Sustained Production of H2O2 Activates Pro-matrix Metalloproteinase-2 through Receptor Tyrosine Kinases/Phosphatidylinositol 3-Kinase/NF-κB Pathway*

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Sang-Oh Yoon, Soo-Jin Park, Sun Young Yoon, Chang-Hyun Yun, and An-Sik Chung‡

From the Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, South Korea

A rate-limiting step of tumor cell metastasis is matrix degradation by active matrix metalloproteinases (MMPs). It is known that reactive oxygen species are involved in tumor metastasis. Sustained production of H2O2 by phenazine methosulfate (PMS) induced activation of pro-MMP-2 through the induction of membrane type 1-MMP (MT1-MMP) expression in HT1080 cells. MMP-2, MMP-9, and tissue inhibitor of metalloproteinase-1 and -2 levels were changed negligibly by PMS. A one time treatment with H2O2 did not induce activation of MMPs. It was also demonstrated that superoxide anions and hydroxyl radicals were not related to PMS action. PMS-induced pro-MMP-2 activation was regulated by the receptor tyrosine kinases, especially the receptors of platelet-derived growth factor and vascular endothelial growth factor, and downstream on the phosphatidylinositol 3-kinase/NF-κB pathway but not Ras, cAMP-dependent protein kinase, protein kinase C, and mitogen-activated protein kinases. PMS did not induce pro-MMP-2 activation in T98G and NIH3T3 cells. This may be related to a low level of MT1-MMP, indicating a threshold level of MT1-MMP is important for pro-MMP-2 activation. Furthermore, PMS increased cell motility and invasion but decreased cell-cell interaction. Cell-matrix interaction was not affected by PMS.

Metastasis is a major cause of death among cancer patients. The metastasis of cancer cells requires several sequential steps, such as changes in cell-ECM interaction, the disconnection of intercellular adhesions and separation of single cells from solid tumor tissue, a degradation of ECM, the locomotion of tumor cells into the extracellular matrix, the invasion of lymph and blood vessels, proliferating of cells, and the induction of angiogenesis (1).

The main groups of proteolytic enzymes involved in tumor invasion are matrix metalloproteinases. The MMPs, a family of zinc-dependent endopeptidases, are involved in tumor invasion, metastasis, and angiogenesis in cancer (2, 3). MMPs are important enzymes for the proteolysis of extracellular matrix proteins such as collagen, proteoglycan, elastin, laminin, and fibronectin (4). MMPs are synthesized as proenzymes, and most of them are secreted from the cells as proenzymes. Among previously reported human MMPs, MMP-2 (gelatinase A/M, 72,000 type IV collagenase) and MMP-9 (gelatinase B/M, 92,000 type IV collagenase) are thought to be key enzymes for degrading type IV collagen, which is a major component of the basement membrane (3). Both MMP-2 and MMP-9 are abundantly expressed in various malignant tumors (5) and contribute to invasion and metastasis (6).

Pro-MMP-2 can be activated by several mechanisms depend on stimulators and cell types. Initially, pro-MMP-2 can be activated by the action of highly expressed MT1-MMP and the adequate expression of TIMP-2 (7–9). In this situation, the balance between MT1-MMP and TIMP-2 is important. At low concentrations, TIMP-2 binds to the catalytic site of some activated MT1-MMP molecules, generating receptors for pro-MMP-2, thereby promoting MMP-2 activation. In this situation, MT1-MMP forms a homophilic complex through the hemopexin-like domain that acts as a mechanism to keep MT1-MMP molecules close together to facilitate pro-MMP-2 activation (10). At high concentrations, TIMP-2 binds and inhibits any active MT1-MMP, thus completely preventing MMP-2 activation. Next, the down-regulation of TIMP-2 by type IV collagen without affecting MT1-MMP can lead to pro-MMP-2 activation (11). In this case, pro-MMP-2 activation involved neither a transcriptional modulation of MMP-2, MT1-MMP, or TIMP-2 expression nor any alteration of MT1-MMP protein synthesis or processing. Finally, activation of pro-MMP-2 in fibroblast culture in a type I collagen lattice was induced intracellularly and is associated with Golgi-enriched intracellular membranes without the help of MT1-MMP (12).

Reactive oxygen species (ROS) are involved in aging and many diseases as follows: cancer, diabetes mellitus, atherosclerosis, neurological degeneration, angiogenesis, and tumor invasion. However, there are few reports on what kinds of ROS and how ROS affect tumor cell invasion. In particular, the specific mechanism of transcriptional regulation of MT1-MMP expression has not yet been understood. Here we report that sustained exposure of H2O2, not a one time exposure of H2O2, to cells increases pro-MMP-2 activation through the induction, adhesion to endothelial cells, extravasation from lymph and blood vessels, proliferation of cells, and the induction of angiogenesis (1).

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‡ To whom correspondence should be addressed: Dept. of Biological Sciences, Korea Advanced Institute of Science and Technology, Taejon 305-701, South Korea.
of MT1-MMP expression, and this activation is mediated via a receptor tyrosine kinase/P38 kinase/NF-κB activation. The sustained production of H₂O₂ also increased cell motility and invasion but decreased cell-cell interaction.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Materials**—HT1080 (fibrosarcoma), T98G (glioblastoma), and NIH3T3 (mouse fibroblast) were grown in DMEM supplemented with 10% HEPES, 50 mg/liter gentamicin (Invitrogen), and 10% heat-inactivated fetal bovine serum. Human endothelial cells were grown in endothelial cell growth media (Clonetics). Peroxynitrite, hydrogen peroxide, sodium nitroprusside (SNP), G64976, indomethacin, SB203580, FPTI III, quinacrine, PD98059, H7, PDTC, NF-κB reporter vectors were purchased from CLONTECH. IsB-α vector was provided by Dr. V. Imbert (Faculte de Medecine, France).

**Transient Transfection and Reporter Gene Assay**—HT1080 cells were plated in 6 wells and incubated at 37 °C. At 70–80% confluency, the cells were washed with DMEM and incubated with DMEM without serum and antibiotics for 5 h. 2 μg of reporter vector and 0.5 μg of β-galactosidase vector were transfected using LipofectAMINE 2000 reagent (Invitrogen). After incubation, cells were lysed, and luciferase activity was measured using a luminometer. β-Galactosidase activity was measured using O-nitrophenyl-β-galactopyranoside as a substrate.

**Cell-Cell Adhesion Assay**—HT1080 cells were plated in 24 wells and incubated at 37 °C to 100% confluency. Other HT1080 cells were radio-labeled with [3H]thymidine overnight and trypsinated. Radioabeled cells were resuspended in DMEM with 10% fetal bovine serum and added to the unlabeled attached 100% confluent 24 wells. After 1–2 h of incubation, nonadherent cells were collected. Then plates were rinsed with PBS, which was collected in the same container. Following this procedure, bound cells were trypsinated completely and collected in other containers.

**Zymography**—All experiments, including zymography, were performed in the absence of serum. Enzymatic activities of MMP-2 and MMP-9 were detected using zymography (NapBio). Samples were electrophoresed on a gel containing 10% SDS-polyacrylamide gel. After electrophoresis, the gel was washed twice with washing buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5% Triton X-100), followed by a brief rinsing in washing buffer without Triton X-100. The gel was incubated with incubation buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM CaCl₂, 0.02% NaN₃, 1 μM ZnCl₂) at 37 °C. After incubation, the gel was stained and destained. In this gel, a clear zone of gelatin digestion appeared, indicating the presence of MMP.

**5-Bromo-2′-Deoxyuridine Incorporation Assay**—Cell proliferation assay was performed using [3H]thymidine incorporation method. Cells were plated on 6 wells and incubated. At 70–80% confluency, cells were treated with various agents, and 12 h later, 1 μCi of [3H]thymidine (Amersham Biosciences) was added to each well and incubated for 24 h. The cells were then washed twice with PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 7H₂O, and 1.4 mM KH₂PO₄) and lysed with 0.4 M diethyldithiocarbamic acid (DDC), and benzoic acid were obtained from Sigma. Sustained production of H₂O₂ also increased cell motility and invasion but decreased cell-cell interaction.

**Flow Cytometric Assessment of H₂O₂**—Cells were plated onto a Lab-Tek chamber slide (Nunc). When cells reached 70–80% confluency, FMS was treated. After incubation, cells were treated with DCFH-DA (5 μM) or dihydroethidium (5 μM) for the detection of H₂O₂ and superoxide anion amount, respectively. Cells were then wash with PBS and subjected to confocal microscopy (Zeiss).

**Flow Cytometric Analysis of H₂O₂**—Flow cytometric analysis for 5-hydroxytryptamine was performed using a flow cytometer. Cells were washed once with sodium phosphate buffer (2.35 g/liter NaHPO₄/7.61 g/liter Na₂HPO₄, pH 7.4) and incubated with medium containing 1 g/liter glucose, 0.2 g/liter CaCl₂, 4.54 g/liter NaCl, 0.37 g/liter KCl in sodium phosphate buffer with 20 μM cytochrome c at 37 °C. The absorbance of the membrane was read spectrophotometrically at 550 nm.

**RESULTS**

**The Effects of ROS on MMPs Expression in HT1080 Cells**—To find the ROS effects on cell viability and MMPs expression, HT1080 cells, various ROS were treated in the cells. Cell viability was tested by a [3H]thymidine incorporation method. Cells were washed once with sodium phosphate buffer (2.35 g/liter NaHPO₄/7.61 g/liter Na₂HPO₄, pH 7.4) and incubated with medium containing 1 g/liter glucose, 0.2 g/liter CaCl₂, 4.54 g/liter NaCl, 0.37 g/liter KCl in sodium phosphate buffer with 20 μM cytochrome c at 37 °C. The absorbance of the membrane was read spectrophotometrically at 550 nm.

**Flow Cytometric Analysis of H₂O₂**—Flow cytometric analysis for the measuring the amount of intracellular H₂O₂ was performed. Briefly, cells were incubated with DCFH-DA (5 μM) for 30 min at 37 °C. Cells were washed with PBS and trypsinated. After washing with PBS, 10 μl of propidium iodide (2.5 mg/ml) was added, and the amount of H₂O₂ was measured by a flow cytometer.

**REFERENCES**

Sustained Production of H₂O₂ Increases Tumor Cell Invasion.
We further tested the effect of a well-known superoxide anion-generating agent, PMS, on cell proliferation and MMPs expression. Non-cytotoxic concentration of PMS induced pro-MMP-2 activation, but not pro-MMP-9, whereas cytotoxic concentration of PMS decreased pro-MMP-2 and pro-MMP-9 expressions and did not induce pro-MMP-2.

**Fig. 1. Effects of ROS on HT1080 cell viability and MMPs activities.** Various concentrations of H$_2$O$_2$ (A), peroxynitrite (B), SNP (C), and PMS (D) were treated to the HT1080 cells. 2 days after the treatment, conditioned media were collected, and gelatin zymography analysis was performed. E, purified commercial pro-MMP-2 and 1 mM 4-aminophenylmercuric acetate (APMA)-treated pro-MMP-2 were used as control. HT1080 cells were incubated for 2 days in the absence or presence of 2 μM PMS. Conditioned media were collected and concentrated. MMP-2 was identified by Western blot analysis using anti-MMP-2 antibody.
activation (Fig. 1D). To identify whether the activated MMP was MMP-2, a Western blot analysis was performed using anti-MMP-2 antibody. Fig. 1E shows that PMS induces pro-MMP-2 activation. To find the direct effects of ROS on pro-MMP activation, ROS and ROS generating agents were added to conditioned media, which were preincubated with HT1080 cells for 2 days and then collected. Both low and high concentrations of \( \text{H}_2\text{O}_2 \), peroxynitrite, and SNP treatments did not affect pro-MMPs activation (data not shown).

Treatment with Intracellular Superoxide Anion-generating Agents Increase pro-MMP-2 Activation through \( \text{H}_2\text{O}_2 \) Generation—Cells were incubated with PMS for various times. PMS induced pro-MMP-2 activation but did not affect pro-MMP-9 expression and activation (Fig. 2A). Menadione and paraquat, which produce intracellular superoxide anion like PMS, also induced pro-MMP-2 activation and was measured using the substrate, and it was found that PMS-, menadione-, and paraquat-treated groups showed higher MMP-2 activity than the untreated group (Fig. 2C). PMS produces superoxide anion in cells, and

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\begin{align*}
\text{Con} & \quad 6 \quad 12 \quad 24 \quad 48 \quad 60 \quad \text{(h)} \\
\text{Menadione} & \quad - \quad + \quad + \quad + \\
\text{Paraquat} & \quad - \quad + \quad + \quad + \\
\end{align*}
\]

\[
\begin{align*}
\text{Con} & \quad 5 \quad 1 \quad 5 \\
\text{PMS} & \quad - \quad + \quad + \\
\text{Menadione} & \quad - \quad + \quad + \\
\text{Paraquat} & \quad - \quad + \quad + \\
\end{align*}
\]

\[
\begin{align*}
\text{Benzoic acid} & \quad (\text{mM}) \\
\text{Mannitol} & \quad (\text{mM}) \\
\text{DMSO} & \quad (\%\text{)} \\
\text{NAC} & \quad (\text{mM}) \\
\end{align*}
\]
this can be converted into $\text{H}_2\text{O}_2$, hydroxyl radical, and $\text{H}_2\text{O}$ by enzymes or metals.

What kinds of ROS are involved in pro-MMP-2 activation? To determine this, the cells were treated with DDC, a potent superoxide dismutase inhibitor, which has been proven to increase superoxide anion but decrease $\text{H}_2\text{O}_2$ (14–16). PMS did not induce pro-MMP-2 activation in the presence of DDC (Fig. 2D), which means that superoxide anion is not involved in pro-MMP-2 activation. Further experiments were done using hydroxyl radical scavengers, benzoic acid, mannitol, and Me$_2$SO. Several times treatment with these agents did not prevent pro-MMP-2 activation by PMS (Fig. 2E). The widely used antioxidant N-acetylcysteine was effective in inhibiting pro-MMP-2 activation but not superoxide anion and hydroxyl radical.

**PMS Increases Superoxide Anion and $\text{H}_2\text{O}_2$ for Long Periods**—Direct treatment with $\text{H}_2\text{O}_2$ did not induce pro-MMP-2 activation (Fig. 1A), but intracellular $\text{H}_2\text{O}_2$ produced by PMS activated pro-MMP-2. To find the differences between the two sources, intracellular and extracellular ROS levels were measured by several methods. Dihydroethidium and DCFH-DA are specific dyes used for the detection of superoxide anion and $\text{H}_2\text{O}_2$, respectively. Direct treatment with $\text{H}_2\text{O}_2$ did not last over 2 h (data not shown), whereas treatment with PMS in-

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**Fig. 3. Effects of PMS on ROS production.** HT1080 cells were incubated for 48 h. During incubation, 2 $\mu$M PMS was treated for the indicated times. After incubation, DCFH-DA (A) and dihydroethidium (B) were added, and confocal microscopy analysis was performed. Con, control. C, the same method was used with A except flow cytometric analysis was used instead of confocal microscopy. D, cells were incubated for 48 h. During incubation 2 $\mu$M PMS was treated for the indicated times. After incubation, media were used for cytochrome c reduction assays.

**Fig. 4. Effects of PMS on transcriptional levels of MMPs and TIMPs.** HT1080 cells were treated with 2 $\mu$M PMS for the indicated times, and RNA was extracted. Northern blot analysis was carried out. RNA loading was normalized using the signal obtained with a glycer-aldehyde-3-phosphate dehydrogenase (GAPDH).
increased intracellular superoxide anion (Fig. 3A) and H$_2$O$_2$ (Fig. 3B) for 2 days after the treatment, which was identified by a confocal microscopy. For the more precise detection of intracellular superoxide anion and H$_2$O$_2$, flow cytometry analysis was used. As shown in Fig. 3C, intracellular H$_2$O$_2$ increased time-dependently, and a similar result was obtained in the case of intracellular superoxide anion, which was measured by a flow cytometer using dihydroethidium (data not shown). Extracellular superoxide anion production by PMS was measured using cytochrome c reduction, and a high level of this anion was present up to 2 days after treatment (Fig. 3D).

The Sustained Production of H$_2$O$_2$ Induces Pro-MMP-2 Activation through Increased MT1-MMP Expression without Affecting Expressions of MMP-2 and TIMP-2—To discover what kinds of changes by PMS induced pro-MMP-2 activation, mRNA levels of MMPs and TIMPs were investigated. As shown in Fig. 4, PMS increased MT1-MMP mRNA. This increased level was continued 60 h after treatment, demonstrating that the sustained production of H$_2$O$_2$ stimulates continuous induction of MT1-MMP but does not affect MMP-2, MMP-9, TIMP-1, and TIMP-2 levels significantly.

PMS Induces Pro-MMP-2 Activation through the PI3-K-dependent Pathway—To find the mechanisms for pro-MMP-2 activation by PMS, various inhibitors of cell signal molecules were used. Appropriate concentrations of inhibitors were determined, and it was found that inhibitors themselves did not affect pro-MMP-2 activation (data not shown). G06976 (calcium-dependent protein kinase C inhibitor), indomethacin (phospholipase A$_2$ inhibitor, cyclooxygenase inhibitor), SB203580 (p38 inhibitor), FPTI III (Ras processing inhibitor), quinacrine (Mepacrine, phospholipase A$_2$ inhibitor), PD98059 (mitogen-activated protein kinase/extracellular signal-regulated kinase-extracellular signal-regulated kinase (MEK-ERK) pathway inhibitor), and H7 (broad serine/threonine kinase inhibitor, protein kinase A inhibitor, protein kinase C inhibitor, and protein kinase G inhibitor) did not have any inhibitory effect on pro-MMP-2 activation by PMS (Fig. 5A). These inhibitors were treated twice during incubation, and similar results were ob-

Fig. 5. Involvement of PI3-K on PMS-induced pro-MMP-2 activation. A and B, various inhibitors were pretreated to HT1080 cells for 3 h, and 2 μM PMS was treated. 2 days after treatment, conditioned media were collected, and zymography was performed. C, wild type or kinase-dead (KD) PI3-K subunit p85 expression vectors were cotransfected with MT1-MMP promoter containing reporter vectors. After 36 h of incubation, luciferase activity was measured. Data represent the mean ± S.D. of three independent experiments. Results were statistically significant (*, $p < 0.01$) using Student’s t test. Con, control.

Fig. 6. Effects of tyrosine kinases on PMS-induced pro-MMP-2 activation. A, genistein and vanadate were pretreated to HT1080 cells for 3 h, and 2 μM PMS was treated. 2 days after treatment, conditioned media were collected, and zymography was performed. B, HT1080 cells were treated with 5 μM genistein. After 3 h, PMS were treated for 36 h, and Northern blot analysis was performed. C, 2 μM PMS was treated and incubated for the indicated times. Western blot analysis was performed using phosphotyrosine-specific antibody. Con, control. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Further experiments were performed using LY294002 (a PI3-kinase inhibitor) and rapamycin (an Akt/mTOR-p70S6K inhibitor). Treatment with LY294002 blocked pro-MMP-2 activation, whereas rapamycin did not show any effect (Fig. 5B). Because PMS-induced pro-MMP-2 activation is processed by MT1-MMP induction, a further experiment was done using a MT1-MMP promoter containing reporter vectors. Wild type p85 (PI3-K subunit) overexpression increased MT1-MMP promoter activity, whereas kinase-dead p85 overexpression decreased the promoter activity (Fig. 5C).

**PMS Induces Pro-MMP-2 Activation via Receptor Tyrosine Kinase-dependent Pathways**—Genistein (a tyrosine kinase inhibitor) inhibited pro-MMP-2 activation as well as pro-MMP-2 expression, whereas treatment with both vanadate (a protein tyrosine phosphatase inhibitor) and PMS led to increased activation of pro-MMP-2 to 62 kDa and a further 43 kDa (Fig. 6A). These results suggest that the protein tyrosine kinase pathway plays a major role in pro-MMP-2 activation by the sustained production of H2O2. To confirm the inhibitory role of genistein in pro-MMP-2 activation, a Northern blot analysis was performed. Genistein decreased transcription levels of MT1-MMP as well as MMP-2 but did not significantly alter the TIMP-2 level (Fig. 6B). To find which tyrosine kinase was involved in PMS-induced pro-MMP-2 activation, total cell extracts were electrophoresed, and Western blotting was performed using a phosphotyrosine-specific antibody. As shown in Fig. 6C, the amount of high molecular weight phosphotyrosine proteins increased with time, but the low molecular weight phosphotyrosine proteins decreased or did not change.

**PMS Increases MT1-MMP Expression through PDGF Receptor**—High molecular weight tyrosine kinases are generally receptor tyrosine kinases, so that various receptor kinases inhibitors were treated before PMS treatment. As shown in Fig. 7A, 0.5 µM AG1295 (a PDGF pathway inhibitor) inhibited pro-MMP-2 activation marginally, but 1 and 5 µM AG1295 inhibited it over 75–85%. SU1498 (a VEGF pathway inhibitor) inhibited it about 20–30%, and 10 µM SU5402 (a fibroblast growth factor pathway inhibitor) showed minimal inhibitory effect on pro-MMP-2 activation, but AG1024 (insulin-like growth factor -1 and insulin pathway inhibitor) and AG1478 (an epidermal growth factor pathway inhibitor) did not inhibit PMS-induced pro-MMP-2 activation. To confirm the PDGF receptor-dependent activation of pro-MMP-2 activation by PMS, the amount of phosphorylated PDGF receptor was measured. As shown in Fig. 7B, PMS increased PDGF receptor phosphorylation.
phosphorylation. AG1295 also decreased MT1-MMP promoter activity (Fig. 7C).

**PMS Induces Pro-MMP-2 Activation through NF-κB**—The receptor tyrosine kinases/PI3-K pathway generally induces NF-κB activation. To conclude if this is the case in PMS-induced pro-MMP-2 activation, NF-κB inhibitors were used. PDTC (general inhibitor) and NF-κB SN50 (specific inhibitor peptide) blocked PMS-induced pro-MMP-2 activation, but NF-κB SN50N (inactive control for SN50) was not effective (Fig. 8A). Further studies were performed using NF-κB subunit p65 vector and inhibitory unit IκB-α vector. As shown in Fig. 8B, p65 vector overexpression increased MT1-MMP promoter activity, whereas IκB-α overexpression decreased the promoter activity. Furthermore, genistein and AG1295 decreased NF-κB activity induced by PMS (Fig. 8C).

**PMS Does Not Activate pro-MMP-2 in T98G and NIH3T3 Cells**—To investigate whether PMS-induced pro-MMP-2 activation is universal to other cells, T98G and NIH3T3, which produce pro-MMP-2, were treated with PMS. PMS did not induce pro-MMP-2 activation in these cells (Fig. 9A). Three days of incubation with PMS showed the same results (data not shown). Transcriptional levels of MT1-MMP, MMP-2, and TIMP-2 were measured by Northern blotting. T98G cells expressed MMP-2 more than HT1080 cells, and NIH3T3 cells produced comparable amounts of MMP-2 to that of the HT1080 cells (Fig. 9B). Levels of TIMP-2 were similar among the three cells. However, NIH3T3 and T98G cells showed a minimal expression of MT1-MMP compared with HT1080. To prove whether these cells express or do not express MT1-MMP, a blotted membrane was exposed for several days, and the MT1-MMP band was detected (data not shown), indicating that these cells express very low amounts of MT1-MMP. NIH3T3 cells were then treated with PMS, and the mRNA level of MT1-MMP was measured by Northern blotting. Although it required a very long exposure time for the detection of the low amount of MT1-MMP expression, the MT1-MMP level in NIH3T3 cells increased over time (Fig. 9C) like HT1080 cells (Fig. 4). We further tested the pro-MMP-2 activation using human endothelial cells that produce a large amount of MT1-MMP. PMS induced pro-MMP-2 activation in endothelial cells (Fig. 9D).

**Effects of PMS on Cell-Cell Interaction, Cell-Matrix Interaction, Motility, and In vitro Invasion**—To find whether PMS affects cell-cell interaction or not, PMS was pretreated or treated at the assay time. HT1080 cells pretreated for 24 h with PMS showed a decrease in the cell-cell interaction by 50%, but cells treated with PMS during the processing time of the assay showed no change (Fig. 10A). PMS had no influence on cell-collagen interaction irrespective of preincubation (Fig. 10B). Treatment with PMS increased cell motility through Transwell (Fig. 10C) and spreading onto plasticware (Fig. 10D). DDC inhibited the cell motility induced by PMS (Fig. 10, C and D). PMS also increased cell invasion, which was inhibited by DDC, but treatment with various concentrations of H₂O₂ did not affect cell invasion (Fig. 10E).
DISCUSSION

ROS are produced by a variety of sources, mitochondrial oxidative phosphorylation, ionizing radiation exposures, cytokines, growth factors, metabolism of exogenous compounds, and pathological metabolic processes. These ROS are involved in many natural and pathological processes, including aging, cancer, diabetes mellitus, atherosclerosis, neurological degeneration, angiogenesis, and metastasis (17). Metastasis requires several sequential steps as described earlier, and a rate-limiting step is degradation of matrix by active MMP-2 and MMP-9 (2). Overproduction of the proenzyme was not sufficient for the acquisition of an invasive phenotype as only activated MMPs can degrade the matrix. However, there are few reports on specific mechanisms of MMPs activation and transcriptional regulation of MT1-MMP expression. Here we try to elucidate what kinds of ROS and how ROS regulate pro-MMPs activation and MT1-MMP expression. To our knowledge, this is the first described intercellular regulation of MT1-MMP by ROS.

PMS and paraquat have been used as superoxide anion-producing agents (14, 18–20). PMS increased pro-MMP-2 activation (Fig. 2), cell motility, and the invasion of cancer cells (Fig. 10), but treatment with $H_2O_2$, peroxynitrite, and SNP (nitric oxide production) did not have an influence on the activation of MMPs and the tumor invasion. The produced superoxide anion turns into $H_2O_2$ by superoxide dismutase and is further catalyzed to $H_2O$ by catalase and glutathione peroxidase or hydroxyl radical by metal ions, such as iron. As shown in Fig. 2, $H_2O_2$ is responsible for pro-MMP-2 activation, raising an important question. Why did direct $H_2O_2$ treatment and PMS-induced $H_2O_2$ show different results? As shown in Fig. 3,
PMS increased intracellular and extracellular superoxide and H$_2$O$_2$ even 48 h after treatment, but one time treatment with H$_2$O$_2$ did not sustain intracellular H$_2$O$_2$ over 2 h, which was assayed by a flow cytometric analysis using DCFH-DA (data not shown).

Regulation of MMP-9 expression is well established, but mechanistic processes of MMP-2 and MT1-MMP expressions and activation of pro-MMP-2 by ROS are not well understood. MMP-9 expression is regulated by c-Jun NH$_2$-terminal kinase, p38, extracellular signal-regulated kinase, protein kinase C,
and Ras pathway, dependent on cell types (3). It has been shown that noncytotoxic H$_2$O$_2$ acts as an intracellular messenger and activates c-Jun NH$_2$-terminal kinase, p38, extracellular signal-regulated kinase, cAMP-dependent protein kinase, protein kinase C, Ras, tyrosine kinases, and various other kinds of signal molecules (21, 22). To find the exact mechanism for H$_2$O$_2$-induced pro-MMP-2 activation, various kinds of inhibitors were treated. In contrast to MMP-9 expression, pro-MMP-2 activation by PMS was not affected by mitogen-activated protein kinases, cAMP-dependent protein kinase, protein kinase C, protein kinase G, Ras, and phospholipase A$_2$ (Fig. 5A). However, genistein, a tyrosine kinase inhibitor, inhibited pro-MMP-2 activation (Fig. 6A) through MT1-MMP down-regulation (Fig. 6B), and specifically PDGF and VEGF receptors, receptor tyrosine kinases, were involved in PMS action about 75–85 and 20–30%, respectively (Fig. 7A). In addition, a protein tyrosine phosphatase inhibitor plus PMS increased more pro-MMP-2 activation (Fig. 5A). Furthermore, it was found that PI3-kinase (Fig. 5) and NF-κB activations (Fig. 8) are also involved in a signal pathway of pro-MMP-2 activation through induction of MT1-MMP expression. It is well established that growth factors induce PI3-K, and in turn NF-κB activation (23), especially PDGF activates NF-κB through Ras and PI3-K (24, 25). In our studies, the Ras inhibitor did not reduce pro-MMP-2 activation (Fig. 5A). It is shown that HT1080 cells express PDGF and the PDGF receptor (26). Therefore, these results demonstrate that the PDGF/PI3-K/NF-κB pathway plays a key role in pro-MMP-2 activation through MT1-MMP induction by the sustained production of H$_2$O$_2$. The tyrosine kinase pathway was also critical for MMP-2 expression (Fig. 6B) as well as pro-MMP-2 activation.

There are several reports on relationships between ROS, protein tyrosine kinase, and NF-κB. It has been suggested that the stimulatory effect of ROS on tyrosine phosphorylation is due to the kinase activation in addition to phosphatase inhibition (27), and ROS also increase expression of the growth factors and the phosphorylation of growth factor receptors, types of receptor tyrosine kinases (21). The activation of receptor tyrosine kinases, such as VEGF receptor and PDGF receptor, increases H$_2$O$_2$ via PI3-K has been reported previously (28–30). Therefore, it can be explained that the sustained production of H$_2$O$_2$ by PMS activates PDGF, VEGF, PI3-K, and the NF-κB pathways. In turn PDGF/PI3-K and VEGF/PI3-K pathways increase H$_2$O$_2$, which is a positive feedback loop, consisting of H$_2$O$_2$, PDGF, VEGF, and PI3-K.

Pro-MMP-2 activation by ROS may depend on cell types. The sustained production of H$_2$O$_2$ induced pro-MMP-2 activation in HT1080 cells and human endothelial cells, but T98G and NIH3T3 cells did not show any effect with the same treatment (Fig. 9A). This indicates that different cells react differently to the same stimulator. Cells having a large amount of MT1-MMP, even weak stimulations, can lead to pro-MMP-2 activation. If cells have a small amount of MT1-MMP such as NIH3T3 and T98G, even strong stimulators cannot activate pro-MMP-2, even though the level of MMP-2 expression is high, as in T98G (Fig. 9B). This implies a threshold of MT1-MMP level for MMP-2 activation. MT1-MMP is overexpressed in certain types of malignant tumor cells (31). Therefore, these cells can readily achieve the MT1-MMP threshold level and activate pro-MMP-2 and further promote metastasis and angiogenesis. However, many cells do not have large amounts of MT1-MMP; therefore, a transient increase in tyrosine kinases activity does not increase MT1-MMP to the threshold level. Only sustained stimulation of tyrosine kinases increases the potential of threshold level of MT1-MMP. HT1080 cells produce a large amount of MT1-MMP even without stimulator (Fig. 9B). This can be explained by constitutively active Akt, downstream target of PI3-K, in HT1080 (32). Further studies were performed on the relationship between PI3-K pathway and MT1-MMP expression in various cells. MT1-MMP not only participates in the processing of pro-MMP-2 but also digests various ECM components in vitro, including collagen (33), thereby profoundly stimulating tumor cell invasion. These data explain why ROS such as H$_2$O$_2$ accelerate metastasis and angiogenesis.

Besides matrix degradation by active MMPs, the cell-cell adhesion, cell-matrix adhesion, and cell motility are closely associated with tumor cell invasion and metastasis (1, 34, 35). For cells to invade the matrix, intercellular adhesions are weakened and tumor cells separate from solid tumor tissue. Therefore, weakening of the cell-cell interaction accelerates tumor cell invasion. In addition, cellular survival and invasion are promoted by cell-matrix interaction via integrins. PMS reduced cell-cell interaction (Fig. 10A) and increased cell motility (Fig. 10, C and D) but did not affect cell-collagen interaction (Fig. 10B). It has been shown that superoxide anion treatment enhances cell motility (36), but our studies showed that sustained production of H$_2$O$_2$ by PMS is responsible for the increased motility and invasion, but not superoxide anion, because DDC treatment in the presence of PMS prevented these phenomena (Fig. 10, C–E).

This study may explain the destructive role of chronic inflammation on tissue. Inflammatory reactions, particularly chronic ones, can be a significant source of oxidative stress. Leukocytes such as activated macrophages and neutrophils release a number of ROS including H$_2$O$_2$ and superoxide anion that can damage the nearby cells, and furthermore, these ROS have the potential to change normal cells to tumor cells. It has been estimated that approximately one-third of the world’s cancers are due to the effects of chronic inflammation (37). In addition, inflammation is found during matrix remodeling in many clinical situations, including wound healing and tumor invasion, thereby increasing tumor cell metastasis, which is still not well understood. Tumor cells produce large amounts of ROS (38), and sublethal amounts of superoxide anion protect cells from apoptosis (14). During inflammation, NF-κB, which is known to be involved in cell survival, invasion, metastasis, and angiogenesis, is activated (39). From this combined information, it can be proposed that the sustained production of H$_2$O$_2$ from chronic inflammation increases tumor cell resistance against the defense system of the body and invasion through 1) a decrease in cell-cell attachment, 2) an increase in matrix degradation by increasing the MT1-MMP expression and activation of pro-MMP-2, and 3) an increase in cell motility. In particular, the protein tyrosine kinase/PI3-K/NF-κB pathway may be most important for pro-MMP-2 activation through MT1-MMP induction by chronic inflammation-induced H$_2$O$_2$. Antioxidants such as N-acetylcysteine and protein tyrosine kinase inhibitors such as genistein can be good candidates for the application of anti-metastatic drugs.

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