

Modulation of matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 in RAW264.7 cells by irradiation

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Received April 6, 2010; Accepted June 2, 2010

DOI: 10.3892/mmr.2010.326

Abstract. Radiation-induced pulmonary injury is a severe complication affecting the quality of life of patients. Although the pathophysiology of the process is not fully understood, we hypothesized that it is potentially related to macrophages and their secretion of matrix metalloproteinase-9 (MMP-9). Macrophages are a type of inflammatory cell that synthesize hundreds of bioactive substances and enzymes. MMP-9 is closely involved in the maintenance of the basilar membrane, and leads to increased extracellular matrix deposition within the lung, which is a characteristic feature of radiation-induced lung fibrosis. We examined the role of ionizing radiation in modulating the production of MMP-9 in a macrophage cell line. RAW264.7 cells were irradiated with various doses of γ -rays, and then MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) levels were determined at several time points. RT-PCR revealed a marked increase in the levels of MMP-9 mRNA, which peaked at 24 h post-irradiation and had begun to decline by 48 h. By contrast, TIMP-1 mRNA experienced only a slight increase at 24 h post-irradiation, reaching significance at 48 h post-irradiation. Western blot analysis demonstrated an increased expression of MMP-9 protein in the irradiated cells, while TIMP-1 protein levels were not notably changed. Dexamethasone inhibited the increased expression of MMP-9 protein induced by ionizing radiation. These results indicate that MMP-9 expression by RAW264.7 cells, and an imbalance between MMP-9 and TIMP-1, may be involved in radiation-induced lung injury.

Introduction

Radiation therapy is very important in the treatment of malignant neoplasms, especially those of the thorax. The lungs are relatively radiosensitive, making them the dose-limiting organ in therapeutic radiation. Radiation-induced pulmonary injury is a common complication of radiotherapy (1), and its precise pathophysiological mechanisms have yet to be established. Since macrophages produce numerous bioactive substances (2), Rubin *et al* (3) proposed that the inflammatory process involves an interplay of cellular interactions, a perpetual cascade of cytokines involved in radiation-induced pulmonary injury, including tumor necrosis factor- α (TNF- α), interleukin-1 β (IL-1 β), interleukin-6 (IL-6) and tumor growth factor- β (TGF- β). It has been suggested that the disruption and alteration of the basement membrane plays a role in radiation-induced pulmonary injury (4). Matrix metalloproteinases (MMPs) are a large family of related proteolytic enzymes (5). MMP-2 and -9 are capable of degrading several components of the basement membrane in the lung interstitial matrix, including type IV collagen and fibronectin. They play a key role in the alteration of the basement membrane and in maintaining the integrity of the lung alveolar wall. MMP-2 is produced by endothelial cells. MMP-9 is produced by inflammatory cells, such as macrophages (6,7), and not only promotes cellular migration, but activates cytokines such as TNF- α and IL-1 β . Yang *et al* (8) suggested that the overexpression of MMP-2 and -9 is involved in the inflammatory response of radiation-induced lung injury *in vivo*.

In previous studies, macrophages were found to be radioresistant and cell function was not affected by high-dose irradiation (9). McKinney *et al* (10) reported that γ -irradiation (0.5-10 Gy) alone did not induce nitric oxide (NO) production in J774.1 and RAW264.7 murine macrophages. However, human alveolar macrophages have been shown to release IL-1 within hours of irradiation *in vitro* (11), and low-dose irradiation to induce IL-1 and IL-6 expression in mouse macrophages *in vitro* (12). Araya *et al* (4) found that ionizing radiation enhanced the expression of MMP-2 in human lung epithelial cells *in vitro*, suggesting that MMP-2 is involved in radiation-induced lung injury. However, whether ionizing radiation stimulates macrophage cells *in vitro* to produce MMP-9 has yet to be determined. Therefore, in the present study, we

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Key words: radiation-induced pulmonary injury, macrophages, matrix metalloproteinase-9, tissue inhibitor of metalloproteinase-1

analyzed the effect of ionizing radiation on the expression of MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1) in RAW264.7 cells.

Materials and methods

Materials. Cell culture dishes were purchased from Corning Inc. (Corning, NY, USA). Anti-MMP-9 (H-129) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-TIMP-1 (G-94) was purchased from Bioworld Technology, Inc. (Minneapolis, MN, USA). A real-time PCR kit was purchased from Toyobo (Osaka, Japan), including ReverTra Ace- α (FSK-100) and SYBR Green real-time PCR master mix-plus-(QPK-212). Dex was purchased from Sigma (St. Louis, MO, USA).

Cell line and culture conditions. RAW 264.7 cells were obtained from the Cell Culture and Collection Center of Wuhan University. The cells were cultured in DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin-streptomycin and 1% glutamine at 37°C in a 5% CO₂ atmosphere. The cells were placed in 35-mm dishes (1x10⁶), washed with phosphate buffered saline (PBS), and then incubated in serum-free DMEM with 1% penicillin-streptomycin and 1% glutamine for 24 h. Upon reaching 80% confluence, cells were used in the following experiments.

Cell irradiation. Monolayer cultures of RAW264.7 cells were irradiated at room temperature using a CO⁶⁰ γ -ray unit at doses of 0, 5, 10 and 20 Gy (at 1.39 Gy/minimum absorbed dose). After irradiation, the RAW264.7 cell cultures were returned to the incubator. The cell lysate was collected at different time points, and the levels of MMP-9 and TIMP-1 were estimated.

RT-PCR of MMP-9 and TIMP-1 mRNA. RT-PCR was used to determine the mRNA expression of MMP-9 and TIMP-1 with GAPDH as an internal control. Total RNA was obtained using a commercial kit (Toyobo) according to the manufacturer's instructions. To obtain cDNA, 2 μ g of total RNA were added to a 20- μ l reaction mixture containing 2 μ l RNA, 4 μ l 5X RT Buffer (25 mM Mg), 2 μ l dNTP (10 mM), 1 μ l Oligo(dT)20 (10 pmol/ μ l), 9 μ l RNase free H₂O, 1 μ l Rnase inhibitor (10 U/ μ l) and 1 μ l ReverTra Ace. PCR primers for mouse MMP-9 and GAPDH were as follows: MMP-9, 5'-AAGGGTACAGCC TGTTCCTGGT-3' (forward) and 5'-CTGGATGCCGTCTAT GTCGTCT-3' (reverse); GAPDH, 5'-CCGTGAAAAGAT GACCCAG-3' (forward) and 5'-TAGCCACGCTCGGTC AGG-3' (reverse); mouse TIMP-1, 5'-CCAGAAATCAACGAG ACCACC-3' (forward) and 5'-TACGCCAGGGAACCAAG AAG-3' (reverse). PCR was performed with 2.5 μ l of cDNA and 0.2 mM primers using the following touchdown PCR protocol: 2 cycles of PCR at 95°C for 15 sec, 58°C for 15 sec and 72°C for 45 sec, followed by 40 cycles (MMP-9) or 40 cycles (GAPDH) of PCR at 95°C for 15 sec, 58°C for 15 sec and 72°C for 45 sec, 2 cycles of PCR at 95°C for 15 sec, 59°C for 15 sec and 72°C for 45 sec, followed by a final 40 cycles (TIMP-1) of PCR. Analysis of relative gene expression data was performed using real-time quantitative PCR and the 2^{- $\Delta\Delta$ CT} method.

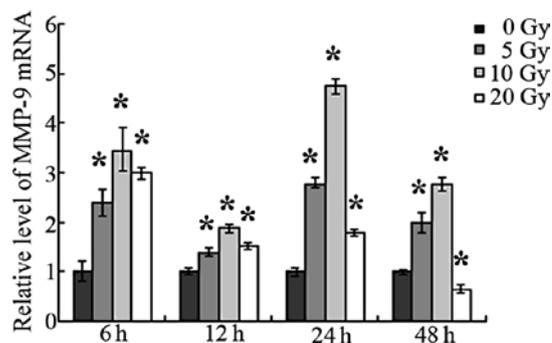


Figure 1. Effect of ionizing radiation on MMP-9 mRNA expression in RAW264.7 cells. Cells were irradiated with various doses of γ -rays (0, 5, 10 and 20 Gy) and cultured at 37°C. Total RNA was harvested at 6, 12, 24 and 48 h post-irradiation. MMP-9 mRNA expression was analyzed using real-time PCR. Values are the means \pm SD (n=3); *P<0.05, significantly different compared to non-irradiated controls.

Measurement of MMP-9 by Western blot analysis. The levels of MMP-9 protein in the cell lysate were measured by Western blotting. Cell lysate was heated at 100°C for 3 min, subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes with incubation overnight at 4°C in Tris buffer containing 5% non-fat dried milk. Subsequently, the membranes were incubated with the primary antibody to MMP-9 (1:200; H-129), followed by secondary antibody (1:3,000; goat anti-rabbit HRP-conjugated Ab) and developed by ECL according to the manufacturer's instructions.

Measurement of TIMP-1 by Western blot analysis. The cell lysate was heated at 100°C for 3 min, separated in 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated first with primary antibody to TIMP-1 (1:500; G-94) and then with secondary antibody (1:3,000; goat anti-rabbit HRP-conjugated Ab), after which the bands were identified with ECL reagent according to the manufacturer's protocol.

Data analysis. All experiments were repeated three times. Comparisons were made using one-way ANOVA with Fisher's *post hoc* test. Differences between means were evaluated with the Student's t-test. A P-value of <0.05 was considered significant.

Results

Radiation enhances MMP-9 mRNA and protein expression in RAW264.7 cells. To determine whether radiation is a sufficient stimulus for the induction of MMP-9 production, RAW264.7 cells were irradiated at 0, 5, 10 and 20 Gy. Total RNA was assayed at 6, 12, 24 and 48 h post-irradiation. After treatment with 5-10 Gy of γ -irradiation, MMP-9 expression in the RAW264.7 cells was significantly increased. At the various time points, total RNA was collected from irradiated and non-irradiated cells. MMP-9 mRNA was analyzed using real-time PCR. Fig. 1 shows that there were increased levels of MMP-9 mRNA in the irradiated compared to the non-irradiated cells. MMP-9 expression was most pronounced in cells treated with 10 Gy irradiation, and began to decline at 20 Gy. A significant

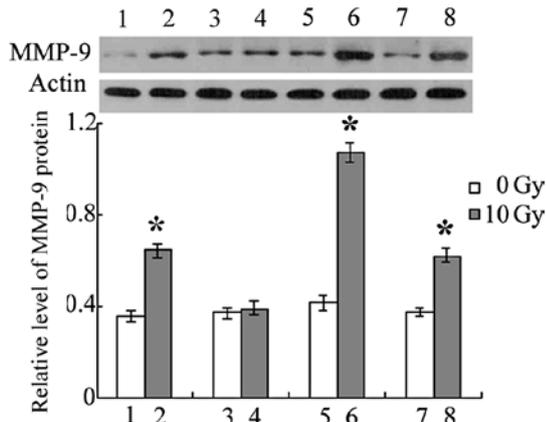


Figure 2. Effect of ionizing radiation on MMP-9 protein expression in RAW264.7 cells. Cells were irradiated with 10 Gy γ -rays and cultured at 37°C. Total protein was harvested at 6 h (lane 2), 12 h (lane 4), 24 h (lane 6) and 48 h (lane 8) post-irradiation. MMP-9 protein expression was analyzed by Western blotting. Lanes 1, 3, 5 and 7 are control cells at 6, 12, 24 and 48 h, respectively. Values are the means \pm SD (n=3); *P<0.05, significantly different compared to non-irradiated controls.

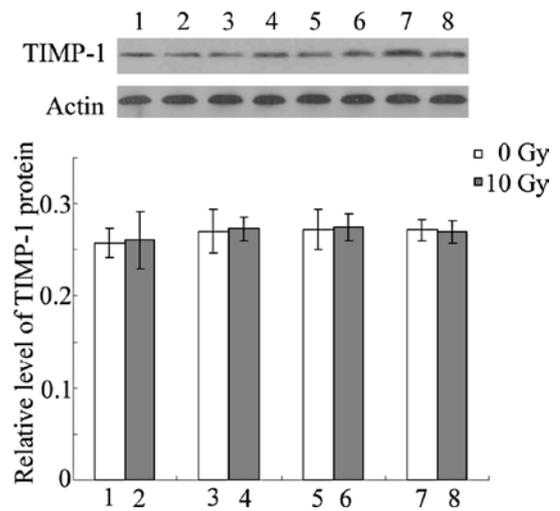


Figure 4. Effect of ionizing radiation on TIMP-1 protein expression in RAW264.7 cells. Cells were irradiated with 10 Gy γ -rays and cultured at 37°C. Cellular total protein was harvested at 6 h (lane 2), 12 h (lane 4), 24 h (lane 6) and 48 h (lane 8) post-irradiation. TIMP-1 protein expression was analyzed by Western blotting. Lanes 1, 3, 5 and 7 are control cells at 6, 12, 24 and 48 h, respectively. Values are the means \pm SD (n=3); *P<0.05, significantly different compared to non-irradiated controls.

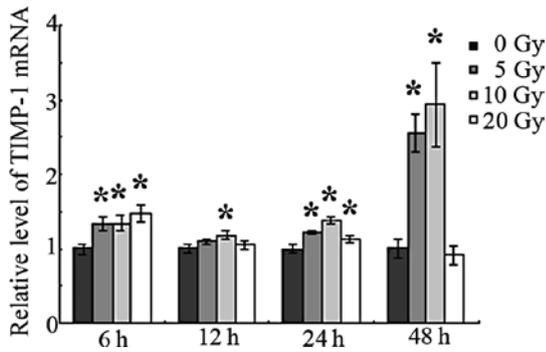


Figure 3. Effect of ionizing radiation on TIMP-1 mRNA expression in RAW264.7 cells. Cells were irradiated with various doses of γ -rays (0, 5, 10 and 20 Gy) and cultured at 37°C. Total RNA was harvested at 6, 12, 24 and 48 h post-irradiation. TIMP-1 mRNA expression was analyzed using real-time PCR. Values are the means \pm SD (n=3); *P<0.05, significantly different compared to non-irradiated controls.

increase in MMP-9 expression was observed starting at 6 h post-irradiation. Expression began to decrease at 12 h post-irradiation, but remained higher than in the non-irradiated cells. Peak levels were reached 24 h after the initial exposure to ionizing radiation. At 48 h, MMP-9 levels began to decline. These results indicate that ionizing radiation increases the expression of MMP-9 mRNA. To confirm the results, MMP-9 protein levels were determined using Western blotting. At the various time points, total protein was collected from irradiated and non-irradiated cells. Fig. 2 indicates that the results were consistent with those of the MMP-9 mRNA.

Radiation moderately increases TIMP-1 mRNA expression, but not TIMP-1 protein expression, in RAW264.7 cells. TIMP-1 transcript levels were determined in RAW264.7 cells irradiated at 0, 5, 10 and 20 Gy. A slight increase in TIMP-1 mRNA transcript levels was observed at 24 h post-irradiation. As shown in Fig. 3, unlike MMP-9, TIMP-1 mRNA transcript

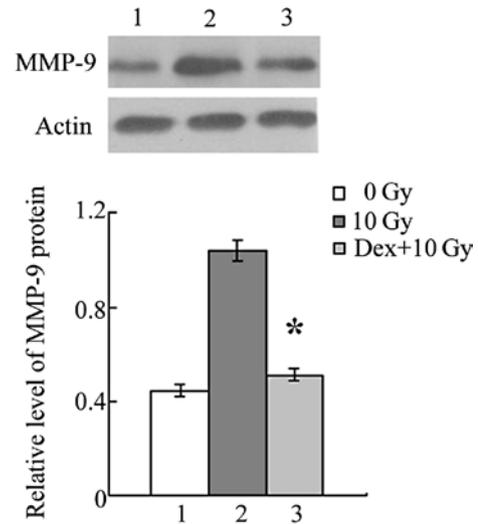


Figure 5. Effect of dexamethasone on MMP-9 production in ionizing radiation-stimulated RAW264.7 cells. Dexamethasone (1 μ M) was added to serum-free defined medium 1 h before treatment with 10 Gy ionizing radiation. Cells were cultured for an additional 24 h, then total protein was collected and was subjected to Western blotting. Lane 1, blank control; Lane 2, irradiation; Lane 3, Dex+irradiation. Values are the means \pm SD (n=3). *P<0.05, significant difference between cells treated with dexamethasone and irradiation compared to cells treated with irradiation alone.

levels were not significantly altered at 6, 12 or 24 h post-irradiation in the irradiated compared to the non-irradiated cells; significance was only achieved at 48 h post-irradiation. To determine whether the changes in TIMP-1 mRNA transcript levels coincided with protein production, TIMP-1 protein levels in serum-free media were determined using Western blotting. Fig. 4 indicates that there was no apparent increase in TIMP-1 protein levels at 48 h post-irradiation (Fig. 4), perhaps due to an *in vitro* post-translational modification of the protein.

Dexamethasone suppresses the increase in MMP-9 expression induced by ionizing radiation in RAW264.7 cells. Since Dex is the primary drug used in the treatment of radiation pneumonitis, we examined its effect on the production of MMP-9 after ionizing radiation. Dex inhibited the increase in MMP-9 production caused by exposure to radiation (Fig. 5) 24 h after the initial exposure at 10 Gy.

Discussion

Pneumonitis involves the aggregation of inflammatory cells and the degradation of extracellular matrix (ECM) components. Macrophages are the most important inflammatory effector cells and accumulate at sites of inflammation (13). MMP-9 and TIMP-1 play an important role in the regulation, turnover and remodeling of the ECM (5), and are therefore considered to play a crucial role in the pathophysiology of pulmonary inflammation and fibrosis (8,14-16), in particular the overexpression of alveolar macrophage MMP-9 (16). In the present study, radiation had a dramatic effect on the up-regulation of MMP-9 protein, but did not affect TIMP-1 protein levels in the RAW264.7 cells. This suggests a disruption in the balance of MMP-9/TIMP-1, which leads to the excessive degradation of the ECM in the local lung environment, facilitating the influx of inflammatory cells. MMP-9 promotes cellular migration (17) and activates cytokines such as TNF- α and IL-1 β (18). IL-1 β (19) and TNF- α (20) play an important role in radiation-induced lung injury and, in RAW264.7 cells, regulate the expression of MMP-9, which may enhance the inflammatory response. We suggest that MMP-9 participates in and aggravates radiation-induced lung injury. In other types of lung inflammation, it was reported that non-selective MMP inhibitor reduced the development of bleomycin-induced fibrosis (21) and ventilator-induced lung injury (22) in mice.

It is believed that the production of MMP-9 is associated with a number of factors, such as the transcription factors AP-1 and nuclear factor- κ B (NF- κ B) (23,24), reactive oxygen species (ROS) (19), IL-1 β (19) and TNF- α (25). For macrophages, ionizing radiation activates transcription genes, such as NF- κ B (26,27), and induces the production of ROS (28), TNF- α and IL-1 (10-12). The MMP-9 gene promoter region contains a NF- κ B motif (29); we therefore speculate that MMP-9 synthesis is closely related to NF- κ B expression. At 24 h post-irradiation, the production of MMP-9 reached its peak. MMP-9 expression may be associated with TNF- α , since TNF- α is a key modulator of MMP-9. McKinney *et al* (10) found that TNF- α was not produced until 24 h post-ionizing radiation, and presented transiently. Nevertheless, in the present study, by 48 h post-irradiation, MMP-9 levels were slightly decreased. We therefore suggest that radiation damages DNA and alters signal transduction, and that these changes may affect the regulation MMP-9 expression. In irradiated cells, there was no apparent increase in TIMP-1 protein levels at 48 h post-irradiation. This may be related to an *in vitro* post-translational modification of the protein. TIMP-1 expression in different cell lines may vary, and the mechanisms of its expression require further study.

According to previous studies, macrophages are radioreistant cells whose cell functions are not affected by high-dose

irradiation (9). McKinney *et al* (10) reported that γ -irradiation (0.5-10 Gy) alone did not induce NO production in J774.1 and RAW264.7 murine macrophages. However, in our experiment, macrophages activated by ionizing radiation led to functional changes.

In this study, we did not investigate the activity of MMP-9. The functions of MMP-9 in relation to TIMP-1 in radiation-induced pulmonary fibrosis require further clarification. However, our findings suggest that ionizing radiation enhances the expression of MMP-9 in RAW264.7 cells, and that cell functions were affected by irradiation. The clinical application of Dex is a fundamental treatment for radiation-induced pulmonary injury (1). Our data indicate that Dex is a potent inhibitor of MMP-9 protein expression induced by ionizing radiation. Taken together, the results of the present study support the hypothesis that MMP-9 production by RAW264.7 cells may be deeply involved in acute lung injury due to radiation exposure. The mechanisms by which radiation stimulates MMP-9 production in RAW264.7 have yet to be elucidated; further study is required to determine the exact biochemical pathways responsible for each step of stimulation.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 30770653).

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