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Doxycycline Suppresses Cerebral Matrix Metalloproteinase-9 and Angiogenesis Induced by Focal Hyperstimulation of Vascular Endothelial Growth Factor in a Mouse Model

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Background and Purpose—A number of central nervous system (CNS) disorders are associated with abnormalities in or activation of angiogenesis, including vascular malformations. To test the hypothesis that the nonspecific matrix metalloproteinase (MMP) inhibitor, doxycycline, suppresses vascular endothelial growth factor (VEGF)-induced cerebral angiogenesis through inhibition of MMPs, we used a mouse model with enhanced cerebral angiogenesis induced by focal hyperstimulation of VEGF from adenovirus DNA (AdVEGF) transduction.

Methods—The time course study of MMP activity was performed at 7 and 14 days after AdVEGF transduction. MMP activity and expression were examined by zymography and immunohistochemistry, respectively. As an index of cerebral angiogenesis, microvessel counting was performed in the brains of 3 groups of mice (n=6): (1) control; (2) AdVEGF only; and (3) AdVEGF plus doxycycline (30 mg/kg per day).

Results—Brain MMP-9 activities increased 4-fold (883 ± 137 versus 179 ± 179 ; 1-sided $P < 0.001$) at 7 days after AdVEGF transduction. VEGF transduction increased vessel counts by 19% (255 ± 27 versus 215 ± 15 , 1-sided $P < 0.01$). Doxycycline treatment decreased MMP-9 activity (89 ± 72 versus 883 ± 137 ; 1-sided $P < 0.001$) and cerebral microvessel number (231 ± 17 versus 255 ± 27 ; 1-sided $P < 0.05$).

Conclusions—Doxycycline is effective in decreasing stimulated cerebral MMP-9 activity and parenchymal angiogenesis. The decrease in MMP-9 activity is associated with decreased microvessel counts. Brain pathophysiological processes that involve abnormally enhanced angiogenesis may be amenable to manipulation by MMP inhibitors, including tetracycline derivatives. (*Stroke*. 2004;35:1715-1719.)

Key Words: drug therapy ■ metalloproteinases ■ angiogenesis

There is growing appreciation that a number of central nervous system (CNS) disorders are associated with abnormalities in or activation of angiogenesis,^{1,2} including ischemic stroke^{3,4} and brain arteriovenous malformations (BAVMs). Little is known about the pathogenesis of BAVMs, in part because there is no good animal model that mimics the human disease of recurrent spontaneous intracranial hemorrhage. Recent data suggests that BAVMs are associated with excessive vascular remodeling caused by pathologically increased angiogenesis.^{5,6,7}

VEGF and MMPs are among the most potent regulators of angiogenesis. VEGF is the specific growth factor for endothelial cells and major regulator of blood vessel formation, ie, angiogenesis. VEGF₁₆₅, typically expressed as a 46-kDa homodimer, is the most biologically active form in in vitro studies.⁸ Angiogenesis also requires degradation of vascular

matrix proteins. MMPs degrade extracellular matrix proteins, cell surface molecules, and other pericellular substances.⁹ Recent findings have indicated that gelatinases, including A (MMP-2, constitutive) and B (MMP-9, inducible), in particular, play a central role during angiogenesis. Studies have shown that VEGF and MMP also influence each other in the process of angiogenesis.¹⁰⁻¹²

Altered expression of VEGF ligand and VEGF receptors have been described in surgical BAVM specimens¹³ and increased VEGF expression has been linked to recurrent BAVMs.¹⁴ We have recently described increased levels of MMP-9 relative to tissue inhibitors of metalloproteinase in surgical BAVM specimens.¹⁵ Taken together, evidence from clinical studies suggests that VEGF and MMPs may contribute to the development or maintenance of the diseased vascular phenotype.

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There is growing evidence that MMP inhibition may be useful in the management of vascular diseases.^{16,17} Tetracycline derivatives, including doxycycline, have nonspecific MMP inhibitory effects that are distinct from their antimicrobial action.¹⁸ To study the effect of MMP inhibition on states of enhanced angiogenesis, we used a murine model newly developed in our laboratory. The model consists of focal hyperstimulation by VEGF₁₆₅ in the brain using adenovirus-mediated DNA transduction, resulting in enhanced formation of cerebral microvessels.^{19,20} Our model is not a model of BAVM, but it is characterized by some of the phenotypic features of BAVM lesional tissue. Therefore, use of this model is a first step to mechanistically examine key angiogenic pathways and their response to pharmacological manipulation.

We hypothesized that the nonspecific MMP inhibitor, doxycycline, can suppress AdVEGF-induced cerebral angiogenesis and the suppression is mediated through inhibition of MMP-9 expression and activity. To test this hypothesis, we examined the effects of doxycycline on MMP activities and microvessel formation in the mouse brain after adenovirus-mediated VEGF transduction.

Materials and Methods

Animals and Treatment

Animal use was approved by the University of California San Francisco Committee of Animal Research. Male CD-1 mice weighing 30 to 35 g were purchased from Charles River Laboratory (Wilmington, Mass). The mice were allowed free access to food and water with a 12-hour alternating light–dark cycle.

Our previous data have shown that VEGF expression increases at day 5 in the mouse brain after AdVEGF transduction compared with the control group, with the peak of microvessel counts occurring later, at ≈3 weeks.¹⁹ This finding suggests that there is significant lag time between the activation of angiogenic factors and the actual formation of new vessels. At day 3, mild inflammatory responses around the needle track was detected in both *AdlacZ* and AdVEGF groups to a similar degree.¹⁹ A time course study at 7 and 14 days after AdVEGF transduction was performed to determine the appropriate time point for assessing MMP expression change induced by VEGF overexpression.

To study the effect of doxycycline treatment on cerebral angiogenesis induced by AdVEGF transduction, the mice were divided into 3 groups: control, AdVEGF, and AdVEGF with doxycycline treatment. The control and AdVEGF groups received *AdlacZ* and AdVEGF injection, respectively. In the treatment group, doxycycline (Sigma) was administered starting on the day of AdVEGF injection, at 30 mg/kg per day via drinking water, a dose shown to inhibit growth of aortic aneurysm in rodents.²¹

Adenoviral-Mediated VEGF Gene Transfer in the Brain

After induction of anesthesia with ketamine and xylazine (intraperitoneally), mice were placed in a stereotactic frame (David Kopf Instruments). A Hamilton syringe was inserted through a burr hole 1 mm lateral to the sagittal suture, 1 mm posterior to bregma, and 3 mm under the cortex; 2 μ L of adenoviral suspension with 2.88×10^9 particles of either AdVEGF or *AdlacZ* was injected stereotactically into the right caudate putamen.

Tissue Collection

Coronal sections of brain tissues including 1 mm anterior and posterior to the injection site were quickly frozen in liquid nitrogen, stored at -80°C , and used for zymography. For microvessel counting and immunostaining, the whole brain was snap-frozen in isopen-

tane with dry ice and stored at -80°C . The tissue was sectioned with a cryostat at 16- μ m intervals.

Microvessel Counting

Mice were sacrificed for brain microvessel counting at 3 weeks after adenoviral-mediated gene transfer. The decision of timing was based on our previous data showing that the number of newly formed brain microvessels peaks at 3 weeks after AdVEGF transduction.¹⁹ Frozen sections were fixed with 100% ETOH at -20°C , then incubated with fluorescein-lycopersicin esculentum lectin (Vector Laboratories) 2 μ g/mL at 4°C overnight. Three areas of microvessels, left, right, and bottom to the needle track, respectively, were chosen in 2 separate brain coronal sections. Microvessel numbers were counted in images captured from these areas by using National Institutes of Health Image J 1.29x. The number of microvessels was calculated as the mean of the numbers obtained from the 6 pictures. Two investigators blinded to the animal treatment condition confirmed the vessel counts manually.

Gelatin Zymography

Equal amounts of sample proteins were separated by electrophoresis on 10% zymogram gels (Invitrogen). The gels were subsequently stained with colloidal blue stain (Invitrogen). Proteolytic bands in zymography were quantified by scanning densitometry using KODAK image analysis software (Eastman Kodak).

Immunohistochemistry

Tissue sections were fixed in 4% paraformaldehyde for 30 minutes. After blocking endogenous peroxidase with 1% hydrogen peroxide in 100% methanol followed by preincubation with 0.5% bovine serum albumin, anti-mouse MMP-9 antibody (R&D Systems) was applied at 4 μ g/mL for overnight at 4°C . The sections were then incubated with biotinylated rabbit anti-goat IgG (Vector Laboratories) for 1 hour at room temperature, followed by incubation with streptavidin-HRP (BioCare). Chromogenic staining was developed using DAB kit (Zymed) and followed by counter staining.

Statistical Analysis

Data are expressed as mean \pm standard deviation. Parameters between different groups in the MMP expression time course study and doxycycline treatment study were analyzed using 2-way ANOVA and Student *t* test. Because theory and previous results distinctly predict an increase of MMP activity and microvessel formation with VEGF administration and a reduction with the added administration of doxycycline, 1-sided *P* values were used for those comparisons; *P*<0.05 is considered statistically significant.

Results

To determine the appropriate time point to examine MMP expression induced by VEGF, the time course of MMP expression after AdVEGF transduction was studied (Figure 1). At 7 days, acute inflammatory response was minimal in all of the animals by H&E staining (data not shown). At day 7 after gene transfer, MMP-9 activities were increased near 4-fold in the AdVEGF transduced mouse brain compared with the ones with *AdlacZ* (883 ± 137 versus 179 ± 179 , arbitrary units [AU] 1-sided *P*<0.001). At day 14, there was little MMP-9 detected in either of the groups (73 ± 18 versus 165 ± 94 AU, 1-sided *P*>0.05), and the differences in MMP-9 levels were greater at 7 days than at 14 days (2-way factorial test for interaction, *P*<0.01). Therefore, day 7 after gene transfer appeared to be an appropriate time for the MMP study. Unlike MMP-9, there was no significant change in MMP-2 expression in response to VEGF either at day 7 (3363 ± 1204 versus 2997 ± 998 AU, 1-sided *P*>0.2) or at day

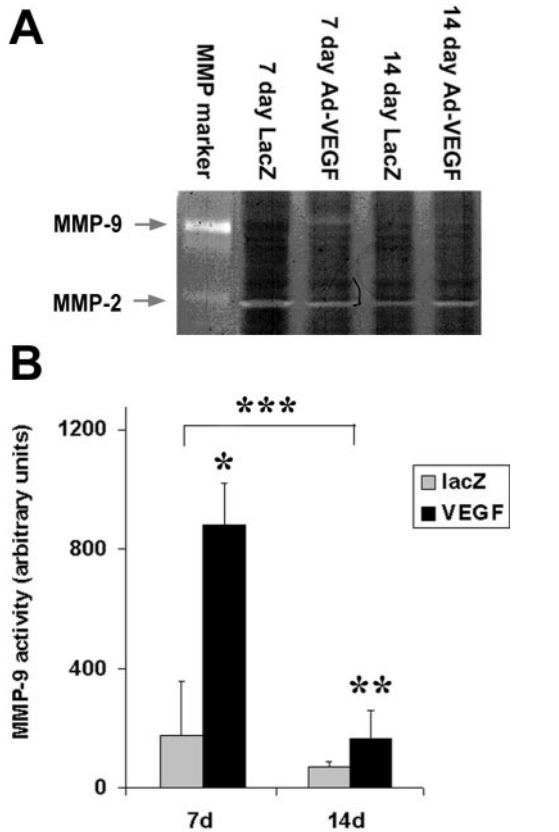


Figure 1. Time course study of MMP expression in the mouse brain after AdVEGF transduction by zymogram. A, Representative photograph of zymogram gel. MMP markers were recombinant human proteins with molecular weight (MW) \approx 92 Kd, and the bands for our specimens slightly lagged behind because of the slightly higher MW of mouse MMP-9 at 105 Kd. B, Bar graph of arbitrary values from densitometry measurement of MMP-9 bands on zymogram gels. *1-sided $P < 0.001$ (Student *t* test), VEGF group versus *lacZ* at day 7. **1-sided $P > 0.05$ (Student *t* test), VEGF group versus *lacZ* at day 14. *** $P < 0.01$ (2-way factorial interaction test), difference in groups at day 7 versus day 14.

14 (2688 ± 615 versus 3329 ± 661 AU, 1-sided $P > 0.2$) (see zymograph in Figure 1A; bar graph not shown).

Immunohistochemistry staining was performed to compare the distribution of MMP-9 expression at day 7 after gene transfer (Figure 2). Diffuse positive staining of MMP-9 was distributed in surrounding areas of the needle track after AdVEGF transduction, as illustrated in Figure 2B, but not in the *AdlacZ* mouse brain (Figure 2A).

Doxycycline suppressed MMP-9 expression at 7 days after AdVEGF transduction in mouse brain (Figure 3). MMP-9 activities were much lower in doxycycline-treated mice than those that did not receive the drug (89 ± 72 versus 883 ± 137 AU, 1-sided $P < 0.001$). In contrast, the *AdlacZ* group was not statistically significantly different from the AdVEGF plus doxycycline group (179 ± 179 versus 89 ± 72 AU, 2-sided $P > 0.4$). In other words, the VEGF-induced MMP-9 activities were completely diminished by doxycycline treatment. Again, unlike MMP-9, there was no change of MMP-2 activity in response to doxycycline treatment (2997 ± 998 versus 2684 ± 821 AU, 1-sided $P > 0.3$) (Figure 3A).

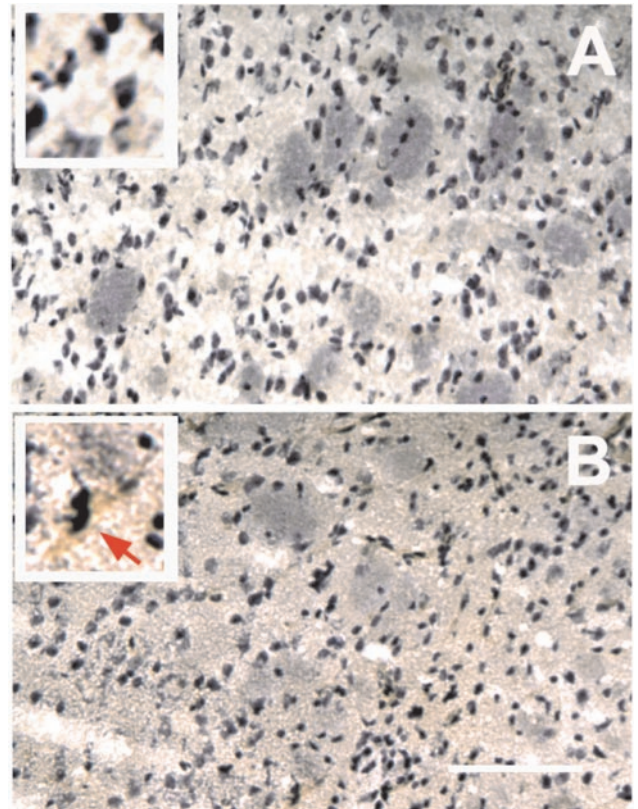


Figure 2. Comparison of MMP-9 expression by immunohistochemistry in the mouse brains after *lacZ* and VEGF gene transfer. (A) *AdlacZ*. (B) AdVEGF. Photographs show portions of the areas surrounding the needle tracks in caudate putamen. There is diffuse dark brown staining in the mouse brain with AdVEGF transduction (B), but not in the *AdlacZ* group (A). Inset is a higher magnification of the field. The red arrow in the inset indicates positive MMP-9 staining surrounding a cell. Bar=50 μ m.

To determine the effect of doxycycline on cerebral angiogenesis, 3 groups of mice ($n=6$ in each group) at 3 weeks after adenoviral DNA transduction were used for comparison. Mice with *AdlacZ* transfer in the brain were used as the control group. Our results showed that doxycycline decreased microvessel counts induced by focal VEGF hyperstimulation in the mouse brain (Figure 4). VEGF transduction increased microvessel formation by 19% in the mouse brain in comparison with the *lacZ* group (255 ± 27 versus 215 ± 15 , number of microvessels; 1-sided $P < 0.01$). The number of microvessels was lower in the doxycycline group than in the VEGF group (231 ± 17 versus 255 ± 27 , number of microvessels; 1-sided $P < 0.05$).

Discussion

Our results have demonstrated for the first time to our knowledge that doxycycline suppresses VEGF-induced cerebral MMP-9 activity in vivo. The doxycycline-induced changes in MMP-9 activity were associated with decreased regional angiogenesis, as evidenced by decreased microvessel counts.

Previous reports have shown that tetracycline derivatives, including doxycycline, influence many aspects of the angiogenesis process.¹⁸ Many of those data, however, were obtained from in vitro systems or from large blood vessels in animals. First, there have been conflicting results of the effect of doxycycline

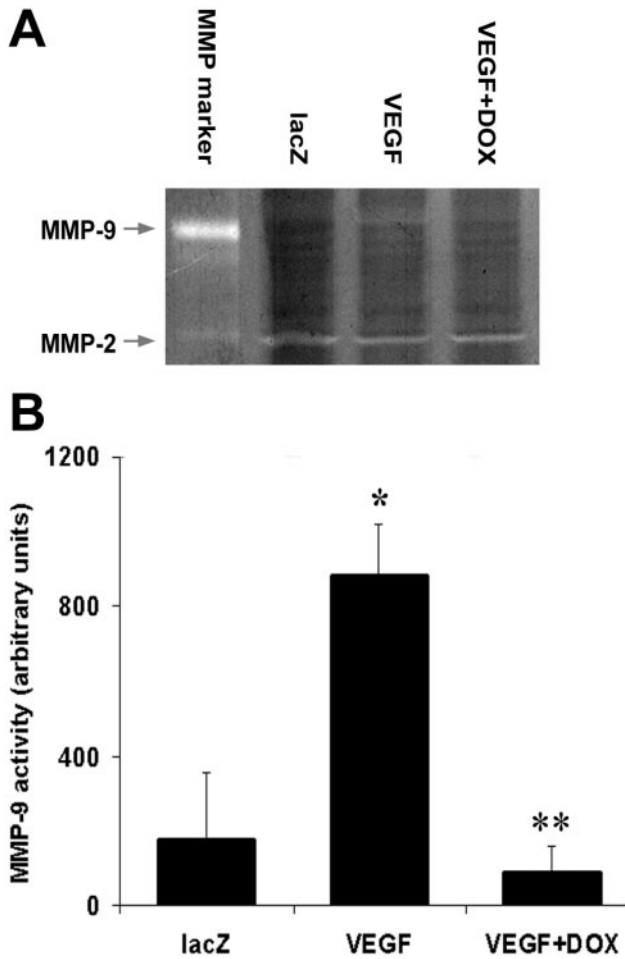


Figure 3. A, Representative photograph of zymogram gel. MMP markers were recombinant human proteins with MW \approx 92 Kd, and the bands for our specimens slightly lagged behind because of the slightly higher MW of mouse MMP-9 at 105 Kd. B, Effect of doxycycline (DOX) on MMP-9 activity in AdVEGF transduced mouse brain by zymogram. Bar graph of arbitrary values from densitometry measurement of MMP-9 bands on zymogram gels. *1-sided $P < 0.001$ (Student *t* test), Ad-VEGF versus AdlacZ; **1-sided $P < 0.001$ (Student *t* test), DOX treatment group versus AdVEGF transduction only.

on cell growth between in vitro and in vivo studies, suggesting the possibility of different mechanisms. For example, in vivo studies on smooth muscle cell proliferation showed that effects of doxycycline differ from known MMP inhibitors, including GM6001²² and BB94.²³ However, in vitro studies using tissue culture showed contradictory results. One reported that doxycycline inhibited angiogenesis whereas GM6001 did not.²⁴ The other one reported that the antiangiogenesis effects of tetracycline derivatives were associated with inhibition of MMP activities.²⁵ Secondly, vascular endothelium in the central nervous system may be functionally distinct from the endothelium of other organ systems. Doxycycline has been shown to inhibit MMP-9 in aorta homogenate cultures and in human and animal aortic aneurysm studies.^{26,27} However, there are significant differences in baseline endothelial MMP activities between the brain microvessel and the aorta. For example, MMP-9 levels increased in the brain microvessel endothelial cells in response to stimulation by inflammatory cytokines, whereas no change

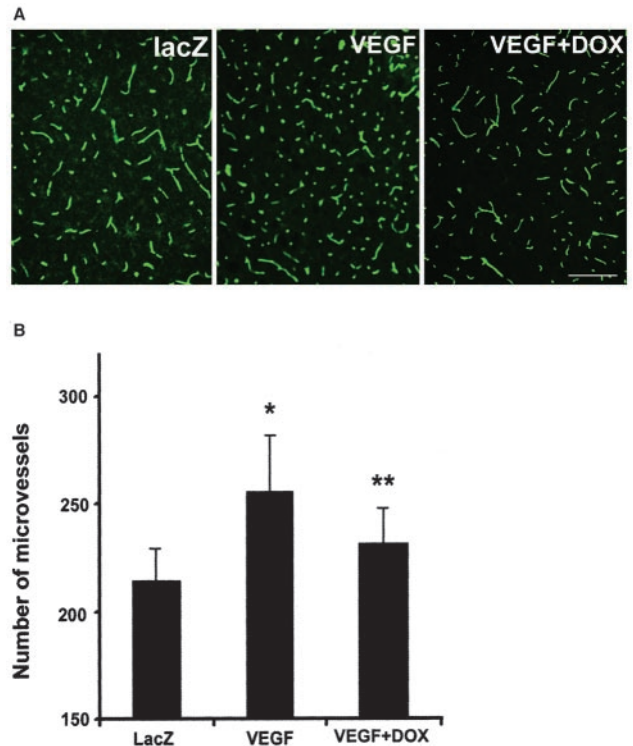


Figure 4. Effects of doxycycline (DOX) on microvessel counts in the mouse brain with AdVEGF transduction. A, Illustrations of lectin staining of brain microvessels near the needle track of adenoviral injection. Bar=500 μ m. B, Values shown in bar graph represent microvessel counts in different treatment groups at 3 weeks after adenoviral injection. Values are mean \pm SD; n=6 in all groups. *1-sided $P < 0.01$ (Student *t* test), AdVEGF versus AdlacZ; **1-sided $P < 0.05$ (Student *t* test), DOX treatment group versus AdVEGF transduction only.

was observed in the aortic endothelial cells.²⁸ Our finding that doxycycline inhibits MMP-9 activity and the formation of capillaries in the mouse brain provides evidence that doxycycline can influence cerebral angiogenesis.

VEGF and MMPs are considered as potent regulators of angiogenesis. Our findings have shown that focal VEGF hyperstimulation is associated with increased MMP-9 expression in the mouse brain. Similarly, Lamoreaux et al reported that VEGF increases the release of another gelatinase, MMP-2, and decreases the release of tissue inhibitors of metalloproteinases by microvascular endothelial cells in vitro.¹⁰ Further, MMP-9 can facilitate the availability of tissue-bound VEGF,^{11,12} which in turn may potentiate angiogenic activities.

There is somewhat contradictory evidence of MMP-9 and MMP-2 in response to stimulatory or inhibitory factors.^{26,28,29} In our study, there was no change in MMP-2 levels with VEGF stimulation or after doxycycline treatment, in contrast to changes in microvessels and MMP-9 levels. In the brain, in addition to endothelial cells and smooth muscle cells, astrocytes constitutively produce MMP-2.³⁰ MMP-2 activities that we detected from all groups of mice regardless of the treatment could be a reflection of constitutive expression of MMP-2, which, in this case, does not respond to either the stimulation from VEGF or the inhibition from doxycycline.

There are several limitations to our study. As pointed out, this model is not a specific model for any particular disease but can

allow mechanistic investigations of various potential interventions in the angiogenic process. Ideally, we would have performed a dose-response study, but the dose used appears to be sufficient to provide proof-of-concept for the hypothesized effects of doxycycline, taken together with other information in the literature. An inflammatory tissue response from adenoviral transduction may confound the direct effects of VEGF overexpression, but our previous studies have demonstrated a minimal degree of acute inflammation with this model.^{19,20} Finally, we have only demonstrated an association between decreased MMP-9 activity and diminished ability of VEGF to induce capillary angiogenesis; further studies can better characterize the causal relationship of the 2 observations.

In conclusion, the present study has demonstrated that doxycycline can reduce MMP-9 activity and angiogenesis induced by focal VEGF hyperstimulation in the mouse brain. The ability to manipulate angiogenesis may have importance in the study of various CNS disorders, and in particular may be of interest in developing models to study the pathogenesis of brain vascular malformations. The mechanism of the effect of doxycycline on brain angiogenesis in relation to its anti-MMP activity remains to be further clarified.

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