Matrix Metalloproteinase-3 Accelerates Wound Healing following Dental Pulp Injury

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Matrix metalloproteinases (MMPs) are implicated in a wide range of physiological and pathological processes, including morphogenesis, wound healing, angiogenesis, inflammation, and cancer. Angiogenesis is essential for reparative dentin formation during pulp wound healing. The mechanism of angiogenesis, however, still remains unclear. We hypothesized that certain MMPs expressed during pulp wound healing may support recovery processes. To address this issue, a rat pulp injury model was established to investigate expression of MMPs during wound healing. Real-time RT-PCR analysis showed that expression of MMP-3 and MMP-9 (albeit lower extent) was up-regulated at 24 and 12 hours after pulp injury, respectively, whereas expression of MMP-2 and MMP-14 was not changed. MMP-3 mRNA and protein were localized in endothelial cells and/or endothelial progenitor cells in injured pulp *in vivo*. In addition, MMP-3 enhanced proliferation, migration, and survival of human umbilical vein endothelial cells *in vitro*. Furthermore, the topical application of MMP-3 protein on the rat-injured pulp tissue *in vivo* induced angiogenesis and reparative dentin formation at significantly higher levels compared with controls at 24 and 72 hours after treatment, respectively. Inhibition of endogenous MMP-3 by *N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid resulted in untoward wound healing. These results provide suggestive evidence that MMP-3 released from endothelial cells and/or endothelial progenitor cells in injured pulp plays critical roles in an-

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Matrix metalloproteinases (MMPs) comprise a zinc-dependent endopeptidase family of at least 24 mammalian members.^{1–4} MMPs act on a variety of substrates, including extracellular matrix (ECM) proteins, proteinases, and their inhibitors, to activate latent growth factors, cytokines, chemokines, receptors, and adhesion molecules or to alter protein function, such as shedding of cell surface proteins.^{5,6} Thus, MMPs have been implicated in a wide range of physiological and pathological processes, including embryonic development, wound healing, tissue remodeling, angiogenesis, morphogenesis, inflammation, and cancer.^{7–9}

Wound healing is a multifactorial process involving the migration, proliferation, and differentiation into several cell populations with subsequent formation of extracellular matrix. MMPs may influence cell migration through extracellular matrix degradation or by altering cellular adhesive properties. MMPs may also stimulate proliferation and/or antiapoptosis by altering the extracellular matrix microenvironment. They further modulate the activity of growth factors and receptors.^{10,11}

On the basis of their ability, MMPs participate in wound healing and angiogenesis.⁴ Several different MMPs play a beneficial role in wound-healing models. During the wound-healing process of skin, CXC chemokine receptor (CXCR) 4⁺ cells migrating around vessels release

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MMP-9 to enhance angiogenesis.¹² MMP-2 increases between 7 and 14 days after spinal cord injury, and MMP-2 deficiency results in an extensive astrocytic scar, suggesting MMP-2 promotes functional recovery after injury.¹³ Increased expression of MMP-2 and MMP-9 is also associated with the healing mechanism of aneurysm.¹⁴ Gelatinolytic activities corresponding to pro-MMP-2 and the active form of MMP-2 are detected at the wound site 3 and 7 days after glaucoma filtration surgery, suggesting its important roles in the degradation of the ECM in the wound-healing process.¹⁵

Dental pulp is encased in dentin, which plays a role as a barrier against bacterial, chemical, and physical stimuli. When the barrier is disrupted by traumatic injury or caries, the dentin-pulp complex has a potential to repair and regenerate. Angiogenesis is essential for this pulp wound-healing process, because blood vessels play an important role in nutrition and oxygen supply, as a conduit for transport of metabolic waste, pulp homeostasis and metabolism, and stem/progenitor cell migration.¹⁶ During pulp wound-healing process, dental pulp stem/progenitor cells migrate to the injured site from perivascular region in the pulp tissue deeper from the injured site.¹⁶ They proliferate and differentiate into endothelial cells for angiogenesis/vasculogenesis or into odontoblasts for reparative dentin formation.¹⁷ The angiogenic signals, such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and transforming growth factor- β , released from injured dental pulp cells, endothelial cells, and ECM by injury contributes to the migration of stem/progenitor cells.^{18–20} EphB/ephrin-B also plays a role in restricting dental pulp stem cells attachment and migration to maintain dental pulp stem cells within their stem cell niche under steady-state conditions.²¹ Precise mechanism for migrating stem/progenitor cells and angiogenesis/vasculogenesis during pulp wound-healing process, however, still remains unclear.

We hypothesized that certain MMPs expressed during pulp wound healing may support recovery processes. MMP-1, -2, -9, and -14 are expressed in healthy dental pulp.²² In inflamed pulp, MMP-9 mRNA level is increased in the odontoblasts, fibroblasts, inflammatory infiltrates, and endothelial cells.²³ Expression and function of MMPs during pulp wound healing, however, have not been systematically studied. To address these issues, we established a rat pulp injury model to investigate the expression of MMPs during wound healing. Our results demonstrated that MMP-3 induced the proliferation, migration, and survival (antiapoptosis) of endothelium *in vitro* and accelerated angiogenesis and pulp wound healing *in vivo*.

Materials and Methods

Antibodies

Goat anti-rat CXCR4 from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-rat MMP-3 from Daiichi Fine Chemical (Toyama, Japan), normal mouse IgG (AbD Se-

rotec, Oxford, UK), rabbit anti-goat IgG Alexa 488, horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody from Invitrogen (Carlsbad, CA), anti-proliferating cell nuclear antigen (PCNA) antibody from Dako (Carpinteria, CA), and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody from Zymed Laboratories (San Francisco, CA) were used.

Experimental Pulp Injury Model

Eight-week male Wistar rats, weighing 220–260 g, were purchased (CLEA, Tokyo, Japan). The experimental protocol was approved by the animal committee of the School of Dentistry, Aichi-Gakuin University, and the National Institute for Longevity Sciences of Japan for animal care. The rats were anesthetized with an i.p. injection of sodium pentobarbital (Schering-Plough, Kenilworth, NJ) at a dose of 30 mg/kg, and 2-mm crowns of maxillary incisors were removed with a diamond point burr (Shofu, Tokyo, Japan), then pulp was cut by a no. 1/2 round burr (Shofu). The amputated pulp was thoroughly washed by PBS and covered with spongel (Astellas Pharma, Tokyo, Japan), and the cavities were filled with light-cured composite resin (UniFil Flow GC, Tokyo, Japan) following treatment with a bonding agent (Clearfil Mega Bond; Kuraray, Kurashiki, Japan). One, 12, 24, 72 hours, and 7 days after injury, rats were perfused with 4% paraformaldehyde (Nakarai tesque, Kyoto, Japan) in PBS (pH 7.4), and the incisors with surrounding tissue were extracted with the animals under anesthesia as described previously. The tissues were fixed in 4% paraformaldehyde at 4°C for overnight and decalcified with 10% formic acid for 2 weeks. The tissues were dehydrated in ascending ethanol series and embedded in paraffin wax (Sigma, St. Louis, MO), and 5- μ m-thick sections were cut and mounted on aminopropyl triethoxysilane-coated slides (Matsunami, Tokyo, Japan). The slides were stored at 4°C until used for *in situ* hybridization and H&E histological examination. The samples for immunohistochemistry were embedded in optimal cutting temperature compound (Sakura, Tokyo, Japan), and 12- μ m-thick frozen sections were cut and mounted on aminopropyl triethoxysilane-coated slides (Matsunami).

Real-Time RT-PCR Analysis

Dental pulp tissues were isolated 0, 12, 24, 48, and 72 hours after injury. The normal pulp tissue from maxillary incisors was used as a control. Total RNA was isolated by TRIzol (Invitrogen) from the freshly excised pulp tissues, and 2 μ g of RNA was reverse transcribed with ReverTra Ace- α (Toyobo, Tokyo, Japan), following the manufacturer's recommendations. The resulting cDNA was then amplified by real-time RT-PCR with Light Cycler-FastStart DNA master SYBR Green I (Roche Diagnostics, Mannheim, Germany). Real-time RT-PCR amplifications were performed at 95°C for 10 seconds, 62°C for 15 seconds, and 72°C for 8 seconds using the primers for β -actin, MMP-2, MMP-3, MMP-9, MMP-10, MMP-13, MMP-14, VEGF, and CXCR4 (Table 1) labeled with Light Cycler-Fast Start DNA

Table 1. Primers for Real-Time RT-PCR

Gene		Sequence	Product size (bp)	Accession number
β -actin	Forward	5'-AAGTACCCCATTTGAACACGG-3'	257	NM_031144
	Reverse	5'-ATCACAATGCCAGTGGTACG-3'		
MMP-2	Forward	5'-GATGGCAAGGTGTGGTGTG-3'	191	NM_031054
	Reverse	5'-AATCGGAAGTTCTTGGTGTAGG-3'		
MMP-3	Forward	5'-TGGCAGTGAAGAAGATGCTG-3'	167	NM_133523
	Reverse	5'-GCTTCCCTGTCATCTTCAGC-3'		
MMP-9	Forward	5'-CGCTTGGATAACGAGTTCTCTC-3'	163	NM_031055
	Reverse	5'-GCAGGAGGTCATAGGTCACG-3'		
MMP-10	Forward	5'-ACCCCACTCACATTCTCCAG-3'	163	NM_133514
	Reverse	5'-CATCGAAGTGAGCATCTCCA-3'		
MMP-13	Forward	5'-GCACTACTTGAAATCATACTACCATCC-3'	183	NM_133530
	Reverse	5'-ACATCAGGCATCCACATCTTG-3'		
MMP-14	Forward	5'-AGTCAGGGTCACCCACAAAG-3'	204	NM_031056
	Reverse	5'-GGTATCCGTCCATCACTTGG-3'		
VEGF	Forward	5'-CTACCTCCACCATGCCAAGT-3'	183	NM_031836
	Reverse	5'-ACACAGGACGGCTTGAAGAT-3'		
CXCR4	Forward	5'-TCCGTGGCTGACCTCTCTT-3'	210	NM_022205
	Reverse	5'-CAGCTTCCTCGGCCTCTGGC-3'		

master SYBR Green I (Roche Diagnostics, Pleasanton, CA) in Light Cycler (Roche Diagnostics). The design of the oligonucleotide primers was based on published rat cDNA sequences. When rat sequences were not available, porcine sequences were used. The RT-PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI) and confirmed by sequencing based on published cDNA sequences. Expression in the pulp tissue immediately, and 12, 24, 48, and 72 hours after injury, was compared with rat normal pulp tissue after normalizing with β -actin. The experiments were repeated three times, and one represented experiment is presented.

Immunohistochemistry

For double-staining immunohistochemistry of CXCR4 and MMP-3, cryotome sections (12 μ m thick) of 24 and 72 hours after injury were used. The endogenous peroxidase activity was blocked with 2% hydrogen peroxide in methanol for 20 minutes. To avoid nonspecific staining, the sections were incubated with 10 mg/ml blocking reagent (PerkinElmer, Boston, MA) for 1 hour at room temperature and then reacted with goat anti-CXCR4 (1/50) in Canget 1 buffer (Toyobo) for 1 hour at room temperature. After three washes in PBT (PBS, 0.05% Tween 20, pH 7.4), sections were incubated with rabbit anti-goat IgG Alexa 488 (1/200) in PBT for 1 hour at room temperature. Unbound antibodies were washed three times with PBT. Sections were incubated with mouse anti-MMP-3 (0.5 μ g/ml) in Canget 1 buffer overnight at 4°C. After three washes in PBT, bound antibodies were reacted with a horseradish peroxidase-labeled goat anti-mouse IgG secondary antibody for 1 hour at room temperature. Color was developed using tyramide signal amplification system Rhodamine-conjugated tyramide (Invitrogen) according to the manufacturer's instructions. All slides were developed in parallel, and the reaction was stopped before detection of nonspecific staining in control preimmune serum-treated sections. Sections were then stained with dye Hoechst 33342 (Sigma) and mounted with Prolong Gold antifade reagent (Invitrogen) and photo-

graphed on a Zeiss Confocal LSM510 microscope (Carl Zeiss, Berlin, Germany). To confirm the localization of MMP-3 in blood vessels, frozen sections were treated with 20 μ g/ml proteinase K (Invitrogen) for 6 minutes at room temperature. After three washes in PBS, sections were stained with 20 μ g/ml Fluorescein Griffonia (Bandeiraea) Simplicifolia lectin (BS1-lectin) (Vector Laboratories, Burlingame, CA) for 15 minutes. This BS1-lectin has been used for specific staining of endothelial cells and endothelial progenitor cells.²⁴ After three washes in PBS, sections were stained with MMP-3 as described above. As negative controls, both only primary antibodies and only secondary antibodies were used. Normal mouse IgG was also used for determination of nonspecific binding of anti-mouse MMP-3 antibody.

In Situ Hybridization Analysis

In situ hybridization staining of sections was done as described previously.²⁵ The cryotome sections obtained 24 and 72 hours after injury were observed by *in situ* hybridization analysis using a rat MMP-3 anti-sense probe. The probes were constructed out of the plasmids after subcloning the PCR product using the same primers designed for real-time RT-PCR (Table 1).

Proliferation, Migration, and Antiapoptosis Analysis

For proliferation analysis, human umbilical vein endothelial cells (HUVECs) (KURABO Industries, Osaka, Japan) at third passage were seeded with 1000 cells per 96-well. They were cultured in endothelial basal medium-2 in the presence of human MMP-3 (50 ng/ml; Chemicon, Temecula, CA) with or without *N*-Isobutyl-*N*-(4-methoxyphenylsulfonyl)-glycylhydroxamic acid (NNGH) (0.13 μ mol/L; Biomol, Plymouth Meeting, PA), NNGH only, MMP-10 (50 ng/ml; R&D Systems, Minneapolis, MN), or VEGF-A (50 ng/ml; Pepro-Tech, London, UK). NNGH is highly specific for MMP-3 at the concentration used.²⁶ Ten microliters of Tetra-color one

(Seikagaku Kogyo, Tokyo, Japan) was added to the 96-well plate, and cell numbers were measured using spectrophotometer at 450 nm absorbance at 2, 12, 24, 36, 48, and 60 hours of culture. As a control, cells were cultured without any supplement. Wells without cells were served as negative controls.

For migration analysis, modified Boyden chamber assays were performed for 24 hours at 37°C with polyethylene terephthalate membrane (BD Biosciences, Franklin Lakes, NJ) in 24-well plates. In brief, HUVECs (10,000/well) were cultured in the upper chamber in the presence of 10 or 100 ng/ml MMP-3 with or without 0.13 μ mol/L NNGH, 50 ng/ml MMP-10 with or without 0.13 μ mol/L NNGH, NNGH only, or 50 ng/ml VEGF-A. As a control, 0.2% bovine serum albumin (Sigma) was used. After the incubation period, the migrated HUVECs attached to the

lower side of the filter were harvested by trypsinization and counted. The numbers of migrated cells were enumerated. The nonmigrated cells on the upper side of the filter were scraped off with a rubber scraper.

To evaluate the effect of MMP-3 on anti-apoptosis, HUVEC at passage 6 or less were grown in endothelial growth medium-2 in a 35-mm dish for 3 days and then incubated with 100 nmol/L staurosporine (Sigma) in EBM-2 supplemented with 50 ng/ml MMP-3 with or without 0.13 μ mol/L NNGH, NNGH only, 50 ng/ml MMP-10, or VEGF-A. NNGH was added 30 minutes before adding MMP-3. As a control, cells were cultured in EBM-2 without any supplement in the presence of staurosporine. After 4 hours, HUVECs were harvested, and the cell suspensions were stained with Annexin V-FITC (Roche) and propidium iodide (Roche) for 15 minutes and then ana-

A

Figure 1. Morphological analysis by H&E staining of wound-healing process in rat-injured pulp. **A:** Schematic diagrams of amputation and filling of rat upper incisor. **Left to right:** rat upper incisor, removing the upper part of crown with a diamond point burr, amputation of pulp with a round burr, washing and stop bleeding, and filling with spongel and resin. **B:** Low power normal pulp. **Arrows** indicate imaginary amputated site. **C:** High power normal pulp; from the boxed area in **B**. **D:** A lower power image of the wound-healing event under the amputated site (**arrows**) at 12 hours after injury. **E** and **F:** One hour after injury, hyperemia, dilatation of blood vessels, and infiltration of neutrophils were observed in upper part of pulp tissue under the amputated site. Twenty-four hours after injury, no inflammatory cells were found, and the denatured tissue was seen under the amputated site. **G** and **H:** A large number of polygonal-shaped cells in pulp can be observed. **I** and **J:** Seventy-two hours after injury, spindle-shaped cells were embedded in collagenous matrix. **K** and **L:** Seven days after injury, odontoblast-like cells and tubular dentin are seen. OD, osteodentin; V, newly formed blood vessels; OB, odontoblast-like cells; TD, tubular dentin. The **upper panels (E, G, I, K)** represent the amputated site. The lower panels (**F, H, J, L**) are magnified from the corresponding boxes in the panel above.

lyzed by flow cytometry (Bay bioscience, Kobe, Japan). The experiments were repeated three times, and one represented experiment is presented.

MMP-3 Protein Application on the Amputated Pulp

After amputation of rat incisor pulp as described above, 50 ng of MMP-3 absorbed in spongel was applied on the amputated pulp. As controls, PBS absorbed in spongel or NNGH together with MMP-3 was used. The cavities were filled with the bonding agent and resin as described above. The paraffin sections (5- μ m thickness) of 24 hours, 72 hours, and 7 days after treatment were morphologically examined after H&E staining or Masson trichrome staining.

For quantitative analysis of newly formed blood vessels, each five frozen sections at 24 hours after treatment, both with MMP-3 and control PBS from four incisors each, a total of 40 sections were stained with Fluorescein Griffonia (Bandeiraea) Simplicifolia lectin (BS1-lectin) as described above. Three rectangles of a standardized size (0.1 mm²) were drawn in the upper part of pulp tissue under the amputated site in every five sections from one sample. The lectin-positive area relative to total area (1.5 mm²) was quantitatively analyzed in a standardized procedure using BZ-II Analyzer (Keyence, Tokyo, Japan) software on a Keyence BZ-9000 fluorescence microscope (Keyence). The data were presented as means $\pm$ SD at four determinations.

To evaluate effect of MMP-3 on proliferation, immunohistochemical analysis of PCNA was performed. Paraffin sections of 24 hours after treatment were deparaffinized, and antigen was retrieved by antigen unmasking solution (Vector Laboratories), according to the manufacturer's instructions. Endogenous peroxidase activity and non-specific staining were blocked as described earlier. The sections were incubated with anti-PCNA antibody (Dako) at dilutions of 1/100 in antibody diluent (Dako) overnight at 4°C and further incubated with a peroxidase-conjugated secondary antibody (ImmPRESS reagent; Vector Laboratories) for 30 minutes at room temperature. After development by DAB+Liquid System (Dako), sections were counterstained with hematoxylin and photographed on an Olympus Vanox-s microscope (Olympus, Tokyo, Japan). For quantitative analysis of proliferating cells, each three paraffin sections at 24 hours after treatment, both with MMP-3 and control PBS from 4 incisors each, were used. Three rectangles of a standardized size (0.1 mm²) were drawn in the upper part of pulp tissue under the amputated site in every three sections. The positive staining cells were counted and quantitatively analyzed. The data were presented as means $\pm$ SD at four determinations.

For quantitative analysis of matrix formation, three fields of each five paraffin sections at 72 hours after treatment, both with MMP-3 and control PBS from four incisors each, and three fields of five sections at 7 days after treatment with MMP-3, MMP-3+NNGH, PBS+NNGH, and control PBS from four incisors each were stained with Masson trichrome

staining. The positive area was quantitatively analyzed in the upper part of pulp tissue under the amputated site, 2 mm in depth from the amputated site, using BZ-II Analyzer software. The data were presented as means $\pm$ SD at four determinations.

Figure 2. Real-time RT-PCR analysis of expression of mRNAs during pulp wound-healing process. **A:** Expression of MMP-2, MMP-3, MMP-9, MMP-13, and MMP-14 during pulp wound-healing process. MMP-3 showed a ninefold peak at 24 hours after injury and decreased to twofold at 72 hours. MMP-9 showed twofold expression at 12 hours. MMP-2 and MMP-14 didn't show any expression change during the healing process. **B:** MMP-13 showed 470-fold expression at immediately after injury. MMP-10 was not expressed both in normal and injured pulp. **C:** Expression of VEGF and CXCR4. VEGF showed 3.5-fold expression immediately after injury, then increased to fivefold at 24 hour after injury. CXCR4 showed 3.5-fold expression at 24 hours after injury. The experiments were repeated three times, and one representative experiment is presented (* $P < 0.05$; compared with 0 hour).

Statistical Evaluation

All data were expressed as means $\pm$ SD at four determinations. *P* values were calculated by using the unpaired Student's *t*-test. Differences were considered to be statistically significant at the *P* < 0.05 level.

Results

Histology of Pulp Tissues during Wound-Healing Process

Pulp normal and wound-healing models were constructed using rat upper incisors (Figure 1, A–D). One hour after injury, red blood cells and dilatation of blood vessels were observed. Acute inflammatory cells, such as neutrophils, infiltrated in the pulp tissue under the amputated site (Figure 1, E and F). Twenty-four hours after injury, the inflammatory cells disappeared, and a large number of fibroblast-like cells and polygonal shaped cells and new blood vessels were found in the pulp tissue under the amputated site (Figure 1, G and H). Seventy-two hours later, spindle-shaped cells were surrounded by immature collagenous matrix to form osteodentin in the upper pulp tissue under the amputated site (Figure 1, I and J). Seven days after injury, one to two layers of well-arranged odontoblast-like cells were found to form tubular dentin under the osteodentin (Figure 1, K and L). Pulp injury completely healed with optimal angiogenesis (Figure 1K) and accompanied by osteodentin/tubular dentin formation. Thus, these results suggested that this pulp injury model was useful for analyzing expression and function of MMPs during pulp wound-healing process.

RNA Expression during Wound-Healing Process

Expression of MMP-2, -3, -9, -10, -13, -14, VEGF, and CXCR4 mRNA was analyzed with real-time RT-PCR at 0, 12, 24, 48, and 72 hours after injury. As shown in Figure 2A, MMP-3 was expressed nearly ninefold at 24 hours compared with control normal pulp tissue, then decreased to fourfold at 48 hours and twofold at 72 hours. MMP-9 expression showed twofold compared with control at 12 hours and decreased to the same level as 0 hour at 72 hours. MMP-13 expression was 470-fold at 0 hour, immediately after injury compared with control normal pulp, and rapidly decreased at 12 hours (Figure 2B). In contrast, expression of MMP-2 and MMP-14/MT1-MMP was continuously at the same level as control normal pulp during the pulp wound-healing process (Figure 2A). MMP-10 mRNA was not detected both in rat normal pulp and the injured pulp.

On the other hand, VEGF showed 3.5-fold expression at 0 and 12 hours after injury, then increased to 5-fold at 24 hours after injury and decreased to basal expression level at 72 hours. CXCR4 showed 3.5-fold increase in expression at 24 hours after injury (Figure 2C).

Localization of MMP-3 and CXCR4 during Pulp-Healing Process

Immediately after injury, neither MMP-3 nor CXCR4 was found in pulp (data not shown). Twenty-four hours after injury (Figure 3J), MMP-3 protein was detected in endothelial cells and/or endothelial progenitor cells in newly formed microvessels and larger vessels under the amputated site (Figure 3, A, D, and K), which positively stained with BS1-lectin (Figure 3, B and C). MMP-3 mRNA expression overlapped CXCR4 in the vascular region (D–I). MMP-3 expression was reduced at 72 hours after injury (N). Arrows indicate the positive signals (A–I, M, N). H&E staining (J–L). Arrowheads indicate the amputated site (J). MV, microvessel; LV, larger vessel.

Double-staining immunohistochemistry showed that MMP-3 expression overlapped CXCR4 in vascular region (Figure 3, D–F). Seventy-two hours after injury (Figure 3L), MMP-3 expression also overlapped CXCR4 in microvascular and larger vessel (Figure 3, G–I). MMP-3 mRNA expression was weakly detected in vascular region (Figure 3N).

MMP-3 Stimulates Proliferation and Migration and Inhibits Apoptosis of HUVECs in Vitro

We next examined whether MMP-3 could induce the proliferation, migration, and survival (antiapoptosis) of HUVECs. As shown in Figure 4A, MMP-3 significantly increased proliferation activity of HUVECs compared with control similarly to the positive control VEGF. The proliferative effect of MMP-3 was significantly blocked by NNGH, a specific MMP-3 inhibitor. HUVECs cultured with NNGH showed a significantly reduced proliferation.

MMP-3 significantly stimulated migration activity of HUVECs in a dose-dependent manner. This effect was two times stronger compared with VEGF as a positive control and significantly blocked by NNGH (Figure 4B).

We also analyzed whether MMP-3 could inhibit the apoptosis of HUVECs. The cells cultured with 100 nmol/L/ml staurosporine for 4 hours caused apoptosis in 52% HUVECs. When cells were treated with staurosporine and MMP-3 coincidentally, only 20% of cells showed apoptosis, indicating reduced apoptosis. This reduced apoptosis was completely blocked with NNGH. HUVECs cultured with staurosporine and VEGF also showed reduced apoptosis (Figure 4C).

However, MMP-10/stromelysin-2, which is a structural homologue of MMP-3, did not have any effect on proliferation, migration, and survival (antiapoptosis) of HUVECs as MMP-3 (Figure 4, A–C).

MMP-3 Induces Angiogenesis and Reparative Dentin Formation during Pulp Wound Healing

Next, we investigated whether MMP-3 induces angiogenesis in injured pulp *in vivo* (Figure 5A; Supplemental Figures S1–S3, see <http://ajp.amjpathol.org>). The rat incisor pulp was treated with MMP-3 in the presence or absence of NNGH. PBS was used as a negative control. Twenty-four hours after treatment, BS1-lectin-stained sections showed that the MMP-3-treated pulp exhibited more newly formed vessels compared with PBS control (Figure 5, B and C). Quantitative analysis indicated 2.0-fold increase in blood vessels in MMP-3-treated pulp compared with PBS control in the upper pulp tissue under the amputated site (Figure 5D). The presence of PCNA, a marker of cell proliferation, was examined in the sections of pulp at 24 hours after treatment. The PCNA-positive staining cells were observed significantly more—a 2.7-fold increase in number in the MMP-3-treated pulp (98.5 ± 17.7 cells/mm²) compared with PBS control (36.0 ± 14.1 cells/mm²) under the amputated site (Figure 5, E, F, and Q). Seventy-two hours after treatment, a large amount of reparative dentin formation was observed in

Figure 4. Effect of MMP-3 on proliferation, migration, and survival in cultured HUVECs. **A:** Enhanced proliferation by MMP-3 and VEGF. HUVECs seeded with 1000 cells per 96-well were cultured in the presence of human MMP-3 (50 ng/ml) with or without NNGH (0.13 μ mol/L), MMP-10 (50 ng/ml), or VEGF-A (50 ng/ml). Cell numbers were measured by adding Tetra-color one. NNGH significantly blocked the effect of MMP-3. **B:** Induced migration by MMP-3 in a dose-dependent manner. Modified Boyden chamber assays were performed for 24 hours with polyethylene terephthalate membrane in a 24-well plate. HUVECs (10,000/well) were cultured in the upper chamber in the presence of 10 or 100 ng/ml MMP-3 with or without 0.13 μ mol/L NNGH, 50 ng/ml MMP-10 with or without 0.13 μ mol/L NNGH, NNGH only, or 50 ng/ml VEGF-A. The induced effect by MMP-3 was blocked by NNGH. **C:** Reduced apoptosis by MMP-3. HUVECs grown in endothelial growth medium-2 for 3 days were incubated with 100 nmol/L staurosporine in endothelial basal medium-2 supplemented with 50 ng/ml MMP-3 with or without 0.13 μ mol/L NNGH, NNGH only, 50 ng/ml MMP-10, or VEGF-A. After 6 hours, the cell suspensions stained with Annexin V-FITC and propidium iodide were analyzed by flow cytometry. The cells cultured with 100 nmol/L staurosporine for 4 hours caused apoptosis in 52% of the cells. When treated together with MMP-3, only 20% of the cells showed apoptosis, indicating reduced effect on apoptosis. This effect was blocked by NNGH. Note no effect by MMP-10, a structural homologue of MMP-3. Data were expressed as means $\pm$ SD at four determinations (* P < 0.05; ** P < 0.01, compared with the control group; # P < 0.05, compared with the MMP-3 group). Statistical analyses were performed by the nonpaired Student's *t*-test. The experiments were repeated three times, and one represented experiments is presented. *** P < 0.01, compared with the MMP-3 group.

the MMP-3-treated pulp compared with the PBS control (Figure 5, G–J). Intense blue-stained collagen by Masson trichrome was observed around newly differentiated odontoblasts/osteodentinoblasts in the MMP-3-treated pulp (Figure 5K). *In situ* hybridization analysis in the series sections showed that *DSPP* mRNA was expressed in the newly differentiated odontoblasts/osteodentinoblasts confined in osteodentin matrix (Figure 5L). There is no obvious odontoblasts/osteodentinoblasts in the pulp that was treated with MMP-3 in the presence of NNGH (Figure

Figure 5. Acceleration of angiogenesis and pulp wound healing by MMP-3. Schematic diagrams of MMP-3 protein application on amputated pulp of rat upper incisor. **A:** The pulp cavity was filled with MMP-3 protein absorbed in spongel and resin filling. p, amputated pulp tissue; m, MMP-3 protein; r, resin (Supplemental Figures S1–S3, see <http://ajp.amjpatol.org>). Twenty-four hours after treatment (**B–F** and **Q**). Seventy-two hours after treatment (**G–N** and **R**). Seven days after treatment (**O, P, S**). BS-1-lectin staining (**B** and **C**), PCNA immunohistochemistry (**E** and **F**), H&E staining (**G–J**), Dentin slalophosphoprotein *in situ* hybridization (**L**), and Masson's trichrome staining (**K** and **M–P**) were used. **B:** Newly formed vessels in the upper part of pulp tissue under the amputated site in MMP-3-treated pulp. **C:** A few newly formed vessels in PBS control pulp. **E:** Many PCNA-positive staining cells were detected in MMP-3-treated pulp. **F:** A few PCNA-positive staining cells in PBS control pulp. **G,** magnified in **H:** A large amount of osteodentin formed under the amputated site. **I,** magnified in **J:** No osteodentin formation. **K:** Intense blue-stained collagen around newly formed osteodentinoblasts (arrows). **L:** Dentin slalophosphoprotein mRNA was detected in newly formed osteodentinoblasts (arrows). **M:** No intense blue-stained collagen and no osteodentinoblasts in PBS control. **N:** NNGH, a specific MMP-3 inhibitor, blocked the MMP-3-induced effect on osteodentin formation. **O** and **P:** Blue-stained collagen was broader in PBS control compared with that in NNGH-treated pulp on day 7. MMP-3-treated pulp (**B, G, H, K**) consisted of more newly formed vessels and more osteodentin compared with PBS control group (**C, I, J, M**). Quantitative analysis indicated that a twofold increase of blood vessels was induced by MMP-3 in the upper part of pulp tissue under the amputated site (**D**). Quantitative analysis of the PCNA-positive staining cells and collagenous matrix formation at 24 hours, 72 hours, and 7 days after treatment (**Q, R, S**). * $P < 0.01$.

5N), as well as in the PBS control pulp (Figure 5, 1, J, and M) at 72 hours after treatment. The dentin matrix formation was observed significantly more—a 3.8-fold increase in area in the MMP-3-treated pulp compared with PBS control under the amputated site (Figure 5R) at 72 hours. Seven days after treatment, NNGH, a MMP-3-specific inhibitor, inhibited the dentin matrix formation significantly more compared with PBS control (Figure 5, O, P, and S).

Discussion

This is the first study to systemically detect the MMPs expressed during pulp wound-healing process and to assess the role of MMPs in injured pulp tissue. Pulp amputation is a commonly performed operation, and it leaves a clean wound, and this is a good representative acute wound model to study the profiles of biological

factors, MMPs. Rat incisor is continuously erupting tooth, and the pulp tissue has a high potential to heal after pulp injury. Rat incisor has also advantages to get many homogenous samples of injured pulp tissue at once, and not take so much time before complete healing compared with canine teeth. Therefore, we have used the rat pulp injury model²⁷ to examine expression of MMPs during pulp wound healing process.

We have recently isolated pulp-derived CD31[−]; CD146[−] side population cells having a highly vasculogenic potential. MMP-3 was highly expressed in CD31[−]; CD146[−] side population cells compared with CD31⁺; CD146[−] side population cells without vasculogenic potential as well as total pulp cells.²⁸ Both MMP-9 and MMP-2 were very weakly expressed in those cell fractions.²⁸ When CD31[−];CD146[−] side population cells were transplanted on the amputated pulp in dogs, the transplanted cells were migrating in the vicinity of the newly formed vasculature and expressed proangiogenic factors, including MMP-3, implying trophic actions on endothelial cells (K. Iohara et al, unpublished data). A previous report has shown that MMPs are induced immediately following injury and show reproducible expression patterns.²⁹ In the present study, MMP-3 significantly increased at 24 hours after pulp injury, and MMP-9 weakly increased at 12 hours. MMP-13 was highly increased immediately after injury and rapidly decreased at 12 hours. MMP-2 and MMP-14 were not found to be changed. Therefore, we have focused our further study on MMP-3 to assess the role of MMPs in angiogenesis and pulp wound healing.

In the present study, MMP-3 was localized in endothelial cells and/or endothelial progenitor cells. MMP-3-positive cells expressed CXCR4, a marker of endothelial cells.³⁰ MMP-3 enhanced proliferation and migration activities and had an antiapoptotic effect on endothelial cells *in vitro*. The similar stimulatory effects of MMP-3 on cell proliferation and migration have also been reported in a human glioblastoma cell line after enhanced gene expression of MMP-3 by platelet-derived growth factor receptor- α .³¹ The high expression of MMP-3 is also reported in the endometrial regenerative cells, a novel stem cell population from the endometrium, which is involved in the angiogenesis phase of the menstrual cycle.³² Furthermore, topical MMP-3 protein application on the injured pulp significantly enhanced angiogenesis and proliferation under the amputated pulp at 24 hours after treatment. Therefore, these results suggest that MMP-3 induces proliferation, migration, and survival of endothelial cells, leading to angiogenesis. On the other hand, angiogenic factors, such as VEGF, basic fibroblast growth factor, and transforming growth factor- β , can be released from extracellular matrix/basement membrane by MMPs degradation,³³ suggesting that MMP-3 also regulates angiogenesis in an indirect way.

Protein application of growth/differentiation factors such as transforming growth factor- β 1, insulin-like growth factor-I and bone morphogenic proteins has been demonstrated to promote reparative dentin formation on the injured/amputated pulp because of their inductive effect on odontoblast differentiation.¹⁶ However, there had

been no report thus far on reparative dentin formation by an endopeptidase catalyzing the turnover and degradation of ECM protein. In the present study, MMP-3 had no stimulatory effect on differentiation of pulp stem/progenitor cells into odontoblasts *in vitro* (data not shown), although it enhanced activity of proliferation, migration, and survival of endothelial cells. Angiogenesis and reparative dentin formation were accelerated in MMP-3-treated pulp compared with PBS control in the rat pulp injury model. Therefore, induced reparative dentin formation might be indirect effect of MMP-3. However, the liberation and modification of the variety of growth/differentiation factors and cytokines embedded in dentin matrix and/or extracellular substrate by MMP-3 might cause some inductive effects on differentiation of pulp stem/progenitor cells into odontoblasts and reparative dentin formation *in vivo*.⁶ MMPs, including MMP-3, are associated with re-epithelialization, which is the regrowth of epithelia over a denuded surface.³⁴ Wound healing is delayed in MMP-3 null mice, suggesting MMP-3 also contributes to wound healing.³⁵ MMP-1 protein application in mouse muscle injury increases myofibril formation and myogenic regeneration through either the digestion of scar tissues or activation of muscle cell migration.³⁶ However, there has been no report on direct topical application of MMP-3 protein in any disease and any injury thus far. Our present results of stimulatory effect on angiogenesis and acceleration of wound-healing events by MMP-3 in the pulp injury might be a useful clue for future clinical treatment for dental pulp. Developing rational therapy for dentin/pulp regeneration requires further identification of specific MMP-3 substrates and characterization of the downstream consequences of MMP-3 proteolytic activity. MMP-3 may also have a more general role in accelerating wound healing or improving angiogenesis.

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