9 is indicated on the right.

Figure 2. Cells of monocyte/macrophage lineage infiltrate the collagen CAM onplants. Collagen onplants supplemented with buffer alone (A, C, and E) or bFGF/VEGF (B, D, and F) were harvested with the underlying CAM at 24 h (A-B), 48 h (C-D), and 66 h (E-F), embedded in OCT compound and frozen. Cryosections were immunostained with a chicken monocyte/macrophage-specific monoclonal antibody (red). The sections were counterstained with YO-PRO-1 iodide, resulting in green-stained cell nuclei. At 24 h (A-B), positively stained cells (arrowheads) were present only in the underlying CAM. By 48-66 h (C-F) positively stained monocytes/macrophages appeared also in the upper areas of onplants as isolated cells or clusters of cells. Bar represents 200 μm. At a higher magnification (Inset in A, bar represents 20 μm), the stained cells display macrophage-like morphological characteristics (rounded or bean-shaped nuclei and a relatively high cytoplasm to nucleus ratio). The lumens of large vessels are denoted with “lu”. Large, unstained, circular areas in the upper portions of the sections represent the sites where the grids of nylon meshes were displaced during the processing of sections.
Figure 3. Kinetics of heterophil influx into collagen CAM onplants. Samples of normal CAM from embryos without onplants (NCAM) and collagen onplants supplemented with angiogenic growth factors (bFGF/VEGF) or buffer alone (control) were excised with the underlying CAM, embedded in OCT compound and frozen. Cryosections were immunohistochemically stained with chMMP-9 antibody and counterstained with Mayer’s hematoxylin. Digital images were collected at 10x, 20x and 40x magnifications and analyzed. (A) Representative sections of control (left panel) and growth factor-supplemented (right panel) collagen onplants harvested at 72 h (original magnification x10). Arrows point to dark brown stained cells and cell clusters confirmed to be chMMP-9-positive heterophils. (B) At a 40x magnification, morphological features of chMMP-9-positive cells are consistent with the characteristics of heterophils, i.e. multi-lobed nuclei (bluish) and granular cytoplasm (brownish). (C) Kinetics of heterophil influx into collagen CAM onplants was determined over the 72 h time course. The 20x magnification images of normal CAM (NCAM), control and growth factor-containing (bFGF/VFGF) onplants were overlaid with a 9x7 square grid and analyzed (data from 9 to 39 images per time point from two independent experiments). ChMMP-9-positive heterophils were counted in the squares occupied with tissue. Data are presented as the mean ± SEM from the numbers of chMMP-9-positive cells per square. Statistical significance (*) was determined in the two-tailed Student’s test (p<0.001).
Figure 4. Angiogenesis and heterophil influx induced by human tumor cells in collagen CAM onplants. (A) Schematic presentation of tissue and cellular components of a collagen onplant containing HT-1080 cells three days after grafting on the CAM. Tumor cells could be visualized in the upper portions of the onplant, around and between two layers of grids from the nylon meshes. The grids are often displaced during tissue processing, leaving empty circles in the tissue sections. Large, pre-existing blood vessels containing nucleated erythrocytes and leukocytes are located in the underlying CAM, which is bordered by the endoderm layer. Newly-formed blood vessels, which are the ones scored in the angiogenic assay, are identified mostly between or directly below the grids and usually are close to tumor cells. Angiogenic vessels are often filled with blood cells, indicating the existence of an established, complete circulation. Stromal fibroblast-like cells of the CAM mesoderm are numerous and infiltrate the entire collagen onplant, including the very top portions of the onplant. Lower and middle portions of the onplant are infiltrated with inflammatory cells such as monocytes/macrophages and heterophils. These cells could be identified morphologically by immunohistochemical staining with chMMP-13- and chMMP-9 specific antibodies, respectively. The matrix components could be identified as a fine network of fibrils present throughout the entire onplant and a thick ribbon-like structure at the collagen/air interface on the top of onplant. (B) Collagen onplants supplemented with buffer alone (control) or 5x10^4 HT-1080 cells (HT-1080) were placed on the CAM and scored 72 h later for levels of angiogenesis. Recombinant chicken TIMP-2 was incorporated into onplants at 2.85 μM. Hydroxamate MMP inhibitor GM6001 was added either topically (top., 5 μl of 25 μM solution) or systemically (syst., 10μl of 1.25 mM solution) at the time of onplant grafting and 48 h later. At 72 h, the angiogenic response was determined as a fraction of grids containing newly formed blood vessels. Statistical significance between the
groups of onplants was determined by comparison with non-treated HT-1080 cell-containing onplants and is indicated by (**) for $p<0.0001$ and (*) for $p=0.004$. (C) At 72 h (day 3), HT-1080 cell-containing onplants were harvested, embedded in OCT compound and frozen. HT-1080 cells were identified in cryosections (left panel) after immunohistochemical staining with mAb 29-7 recognizing a human cell surface antigen (original magnification x20). Human tumor cells (brown) are located at the top and in between the grids of onplants, while underlying CAM appears mainly devoid of tumor cells. Displaced nylon mesh grids appear as empty circular structures spaces. Some newly-formed blood vessels are indicated by arrows. By day 6 of incubation, highly vascularized HT-1080 tumors are generated on the top of the onplant grids (right panel). (D) Kinetics of heterophil influx into collagen CAM onplants supplemented with HT-1080 cells. The numbers of chMMP-9-positive heterophils were determined over a 72 h time course in the 20x images of tissue cryosections stained with the chMMP-9 antibody as described in Figure 3. For comparison, samples of 72 h control onplants (control) and normal CAM (NCAM) were included in quantitation. Data are presented as the mean ± SEM of heterophil numbers per square scored in 7 to 14 images per time point. Statistical significance is indicated by (**) for $p=0.0001$. 
**Figure 5. Inhibition of heterophil influx and angiogenesis in HT-1080-onplants by cortisone.** Onplants containing HT-1080 cells (5x10^4 cells per onplant) were placed on the top of the CAM of 10 day-old chick embryos, non-treated or injected with cortisone (0.5 mg/embryo). (A) At 72 h, non-treated (left panels) and cortisone-treated (right panels) HT-1080-onplants with the underlying CAM were harvested, embedded in OCT compound, and frozen. Cryosections were immunohistochemically stained with mAb 29-7 (upper panels) or anti-*chMMP-9* antibody (lower panels). As judged by the 29-7 staining (original magnification x4), expanding HT-1080 cells (brown) are localized at the top of the onplants and in between grids. Boxed areas are presented at higher magnification (original magnification x40) of the adjacent sections stained with the *chMMP-9*-specific antibody. The *chMMP-9*-positive staining is associated with single heterophils and heterophil clusters (arrows). (B) Cortisone reduces the heterophil influx (upper graph) and angiogenic response (lower graph) in the collagen onplants containing HT-1080 cells. Cryosections of non-treated and cortisone-treated collagen HT-1080-onplants harvested at 72 h were stained with the *chMMP-9* antibody. The *chMMP-9*-positive heterophils were scored in 20x images. Data are presented as the mean ± SEM of *chMMP-9*-positive heterophil numbers per square. Levels of angiogenesis were scored at 66 h and presented as percentage of grids with newly formed blood vessels. Statistical significance (*) is indicated for *p*<0.0001. (C) Cortisone diminishes *chMMP-9* protein levels in collagen CAM onplants containing HT-1080 cells. Non-treated (HT-1080) and cortisone-treated (HT-1080+cortisone) onplants were harvested at 72 h and snap frozen on dry ice. Extracted proteins were separated by SDS-PAGE on gelatin-containing 8% polyacrylamide gels for zymography analysis (5 μg/lane, upper panel) or separated by 8% SDS-PAGE, transferred to a membrane support and immunoblotted with anti- *chMMP-9* antibody (25 μg/lane, lower panel).
Figure 6. Modulation of angiogenesis in HT-1080-onplants: Inhibition of inflammatory cell influx by ibuprofen and its rescue by exogenous heterophils. (A) Collagen onplants containing $5 \times 10^4$ HT-1080 cells were incubated on the CAM of non-treated embryos (left panels) or embryos treated systemically with ibuprofen (middle and right panels). In addition, a subset of ibuprofen-treated embryos was engrafted with HT-1080-onplants containing $5 \times 10^4$ purified heterophils isolated from peripheral blood (right panels). At 72 h, onplants were harvested and frozen in OCT compound or fixed in formalin. Cryosections were immunohistochemically stained with mAb 29-7 to visualize human cells (upper panels) or chMMP-9-specific antibody to visualize heterophils (arrows, middle panels). Paraffin-embedded sections were immunohistochemically stained with chMMP-13-specific antibody to visualize monocytes/macrophages as brown rounded cells (lower panels). (B) Angiogenic response and the heterophil and monocyte influxes were scored at 72 h in control onplants (ctrl) and onplants containing $5 \times 10^4$ HT-1080 cells (HT), which were placed on non-treated embryos or embryos systemically treated with ibuprofen (IB). Isolated heterophils (Het) or erythrocytes (Ery) were added at a concentration of $5 \times 10^4$ cells per onplant to the two subsets of HT-1080-onplants grafted on the CAM of ibuprofen-treated embryos. Angiogenic response was determined as a fraction of onplant grids containing the newly formed blood vessels (upper graph). Influx of heterophils was determined as a tissue density of chMMP-9- positive cells scored in a 20x tissue images (middle graph). Influx of monocytes was determined as a tissue density of chMMP-13- positive cells scored in a 20x tissue images (lower graph). Shown is a representative experiment out of three independent experiments. Statistical significance was confirmed ($p<0.05$) for each variable in comparison with the previous experimental group as depicted in the scatter and bar graphs.
Figure 7. Angiogenic effects of purified chMMP-9. Control and HT-1080 cell-containing onplants were supplemented with either buffer or 100 ng purified chMMP-9 and placed on the CAM of non-treated embryos or embryos treated with ibuprofen. At 72 h, angiogenesis was scored in the onplants to determine fraction of grids containing newly-formed blood vessels. Statistical significance is indicated by (*, \(p=0.009\)) and (**, \(p=0.001\)).

Figure 8. Induction of angiogenesis and inflammatory cell influx by purified heterophils. (A) Collagen onplants supplemented with buffer alone (control) or 5x10^4 heterophils isolated from peripheral blood were engrafted on the CAM of 10 day-old embryos incubated ex ovo. Onplants were harvested and fixed in formalin or frozen in OCT compound at 72 h, after the angiogenic response was scored. Paraffin sections stained with H&E indicate similar tissue composition of the onplants (upper panels). Arrows point to some of newly formed angiogenic vessels visualized more frequently, especially in between the grids, in the onplants supplemented with heterophils. Cryosections were immunohistochemically stained with chMMP-9-specific antibody to visualize heterophils (arrows, middle panels). In addition, paraffin sections were immunohistochemically stained with anti-chMMP-13 antibody identifying brown rounded cells of monocyte/macrophage lineage (lower panels). (B) Control onplants with and without exogenously added heterophils were scored for angiogenesis (upper graph), heterophil influx (middle graph) and infiltration by monocytes (lower graph) as described in Figure 7. One of three independently performed experiments is shown. A statistically significant difference was confirmed (\(p<0.01\)) for the two experimental groups depicted in the scatter and bar graphs.
Reference List


(53) Hahn-Dantona EA, Aimes RT, Quigley JP. The isolation, characterization, and molecular cloning of a 75-kDa gelatinase B-like enzyme, a member of the matrix metalloproteinase (MMP) family. An avian enzyme that is MMP-9-like in its cell expression pattern but diverges from mammalian gelatinase B in sequence and biochemical properties. J Biol Chem. 2000;275:40827-40838.


Figure 1

Angiogenesis in 66 h collagen onplants

H

Incubation, h: 3 17 26 50 66
bFGF/VEGF: − − − − − + + + + + +

chMMP-9

1 2 3 4 5 6 7 8 9 10 11
Figure 3

A  72 h control onplant  72 h growth factor-supplemented onplant

B  chMMP-9-positive heterophils in onplant tissue

C  Heterophil influx into collagen CAM onplants

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Figure 4

A. HT-1080 cell-induced angiogenesis

B. Angiogenesis induced by HT-1080 cells

C. HT-1080 cells in collagen onplants

D. Heterophil influx induced by HT-1080 cells
Figure 5

A) HT-1080 and HT-1080 + cortisone

B) Heterophil Influx

C) Zymogram

Western Blot

**hu 29-7** (tumor cells)  

**chMMP-9** (heterophils)

**chMMP-9-positive heteropils per square**

**Angiogenic Response**

**Angiogenic grids, fraction**

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**Figure 6**

Panel A: Immunohistochemical staining for hu29-7 (tumor cells), chMMP-9 (heterophils), and chMMP-13 (monocytes) in HT-1080, HT-1080+Ibuprofen, and HT-1080+Ibuprofen + isolated heterophils

Panel B: Bar charts showing the Angiogenic Response, Heterophil Influx, and Monocyte Influx for different treatments: Ctrl, HT, HT+IB, HT+IB+Het, and HT+IB+Ery.
Figure 7

Angiogenic effects of purified chMMP-9

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Figure 8

A

Control

Control + isolated heterophils

H&E

chMMP-9 (heterophils)

chMMP-13 (monocytes)

B

Angiogenic Response

Angiogenic grids, fraction

Control
Control + Heterophils

Heterophil Influx

chMMP-9-positive cells per square

Control
Control + Heterophils

Monocyte Influx

chMMP-13-positive cells per square

Control
Control + Heterophils
Pro-angiogenic role of neutrophil-like inflammatory heterophils during neovascularization induced by growth factors and human tumor cells

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