Gamma-Irradiation Induces Matrix Metalloproteinase II Expression in a p53-Dependent Manner

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Matrix metalloproteinases (MMPs) are a family of proteinases that degrade the basement membrane and have been implicated in promoting tumor metastasis. MMP-2, one member of this family, was recently found to be a p53 target and subject to p53 upregulation. In this study, we examined the correlation between the expression of MMP-2 and the increased expression of p53 after γ-irradiation. Three human p53-positive cell lines that express wild-type p53, including U2-OS (osteosarcoma), RKO (colon carcinoma), MCF-7 (breast carcinoma), one mouse p53 positive cell line and HepG2 (liver carcinoma), and two p53-negative human cell lines, SAOS-2 (osteosarcoma) and RKO-E6 (colon carcinoma), were used in this study. The MMP-2 activity was analyzed by using gelatin zymography. The p53 level was measured by western blot analysis. Our results show that wild-type p53 induced by ionizing radiation caused a subsequent increase of MMP-2 activity in U2-OS and RKO cells but not in MCF-7, HepG2, SAOS-2, or RKO-E6 cells. These results suggest that the γ-radiation–induced expression of MMP-2 is dependent on the cell type and presence of functional p53. Thus, ionizing radiation could activate MMP-2 activity in a subset of human cancer cells and may lead to an increase in their metastatic potential. *Mol. Carcinog. 27:252–258, 2000.*   © 2000 Wiley-Liss, Inc.

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INTRODUCTION

The degradation of the basement membrane is catalyzed by a family of matrix metalloproteinases (MMPs) [1]. MMPs play an important role in regulating various physiological processes, such as embryonic development and wound healing, as well as pathological processes such as cancer metastasis and arthritis [2,3]. Studies have shown that the basement membranes of the extracellular matrix are composed mostly of a type IV collagen [4]. The integrity of the basement membrane surrounding a solid tumor is crucial in constraining the invasion and metastasis of the tumor. Invading cancer cells must break down the basement membrane and infiltrate the bloodstream and/or lymphatic system in order to invade distant organs. These invading cancer cells penetrate the basement membrane by secreting proteolytic enzymes that catalyze the degradation of the collagen matrix in the basement membrane [5]. One of the proteolytic enzymes that degrade the basement membrane is the 72-kDa type IV collagenase known as MMP-2 or gelatinase A [5,6]. Increased expression of MMP-2 is detected in human colonic adenocarcinoma [7], whereas MMP-2 is knockout mice have reduced angiogenesis and tumor progression capabilities [8]; these experiments support the notion that MMP-2 promotes tumor progression and metastasis.

p53 is a tumor suppressor gene as well as a transcriptional factor that controls cell growth by the induction of apoptosis and G1 arrest. This is mainly achieved by p53-induced induction of *bax* and *p21* [9–11]. Intracellular p53 levels can be altered through the exposure of the cells to various insults such as chemical agents [12], ultraviolet radiation [13], and ionizing radiation [14]. We have recently shown that MMP-2 is a p53 target that is subjected to p53 upregulation [15]. Indeed, induction of *p53* by the DNA-damaging chemotherapeutic agent etoposide increases the level of *MMP-2* mRNA expression [15]. This study implied that ionizing radiation that activates p53 would increase MMP-2 activity in a subset of human cell lines and may lead to increases in their metastatic potential [16]. To extend this study, we investigated the correlation between p53 activation and MMP-2 induction in multiple cultured human and mouse

carcinoma cell lines harboring functional p53 or inactive p53. We chose γ-radiation as a p53 activator, since radiation is such a widely used treatment modality in cancer therapy. We report here that ionizing radiation induced MMP-2 expression in a p53- and cell line-dependent manner.

MATERIALS AND METHODS

Cell Culture and γ-Irradiation

U2-OS and SAOS-2 cells were cultured in McCoy's 5A medium containing 10% fetal calf serum (FCS), L-glutamine, and antibiotics. The RKO and RKO-E6 cells were obtained from Dr. Theodore Lawrence's lab and were cultured in RPMI 1640 containing 10% FCS and penicillin/streptomycin (100 U/mL). MCF-7 cells were cultured in RPMI 1640 containing 10% FCS and penicillin/streptomycin (100 U/mL). HepG2 were cultured in Dulbeccos' modified Eagle's medium containing 10% FCS and penicillin/streptomycin (100 U/mL). The cells were cultured in 60-mm tissue culture plates in an incubator supplied with 5% CO_2 at 37°C. The cells were irradiated with an absorbed dose of 5 Gy by using a ^{60}Co teletherapy unit with a dose rate of 1.49 Gy/min (Atomic Energy of Canada, Missisauga, Ontario).

Collection of Condition Media and Preparation of Cell Lysates

After the cells were γ-irradiated, the cell culture media were replaced with fresh serum-free media. The conditioned media from both γ-irradiated and nonirradiated cells were collected at 0, 4, 8, 12, and 24 h postirradiation. In parallel, the cell lysates were prepared by using Tris-glycine sample buffer [0.2 M Tris–HCl, pH 6.8, 5% (v/v) glycerol, 0.1% bromophenol blue, and 5% sodium dodecyl sulfate (SDS)]. Cells from each six-well plate were lysed in 200 μL of the Tris–glycine sample buffer. The cell lysates were subsequently sonicated by using a sonicator dismembranator (Fisher Scientific, Pittsburgh, PA), aliquoted, and stored at −20°C.

Western Blot Analysis

The proteins in the cell lysates were resolved on a 10% SDS-polyacrylamide gel and electrophorized for 50 min at 200 V. The proteins were then transferred to nitrocellulose membranes (BioTrace HP, Gelman Sciences, Ann Arbor, MI) at 4°C (65 V, 3 h). The membranes were incubated with a p53-Ab2 mouse monoclonal antibody (Calbiochem, La Jolla, CA) at a 1:2000 dilution in TBST (0.1 M Tris–HCl, pH 6.8, 137 mM NaCl, and 0.1% Tween 20) with 1% gelatin. The membranes were then probed with an anti-mouse horseradish peroxidase–conjugated secondary antibody and visualized by using a supersignal chemiluminescent kit according to the manufacture's procedure (Pierce, Rockford, IL). Protein loading was standardized through a western blot by using mouse anti–human β-actin monoclonal antibodies (Sigma, St. Louis, MO) at a 1:10 000 dilution. Following the same procedure, the expression levels of p21 and bax were probed with a mouse anti-p21/WAF antibody (Santa Cruz Biotech., Santa Cruz, CA) and a rabbit anti-bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Gelatin Zymography

MMP-2 levels were measured by using gelatin zymography. A 10% SDS-polyacrylamide gel was cast in the presence of 0.1% gelatin. The conditioned medium was mixed with an equal volume of the 2 × Tris–glycine sample buffer described above. The samples were loaded and electrophorized at 125 V for 90 min. After the electrophoresis, the gel was incubated in a renaturing buffer (2.5% Triton X-100) for 30 min at room temperature and then incubated for an additional 30 min at room temperature in developing buffer (5 mM Tris, 20 mM NaCl, 0.5 mM $CaCl_2$, and 0.02% Brij 35, pH 7.6). The gels were then incubated in fresh developing buffer for 18 h at 37°C. The gels were stained (with 0.5% Coomassie Blue, 40% MeOH, and 10% acetic acid), destained (40% MeOH, 10% acetic acid). The intensities of the MMP-2 bands were quantified by using NIH image (Version 1.65).

RESULTS

Expression of MMP-2 Induced by γ-Irradiation in U2-OS and RKO Cells but Not in MCF-7 and HepG2 Cells

We have previously shown that p53 positively regulates the expression of MMP-2 [15]. Since γ-irradiation induces p53 expression [17], we examined whether γ-irradiation could also induce the expression of MMP-2 in cell lines that express wild-type p53. Three human cancer cell lines and one mouse cancer cell line (U2-OS, RKO, MCF-7, and HepG2) were used in the experiments. The cells were γ-irradiated with 5 Gy (at 1.49 Gy/min absorbed dose rate). At the time indicated in the figures (Figure 1A), the conditioned media were collected from irradiated and nonirradiated cells. The MMP-2 activity in the conditioned media were analyzed by using a gelatin zymography assay. The results show that there were increased levels of MMP-2 in irradiated U2-OS and RKO cell lines as compared with their nonirradiated equivalents (Figure 1A and B). The quantitative data suggest that MMP-2 activity began to increase approximately 4–8 h postirradiation and peaked at 12–24 h (Figure 2A and B). A 40% increase of MMP-2 was observed at 24 h postirradiation for U2-OS cells (Figure 1A) and a 25% increase was observed for RKO cells (Figure 1B) as compared with their nonirradiated controls. In contrast to U2-OS and RKO cells, the MMP-2 activity was not changed after

A   U2-OS Cells   **B**   RKO Cells

0 Gy   ← MMP-2

5 Gy   ← MMP-2

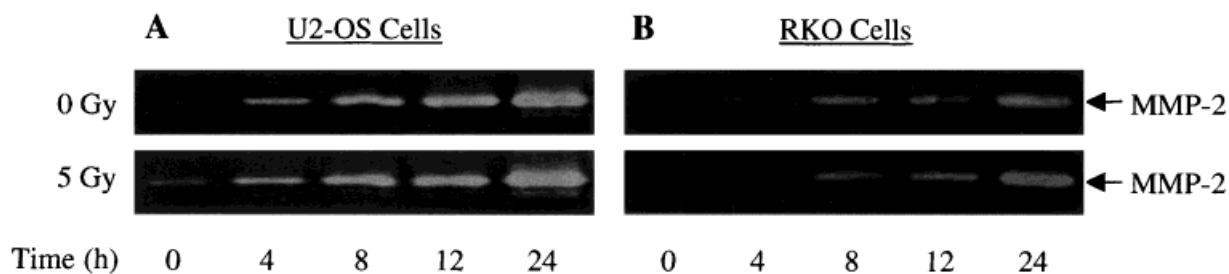Time (h)   0   4   8   12   24   0   4   8   12   24

Figure 1. Conditioned media from U2-OS and RKO cells show an increased activity of MMP-2 in the irradiated fractions. U2-OS cells were cultured in McCoy's 5A medium containing 10% FCS, and the RKO cells were cultured in RPMI 1640 containing 10% FCS. One day before irradiation, the cells were seeded into 60-mm tissue culture plates at 50% confluency. The cells were irradiated with an absorbed dose of 5 Gy. The conditioned media from the irradiated and nonirradiated U2-OS (A) or irradiated and nonirradiated RKO cells (B) were collected at various timepoints as indicated. Zymography was performed on the media to determine the MMP-2 activity present. The levels of MMP-2 were quantitated by using NIH image (version 1.61).
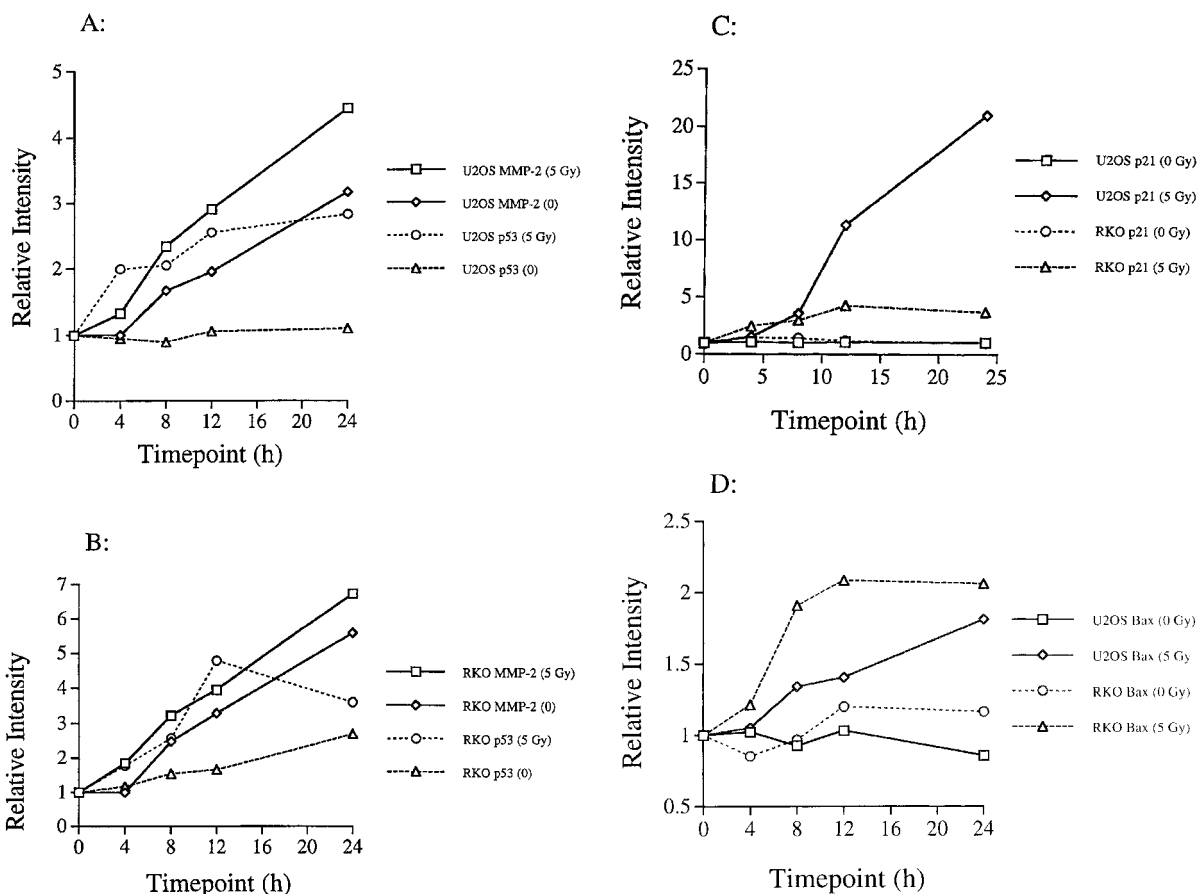
A:

B:

C:

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Figure 2. Normalized graphs of U2-OS and RKO cell lines indicating an increase in MMP-2 and p53 levels after γ-irradiation. These graphs depict the levels of MMP-2 in media over a course of time compared with the levels of p53 for U2-OS (A) and RKO cells (A) The MMP-2 and p53 levels were normalized with respect to control time points taken at the zero-hour time point. The graphs also indicate the net difference between the irradiated and nonirradiated control cell extracts and conditioned media that were collected synchronously during the specified timepoint. The resultant intensity quantification for the nonirradiated fractions was subtracted from the intensity quantification fractions, for the irradiated fraction, giving rise to the net increase of p53 and MMP-2 levels seen on the graphs. The numbers are the average of three to five sets of data. The calculated errors for the MMP-2 graphs ranged from ±0.06 to ±0.43. The calculated errors for the p53 graphs in ranged ±0.07 to ±0.32. The western blots for p21 and bax were also quantified and graphed (B and C). The calculated errors for the p21 and bax graphs range from ±0.11 to ±0.85 and ±0.08 to ±0.26, respectively.

irradiation in the conditioned media from MCF-7 and HepG2 cells, even though the p53 levels in these cell lines increased after γ-irradiation (data not shown). The results suggest that MMP-2 expression can be increased by γ-irradiation in certain types of cell lines.

Radiation-Induced Expression of MMP-2 Correlated with the Increased Expression of p53

It has been well documented that exposure of cells to varying doses of γ-radiation can increase the activity of the p53 protein [17]. Further studies have shown that wild-type p53 plays a role in the regulation of metastatic factors such as MMP-2 [15]. The U2-OS (human osteosarcoma) and RKO (human colon carcinoma) tumor cell lines used in this study both express wild-type p53. The same dosage (5 Gy) of ionizing radiation that induced MMP-2 expression was used to treat the cells. The p53 expression in the γ-irradiated and nonirradiated cells was determined by using western blot analysis.

As shown (Figure 2A and B), this dose of irradiation also increased p53 expression in those cell lines over a 24-h time course as compared with nonirradiated controls. The protein amount loaded on the gel was monitored and normalized with a β-actin antibody, a housekeeping protein (Figure 3B). As shown (Figure 3A and B), the level of p53 protein increased at 4–8 h postirradiation and peak levels were reached at 12–24 h after the initial exposure to ionizing radiation. These results indicate that ionizing radiation can increase the expression of p53, which correlates with the activation of MMP2.

In order to confirm that the expressed p53 is active, we examined the expression of p21 and bax upon γ-irradiation. Previous data have shown that activated p53 can induce the increased production of p21 [18] and bax [19]. The blots were performed on the p53-expressing U2-OS and RKO cell lines as well as the p53-negative SAOS and RKO-E6 cell-lines. Our results indicate that in the p53-positive
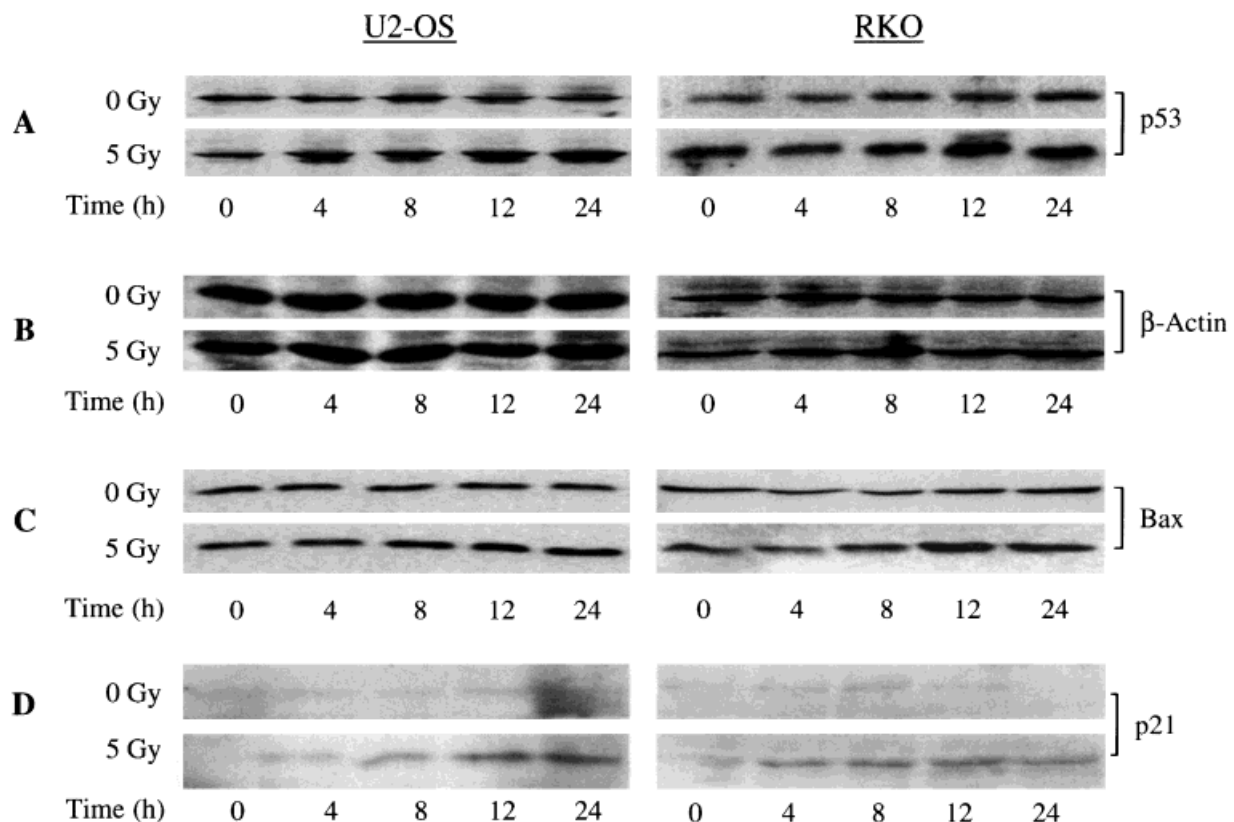

Figure 3. Intracellular p53 levels increased in response to ionizing radiation. The cell lysates were prepared as described in the Materials and Methods. Equal volumes (8 µL) of lysates were resolved on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated accordingly with a first antibody, then probed with a horseradish peroxidase–conjugated secondary antibody and visualized by using a chemiluminescent kit according to the manufacture's procedure (Pierce, Rockford, IL). (A) p53 expression in irradiated and nonirradiated U2-OS and RKO cells. A mouse anti-p53 monoclonal antibody (Ab-2, Calbio- chem, La Jolla CA) was used to detect the level of p53. (B) β-actin expression in irradiated and nonirradiated U2-OS and RKO cells. The membrane was incubated with a mouse anti-β-actin polyclonal antibody (Sigma, St. Louis). (C) bax expression in irradiated and nonirradiated U2-OS and RKO cells. A rabbit anti-bax antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used in this experiment. (D) p21 expression in irradiated and nonirradiated U2-OS and RKO cells. The blots were performed by using (α)anti21/WAF antibody (Santa Cruz Biotechnology).

cell lines, the cells that were irradiated showed increased levels of p21 and bax, while the cells that were not irradiated had constant levels of p21 and bax (Figure 3C and D) with quantitation, (Fig. 2C and D). The increased expressions of p21 and bax are in accordance with the increase of p53 in the cells (Figure 3A) indicating that the increased p53 level correlates to the increased activity of p53. The p53-negative cell lines (SAOS-2 and RKO-E6) expressed very low levels of p21 or bax, and γ-irradiation has no effect on their expression (data not shown).

MMP-2 Secretion Dependent on Functional p53

To determine whether wild-type p53 is necessary for the expression of MMP-2, we examined MMP-2 expression in two p53-negative cell lines. In the first cell line were SAOS-2 cells, which had the *p53* gene deleted [20]. Previous studies indicate that SAOS-2 cells are devoid of p53 [21]. The second cell line was the RKO-E6 cells, which are derived from RKO cells infected with a human papillomavirus E6 gene that results in the degradation of p53 through an ubiquitin-mediated pathway [22]. Using these two cell lines, we analyzed the activity by zymography and the expression of MMP-2 by western bloting in both the irradiated and nonirradiated cells. Interestingly, the MMP-2 activities in the conditioned media from these two cell lines were undetectable (Figure 4, lanes 2 and 4). The MMP-2 activity in conditioned media after irradiation was also undetectable (data not shown). However, MMP-2 protein was expressed in both SAOS-2 and RKO-E6 cells (Figure 5, lanes 2 and 4). Interestingly, the ratio of pre-MMP-2 to MMP-2 is much higher in SAOS-2 and RKO-E6 cells than in U2-OS cells (Figure 5, lanes 2 vs 1) and RKO cells (Figure 5, lane 4 vs 3). The pre–MMP-2 level was 43% in U2-OS cells, 64% in SAOS-2 cells, 65% in RKO cells, and 79% in RKO-E6 cells. Our results suggest that p53 may not only be involved in radiation-induced expression of MMP-2 but may also be involved in MMP-2 processing, activation, and secretion.
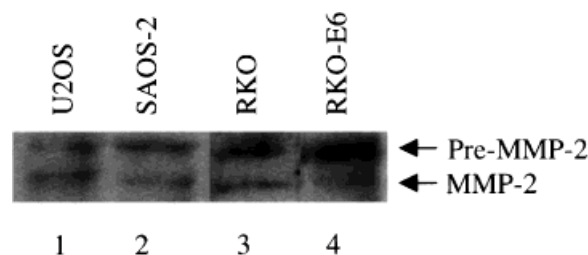
Figure 5. Higher pre-MMP-2 level in p53-negative cells. The cell lysates were prepared as described in the Materials and Methods. Equal volume (8 µL) of lysates was resolved on a 10% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. The level of MMP-2 was determined by western blot analysis by using an MMP-2 Ab-3 antibody (Calbiochem, La Jolla CA). The data indicate that there was a higher percentage of pre-MMP-2 in p53-negative cells (lanes 2 and 4) than in p53-positive cells (lanes 1 and 3).

DISCUSSION

Tumor metastasis is a complex process and can be largely divided into three phases: (i) escape of the tumor cells from the mass of primary tumor and dissemination of the cells through the lymphatics or blood vessels, (ii) penetration through vessel walls and other tissue barriers, and (iii) growth at the new site [2]. Many genes have been implicated in this process. Some of the metastasis-associated genes are regulated either positively or negatively by p53. Examples include *MMP-1*, *MMP-2*, epidermal growth factor receptor, *cathepsin D*, basic fibroblast growth factor, *MDR1*, *thrombospondin-1*, and *KAI-1* [16,23]. Since radiation has been shown to induce *p53*, it has been hypothesized that radiotherapy may promote tumor metastasis in a subset of human cancers harboring wild-type p53 [16]. It has also been found that increased expression of p53 affects the metastatic potential of certain types of cancers [24].

Using three human cancer cell lines and one mouse cancer cell line that harbor wild-type p53, as well as two p53-negative cell lines, we showed that the MMP-2 activities in conditioned media harvested from U2-OS and RKO cells were induced by γ-irradiation. Interestingly, the MMP-2 activities did not change in conditioned media harvested from irradiated HepG2 and MCF-7 cells even though they showed a significant increase in p53 levels after radiation treatment (data not shown). Thus, it appears that MMP-2 activity can only be induced by γ-irradiation in certain types of cells. These results may explain the previous observations that radiation therapy can increase hematogenous metastasis in some tumors but not in others [25]. We have also shown that the radiation-induced expression of MMP-2 correlated with the expression level of p53. In U2-OS cells, p53 was induced within 4 h, and MMP-2 activity was increased from 4 to 24 h. Likewise, in RKO cells, p53 was induced at 12 h, postirradiation, and MMP-2 activity reached its peak at 24 h. These results further suggest that MMP-2 increase is a consequence of increased levels of wild-
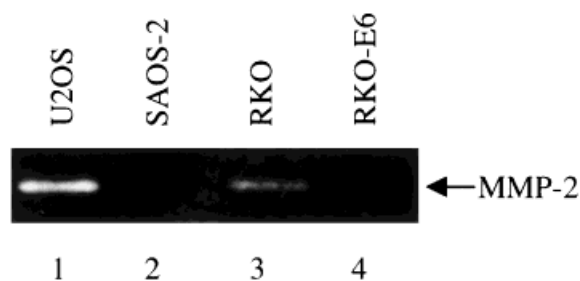

Figure 4. MMP-2 secretion dependent on p53. Zymography of conditioned media taken from the 24-h time points in four different cell lines (U2-OS, RKO, SAOS, and RKO-E6) indicated the presence of MMP-2 activity in the p53-positive cell lines (lanes 1 and 3) but little or no MMP-2 activity in the p53-negative cell lines (lanes 2 and 4).

type p53 induced by exposure to γ-radiation. It is, however, not clear why p53-mediated MMP-2 activation by irradiation is cell-line dependent.

To further confirm that the increased expression of MMP-2 is p53 dependent, we examined the MMP-2 expression after γ-irradiation in p53-negative cell lines. Two p53-negative human cancer cell lines, SAOS-2 (human osteosarcoma) and RKO-E6 (human colon cancer), were also used in the study. Indeed, MMP-2 expression was not induced in these two cell lines, indicating that MMP-2 induction by γ-irradiation is p53-dependent. We also measured the amounts of p21 and bax in the cell lysates. This was done because p21 and bax protein levels correlated with the level of p53 being expressed. Our data suggest that the levels of p21 and bax increase as the levels of p53 increase in response to radiation in U2-OS and RKO cells. The expression levels of p21 and bax did not increase in SAOS-2 and RKO-E6 cells (data not shown), which indicates that the γ-irradiation–induced p21 and bax expressions are p53-dependent. In addition, it was interesting to find that the expressed MMP-2 was not efficiently secreted into the conditioned media by these two cell lines. Our data suggest that the pre–MMP-2 is not efficiently matured in SAOS-2 and RKO-E6 cells. These results suggest that p53 may even play a role in the maturation of MMP-2.

In conclusion, we have shown that wild-type p53 activated by γ-irradiation induces an increased level of MMP-2 expression. The p53 ''status'' of a certain cancer appears to play a role in the determination of whether MMP-2 is activated by DNA damage. Thus, it is possible that a subset of human cancer with wild-type p53 may undergo metastasis upon radiotherapy via MMP-2 activation. If this is the case, the next challenge will be how to determine the metastatic potential of a patient's tumor when exposed to radiation. This will require an in-depth understanding of in vivo regulation of MMP-2 and other metastasic genes [16] and the tumor context. Nevertheless, the finding reported here serves as the first step in understanding regulation of MMP-2 expression by radiation in the context of p53 status. MMP-2 status of cancer cells can be potentially used as a prognostic factor for predicting the outcome of certain forms of bladder cancer [26]. Thus, a better understanding of the activation of MMP-2 and p53 by various factors will not only lead to an improved disease prognosis but also aid in the development of treatment strategies that are more safe and effective.

ACKNOWLEDGMENTS

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