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Inhibition of Matrix Metalloproteinase-2 Enhances Radiosensitivity by Abrogating Radiation-induced FoxM1-mediated G2/M Arrest in A549 Lung Cancer Cells

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Abstract

Matrix metalloproteinase-2 (MMP-2) is known to degrade the collagen IV, play a role in radiation-induced lung injury. We therefore investigated the anti-tumor effects of combining MMP-2 inhibition using an adenovirus expressing siRNA against *MMP-2* (Ad-MMP-2-Si) with radiation therapy (IR) on A549 lung cancer cells *in vitro* and *in vivo*. IR increased MMP-2 mRNA, protein and activity in lung cancer cells. MMP-2 inhibition along with IR enhanced radiosensitivity as determined by clonogenic assay, flow cytometry, and TUNEL assay. We show that MMP-2 inhibition prior to irradiation reduced p53 phosphorylation, with a corresponding reduction in the expression of the p53 downstream target gene *p21^{Cip1/Waf1}*. Irradiated tumor cells induced the FoxM1-mediated DNA repair gene, XRCC1 and Checkpoint-2/1, which were abrogated with combined treatment of Ad-MMP-2-Si and IR. Further, the combination of Ad-MMP-2-Si with radiotherapy significantly increased anti-tumor efficacy *in vivo* compared to either agent alone. Indeed, histological analysis of tumor sections collected from the combination group revealed more apoptotic cells. These studies suggest that MMP-2 inhibition in combination with radiotherapy abrogates G2 cell cycle arrest leading to apoptosis and provide evidence of the anti-tumor efficacy of combining MMP-2 inhibition with irradiation as a new therapeutic strategy for the effective treatment of NSCLC patients.

Keywords

MMP-2; FoxM1; G2/M arrest; radiosensitization; A549

INTRODUCTION

NSCLC patients with unresectable stage III disease or medically inoperable disease account for about 40% of all patients diagnosed with NSCLC. Currently, radiation therapy plays an important role in achieving local control of the tumor and in the relief of symptoms resulting from metastatic disease. However, complete response rates are low and long-term survival

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Ionizing radiation has been shown to upregulate several proteolytic enzyme molecules such as MMPs, which have been strongly implicated in tumor invasion, angiogenesis and tumor progression. We show that the concomitant use of MMP-2 inhibitor during radiotherapy could be a potential therapeutic approach to improve the efficacy of radiotherapy for lung cancer patients.

remains poor. In addition, therapeutic efficacy is limited when cancer cells develop resistance to radiation. This underscores an urgent need for the development of more effective and less toxic treatments.

Matrix metalloproteinases (MMPs) comprise a large family of related proteolytic enzymes that includes collagenases, gelatinases, stromelysins, elastases, and membrane-type (MT-MMPs).¹ The expression and activity of MMPs increase in almost all human cancer types and are associated with advanced tumor stage and poor survival.² MMP-2 belongs to the gelatinase sub-family and is believed to play an important role in extracellular matrix (ECM) turnover. MMP-2 can degrade denatured interstitial collagens I and III as well as native collagen IV, which is an important component of the basement membrane.

Previous reports have described the increased expression of MMP-2 after irradiation. Ionizing radiation increased MMP-2 activity in rat astrocytes,³ rat mesangial cells,⁴ rat kidney tubule epithelial cells,⁵ human bronchial epithelial cells, and A549 lung cancer cells.⁶ In addition, irradiation enhanced secretion of MMP-2 in subcutaneous Lewis lung carcinoma (LLC-LM) tumors⁷ and pancreatic cancer cells.⁸ Analogous to its surgical removal, radiation therapy of a primary LLC-LM tumor is followed by the explosive growth of previously dormant metastases.⁹ The inflammatory process of radiation-induced lung injury includes infiltration by various inflammatory cells known to release high amounts of MMP-2.¹⁰ These studies suggest that a subset of patients may be at increased risk of metastatic growth after the surgical resection or radiation treatment of their primary tumors due to increased MMP-2 expression. These patients could benefit from treatment that includes the addition of MMP-2 inhibitors to radiotherapy.

Ionizing radiation leads to direct apoptotic events in the tumor cell population and/or induces arrest at cellular interphase checkpoints. This arrest allows the cell to repair DNA strand breaks before continuing the cell cycle or induces apoptosis if DNA repair is not possible.¹¹ The present study was designed to examine the effects of the combining MMP-2 inhibition and radiation in lung cancer cells *in vitro* and *in vivo*. Our results demonstrate that MMP-2 inhibition enhances the therapeutic response of lung cancer cells to irradiation. We also show MMP-2 inhibition abrogates FoxM1 transcriptional pathways and its downstream signaling effector checkpoint kinase (Chk2)-mediated DNA repair response in irradiated lung cancer cells.

MATERIALS & METHODS

Antibodies and reagents

The following antibodies and reagents were used in this study: anti-Cyclin-B1 (Biomed, Foster City, CA); anti-phospho-p53 (Ser-15) and anti-phospho-Cdc-25C (Ser-216) (Cell Signaling, Boston, MA); anti-FoxM1 (Affinity BioReagents, Golden, CO), anti-FoxM1 (for IP), anti-MMP-2, anti-p53, anti-p21^{Cip1/Waf1}, anti-Cdc2, anti-phospho-Cdc2 (Thr-14/Tyr-15), anti-Chk1, anti-Chk2, anti-XRCC1, and anti-GAPDH primary antibodies, HRP-conjugated secondary antibodies, mouse IgG, p53 siRNA, FoxM1 siRNA, and control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho- γ -H2AX (Ser-139), anti-phospho-Ser/Thr-Proline-MPM-2 antibodies and human recombinant MMP-2 protein (Calbiochem, San Diego, CA), ECL reagent (Amersham Pharmacia, Piscataway, NJ), Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA), DAB peroxidase substrate (Sigma, St Louis, MO), TUNEL Detection kit (Roche Molecular Biochemicals, Indianapolis, IN), Annexin-V-FITC Apoptosis Detection Kit (BioVision Mountain View, CA); MTT cell growth assay kit (Millipore Corporation, Billerica, MA); and CytoTox-96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI).

Cell lines and culture conditions

A549 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and maintained as a monolayer in RPMI 1640 medium (ATCC) supplemented with 10% fetal bovine serum (FBS; Japan), 50units/mL penicillin, and 50µg/mL streptomycin (Life Technologies Inc., Frederick, MD), hereafter referred to as complete medium, at 37°C in a humidified 5% CO₂ atmosphere.

Adenoviral siRNA constructs, infection and radiation

Adenoviral siRNA for MMP-2 (Ad-MMP-2-Si) and scrambled vector (Ad-SV) were constructed and amplified as described previously.¹² Viral titers were quantified as pfu/mL following infection of 293 cells. Titters obtained for the viruses used in this work are as follows: Ad-SV (7.6×10^{11} pfu/mL) and Ad-MMP-2-Si (5.0×10^{11} pfu/mL). The amount of infective adenoviral vector per cell in culture media was expressed as multiplicity of infection (MOI). Viral constructs were diluted in serum-free medium to the desired concentration, added to infected cells, and incubated at 37°C for 1h. The necessary amount of complete medium was then added; cells were incubated for 24h and irradiated with X-ray radiation (a single dose of 10Gy). After addition of complete medium, cells were incubated for 12h.

Gelatin zymography

To prepare tumor-conditioned medium, A549 cells were infected with mock (PBS), 50MOI of either Ad-SV or Ad-MMP-2-Si. After 24h of incubation, cells were irradiated with 10Gy and cultured for 12h in serum-free DMEM/F-12 medium. The tumor-conditioned medium was collected and equal amounts of proteins were used to determine MMP-2 activity. Gelatin zymography was performed as described previously.¹³

Western blot analysis

A549 cells were infected with mock, 50MOI of Ad-SV or the indicated MOI of Ad-MMP-2-Si and irradiated as described above. Whole cell lysates were prepared by lysing cells in RIPA lysis buffer [50mM Tris-HCl (pH 7.4), 150mM NaCl, 1% IGEPAL, 1mM EDTA, 0.25% sodium deoxycholate, 1mM sodium fluoride, 1mM sodium orthovanadate, 0.5mM PMSF, 10µg/mL aprotinin, 10µg/mL leupeptin]. Equal amounts of protein were resolved by SDS-PAGE and transferred to a PVDF membrane as per the manufacturer's instructions. After blocking with 5% nonfat dry milk and 0.1% Tween-20 in PBS, membranes were incubated with 1:1000 dilutions of primary antibodies. HRP-conjugated secondary antibodies were used to detect chemiluminescent signals with the ECL system. GAPDH levels served as a loading control. For detection of phospho-FoxM1 total cellular extracts were immunoprecipitated with FoxM1 antibody and immunocomplex was subjected to western blotting with antibodies for MMP-2, which recognizes the phosphorylated protein sequence phosphoserine/phosphothreonine-proline.

Reverse transcription-PCR (RT-PCR)

Total RNA was extracted using TRIZOL reagent (Life Technologies, Grand Island, NY) according to manufacturers' protocol, and RT-PCR was performed as described previously.¹² PCR products were resolved on 2% agarose gels and were visualized by ethidium bromide staining. To normalize for the amount of input RNA, RT-PCR was performed with primers for the *GAPDH*. The specific primers used in this study were as follows: *MMP-2*, forward 5'-GTGCTGAAGGACACACTAAAGAAGA-3' and reverse 5'-TTGCCATCCTTCTCAAAGTTGTAGG-3'; *GAPDH*, forward 5'-TGAAGGTCGGAGTCAACGGATTGGT-3' and reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3'. To determine the quantity of PCR products on the agarose gel, images

were generated by Alpha Innotech Image Acquisition and Analysis Software and processed for display.

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out in 96-well plates using a MTT cell growth assay kit. Cells (5×10^3 /well) were plated and infected with mock, 50MOI of Ad-SV or Ad-MMP-2-Si in 16-wells/sample for 24h and then irradiated with 10Gy. The proliferation rate was measured after irradiation at 12, 24, 36, and 48h. At the end of various treatments, 20 μ L of MTT solution was added to each well and the plates were further incubated for 1h. Samples were read on a microplate reader using test wavelength 550nm and reference wavelength 655nm. The % of viability was calculated as $100 \times \text{mean absorbance of sample} / \text{mean absorbance of mock}$. Values were plotted accordingly.

Cytotoxicity assay

Cells (1×10^4 cells/well) were plated in 96-well plates, infected and irradiated as described above. 48h after irradiation, medium from each well was collected to measure the amount of released lactate dehydrogenase (LDH). Separate wells were exposed to lysis buffer (9% Triton X-100) and medium was collected to measure the total amount of cellular LDH. The amount of cell death was calculated as a percentage of released LDH over total intracellular LDH.

Clonogenic assay

Cells were infected with 50MOI of either Ad-SV or Ad-MMP-2-Si for 24h and irradiated with 10Gy. Cells were trypsinized and seeded (200 cells) in 100mm Petri dishes. On day 10 after irradiation, cells were fixed in methanol and stained with Giemsa and colonies (>50 cells) were counted. Survival fraction was defined as number of colonies divided by the number of plated cells.

Terminal deoxynucleotidyl transferase-mediated nick labeling (TUNEL) assay

We tested for apoptotic nuclei using the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) enzyme reagent (Roche Molecular Biochemicals, Indianapolis, IN) as per the manufacturer's instructions. To determine the number of apoptotic cells, fluorescent-labeled nuclei were counted and imaged. The apoptotic index was calculated as follows: $\text{apoptotic index (\%)} = 100 \times (\text{apoptotic cells} / \text{total cells})$.

FACS analysis

Propidium iodide (PI) positive cells were determined as described earlier.¹⁴ Briefly, A549 cells were infected and irradiated as described earlier. Cells were trypsinized, washed with cold PBS, and incubated with 5 μ L of PI in 1X binding buffer at room temperature for 5–10min in the dark. FACS analysis was performed using a FACS Calibur Flow Cytometer (BD Biosciences, San Jose, CA) with an excitation wavelength of 488nm and emission wavelength of 530nm. Data acquisition and analysis were performed using CellQuest software (BD Biosciences).

Animal experiments

Harvested A549 cells in exponential growth phase by brief exposure to trypsin-EDTA solution and cell viability was determined by trypan blue exclusion and only cells with >95% viability were used. To determine tumorigenic ability, tumor cells (5×10^6 cells/mouse) were injected s.c. into the flank of 4–6-week-old female nude mice. When mean tumor diameter reached 3–6mm (12th day), the mice were separated into groups of five animals per group, and each group was injected intratumorally with PBS (mock) or 5×10^7 pfu of either Ad-SV or Ad-

MMP-2-Si (10–25 μ L volume) and were given three doses on alternate days. Between the first and the second injections, and the second and the third injections, one group were radiated with a dose of 5Gy, each time. Subcutaneous tumor growth was measured every third day and tumor volume was calculated as $\pi/6 \times (R_{\max} \times R_{\min}^2)$. Regression of the subcutaneous tumor growth was followed for up to 4 weeks. Mice were euthanized when the tumor diameter in control mice measured between 13–14mm. The subcutaneous tumors were removed and fixed in 10% phosphate-buffered formaldehyde.

Immunohistochemical analysis

The fixed tissue samples were then processed into paraffin blocks. Tissue sections (4–5 μ m-thick) were subjected to immunostaining for MMP-2 and FoxM1 as described previously.¹⁴ Briefly, after deparaffinization and rehydration, tissue sections were permeabilized in 0.1% TritonX-100 in PBS. Endogenous peroxidase activity was quenched by 3% H₂O₂ in methanol for 20min followed by PBS rinses. Non-specific binding was blocked for 1h at RT with 1% BSA in PBS followed by incubated for 1h at RT with anti-MMP-2, anti-FoxM1 or anti-mouse IgG (negative control) at a concentration of 1.0 μ g/mL. The slides were rinsed in PBS and incubated for 1h with HRP-conjugated secondary antibody (1:200). The slides were washed and stained with DAB peroxidase substrate solution followed by counterstained with hematoxylin and mounted. Mounted specimens were observed and photographed using light microscopy.

Statistical analysis

All data are presented as means $\pm$ SE of at least three independent experiments, each performed at least in triplicate. In the animal experiments, the mean differences in tumor volumes were compared among treatment groups using a one-way analysis of variance (ANOVA). Statistical differences are presented at probability levels of $p < 0.05$, $p < 0.01$ and $p < 0.001$.

RESULTS

Ad-MMP-2-Si infection inhibits radiation-induced MMP-2 expression

To examine the effect of radiation on MMP-2 expression, we determined MMP-2 activity, protein and mRNA levels in A549 lung cancer cells irradiated with various doses of X-ray. Radiation induced MMP-2 activity increased up to 10Gy and decreased thereafter. The decreased levels of MMP-2 activity beyond the 10Gy could be due to severe damage of DNA and other proteins. Maximal induction in MMP-2 activity was observed at 10Gy with a ~ 3 fold increase compared to the control (0Gy). Western blot and RT-PCR analyses indicated that MMP-2 protein and mRNA levels increased up to ~ 3 fold in 10Gy-irradiated cells compared to mock (non-irradiated) controls (Fig. 1A). It has been hypothesized that MMPs may play a role in the induction of a malignant phenotype after ionizing radiation. Therefore, we first determined whether MMP-2 inhibition at 10Gy enhances the radiosensitivity of A549 lung cancer cells. We have used an adenovirus carrying siRNA against MMP-2 (Ad-MMP-2-Si) to inhibit MMP-2 expression. Our previous studies clearly demonstrate that Ad-MMP-2-Si specifically inhibits MMP-2 expression without inducing the interferon pathway.^{12,14} Figure 1B demonstrates cytotoxicity following Ad-MMP-2-Si infection at 10–50MOI with 10Gy radiation. Cytotoxicity derived by LDH release assay 48h after treatment is shown. Maximal effect was observed with the combination of 50MOI and 10Gy radiation ($p < 0.01$). The MOI of Ad-MMP-2-Si that gave the greatest radiosensitization at 10Gy in A549 cells (50MOI) was used to determine the underlying mechanism in Ad-MMP-2-Si-mediated radiosensitivity. Figure 1C shows the cell survival rate after exposure to Ad-MMP-2-Si and irradiation alone and in combination as determined by MTT viability assay. The combination of Ad-MMP-2-Si and radiation caused a slight decrease in cell viability ($\sim 80\%$) at 12h. By 48h, there was a dramatic reduction in viability to $\sim 20\%$ as compared to the control population. No significant

changes in cell growth were observed for the combination of Ad-SV (scrambled vector) and radiation as compared to treatment with radiation alone. We next determined the effect of Ad-MMP-2-Si on irradiation-induced MMP-2 expression. Figure 1D indicates that Ad-MMP-2-Si infection prior to radiation inhibits irradiation-enhanced MMP-2 activity, expression and mRNA levels by more than 90%.

Ad-MMP-2-Si infection inhibits colony formation and induces DNA damage and apoptosis in A549 cells

To determine whether the combination of MMP-2 inhibition and radiation has an effect on the kinetics of cell death as compared to cells treated with single agents alone, we carried out the colony-forming assay. As shown in Figure 2A, treatment of A549 cells with either Ad-MMP-2-Si or radiation decreased the colony forming ability of cells by ~40%. A 95% inhibition of colony forming ability was observed in cells treated with a combination of Ad-MMP-2-Si and radiation as compared to non-irradiated cells. In contrast, the number of colonies in the Ad-SV-treated group was not affected. We next evaluated whether the combination treatment caused increased DNA damage in A549 cells. The histone variant H2AX is phosphorylated on Ser-139 (γ -H2AX) during DNA damage.¹⁵ Figure 2B shows a 2-fold increase in p- γ -H2AX in cells treated with Ad-MMP-2-Si and radiation treatments as compared to irradiated cells indicating an increase in DNA damage in the cells that received the combined treatments compared to radiation alone. We performed the TUNEL assay, to determine if the cell death observed in Ad-MMP-2-Si plus radiation-treated cells, was associated with apoptosis. Indeed, an increase in the number of apoptotic cells was observed with combination treatment of radiation and Ad-MMP-2-Si when compared to cells treated with radiation (Fig. 2C). Quantitative analysis indicated that the percentage of apoptotic cells was ~12–12.5% in radiation or radiation plus Ad-SV-treated cells when compared to controls. However, there was a remarkable increase of ~65% in TUNEL-positive cells in cells infected with Ad-MMP-2-Si prior to radiation.

Ad-MMP-2-Si infection abrogates radiation-induced G2/M arrest

Most mammalian cells exhibit transient delays in the G₁ and G₂ phases of the cell cycle after treatment with radiation to allow the cell to correct possible defects.¹⁶ To identify whether the growth inhibitory effect in cells that received the combined treatment of radiation and Ad-MMP-2-Si is caused by specific perturbation of cell cycle-related events, DNA contents of A549 cells were measured by flow cytometric analysis. As shown in Figure 3A, treatment of A549 cells with radiation resulted in an increase in the percentage of G2/M cells (from 13% to 50%) as compared to control cells. In cells infected with Ad-MMP-2-Si prior to radiation, the percentage of cells in G2/M phase decreased, the percentage of cells in sub-G1 increased to a maximum of 33%, and the majority of cells were in the S phase at 24%.

Ad-MMP-2-Si suppresses p53 activation in response to radiation

To gain insights into the molecular mechanism(s) for Ad-MMP-2-Si and radiation-mediated attenuation of G2/M, we measured changes in the levels of proteins that are implicated in the G2/M transition. In response to genotoxic stress, the p53 protein is stabilized and activated by phosphorylation and, via its transcription activity, it induces the expression of numerous genes.¹⁷ Among them, p21^{Cip1/Waf1}, an inhibitor of cyclin-dependent kinases, negatively regulates the G1/S and the G2/M phases of the cell cycle.^{18,19} To study the effect of MMP-2 inhibition on radiation-induced p53 expression, cells were infected with Ad-MMP-2-Si before exposure to radiation and were analyzed by western blotting at 12h post-radiation (before the onset of apoptosis). Figure 3B shows that p53 expression and phosphorylation on serine-15 residues were increased in irradiated cells. Further, Ad-MMP-2-Si infection prior to radiation strongly inhibited radiation-induced p53 phosphorylation (Fig. 3B).

MMP-2 inhibition reduces radiation-induced Chk2 expression and phospho-Cdc25C (Ser-216)

Mammalian cells respond to DNA-damaging agents by activating cell cycle checkpoints. Chk2 is required for the maintenance of DNA damage-induced G₂ arrest.²⁰ Irradiated cells exhibited a significant increase in Chk2 levels, whereas Chk1 activity displayed very small changes. Further, radiation-induced Chk2 levels were decreased in cells infected with Ad-MMP-2-Si prior to irradiation (Fig. 3C). Chk2 phosphorylates Cdc25C on serine-216,²¹ which creates a binding site to 14-3-3.²² The 14-3-3-bound Cdc25C phosphatase is then sequestered in the cytoplasm and prevents it from activating its nuclear substrate Cdc2 (Cdk1), a critical kinase for driving cells through G₂.²³ Corresponding to these studies, we show that radiation-induced phosphorylation of Cdc25C on serine-216. Activation of Cdc2 kinase requires dephosphorylation at Thr-14/Tyr-15 residues.²⁴ We therefore determined the Thr-14/Tyr-15 phosphorylation status of Cdc2 in cells treated with radiation and Ad-MMP-2-Si. Figure 3C indicates that the accumulation of the phosphorylated-Cdc2 (Thr-14/Tyr-15) (negatively regulates cell cycle progression) in irradiated cells. However, MMP-2 inhibition in combination with radiation decreased radiation-induced phosphorylation of Cdc25C, which in turn results in activation of Cdc2 (dephosphorylates Cdc2) leading to cell cycle progression through G₂ phase. Cyclin-B1 is the regulatory subunit of Cdc2 serine/threonine kinase and accumulation of cyclin-B1 in the late G₂ phase of the cell cycle is a prerequisite for mitotic initiation in mammalian cells.²⁵ Others have shown that induction of cyclin-B1 after radiation abrogated the G₂ delay.²⁶ Therefore, we next determined the cyclin-B1 expression. Our studies show that cyclin-B1 expression decreased in irradiated cells, whereas cyclin-B1 levels were induced in cells infected with Ad-MMP-2-Si prior to radiation (Fig. 3C). These results suggest that MMP-2 inhibition removes the radiation-induced phosphorylation of Cdc2 at Thr-14/Tyr15, thereby activating Cdc2 and inducing cyclin-B1 and leading to cell cycle progression.

Ad-MMP-2-Si infection decreases radiation-induced FoxM1 expression

Since FoxM1 transcription factor regulates the expression of cell cycle genes essential for DNA replication and mitosis during organ repair and cancer progression, we next determined the role of FoxM1. Figure 4A indicates an increase in FoxM1 expression and phosphorylation in irradiated cells compared to controls. However, Ad-MMP-2-Si infection prior to irradiation inhibited FoxM1 expression and radiation induced phosphorylation. As FoxM1 is involved in regulating transcription of DNA repair factor XRCC1, we determined the expression of XRCC1 in the irradiated cells and those that received both Ad-MMP-2-Si and radiation. XRCC1 expression was induced in irradiated cells, but we could not detect XRCC1 in cells infected with Ad-MMP-2-Si prior to irradiation (Fig. 4A).

To evaluate the functional role of FoxM1 in radiation-induced cell cycle arrest, we downregulated the expression of endogenous FoxM1 in cells using RNA interference. A pool of siRNA oligonucleotides, directed against FoxM1, efficiently depleted cells of endogenous FoxM1. Irradiated cells transfected with FoxM1 siRNA displayed decreased levels of the XRCC1 repair gene and high levels p-γH2AX compared to cells subjected to radiation alone. The increased number of DNA breaks in FoxM1 decreased A549 cells was correlated with decreased phosphorylation of p53 (ser-15) and p21^{Cip1/Waf1} expression (Fig. 4B), thereby suggesting that FoxM1 functions upstream of p53 expression. Taken together, these results suggest that MMP-2 inhibition stops radiation-induced FoxM1 expression, which in turn inhibits cell cycle arrest and DNA repair.

To further test the hypothesis that MMP-2 inhibition leads to FoxM1 reduction, mock and Ad-MMP-2-Si-infected cells were cultured in the presence of recombinant human-MMP-2. As shown in Figure 4C, FoxM1 expression was induced by 1.61- and 2.71-fold in the presence of 50 and 100ng/mL of MMP-2 compared to control (mock) cells. Similarly, addition of MMP-2

restored FoxM1 expression in Ad-MMP-2-Si-infected A549 cells. Our results demonstrate that MMP-2 induction contributes directly to FoxM1 expression in irradiated cells.

MMP-2 inhibition enhances radiosensitivity in vivo

Based on the encouraging results from the *in vitro* evaluation of enhancement of radiosensitivity, we evaluated the effects of MMP-2 inhibition in combination with radiation on A549 tumors propagated in the flanks of athymic nude mice. Treatment began on day 12 when the A549 tumors had reached 4–5 mm diameter. Tumor growth was measured and the change in tumor size determined over time. As shown in Figure 5A, on day 24 post-treatment, radiation alone ($2 \times 5\text{Gy}$) and Ad-MMP-2-Si alone delayed A549 tumor growth more than mock (control) by $\sim 17\%$ (750 ± 39.54 vs. $900 \pm 53.21 \text{ mm}^3$) and 56% (400 ± 37 vs. $900 \pm 53.21 \text{ mm}^3$), respectively. Notably, MMP-2 inhibition in combination with radiation reduced A549 tumor size completely.

One set of tumors from each treatment was excised at day 5 post-treatment and the tissue sections were stained to determine whether radiation induced MMP-2 and FoxM1 *in vivo*. Tumor sections from mice that received radiation alone showed intense staining for MMP-2 and FoxM1. However, MMP-2 expression and FoxM1 nuclear translocation were decreased significantly in tissue sections from mice treated with Ad-MMP-2-Si and radiation, thereby confirming our *in vitro* results (Fig. 5B; Middle panel). Furthermore, we carried out the TUNEL assay to detect apoptotic cells (Fig. 5B; Bottom panel). Corresponding to their growth characteristics, we found few apoptotic cells ($\sim 40 \pm 6.75$ cells/field) in irradiated tumors. In contrast, we found abundant TUNEL-positive cells in the tumor sections from mice that received Ad-MMP-2-Si and radiation (210 ± 11.58 cells/field) (Fig. 5C).

DISCUSSION

The combination of radiotherapy and biological therapies, which interfere with cancer cell growth signaling, provides several potential benefits for improving therapeutic outcome. The combination may increase tumor response, protect normal tissues, and exhibit non-overlapping toxicities. Additionally, two partially effective therapeutic modalities may be combined without having to significantly reduce their dose levels to avoid treatment-related toxicities. In this study, our data demonstrated that radiation enhanced a dose-dependent increase in MMP-2 expression of A549 lung cancer cells. Infection with adenovirus carrying siRNA against MMP-2 (Ad-MMP-2-Si) markedly decreased MMP-2 expression and viability of irradiated lung cancer cells. We also demonstrated that MMP-2 inhibition abrogated radiation-induced cell cycle arrest and enhanced radiation-induced apoptosis.

We next sought to determine the mechanisms underlying MMP-2 inhibition-mediated, radiation-induced abrogation of G2/M arrest and apoptosis. Progress through the cell cycle is controlled by various surveillance mechanisms that block or delay transitions until each phase of the cell cycle is accurately completed. Integrity of the control pathways, which are termed “check points,” are critical for genomic integrity²⁷ as well as for the repair and survival of cells exposed to DNA-damaging agents.²⁴ A well-known tumor suppressor, p53 protein, senses DNA damage at several stages of the cell cycle and accordingly determines whether the cell needs to arrest at the subsequent checkpoint to undergo DNA repair or proceed through it.²⁸ Recent studies have shown evidences that p53 mediated pathways promote radiation-induced cell cycle arrest in G2 and that the duration of G2 arrest was longer in cell lines with wild type p53²⁹⁻³². It has been shown that p21^{Cip1/Waf1} could also be involved in p53-mediated G2 arrest.³³ p53-defective cells fail to maintain the DNA damage-induced arrest, and cells gradually escape the checkpoint and progress into the M phase, which is when they eventually undergo aberrant mitoses.³⁴ Consistent with these observations, we show that MMP-2 inhibition reduces radiation-induced p53 and p21^{Cip1/Waf1} expression in A549 cells.

Activated p53 transcriptionally upregulates genes that are involved in DNA repair and/or cell cycle arrest.³⁵ Checkpoint kinase-2 (Chk2) activation is most pronounced in response to DNA damage caused by DNA double-strand breaks such as those produced by radiation.³⁶ Our studies clearly indicate that radiation-induced Chk2 is more pronounced than Chk1. Further, the radiation-induced Chk2 levels were reduced to lower than control levels in cells infected with Ad-MMP-2-Si prior to radiation. These observations are consistent with previous reports indicating that Chk2 rather than Chk1 is the primary checkpoint kinase responsive to radiation treatment.³⁷ G2 delay after radiation is marked by delayed expression of cyclin-B1.²⁶ Previous studies indicate that the cellular decision to enter into apoptosis is regulated, at least in part, by the abundance of cyclin-B1 protein. Antisense inhibition of cyclin-B1 accumulation prevents γ -radiation-induced apoptosis in human hematopoietic cells and ectopic cyclin-B1 expression is sufficient to induce apoptosis.³⁸ In human cells, an inhibition of cyclin-B1 transcription by the p53 tumor suppressor prevents G2/M transition.³⁹ Accordingly, our data also indicates that radiation inhibited cyclin-B1 expression whereas MMP-2 inhibition induced cyclin-B1 expression in irradiated cells. Taken together, our studies indicate that MMP-2 inhibition enhances radiosensitivity by abrogating the radiation-induced G2/M arrest.

FoxM1 signaling is involved in cell proliferation and apoptosis, which affects the development and function of many organs.⁴⁰ Loss of FoxM1 expression generates mitotic spindle defects, delays cells in mitosis, and induces mitotic catastrophe.⁴¹ We show the involvement of FoxM1 in the transcriptional response during DNA damage/checkpoint signaling in cells treated with Ad-MMP-2-Si and radiation. MMP-2 inhibition prior to radiation abrogated radiation-induced FoxM1 expression. We also demonstrated that siRNA silencing of FoxM1 levels in irradiated A549 cells reduced p53 and p21^{Cip1/Waf1} expression. The depletion of FoxM1 also increased cell sensitivity to radiation as determined by decreased expression of the repair gene XRCC1 and increase in DNA damage as determined by increased expression of phosphorylated- γ -H2AX. Interestingly, we observed that addition of recombinant MMP-2 induces FoxM1 expression in A549 cells. Previous reports indicate that FoxM1 induces MMP-2 expression and has a binding site for the MMP-2 promoter.⁴² Currently, we do not fully understand how MMP-2 alters FoxM1 expression, and as such, it is the focus of our ongoing investigation. Consistent with *in vitro* findings, A549 tumor xenografts treatment with the combination of Ad-MMP-2-Si and radiation suppressed tumor growth significantly more than either Ad-MMP-2-Si or radiation alone. Further, tumor sections from mice that received the combination treatment of Ad-MMP-2-Si and radiation showed less immunoreactivity for MMP-2 and FoxM1 as compared to non-irradiated and irradiated tumors. These results identify a novel role for FoxM1 in the transcriptional response during DNA damage/checkpoint signaling in irradiated lung cancer cells. Previous studies have shown that a cell-penetrating ARF peptide inhibitor of FoxM1 selectively induced apoptosis in human hepatocellular carcinoma cell lines and mouse models.⁴³ Downregulation of FoxM1 by FoxM1 siRNA inhibited the expression of MMP-2, MMP-9, and VEGF and inhibited cell growth in pancreatic cancer.⁴⁴ Partial deletion of mouse FoxM1-floxed targeted allele *in vivo* was sufficient to significantly reduce the number and size of lung adenomas induced by urethane.⁴⁵ Thus, inhibition of FoxM1 in along with radiation represents potential new clinical strategies and a worthwhile target for therapeutic intervention in lung cancer

In summary, MMP-2 inhibition can overcome the G₂/M arrest in irradiated cells, thereby driving cells through a lethal mitosis (Fig. 6). As such, it is potentially useful as a radio-sensitizer in lung cancer therapy. These findings suggest that the concomitant use of MMP-2 inhibitor during radiotherapy could be a potential therapeutic approach to improve the efficacy of radiotherapy for lung cancer patients.

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Abbreviations used

MMP, matrix metalloproteinase
 siRNA, short interfering RNA
 IR, irradiation
 Gy, Gray
 RT-PCR, reverse transcription polymerase chain reaction
 MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
 TUNEL, terminal deoxynucleotidyltransferase-mediated nick end labeling
 Cdc, cell division cycle
 Chk, checkpoint kinase
 FoxM1, forkhead box M1
 XRCC1, X-ray repair cross complementing group-1

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Figure 1. X-Ray radiation induces MMP-2 and Ad-MMP-2-Si inhibits radiation-induced MMP-2 activity and expression in the A549 lung adenocarcinoma cell line

(A) (*Upper Panel*) A549 cells were irradiated with 0–20 Gy X-ray, incubated for 12h, and conditioned medium was collected. MMP-2 activity was determined by gelatin zymography. The band intensities of MMP-2 activity were quantified by densitometry. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from non-irradiated (0 Gy) conditioned medium. (*Lower Panel*) A549 cells were irradiated with 5 and 10Gy. After 12h of incubation, total cell lysates were used to determine MMP-2 levels by western blotting, and total RNA was used to determine MMP-2 mRNA transcription levels by semi-quantitative RT-PCR with gene-specific primers. The band intensities of MMP-2 protein and amplified cDNA were quantified by densitometry and normalized with the intensity of the mock bands. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from individual treatments.

(B) Combination therapy with 10Gy radiation and Ad-MMP-2-Si as compared with single-agent therapy alone across a wide range of doses in A549 cells. Standard lactate dehydrogenase colorimetric assay was used to assess cytotoxicity. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock.

(C) Effect of either radiation or Ad-MMP-2-Si alone or in combination on A549 cell proliferation as determined by MTT assay. A549 cells were infected with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si. After 24h of incubation, cells were irradiated with 10Gy and incubated for a further 12–48h. The % of viability was calculated as a ratio of mean absorbance of sample to mean absorbance of mock $\times 100$ and values were plotted. *Points*, means of three experiments; *bars*: SE.

(D) A549 cells were infected with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si. After 24h of incubation, cells were irradiated with 10Gy and incubated for a further 12h. Conditioned media was used to determine MMP-2 activity by gelatin zymography, total cell lysates were used to determine MMP-2 levels by western blotting, and total RNA was used to determine MMP-2 mRNA transcription levels by RT-PCR with gene-specific primers. The band intensities of MMP-2 activity, protein and amplified cDNA were quantified by densitometry and normalized with the intensity of the mock bands. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.01$, significant difference from mock; ** $p < 0.001$, significant difference from radiation.

Figure 2. Ad-MMP-2-Si infection enhances DNA damage and radiosensitivity

(A) Clonogenic assay was performed as described in Materials & Methods. The cells were cultured, and colonies larger than 50 cells were counted. * $p < 0.05$, significant difference from mock; ** $p < 0.05$, significant difference from radiation. (B) A549 cells were infected with mock (PBS), or 50 MOI of either Ad-SV or Ad-MMP-2-Si, incubated for 24h, irradiated with 10Gy, and incubated for another 12h. To detect DNA damage, cellular extracts were subjected to western blotting with antibody for p- γ -H2AX, which is a marker for DNA double strand breaks. Densitometric analysis of p- γ -H2AX band density is shown. Columns: mean of quadruplicate experiments; bars: SE; * $p < 0.001$, significant difference from mock; ** $p < 0.001$, significant difference from radiation.

(C) A549 cells were infected with mock (PBS), or 50 MOI of either Ad-SV or Ad-MMP-2-Si, incubated for 24h, irradiated with 10Gy x-ray, and incubated for another 24h. Apoptosis was detected using the TUNEL assay. *Columns*: mean of quadruplicate experiments; *bars*: SE; * $p < 0.001$, significant difference from mock.

Figure 3. Ad-MMP-2-Si infection reverses radiation-induced G2M arrest

A549 cells were infected with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si, incubated for 24h, irradiated with 10Gy x-ray, and incubated for another 12h.

(A) DNA contents of A549 cells were measured by flow cytometric analysis to determine cell cycle arrest.

(B) Western blot analysis from total cell lysates for determination of total p53, phospho-p53 (ser-15) and p21^{Cip1/Waf1}. Densitometric quantification of protein bands for p53, phospho-p53 (Ser-15) and p21^{Cip1/Waf1} is shown. Columns: mean of triplicate experiments; bars: SE; * $p < 0.001$, significant difference from mock; ** $p < 0.001$, significant difference from radiation.

(C) Western blot analysis from total cell lysates for Chk2, Chk1, Cdc2, phospho-Cdc2 (T-14/Y-15), cyclin-B1 and phospho-Cdc25C (S-216). Densitometric analysis of Chk2 and Chk1, and phosphor-Cdc2 (T-14/Y-15) and phospho-Cdc25C (S-216) band density is shown. Columns: mean of quadruplicate experiments; bars: SE; * $p < 0.001$, significant difference from mock; ** $p < 0.001$, significant difference from radiation.

Figure 4. Ad-MMP-2-Si infection decreases radiation-induced FoxM1 expression

A549 cells were infected with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si, incubated for 24h, irradiated with 10Gy x-ray, and incubated for another 12h.

(A) Total cellular extracts were subjected to western blotting with antibodies for FoxM1 and XRCC1, a DNA repair gene. For detection of phospho-FoxM1, total cellular extracts were immunoprecipitated with FoxM1 antibody and immunocomplex was subjected to western blotting with antibodies for MPM-2, which recognizes the phosphorylated protein sequence phosphoserine/phosphothreonine-proline. Densitometric analysis of FoxM1, phospho-FoxM1 and XRCC1 band density are shown in columns. Columns: mean of triplicate experiments; bars: SE; * $p < 0.001$, significant difference from radiation.

(B) A549 cells were transfected with FoxM1 siRNA prior to infection with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si. After 24h of infection, cells were irradiated with 10Gy X-ray and incubated for another 12h. Total cellular extracts were subjected to western blotting with antibodies for FoxM1, p53, p21^{Cip1/Waf1}, Chk2, Chk1 phospho- γ -H2AX and XRCC1.

(C) A549 cells were incubated with recombinant human MMP-2 protein (50 and 100 ng/mL) after 24h of transfection with mock or 50 MOI of either Ad-SV or Ad-MMP-2-Si. Total cellular extracts were subjected to western blotting with FoxM1 antibody. GAPDH was used as a loading control. Densitometric analyses of FoxM1 band densities are shown in columns. *Columns*: mean of triplicate experiments; *bars*: SE; * $p < 0.001$, significant difference from mock. ** $p < 0.05$, significant difference from respective rhMMP-2.

Figure 5. MMP-2 inhibition enhances radiosensitivity *in vivo*

(A) Graph showing the mean tumor volumes of A549 lung adenocarcinoma xenografts from mice that received treatment with mock, radiation (IR), IR+Ad-SV, Ad-MMP-2-Si, or IR+Ad-MMP-2-Si. (B) Representative sections (600X magnification) from mock, IR-, IR+Ad-SV-treated tumors as well as those treated with IR+Ad-MMP-2-Si. H&E staining of A549 lung adenocarcinoma xenograft sections (600X) (upper panel). Immunohistochemical analysis of MMP-2 and FoxM1 expression are shown in the middle panels. Lower panel indicates TUNEL staining of tissue sections. Also shown is the negative control where the primary antibody was replaced by non-immune serum (Insert).

(C) Quantitation of apoptotic cells indicated an increase in number of TUNEL-positive cells. *Columns*: mean of triplicate experiments; *bars*: SE; of 8–10 sections from five animals in the same treatment group. * $p < 0.05$ and ** $p < 0.001$, significant difference from mock.

Figure 6. Schematic representation of the effect of MMP-2 inhibition on radiation-induced DNA damage response

Following DNA damage by radiation, FoxM1 stimulation leads to a classical stress response and cell cycle arrest via Chk2 and Cdc25C. MMP-2 inhibition inhibits FoxM1 expression, resulting in abrogation of the G2/M transition.