Collagenolytic-dependent Angiogenesis Mediated by Matrix Metalloproteinase-13 (Collagenase-3)*

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We have demonstrated previously that new blood vessel formation induced by angiogenic growth factors in onplants placed on the chorioallantoic membrane (CAM) of the chick embryos is critically dependent on the cleavage of fibrillar collagen by a previously unidentified interstitial collagenase. In the present study we have used a quantitative CAM angiogenesis system to search for and functionally characterize host avian collagenases responsible for the collagen remodeling associated with angiogenesis. Among the matrix metalloproteinases (MMPs) identified in the CAM onplant tissue, the chicken MMP-13 (chMMP-13) was the only enzyme whose induction and expression coincided with the onset of angiogenesis and blood vessel formation. The chMMP-13 cDNA has been cloned and recombinantly expressed. The chMMP-13 protein has been purified, characterized in vitro, and examined in situ in the CAM. MMP-13-positive cells appear in the CAM shortly after angiogenic stimulation and then accumulate in the collagen onplant tissue. Morphologically, the chMMP-13-containing cells appear as hematopoietic cells of monocytic/macrophage lineage. In vitro, the chMMP-13 proenzyme is rapidly and efficiently activated through the urokinase plasminogen activator/plasminogen/plasmin cascade into a collagenase capable of cleaving native but not the (r/r) mutant collagenase-resistant collagen. Surprisingly, nanogram levels of purified chMMP-13 elicit an angiogenic response in the CAM onplants comparable with that induced by the angiogenic growth factors. The chMMP-13-mediated response was efficiently blocked by select protease inhibitors indicating that plasmin-activated chMMP-13 can function as an angiogenic factor in vivo. Altogether, the results of this study extend the physiological role of MMP-13, previously associated with cartilage/bone resorption, to the collagen remodeling involved in the angiogenic cascade.

Angiogenesis, the formation of new vasculature from preexisting vessels, involves extensive tissue remodeling (1). Sprout-
types produce the enzyme(s). By using a variety of in vitro angiogenic models, it has been shown that specific MMPs, in particular MMP-14 and MMP-2, are up-regulated in both endothelial and stromal cell cultures (1, 7–9). In contrast, a number of in vivo studies using gene-depleted null mice have indicated that MMP-9 expression and activity correlate positively with the progression of angiogenesis (10, 11). However, MMP-9, although a potent gelatinase, is clearly not an interstitial collagenase (12) and therefore could not initiate the rate-limiting cleavage of fibrillar collagen in vivo. Thus, it would appear that additional in vivo studies are needed to evaluate the angiogenic involvement of specific collagenases. For in vitro modeling of angiogenesis, the corneal alloplastic membrane (CAM) of the developing chick embryo historically has been a frequently used model system (13, 14). It provides an accessible, facile in vitro system to examine molecular events that occur during new blood vessel formation induced by angiogenic growth factors or tumors. The CAM system has implicated MMP-2 as one possible contributory proteolytic enzyme (9, 15). This enzyme has the catalytic potential to cleave interstitial collagen (12) but mainly has been examined in the CAM as a potential modulator of specific integrins (15).

Our laboratory has developed and utilized a quantitative CAM angiogenesis assay that involves placing select collagens onto the CAM in the absence or presence of angiogenic factors (16). Within 24 h various stromal cells, macrophage-like cells, and endothelial cells infuse the collagen ontplant from the underlying CAM, thereby expanding the vascular tissue. At the end point of the assay, only the new blood vessels that have formed in the collagen/ontplant are scored. It has been demonstrated that the new blood vessel formation in the CAM is MMP-dependent (16). Furthermore, the use in this assay of a specific, collagenase-resistant, mutant collagen (r/r) has implicated an undefined interstitial collagenase as the rate-limiting angiogenic MMP (16).

In the present study we have used the CAM angiogenesis system to screen for host avian collagens responsible for the collagen cleavage and remodeling associated with new blood vessel formation. We have identified chicken MMP-13 (chMMP-13), induced in the CAM angiogenic onplants, as a likely candidate. The chMMP-13 cDNA has been cloned from the total RNA isolated from the onplant tissue and recombantly expressed. The chMMP-13 protein has been purified, characterized in vitro, and then examined in situ in the CAM and in other chick tissues. We demonstrate that the chMMP-13 zymogen is rapidly and efficiently activated into an interstitial collagen-cleaving enzyme by the uPA/plasminogen cascade. Moreover, purified chMMP-13, when added at nanogram levels onto the CAM, can function directly as a stimulator of new blood vessel formation.

MATERIALS AND METHODS
CAM Angiogenesis Assay—Angiogenesis assays were performed as described previously (16). Fertilized White Leghorn chicken eggs were received from SPAFAS (North Franklin, CT) and incubated in a humidified incubator at 38 °C. At day 4, eggshells were carefully removed, and embryos were incubated throughout the length of the experiment under shell-less conditions, in a covered dish placed in a humidified air incubator at 38 °C and 60% humidity. Onplants were generated by overlaying two grided plastic meshes and embedding them into 30 μl of 1.6 mg/ml collagen of different types and origin. Where indicated, collagen was supplemented with basic fibroblast growth factor (bFGF) at 16.7 μg/ml and VEGF at 5 μg/ml (PeproTech Inc., Rocky Hill, NJ). Collagen onplants were placed on the CAM of 10-day-old shell-less embryos. Test proteins or reagents were incorporated into the onplants at the concentrations indicated in the text.Ontplant-bearing embryos were incubated for an additional 66 h at which time the extent of ontplant vascularization was quantified. Newly formed vessels were identified by analyzing the upper plane of the onplant with a dissecting microscope (i.e. scoring only those vessels that sprouted up from the CAM and reached the plane of the upper mesh). The angiogenic index of the ontplant tissue was determined as the percentage of grids that contained newly formed blood vessels from the total number of grids in the upper mesh. Data were processed using GraphPad Prism software (Graphpad Software Inc. San Diego). Statistical significance was estimated with the Mann-Whitney test. To facilitate angiogenic scoring in some experiments, 100 μl of india ink were injected intravenously to visually enhance the appearance of the vasculature. Images were taken at ×6.3 by using a digital video camera mounted on an Olympus SZ60 dissecting microscope (Olympus, Melville, NY).

Immunohistochemistry and Histology—Ontplants and embryo tissues were collected immediately after scoring, fixed in 10% zinc formalin, and paraffin-embedded. 4-μm-thick sections were prepared and processed either for histological or immunohistochemical analyses. For routine histology, the sections were stained with Gills III hematoxylin and eosi. Giemsa-based dyes were used to discriminate the cells of hematopoietic origin. For immunohistochemistry, the chMMP-13 antigen was retrieved by heating the sections in 0.01 m citrate buffer, pH 6.0, for 5 min in a microwave oven. Nonspecific binding was blocked with 3% bovine serum albumin and 5% normal goat serum in phosphate-buffered saline. The sections were subsequently probed with a polyclonal huMMP-13-specific antibody (Triple Point Biologics Inc., Forest Grove, OR), which is directed to the highly conserved hinge domain of the enzyme and cross-reacts with chMMP-13. Bound antibody was visualized with the chromogen diaminobenzidine, resulting in dark brown staining. Counterstaining was performed with Gills III hematoxylin. Images were taken using a DVC digital camera mounted on an Olympus BX60 microscope (Olympus, Melville, NY) and processed with Adobe Photoshop (Adobe Systems, Seattle, WA) and Macromedia Freehand software (Macromedia Inc., San Francisco).

RNA Isolation—RNA was isolated from cells and tissues using either Trizol reagent (Invitrogen) or RNase-free water and stored at −80 °C.

Screening for Expression of chMMPs in the CAM Angiogenic Tissue—The full-length sequence of the chMMP-13 cDNA was determined by screening of a gene-enriched cDNA library (17) generated in p5'-RACE. Collagen onplants were dissected from the CAM and snap-frozen in liquid nitrogen. Total RNA was purified from the onplants, and 1 mg of RNA was reverse-transcribed to first strand cDNA with random hexamer primers. After cDNA synthesis, the PCR was carried out with degenerate primers from the two most conserved domains found in MMPs: the Cys-switch and the zinc-binding regions. The forward primer was 5′-AGCCGFMGTTGGGRWICCGA-3′; the reverse primer was 5′-GATGICCIADYTCRGTCIACG-3′ (I = deoxyinosine, Y = C + T, R = A + G). The reaction was cycled at 95 °C for 5 min, followed by 35 1-min cycles at 94 °C for denaturation, 1 min at 40 °C for annealing, and 2 min at 72 °C for elongation. The PCR products were cloned into pcR4-Topo vector. Those clones containing an insert of ~450 bp were sequenced.

Cloning of chMMP-13 Full-length cDNA—The 5′-279 bp fragment coding for the first 93 amino acids of the chMMP-13 cDNA was cloned using the SMART RACE cDNA amplification system (Clontech, Palo Alto, CA). Briefly, mRNA was purified from FGF-treated chick embryonic fibroblasts and then reverse-transcribed using Superscript II (Invitrogen) at 42 °C for 1.5 h primed with SMART II oligonucleotides and 5′ coding sequences. The first strand cDNA was subjected to touchdown PCR by using universal primers provided in the kit and gene-specific primer 5′-AGATGCTGGTGCAACATGTCGG-3′. The PCR was carried out under the following cycling conditions: 5 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min followed by 27 cycles 94 °C for 5 s, 68 °C for 1 min, and 72 °C for 2 min, followed by 27 cycles 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 2 min. This reaction yielded a 1-kb fragment that was cloned into pcR4-Topo vector and sequenced.

RT-PCR—Reverse transcription was performed on 4-μg samples of total RNA using SuperScriptII kit following the manufacturer’s instructions (Invitrogen). PCRs were performed with specific primer 5′-AGATGCTGGTGCAACATGTCGG-3′. The PCR was performed under the following cycling conditions: 5 cycles at 94 °C for 30 s, 55 °C for 1 min and 72 °C for 2 min followed by 27 cycles 94 °C for 5 s, 68 °C for 1 min, and 72 °C for 2 min, followed by 27 cycles 94 °C for 5 s, 68 °C for 10 s, and 72 °C for 2 min. This reaction yielded a 1-kb fragment that was cloned into pcR4-Topo vector and sequenced.

Western Blot—Western blots were performed using specific antibody 5′-GGTCAGATGATCTTAGAGGGT-3′ and antisense, 5′-CAACTAGTGTGATCACCTATCAG-3′, which generated a fragment of 489 bp, chicken GAPDH (sense, 5′-GGAGAGGTTGCG-CTGGTGGATCG-3′ and antisense, 5′-GGTGGAGCAAGCTGTGAAGAATTTGGCTTGG-3′, which produced a 138-bp fragment), chicken MMP-13 (sense, 5′-GAATGACCCCAAAAGGTTGGT3′ and antisense, 5′-GATCATCAACCTTCCTGAGATGAGG-3′, which generated a 327-bp product), and chicken MMP-2 (sense, 5′-TGGTGCAACAGCAGTGG-3′ and antisense, 5′-ATGGGATGGGCACTTGGAGG-3′, which generated a 923-bp fragment). The PCR was performed at 94 °C...
for 2 min, followed by 35 cycles of 30 s at 94 °C for denaturation, 30 s at 60 °C for annealing, and 1 min at 72 °C for elongation. PCR samples were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The intensity of ch-MMP-13, ch-MMP-2, and chGAPDH bands was determined by densitometric scanning, and analysis was performed on Alphalager 3.3 (Alpha Innotech Corp., San Leandro, CA).

Expression of Recombinant chMMP-13 Protein—The cDNA containing the entire coding sequence for chMMP-13 was cloned into the expression vector pEE12 and used to transfect the murine myeloma cell line NS0 as described (18). Wells containing NS0 cells expressing the chMMP-13 protein were identified by using gelatin and casein zymography of conditioned medium, and then cell lines were isolated and cloned by limiting dilution.

Purification of Recombinant Proteins—For isolating recombinant chMMP-13, serum-free conditioned medium from NS0 cell clones expressing ch-MMP-13 was dialyzed against 20 mM Tris-HCl, pH 7.5, 10 mM CaCl2 buffer and then applied to a Unos columns, equilibrated with the same buffer, and attached to a Biologic DuoFlow FFLC system (Bio-Rad). Bound protein was eluted with a linear gradient of 0–1 M NaCl in the same buffer and analyzed by SDS-PAGE, Western blotting, and zymography. Chicken MMP-2, MMP-9, and TIMP-2 were purified as described previously (12, 19, 20).

Collagenase Assays—Assays for the cleavage of native, acid-soluble collagens by specific MMPs were performed essentially as described (12). Briefly, collagen solutions were solubilized in 10 mM acetic acid and diluted to the indicated concentrations in 40 mM Tris-HCl, pH 7.5, 200 mM NaCl, 10 mM CaCl2, 0.05% Brij-35 buffer (calcium assay buffer). Individual MMPs or organonemural p-aminophenylmercuric acid (APMA) was added to collagen mixtures at the concentrations indicated in the text. Reactions were performed at 23 °C to maintain the native triple helical collagen status and then terminated by the addition of 10× reducing SDS sample buffer. The samples were analyzed for specific collagen cleavage by SDS-PAGE.

SDS-PAGE, Western Blotting, and Zymography—Proteins separated by SDS-PAGE were detected in the gels by either Coomassie Blue R-250 calcium assay buffer, and stained with Coomassie Blue R-250. Washed twice in 2.5% Triton X-100, incubated overnight at 37 °C with horseradish peroxidase-conjugated goat anti-rabbit IgG, and MMP-13 antibody. Bound protein was eluted with a linear gradient of 0–1 M NaCl in the same buffer and analyzed by SDS-PAGE, Western blotting, and zymography. Chicken MMP-2, MMP-9, and TIMP-2 were purified as described previously (12, 19, 20).

Activation of ch-MMP-13 Zymogen by Plasmin—Chicken plasmin was generated by incubation of purified plasminogen (2.5 μg) with recombinant uPA (5% conditioned medium from HEla cells transfected with the chicken uPA CDNA) in 25 mM Tris, pH 8.0, 0.1% Triton X-100 (TX buffer) for 1 h at 37 °C. Where indicated, aprotinin was added at a final concentration of 10 μg/ml to block the activity of generated plasmin. To analyze whether plasminogen or uPA alone could induce ch-MMP-13 activation, these proteins were preincubated individually and thereafter mixed with the ch-MMP-13 zymogen. The ch-MMP-13 zymogen was added at 33 nM to each of reaction mixtures, which were further incubated at 37 °C. At the indicated time intervals, the reaction was stopped with ×10 SDS sample buffer. Proteins from 20-μl aliquots were separated on SDS-PAGE on 10% gels under reducing conditions, transferred to a PVDF membrane, and probed with 1 μg/ml anti-MMP-13 rabbit antibody. Bound antibodies were detected with the secondary horseradish peroxidase-conjugated goat anti-rabbit IgG, and MMP-13 bands were visualized by ECL.

To determine the actual amount of plasmain generated from 2.5 μg plasminogen upon incubation with recombinant chuPA, a standardized assay for plasmin-mediated cleavage of a specific tripeptide substrate was employed. The plasmain substrate S2251 (American Diagnostics, Greenwich, CT) was incubated with serial dilutions of purified human plasmain (1.5–100 nM), and S2251 cleavage was monitored during a 1-h incubation at 37 °C according to the manufacturer’s instructions. In parallel, the chicken plasmin plasminogen (100 μg/ml) was incubated with recombinant chuPA incubation mixture and was serially diluted and similarly assayed with the plasmin substrate S2251. The concentration of plasmin in the incubation mixture was then determined based on a kinetic analysis of the ensuing cleavage reaction compared with the standardized cleavage reaction with purified plasmin. The analysis confirmed that chuPA was the rate-limiting factor in conversion of plasminogen to plasmain, yielding ~10–25 nM of plasmain from 2.5 μg chicken plasminogen. In addition, it was also determined that at a concentration as low as 2 nM, purified human uPA was capable of generating 1 nM plasmain from 250 μM of human plasminogen (data not shown), indicating that the chicken uPA/plasminogen cascade operates at a similar efficacy in generating plasmin as the human uPA/plasminogen cascade.

Collagenolytic Activity of the Plasmin-activated ch-MMP-13 Enzyme—The ch-MMP-13 zymogen was activated by plasmin as described above. After incubation for 1 h at 37 °C, aprotinin was added at 10 μg/ml to all reaction mixtures to block any residual collagenolytic activity of plasmin or plasmin-generating reagents. Where indicated, recombinant chuPA was added at 30 ng/ml concentration of 33 nM to inhibit proteolytic activity of the generated ch-MMP-13 enzyme. The collagenolytic ability of the plasmin-activated ch-MMP-13 was assessed by the 3A-4/14 cleavage of type I collagen. A 20-μl aliquot of the above described reaction mixtures was mixed with 20 μg of type I rat collagen in 30 μl of calcium assay buffer and incubated for 18 h at 23 °C. Proteins were separated by SDS-PAGE on a 10% gel, and protein bands were visualized by Coomassie staining.

RESULTS

MMP-13 Expression in the Chicken CAM Correlates with Growth Factor-induced Angiogenesis—A quantitative method for scoring new blood vessel formation in the chick embryo was used to examine the involvement of specific MMPs in angiogenesis. The method involves placement of collagen-ensheathed grids on the CAM and then scoring for the newly formed blood vessels that distinctly appear in the upper grids of the collagen onplant (see Ref. 16 and “Materials and Methods”). The development of functional, blood-bearing vessels in the collagen onplants was stimulated by the addition of the angiogenic growth factors FGF and VEGF. The number of grids containing newly formed blood vessels increased 5–10-fold in the presence of FGF/VEGF as compared with control conditions lacking exogenous angiogenic factors (Fig. 1A). This growth factor- induced blood vessel formation was inhibited almost completely by purified chTIMP-2 (Fig. 1A). In conjunction with the reported sensitivity of CAM angiogenesis to synthetic broad spectrum MMP inhibitors (16), the latter result with a natural MMP inhibitor strongly indicated an involvement of avian MMPs in the formation of new vessels in the CAM. Moreover, CAM angiogenesis was substantially reduced when the normal wild type (wt) collagen in the onplants was substituted with a collagenase-resistant mutant (r/r) collagen (Fig. 1A). This finding suggests that during angiogenesis, one of the rate-limiting MMPs is an interstitial collagenase that specifically cleaves wt collagen at the site mutated in the r/r collagen.

To identify potential MMP candidates, the temporal expression of various chicken MMPs was examined in both control and growth factor-containing onplants. Total RNA was extracted from the CAM onplants at 15 h (before any new blood vessels have formed), at 45 h (when new vessels have begun to sprout), and at 66 h (the peak of new vessel formation) (16). The RNA was reverse-transcribed and subjected to PCR analysis by using degenerate primers targeting the two most conservative domains found in MMPs, the cysteine switch and zinc-binding regions.

The chicken MMP-1, -8, and 14 mRNAs were not detected in the CAM extracts. In contrast, the mRNA for chMMP-2 (gelatinase A), chMMP-13 (collagenase-3), and chMMP-16 (MT3-MMP) were readily detected. However, mRNA expression levels of chMMP-2 and chMMP-16 were not affected by the angiogenic growth factors and did not correlate with the kinetics of CAM angiogenesis (Fig. 1B). Furthermore, as shown previously, the tissue distribution of chMMP-2, which can function as an interstitial collagenase (12), did not correlate with the sites of new blood vessel development in the CAM (16). In contrast to chMMP-2 and chMMP-16, chMMP-13 mRNA ex-
pression was up-regulated by the angiogenic growth factors and exhibited a temporal pattern that closely correlated with the kinetics of new blood vessel formation (Fig. 1B). Thus, among identified MMPs, an interstitial collagenase, i.e. chMMP-13, was the only MMP candidate whose expression correlated positively with angiogenesis.

Cloning of Full-length Chicken MMP-13 cDNA from CAM Onplants—To examine in more detail the possible role of chMMP-13 in angiogenesis, we cloned, expressed, isolated, and biochemically characterized the recombinant enzyme. A previously reported partial cDNA sequence of chMMP-13 (22) covered the region from the beginning of the catalytic domain to the 3′-untranslated region. In order to generate a full-length chMMP-13 cDNA, total RNA was isolated at 66 h from growth factor-treated CAM onplants. The reverse-transcribed RNA was then employed as a template for 5′-RACE using primers specific for known regions of the chMMP-13 cDNA. The resulting full-length coding sequence and deduced amino acid sequence are shown in Fig. 1A. The newly identified 360 nucleotides in the 5′ end of the transcript contained an initiation ATO at the beginning of a complete open reading frame of 1413 nucleotides. Our sequence is nearly identical to the partial sequence in the overlapping regions as reported previously (22). One difference involves an A to C change at nucleotide 310, which results in a predicted activation site for chMMP-13 at VGE \( \rightarrow \) YNF instead of VGE \( \rightarrow \) YKP as reported previously (22). The newly deduced activation site in chMMP-13 is identical to that in huMMP-13 (23). Fig. 1B shows an alignment of the chicken and human MMP-13 deduced amino acid sequences with the different domains and regions of the enzyme delineated. Overall chMMP-13 is 71% identical to huMMP-13 at the protein level (Table I). The highest homology exists between the catalytic domains (82%), whereas the propeptide and hemopexin domain homology is only 58 and 67%, respectively. The overall homology and interdomain homology between chMMP-13 and the six known mammalian MMP-13 molecules is presented in Table I. The interspecies comparison indicates that chMMP-13 is as similar (or dissimilar) to other mammalian MMP-13 as it is to huMMP-13.

Expression of chMMP-13 in Embryonic Tissues—The expression of chMMP-13 mRNA in various embryonic tissues was examined by RT-PCR. A specific signal for chMMP-13 could be detected after 35 cycles in most embryonic tissues tested, with the highest levels observed in lungs, intestine, and stomach (Fig. 2A). In contrast, chMMP-2 mRNA levels appeared to be abundant and are readily detected in all of the tested tissues, even at 30 cycles.

To confirm the tissue-dependent expression of chicken MMP-13 at the protein level, we examined the distribution of chMMP-13 antigen by immunohistochemistry. Fig. 2B shows strong MMP-13-positive staining in lung and stomach and weaker staining in intestine, i.e. tissues that yielded the highest RT-PCR signals (Fig. 2A). The specific immunohistochemical signal appeared to localize in a select population of round, mononuclear cells in all three tissues. Distinct staining for the chMMP-13 antigen also appeared in regions of bone development (data not shown), consistent with previous reports (24, 25) that MMP-13 is associated with cartilage resorption and bone remodeling.

Expression, Isolation, and Partial Characterization of Recombinant chMMP-13—To analyze the putative angiogenic properties of chMMP-13, the full-length cDNA for chMMP-13 was recombinantly expressed in the mouse NSO myeloma cell line, using vectors and methods that previously had been used successfully to produce 0.1–1 mg quantities of avian MMPs (17, 19). In contrast to mock-transfected cell cultures, the serum-free conditioned medium isolated from chMMP-13-transfected NSO cells demonstrated a major 60-kDa protein (Fig. 3A, lanes 1 and 2), which strongly reacted with the MMP-13-specific antibody in Western blotting (Fig. 3B, lane 2). The 60-kDa protein also exhibited caseinase and gelatinase activities in the respective substrate gel zymographs (lanes 2 in Fig. 4, C and D). A weakly stained 50-kDa protein, also immunologically reactive with anti-MMP-13, was present in the conditioned medium. This protein was resolved better in the substrate gels and manifested distinctly enhanced gelatinase and caseinase activities, indicating that it represented the processed, active form of chMMP-13, whereas the 60-kDa protein appeared to be the zymogen form of chMMP-13.
The conditioned medium from the chMMP-13 transfected cultures was passed through a fast protein liquid chromatography cation exchange column, and bound proteins were eluted with a 0.1–1.0 M NaCl gradient. Thirty five fractions were collected, and greater than 80% of the total MMP-13 enzymatic activity was isolated in four fractions (Fig. 4, lanes 3–6). The two central fractions contained a mixture of ~75% chMMP-13 zymogen and 25% active enzyme (Fig. 4B, lanes 4 and 5), whereas the leading fraction included mainly zymogen (Fig. 4B, lane 3), and the trailing fraction contained mainly active enzyme (Fig. 4B, lane 6). A 25–30-kDa immunologically reactive fragment of chMMP-13, identified in the conditioned medium, was distinctly resolved in one of the fractions (Fig. 4B, lane 4) but was not enzymatically active in the zymographs.

In order to activate the proenzyme form of chMMP-13, the isolated fraction that contained mainly the 60-kDa chMMP-13 zymogen was treated with 1.0 mM APMA. Aliquots were removed at various times and analyzed for conversion to the 50-kDa active form (Fig. 5A). Conversion to the lower molecular weight, processed form of chMMP-13 was detectable within 5–10 min, and nearly complete conversion occurred by 90–120 min.

To assess the interstitial collagenase activity of chMMP-13, the APMA-generated, active form of the enzyme was then incubated at 23 °C with either wild type (wt) or mutant (r/r) collagenase-resistant mouse type I collagen, generating 3/4 and 1/4 fragments but failed to cleave type III collagen (Fig. 5C). This result, in combination with the

The deduced amino acid sequence of chicken with human MMP-13. The standard arrow indicates the amino terminus (Val 1) of the chicken MMP-13 proenzyme. The unpaired cysteine (boxed) in the PRC sequence of the proenzyme, the three histidines (*), which coordinate the zinc ion at the active site, and the catalytic glutamic acid (boxed) are indicated. The hinge region is shown in boldface type, and the hemopexin domain is overlined. Potential N-linked glycosylation sites are indicated by (∇).

Table I

| Species | Full-length sequence | Domain sequence |
|---------|----------------------|-----------------|
| Human   | 71                   | Propeptide 82   |
| Mouse   | 71                   | Catalytic 82    |
| Rabbit  | 71                   | Hinge 82       |
| Rat     | 71                   | 82             |
| Horse   | 72                   | 82             |

The full-length and domain homology between chicken and mammalian MMP-13 proteins

The deduced amino acid sequences of MMP-13 from the indicated species are aligned with the deduced amino acid sequence of chMMP-13 by using BLOSUM 62 matrix software. Domains were delineated as described previously (39). Homology is indicated in percent.

The GenBank accession numbers for MMP-13 are as follows: human, AF034087; and bovine, AF072685. NM_002427; mouse, NM_008607; rabbit, AF059201; rat, XM_217083; and is assigned negative numbers. The deduced amino acid sequence is presented in the upper line. Underlined is the new sequence determined in the present study. The nucleotide number is indicated at the right with the A of the initiation ATG being +1. The 5'-non-coding sequence is indicated in lowercase and is assigned negative numbers. The deduced amino acid sequence is presented in the lower line. The asterisk indicates the stop codon after nucleotide 1413. B, alignment of the deduced amino acid sequence of chicken with human MMP-13. Vertical lines between the chicken (top line) and human (bottom line) sequences indicate identical amino acids. Lowercase letters indicate amino acids, which differ in the human sequence from those in the chicken sequence. The raised arrow indicates the first amino acid (Val 1) of the chicken MMP-13 proenzyme. The hemopexin domain is overlined.
enhanced expression of chMMP-13 during FGF/VEGF-mediated CAM angiogenesis (Fig. 1), provided further evidence for chMMP-13 as a critical collagen-remodeling enzyme during new blood vessel formation.

Efficient and Rapid Activation of the chMMP-13 Proenzyme through the uPA/Plasminogen Cascade—Several reports (26, 27) have indicated that huMMP-13 can be activated by members of the MMP family, including MMP-2, MMP-3, and MMP-14. Of the activating MMPs, chMMP-2 and chMMP-9 were the only chicken homologues available in purified form (17, 19) for testing as possible activators of chMMP-13. When active chMMP-2 and chMMP-9 were incubated for up to 16 h with the chMMP-13 proenzyme, no processing of the 60-kDa MMP-13 zymogen was detected (Fig. 6A). Even if an active form of huMMP-3 was incubated for 16 h with the chMMP-13 proen-
zyme, no apparent processing occurred. However, if chMMP-13 zymogen was incubated with purified chicken plasminogen plus chicken uPA that can efficiently generate plasmin from the plasminogen, rapid processing of the 60-kDa chMMP-13 proenzyme was observed in less than 1 min and was nearly completed by 30 min (Fig. 6B). The 50-kDa processed form of chMMP-13 was quite stable under these plasmin-generating conditions; however, some proteolytic fragmentation of
The reaction samples (20 μl) were separated by SDS-PAGE under reducing conditions followed by Western blotting with anti-MMP-13 antibody (1 μg/ml). The positions of the 60-kDa proenzyme and the 50-kDa active enzyme are indicated with the arrow and arrowhead, respectively. The position of molecular mass markers in kDa is indicated.

The concentration of plasminogen (2.5 μM) utilized for the processing of the chMMP-13 zymogen (33 nM) in Fig. 6B, although in large molar excess over the zymogen, is the natural physiological concentration of plasminogen found in plasma and other tissue fluids (28). In addition, the actual enzyme/substrate molar ratio is not unusually high because the conversion efficiency of plasminogen to plasmin by uPA under the conditions used was only 0.5–1.0%, as determined by the measuring of plasmin activity after 1 h of preincubation of 2.5 μM plasminogen with uPA (data not shown, see "Materials and Methods"). Thus, not more than 25 nM of active plasmin is present during the processing of 33 nM of chMMP-13 zymogen.

In order to determine whether even lower concentrations of plasmin could efficiently process the chMMP-13 zymogen, thereby approaching expected physiological enzyme/substrate ratios, the chicken plasminogen was added at decreasing concentrations after activation with uPA (Fig. 6C). Levels of plasminogen as low as 20 nM (yielding plasmin at 0.2 nM) in 1 h converted almost all of the 60-kDa chMMP-13 zymogen to a major 50-kDa processed form with trace levels of a 53–55-kDa processed form (Fig. 6C, left panel). The 4 nM plasminogen concentrations caused ~50% conversion of chMMP-13 zymogen after 1 h. Further incubation under these conditions (4 nM plasminogen), representing an enzyme (0.04 nM plasmin) to substrate (33 nM proMMP-13) molar ratio of 1:825, resulted in almost complete conversion of chMMP-13 zymogen to the 50-kDa processed form in 2 h (Fig. 6C, right panel). That plasmin is indeed the converting enzyme is indicated by the complete abrogation of the processing events by aprotinin, a potent inhibitor of plasmin (Fig. 6C, last lane). Thus conversion of chMMP-13 proenzyme to its 50-kDa processed form in 2 h, at enzyme to substrate ratios approaching 1:1000, demonstrates that plasmin is a highly efficient, physiological processing enzyme for chMMP-13.

The requirements for the chMMP-13 activation cascade and a demonstration that generated chMMP-13 enzyme was catalytically active are presented in Fig. 7. The activation status of the chMMP-13 protein was analyzed by Western blotting after a 1-h incubation of the zymogen with the various components of the activating cascade (Fig. 7, upper panel). Both uPA and plasminogen were required for efficient chMMP-13 processing (lanes 2 and 6), because either one alone failed to reduce significantly the levels of the 60-kDa chMMP-13 zymogen (Fig. 7, lanes 3 and 4). Plasminogen alone generated a small amount of the 50-kDa species (lane 3), but this was because of trace levels of plasmin present in the plasminogen preparation (data not shown). That the generated plasmin is the main activator of the chMMP-13 proenzyme in this system again is indicated by results showing that the plasmin inhibitor, aprotinin, completely prevented the generation of the 50-kDa chMMP-13 form (Fig. 7, lane 5).

To verify the interstitial collagenase activity of the generated 50-kDa form of chMMP-13, all the above-indicated samples were added to triple helical collagen and further incubated for 24 h at 23 °C. The results are illustrated in corresponding lanes of the lower panel of Fig. 7. Only when pro-MMP-13 was incubated with uPA and plasminogen without the plasmin inhibitor did the generated 50-kDa enzyme mediate a 3/4–1/4 cleavage of triple helical collagen (Fig. 7, lane 2). If TIMP-2 was added to the active 50-kDa protein, no collagen cleavage was observed (Fig. 7, lane 6), indicating that the cleavage of triple helical collagen was because of active MMP-13 and not plasmin or uPA.

Monocyte-like Cells Containing chMMP-13 Are Present at Elevated Levels in the Angiogenic CAM Tissue—The presence of active MMP-13 occurred after 60–120 min.
of chMMP-13 protein during angiogenesis was examined using immunohistochemical analysis of normal CAM and CAM with collagen onplants (untreated and growth factor-containing) (Fig. 8). A few chMMP-13 positive cells were present in normal CAM tissue from day 13 embryos (on average less than 1.0 cell per field). Control onplants undergoing low to moderate levels of angiogenesis contained about 10–15 chMMP-13 positive cells per field. In contrast, 35–40 chMMP-13 positive cells per field could be found in the FGF/VEGF onplants, undergoing extensive formation of new blood vessels (Fig. 8A). Thus, growth factor-induced angiogenesis in the CAM is accompanied by the appearance of cells carrying MMP-13 protein.

A lower power view of a representative FGF/VEGF CAM onplant is shown in Fig. 8B, panel a. Numerous chMMP-13 positive cells are scattered among the mesenchymal cells in the vascularized CAM tissue. The cells with chMMP-13 protein are often clustered near small blood vessels. At higher magnification, chMMP-13 positive cells adjacent to blood vessels (Fig. 8B, panels c and d) can be easily distinguished from the wispy, pale-stained stromal cells that make up the majority of the CAM tissue and from the endothelial cells, which line the blood vessels and do not exhibit any chMMP-13-specific staining. In contrast, MMP-13-positive cells are round, characterized by a relatively high volume of condensed cytoplasm, and distinct oval or bean-shaped nuclei with large nucleoli. Differential staining of adjacent sections with Giemsa dyes indicated that the chMMP-13 positive cells are of hematopoietic origin and appear to belong to the monocyte/macrophage hematopoietic lineage. These cells are not stained in the absence of MMP-13-specific antibody (b). MMP-13 positive cells are often found around newly formed blood vessels (*). The plastic mesh from an onplant is marked with #. Magnification is ×20 in panels a and b and ×100 in panels c and d.

FIG. 7. Plasmin-generated chMMP-13 is an active collagenase. Reaction mixtures containing TTX buffer alone (lane 1) or supplemented with chicken plasminogen (plgn) (2.5 μg) and uPA (lanes 2, 5, and 6), plasminogen alone (lane 3), or uPA alone (lane 4) were incubated for 1 h at 37 °C to allow the generation of plasmin. Purified chMMP-13 proenzyme (33 nM) was then added to each reaction. Where indicated, 10 μg of aprotinin (aproti) was added to block the activity of the generated plasmin (lane 5). Following an additional incubation for 1 h at 37 °C, aprotinin (10 μg) was added to all samples to block further plasmin activity. Where indicated, recombinant chTIMP-2 (33 μg) was added to inhibit the proteolytic activity of the chMMP-13 enzyme (lane 6). Thereafter, 20 μl from each reaction were added to 20 μg of type I collagen in 30 μl of calcium assay buffer. Following incubation at 23 °C for 24 h, the samples were processed by SDS-PAGE under reducing conditions (lanes 1–6). The gels were then analyzed for the cleavage status of collagen. In the upper panel the status of chMMP-13 activation was analyzed by Western blotting (WB) as described in Fig. 6. In the lower panel the collagenolytic activity of the plasmin-activated chMMP-13 was assessed by monitoring the 3/4–1/4 cleavage of type I collagen by Coomassie Blue staining of the gel. The chMMP-13 proenzyme (arrow), active enzyme (arrowhead), intact collagen (Col), and the cleaved collagen fragments (3/4 and 1/4) are indicated on the left.

FIG. 8. Localization of chMMP-13 expressing cells in collagen CAM onplants during growth factor-induced angiogenesis. A, angiogenic growth factors induce an increase in the number of MMP-13-positive cells in collagen CAM onplants. Collagen onplants with the underlying CAM were fixed, processed, and immunostained with MMP-13-specific antibody. The number of MMP-13-positive cells per microscopic field was determined in normal CAM, control onplants (no angiogenic factors), and growth factor-containing onplants (FGF/VEGF). Mean number of MMP-13-positive cells is indicated in parentheses. B, morphology of MMP-13-positive cells in CAM collagen onplants. Microscopic examination of collagen CAM onplants stained with MMP-13 specific antibody (panels a, c, and d) indicate that, similar to staining in the chick organs, MMP-13-positive cells are round mononuclear cells (arrows), which appear to belong to the monocyte/macrophage hematopoietic lineage. These cells are not stained in the absence of MMP-13-specific antibody (b). MMP-13 positive cells are often found around newly formed blood vessels (*). The plastic mesh from an onplant is marked with #. Magnification is ×20 in panels a and b and ×100 in panels c and d.
sues undergoing growth factor-mediated angiogenesis (Fig. 8) and that specific inhibitors of active MMPs substantially reduce the new blood vessel formation in the CAM (Fig. 1) suggested that active chMMP-13 may be directly involved in this angiogenesis model system. This hypothesis implies that in vivo the chMMP-13 zymogen would have to be activated in order to exert its angiogenic action.

The zymogen form of chMMP-13 has been efficiently activated in vitro by physiological concentrations of plasmin (Fig. 6). Because this activation could be completely inhibited by aprotinin (Fig. 7), we further analyzed the effects of this inhibitor on the in vivo angiogenic response in the CAM. Therefore, aprotinin was incorporated into the FGF/VEGF-containing onplants or added directly onto the onplants placed on the CAM. The aprotinin-treated embryos exhibited a complete reduction in growth factor-stimulated angiogenesis down to control levels of untreated animals (Fig. 9A).

To test directly the angiogenic capability of chMMP-13, the purified zymogen was mixed with the collagen in control onplants. The onplants containing only 15 ng of the chMMP-13 exhibited a 2–3-fold enhancement in the levels of new blood vessel formation (Fig. 9B). The enhanced angiogenesis mediated by purified chMMP-13 was prevented by aprotinin, indicating that in order to exert its angiogenic activity, the exogenous chMMP-13 zymogen has to be activated in vivo by endogenous plasmin or a plasmin-like enzyme. Most important, when wt(+/-) collagen was substituted with the collagenase-resistant (r/r) collagen in the onplants, chMMP-13 did not stimulate angiogenesis (Fig. 9B), a result consistent with the notion that the interstitial collagenase activity and collagen remodeling ability of chMMP-13 indeed provide the angiogenic stimuli.

The angiogenesis stimulated by purified chMMP-13 was examined microscopically. The upper panels of Fig. 9C, which are planar views of the upper grids in the collagen onplants, illustrate enhanced levels of new blood vessel formation in vivo within those collagen onplants containing chMMP-13 (compare Fig. 9C, panels a and b). The injected India ink contrast enhances the new blood vessels (Fig. 9C, arrows), and they appear as dark channels or capillary beds within the opaque collagen and are clearly more numerous and more dense in Fig. 9C, panel b. A dramatic reduction in the number of these chMMP-13-induced blood vessels resulting from the addition of aprotinin also is clearly observed (Fig. 9C, panel c). Histological sections cut through the onplants and stained with hematoxylin and eosin are shown in the lower panel of Fig. 9C as cross-sections of onplants and the engulfing CAM tissue. In contrast to control onplants (Fig. 9C, panel a), small angiogenic blood vessels (indicated by arrowheads) are observed within the upper regions of the onplants supplemented with purified chMMP-13 (Fig. 9C, panel b). Remarkably, very few or no vessels are observed in the onplant treated with aprotinin (Fig. 9C, panel c). However large numbers of viable stromal cells have accumulated in both of the chMMP-13-containing onplants (Fig. 9C, panels b and c); thus indicating that aprotinin did not exert cytotoxic or cytostatic effects. The lack of onplant vascularity in the presence of aprotinin (Fig. 9C, panel c) indicates that this inhibitor of plasmin could specifically prevent the formation of those vascular structures that are induced in the CAM by chMMP-13 zymogen incorporated into collagen onplants. This finding is consistent with a requirement for specific activation by plasmin of chMMP-13 in order to exert the angiogenic effects of the enzyme.

**DISCUSSION**

In this study, we have shown that the enzymatic activity of an endogenous interstitial collagenase is required for the initiation of the extracellular matrix remodeling events that accompany new blood vessel formation in the CAM. The evidence for the specific requirement of a collagenase in angiogenesis is based on the observations demonstrating that the migration of endothelial cells and the completion of vessel formation are prevented or substantially diminished when wild type collagen was substituted with collagenase-resistant (r/r) collagen in the CAM onplant matrix (Fig. 1A) (16). A limited subset of the MMP family represents the enzymes that specifically can cleave triple helical collagens, i.e., the true collagenases, including MMP-1, -8, -13, -14, -16, and possibly MMP-2 (2).

Among the avian collagenase homologues, chicken MMP-1, MMP-8, and MMP-14 were not found in the CAM angiogenic tissue despite an extensive search using a combination of PCR with degenerate primers, data base analysis, and potentially cross-reacting antibodies. On the contrary, chMMP-2, chMMP-13, and chMMP-16 were readily detected in the angiogenic CAM by RT-PCR or immunohistochemistry (Fig. 1B) (16). However, the mRNA levels for chMMP-16 did not correlate with the progression of CAM angiogenesis (Fig. 1B). Although chMMP-2 has been shown previously to efficiently hydrolyze fibrillar collagen (12), the expression levels, tissue distribution, and time and growth factor dependence of chMMP-2 did not correlate with angiogenesis induced in the CAM. Therefore, chMMP-13 would be the likely enzyme initiating specific collagen cleavage in growth factor-induced angiogenic remodeling, although it is possible that the endogenous chMMP-16 and chMMP-2 could contribute to CAM angiogenesis in other catalytic manifestations.

Several lines of evidence implicate chMMP-13 as the endogenous collagenase responsible for initiating the critical matrix remodeling events in the CAM. First, the chMMP-13 expression is characterized by time-, tissue-, and growth factor-dependent induction. Second, activation of the chMMP-13 proenzyme in vivo can be achieved through a physiologically relevant mechanism involving the uPA/plasminogen/plasmin cascade. Third, an active chMMP-13 efficiently and specifically cleaved interstitial collagens, while being incapable of cleaving mutant r/r collagen (Figs. 1 and 8). Finally, in addition to the close coordination between CAM angiogenesis and endogenous chMMP-13 expression, purified chMMP-13 added directly to the CAM onplants at nanogram quantities induced substantial levels of new blood vessel formation (Fig. 9). To our knowledge, the latter finding represents a novel demonstration that a single exogenous MMP can induce angiogenesis in vivo. Remarkably, exogenous chMMP-13 stimulated blood vessel formation in the CAM onplants in the absence of supplemental FGF/VEGF.

The precise mechanisms underlying angiogenic stimulation by active chMMP-13 have yet to be identified. It is unlikely that the chMMP-13 activity simply substitutes for the effects of FGF and/or VEGF. The more likely event(s) is that exogenous chMMP-13, upon activation in the CAM tissue, cleaves the fibrillar collagen and further initiates a cascade of events that result in the accessibility of endogenous FGF and VEGF or other angiogenic stimulatory factors. However, the cleavage of fibrillar collagen has to be the initiating event because the addition of chMMP-13 to CAM onplants containing collagenase-resistant r/r collagen failed to induce angiogenesis (Fig. 9B). The cleavage of wild type interstitial collagens by chMMP-13 could either expose angiogenic cryptic sites or generate angiogenic collagen fragments. An even more complex scenario may involve chMMP-13-dependent cleavage of collagen thereby creating a matrix pathway for endothelial cells as well as chMMP-13-induced cleavage of indeterminate compo-
components that allows for the subsequent release of angiogenic factors associated with the matrix components.

The above proposed hypotheses involving the cleavage of specific proteins by chMMP-13 and the subsequent release or generation of angiogenic products are supported by the existence of MMP-mediated fragmentation of specific bioactive targets (reviewed in Refs. 3, 29, and 30). A few findings that relate to the specific growth factor-induced angiogenesis described herein include the increase of VEGF expression and production in MMP-14-transfected tumor cells (31), the release of bioactive
VEGF by MMP-9 (11), the exposure of an angiogenic cryptic site in type IV collagen by MMP-9 (32), or the generation of active growth factors by MMP-9 (33), MMP-2 (34), or MMP-13 (35). The latter study demonstrated that chMMP-13 in vitro is capable of generating directly the active form of a potent growth factor.

It is interesting that endogenous chMMP-13 is detected in the CAM vascular tissue at the time of angiogenic induction. Neither chMMP-13 protein nor cells expressing the chMMP-13 antigen were found in the normal, untreated CAM, whereas abundant chMMP-13-containing cells were identified in the vascularized CAM 2.5 days after the addition of FGF/VEGF (Fig. 8A). Despite expectations, our immunohistochemical analyses have not identified mesenchymal stromal cells and endothelial cells in the explants as the purveyors of the chMMP-13 collagenase. Instead, a distinct population of cells whose morphology suggested a hematopoietic origin was surprisingly the major if not the only source of chMMP-13 (Fig. 8B). These chMMP-13-positive cells apparently enter the vascular CAM in response to FGF or VEGF. We suggest that other forms of angiogenic induction such as tumor formation, wound repair, female reproductive cycling, and acute and chronic arthritic occurrences may also involve the influx of monocyte-like responder cells containing varying levels of specific proteolytic enzymes that could directly contribute to vascular tissue remodeling. In this regard, inflammation-responsive hematopoietic cells such as monocytes, macrophages, and neutrophils have been shown to import specific MMPs catalytically involved in matrix remodeling of tumor tissue (30, 36). Therefore, our discovery of the interstitial collagenase, chMMP-13, in hematopoietic cells of the monocyte-macrophage lineage, which enter the CAM tissue in response to angiogenic factors, is consistent with recent findings in tumor biology.

The chMMP-13 molecule, like all members of the MMP family is expressed as an inactive zymogen, which must be converted to its active form in order to remodel the collagen-enzriched vascular tissue. Extensive studies have demonstrated that huMMP-13 (collagenase-3) is activated by a select number of other MMP family members. By using in vitro cell culture models and purified reactants in solution, it has been shown that MMP-9, MMP-2, and MMP-14 efficiently convert the 60-kDa huMMP-13 zymogen to the 48-kDa active enzyme, proceeding through a 50–55-kDa autolytic intermediate (26, 27, 37, 38). Surprisingly, the plasminogen/plasmin cascade was excluded from the physiologic pathway of huMMP-13 activation because plasmin generated by concanavalin A-stimulated human fibroblasts did not activate the huMMP-13 proenzyme. In agreement, the plasminogen activator failed to prevent huMMP-13 activation by cells that expressed active MMP-2 and -14 (27). Our results with the chMMP-13 zymogen are in sharp contrast. The 60-kDa chMMP-13 proenzyme resisted activation in vitro by the tested chicken and human MMPs, including soluble chMMP-2 and huMMP-3 (Fig. 6A), and in culture by cell surface-expressed huMMP-14,2 but was very efficiently activated by the plasminogen/plasmin cascade (Fig. 6, B and C).

The processing of the chMMP-13 zymogen by 10–25 nm chicken plasmin proceeds immediately, within 1 min, is complete in 30 min, and generates a 48–50-kDa form of chMMP-13 capable of cleaving triple helical interstitial type I collagen into its signature 3/4/1/4 fragmentation polypeptides (Fig. 7). Even much lower concentrations of plasmin (<1.0 nM) can generate the 48–50-kDa active form of chMMP-13 in 1–2 h (Fig. 6C). Furthermore, plasmin appears to be involved in the activation of chMMP-13 in vivo because aprotinin, added to the CAM onplants, completely abolished the angiogenic response induced either by FGF/VEGF or by the chMMP-13 zymogen (Fig. 9). These findings clearly indicate that the uPA/plasminogen/plasmin cascade indeed is involved in the activation of chMMP-13 under physiological conditions.

The activation pathways of huMMP-13 and chMMP-13 may contrast because of specific differences in the sequence and structure of the two homologues. Both the APMA activation site, 55FFG \ LEV \ 60 where cleavage results in an activation intermediate, and the autoactivation site, \ 81VEG \ YN \ 85, where cleavage generates a fully processed enzyme, are identical in human and chicken zymogens (Fig. 2B). However, the regions harboring the initiating MMP-14 and plasmin cleavage sites are not homologous in the two proenzymes: \ 33LAG \ ILK \ EN \ 40 for huMMP-13 (26, 27) and \ 33PIGIMKKKS \ 40 for chMMP-13 (where G35 and K38 are the putative P1 amino acids involved in the MMP-14 and plasmin cleavages, respectively). Thus, in sharp contrast to the 100% homology of the APMA and autoactivation sites, these regions in the human and chicken MMP-13 zymogens are only 38% homologous. Therefore, effectiveness of MMP-13 activation by MMP-14 or by plasmin may depend on how these nonhomologous, nonconserved regions of the propeptide domains are presented contextually to tissue environments enriched in either MMP-14 or plasmin. Evidently, those environments could be quite different for the human and chicken MMP-13 zymogens. In this regard, activation of the huMMP-13 proenzyme, expressed mainly in chondrocytes and tumor cells (24), was suggested to be mediated predominantly by MMP-14, whereas a physiological role for MMP-13 activation by plasmin was excluded (27).

chMMP-13 also has been identified in chondrocytes from the developing long bones of 15-day-old embryos (25). However, the findings of the present study show that the majority of chMMP-13-positive cells in other chick embryo organs, including lungs, intestine, and stomach, appear to belong to the monocyte/macrophage lineage (Fig. 8D). Similarly appearing cells were virtually absent in the normal CAM at day 13 of development, yet a significant number of cells entering the CAM tissue in response to FGF/VEGF indeed express MMP-13 and morphologically belong to the monocyte/macrophage lineage (Fig. 8D).

With a view that activated chicken monocytes are also able to produce and secrete uPA,2 activation of the chMMP-13 zymogen could then be accomplished very efficiently in a tissue environment that is enriched in plasminogen. The highly vascularized embryonic CAM with a circulating concentration of plasminogen at 100–150 μg/ml (28) indeed can provide such a setting. Thus, chMMP-13-positive monocyte/macrophage cells in the CAM collagen onplants could serve as a source for both chMMP-13 zymogen and uPA, the physiological initiator of the avian plasmin activation cascade. Most important, mammalian MMP-13 generated through the plasmin pathway has been implicated in the dissolution of fibrillar collagen by MMP-14-deficient murine keratinocytes (6), thus representing a clear precedent for remodeling of interstitial collagen not by MMP-14 but by a plasmin-activated MMP-13. The characterization herein of chMMP-13 as a plasmin-activated interstitial collagenase is a unique example in vascular tissue upon angiogenic stimulation and its demonstrated ability to directly contribute to new blood vessel formation, now clearly extends the collagen-remodeling role of this MMP to neovascularization.

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Collagenolysis-dependent Angiogenesis Mediated by Matrix Metalloproteinase-13 (Collagenase-3)
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