Restoration of Transforming Growth Factor-β Signaling Enhances Radiosensitivity by Altering the Bcl-2/Bax Ratio in the p53 Mutant Pancreatic Cancer Cell Line MIA PaCa-2*

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In this study, we investigated whether lack of transforming growth factor β (TGF-β) type II receptor (RII) expression and loss of TGF-β signaling played a role in radiation resistance of pancreatic cancer cells MIA PaCa-2 that possess a mutated p53 gene. Transfection of this cell line with a RII cDNA led to a stimulation of the transcriptional activity of p3TP-Lux, a TGF-β-responsive reporter construct. The RII transfectants (MIA PaCa-2/RII) showed a significant increase in sensitivity to radiation when compared with MIA PaCa-2/vector cells. The increase in sensitivity to radiation was reversed by neutralizing antibodies to TGF-β, indicating that these changes were dependent on TGF-β signaling. Compared with MIA PaCa-2/vector cells, MIA PaCa-2/RII cells showed a greater than 3-fold increase in apoptosis after radiation. Enhanced radiation sensitivity of MIA PaCa-2/RII cells was associated with an induction of Bax mRNA and protein that was followed by a release of cytochrome c and activation of caspase-3 and poly(ADP-ribose) polymerase cleavage after radiation exposure. Overexpression of Bcl-xL or treatment with antisense oligodeoxynucleotides targeted against Bax significantly inhibited radiation-induced apoptosis in MIA PaCa-2/RII but not in MIA PaCa-2/vector cells, suggesting that Bax induction is necessary for radiation-induced TGF-β signaling-mediated apoptosis. Thus, restoration of TGF-β signaling sensitized these cells to ionizing radiation, although these cells possess a mutated p53 gene. In addition, disruption of RII function by dominant negative mutant of RII inhibited the radiation-induced TGF-β signaling and apoptosis in primary cultures of mouse embryonic fibroblasts. Together, these observations imply that RII is an important component of radiation-induced TGF-β signaling, and loss of function of RII may enhance resistance to radiation-induced apoptosis.

Cellular proliferation is a complex process involving both stimulatory and inhibitory signals. Abnormal proliferation observed in cancer cells is caused by mutations that either increase positive growth signals or decrease negative growth control signals or do a combination of both. Molecular analysis reveals that greater than 90% of pancreatic carcinomas harbor point mutations of the e-Ki-ras gene (1). The p16 gene, which encodes a cyclin-dependent kinase inhibitor is reported to be mutated in greater than 80% of pancreatic cancers, and ~75% of pancreatic carcinomas also possess mutations of the p53 gene (2). The DPC4 gene, a putative tumor suppressor gene, is reported to be homozygously deleted in about 30% of pancreatic cancers and to be inactivated by intragenic mutations in another 20% of these cancers (3). Previous studies from our laboratory (4, 5) indicate that another common feature of pancreatic cancer cells is a lack of negative growth response to TGF-β. Lack of response to TGF-β can be caused by mutations of its receptors (6), epigenetic changes that down-regulate receptor expression, or mutations affecting downstream targets of TGF-β including DPC4. Thus, pancreatic cancer is a molecularly complex disease. The role that the lack of TGF-β signaling plays in the resistance to therapy in pancreatic cancer is not well understood.

TGF-β isoforms (TGF-β1, TGF-β2, and TGF-β3) are 25-kDa homodimer polypeptides that regulate cell proliferation and differentiation (7–9). TGF-β molecules are potent inhibitors of growth in a variety of cells including epithelial, endothelial, and lymphoid cells (10). TGF-β is expressed in growth-arrested cells and in the G1 phase of the cell cycle and contributes to an orderly progression through the cell cycle (11). TGF-β signals by binding to transmembrane serine-threonine kinases termed type I receptor (RI) and type II receptor (RII) (12, 13). Genetic evidence shows that both receptors are required for TGF-β signaling (13). A third receptor, type III (RIII), is not believed to be involved directly in TGF-β signaling but acts to present TGF-βs to RII (13).

The TGF-β ligand binds first to the RII, and this receptor-ligand complex recruits and homodimerizes with RI. Subsequently, RI is activated by the type II receptor kinase that phosphorylates the glycine-serine-rich domain of RI. The activated type I receptor kinase then phosphorylates Smad2 or Smad3. Upon phosphorylation, a Smad2 or Smad3 homotrimer associates with the Smad4 (DPC4) homotrimer to form a heteroexamer that translocates to the nucleus (14). The Smad

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1 The abbreviations used are: TGF, transforming growth factor; RI, RII, and RIII, TGF-β type I, II, and III receptor, respectively; MEF, mouse embryonic fibroblast; TUNEL, terminal transferase-mediated dUTP-digoxigenin-end labeling; ELISA, enzyme-linked immunosorbent assay; PARP, poly(ADP-ribose) polymerase; AS, antisense; NS, nonsense.

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complex then associates with other co-factors including Fast-1, p300/CBP, and AP-1, forming a transcriptional active complex (14–17).

Loss of growth-inhibitory response to TGF-β due to a lack of RII expression has been reported in a variety of carcinoma cells (18–20). Further studies demonstrated that genetic alterations and/or down-regulation of TGF-β receptors play a critical role in altering responsiveness to TGF-β in cancer cells and indicate that RII is a tumor suppressor gene (6, 21). The mutations in simple repeat sequences of RII are caused by microsatellite instability in hereditary nonpolyposis colon cancer (22) and in gastric cancers (23, 24). However, in contrast to hereditary nonpolyposis colon cancer, we did not find a significant number of mutations of the RII gene in pancreatic adenocarcinoma (25). Although microsatellite instability-directed mutations appear rare in the RII gene of pancreatic cancers (25), down-regulation of TGF-β receptors could be caused by epigenetic alterations. We found that all three receptors are expressed in most non-tumor pancreas tissues (5). RII was absent or expressed in low levels in more than half of the pancreatic cancer cell lines and tumor tissues analyzed (5). Therefore, lack of expression of RII may be responsible for a loss in response to negative growth regulation by TGF-β in pancreatic cancer cells that express the wild-type DPC4 gene.

One of the molecular determinants regulating the response to ionizing radiation is the tumor suppressor protein p53, which serves as a pivotal component of the apoptosis pathway(s) in diverse cell types (26, 27). Radiation causes an accumulation of p53 protein that initiates apoptosis (31–34). p53 is mutated and non-functional in the majority of pancreas tumors (1, 35, 36). Mutation of p53 is recognized as an important factor in poor prognosis of this disease and plays a role in the resistance of cancers to radiation and chemotherapy (1, 35, 36). Thus, the identification of p53-independent pathways that cause radiosensitization may result in a novel approach for therapy for radiation-resistant tumors. Similar to p53, TGF-β regulates cell growth and mediates growth control through the induction of cyclin-dependent kinase inhibitors (14, 37).

The human pancreatic cancer cell line MIA PaCa-2 is highly tumorigenic and lacks RII expression (4), but it expresses the other two TGF-β receptors, RI and RIII (5). These cells also express DPC4 (5, 38) and are reported to lack mutations of the DPC4 gene (39). In this study, we determined whether TGF-β signaling played a role in the radiation resistance of MIA PaCa-2 cells that possess a mutated p53 gene. The results shown here demonstrate that restoration of TGF-β signaling by RII expression led to radiosensitization in the p53 mutant pancreatic cancer cell line MIA PaCa-2.

MATERIALS AND METHODS

Cell Culture, Transfections, and Selection of Clones—The pancreatic cancer cell line MIA PaCa-2 was obtained from the American Type Culture Collection (ATCC; Manassas, VA). MIA PaCa-2 is a hypotriplid cell line established from a 65-year-old Caucasian male (40). We established limiting dilution clones from this cell line. We previously showed that one of these clones lacks expression of RII (4). This limiting dilution clone was used in the present study. The nontransformed human epithelial cell line, Ha578N, obtained from ATCC was used as a normal control for mRNA expression of the TGF-β RII gene (5). Primary cultures of mouse embryonic fibroblast (MEF) cells from normal mice at passage three (kindly provided by Dr. Tyler Jacks, Howard Hughes Medical Institute) were grown in Dulbecco’s modified Eagle medium supplemented with 1% glutamine, 10% fetal bovine serum, and 1% penicillin/streptomycin at 37 °C and 5% CO2.

Transfection and selection of stable RII transfectants were done as previously described (21). The RII cDNA was cloned into a pRClCMV expression vector. The plasmid (10 μg) was linearized with SacI and transfected by electroporation into the limiting dilution clone from MIA PaCa-2 cells. Electroporation was carried out at 250 V, 960 microfarads with a gene pulsar machine (Bio-Rad). Parental MIA PaCa-2 cells were similarly transfected with an empty vector pcRClCMV (10 μg). Cells were then selected based on neomycin resistance, and individual colonies were ring-cloned.

Plasmid ΔRII, which functions as a dominant negative mutant for RII, contains serine kinase COOH terminus-defective RII cDNA cloned in a tetracycline-controllable expression system (41). Transient transfection in primary MEF cells using ΔRII or ΔRII plus p3TP-LUC reported was performed as described previously (42). Full-length human RII cDNA was transfected in MIA PaCa-2 transfectants using the calcium phosphate precipitation method.

mRNA Expression Analysis of RII in MIA PaCa-2 Cells Stably Transfected with RII cDNA—The clonally transfected were screened for RII mRNA expression by reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was extracted from cultured cells using TRIZOL reagent (Invitrogen) according to the manufacturer’s protocol. RT-PCR was performed using a commercially available kit (PerkinElmer Life Sciences) following the protocol given by the manufacturer. The specific primers used to amplify regions of the RII gene are described by us elsewhere (5). Total mRNA from a normal epithelial cell line, Ha578N, was used as a positive control for RII gene expression. Amplification of the β-actin gene using specific primers (44) was used as an internal control and to normalize expression levels. The PCR products were run on 2% agarose gels in TBE stained with ethidium bromide.

Transient Transfection and Luciferase Assay—To determine whether RII expression restored TGF-β signaling, we measured TGF-β-responsive promoter activity using the p3TP-Lux assay (12). Cells were transiently co-transfected with the p3TP-Lux reporter construct and Renilla luciferase reporter construct by LipofectAMINE Plus (Invitrogen). Twenty-four hours later, the cells were treated with recombinant TGF-β or irradiated at 5 Gy. After 24 h of incubation, the cells were lysed in reporter lysis buffer (Promega) using one freeze-thaw cycle. The levels of Firefly and Renilla luciferases in the cell extract were assayed using a dual luciferase reporter assay system (Promega). TGF-β-responsive promoter activity was expressed as a ratio of firefly luciferase activity and Renilla luciferase activity.

Irradiation—A 100-kV industrial x-ray machine (Phillips) was used to irradiate the cultures at room temperature. The dose rate with a 2-mm aluminum plus 1-mm beryllium filter was ~1.85 Gy/min at a focus-surface distance of 30 cm (44).

Quantiﬁcation of Apoptosis—To quantitate apoptosis, the ApoTag in situ apoptosis detection kit (Oncor) was used as described (45). The ApoTag kit detects the DNA strand breaks in single cells by terminal transferase-mediated dUTP-digoxigenin end labeling (TUNEL). To determine the response of MIA PaCa-2 transfectants to TGF-β–induced apoptosis, cells were grown in Dulbecco’s modiﬁed Eagle’s medium supplemented with 10% fetal bovine serum. They were made quiescent by serum deprivation for 3 days and then exposed to exogenous TGF-β1 (10 ng/ml) or 500 ng/ml anti-TGF-β-neutralizing antibody, AP-100-NA (R & D Systems), for 24 h. After 24 h, the DNA was tagged with digoxigenin-dUTP and conjugated with an anti-digoxigenin ﬂuorescein. The cells were counterstained with propidium iodide and antifade. For a negative stained control, the cells were incubated with phosphate-buffered saline instead of terminal transferase. The stained specimen was observed in a triple band pass filter using a Nikon-MicrophotFX epifluorescence microscope. To determine the percentage of cells showing apoptosis, 2000 cells were counted. Mean values were calculated from four replicates.

To determine radiation-induced apoptosis, MIA PaCa-2 transfectants or transient ΔRII MEF transfectants were seeded in chamber slides and were exposed to a 5-Gy dose of radiation. Cell death was measured after 24 h by TUNEL staining as described above. Mean values were calculated from four replicates.

For flow cytometry, cells were lifted by using nonenzymatic cell dissociation medium (Sigma), washed with phosphate-buffered saline, and fixed in 70% ethanol. After centrifugation, cells were resuspended in 5 μg/ml RNase and stained in 10 μg/ml propidium iodide and analyzed by flow cytometry using a FACScan Plus™ cell sorter. Each histogram was constructed with data from at least 10,000 events. Data from three independent experiments were analyzed to calculate the percentage of sub-G0 population (apoptosis) using the CellQuest software (Becton Dickinson).

Enzyme-linked Immunosorbent Assay (ELISA) for Detection of Activated TGF-β Protein—The levels of radiation-induced TGF-β1 protein secreted in the culture supernatants of untreated and irradiated cells

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from MIA PaCa-2 transfectants were measured by ELISA. Microtiter
ELISA plates were coated with anti-human TGF-β1 purified mono-
clonal antibody (R & D Systems) (capturing antibody). Then 100 μl of
the culture medium supernatants were added to the plates and incu-
bated for 2 h at room temperature. The unbound materials were
washed away, and the plates were incubated with biotin-labeled anti-human
TGF-β1 monoclonal (R & D Systems) secondary antibody for 1 h at
room temperature. After washing, the complex was conjugated with
streptavidin peroxidase for 30 min at room temperature. 3,3',5,5'-Tet-
ramethylbenzidine substrate solution was added for color reaction, and
the optical density was measured at 450 nm using an ELISA plate
reader.

Colonies-forming assay—For clonogenic cell survival studies, two dif-
ferent cell concentrations in quadruplicate sets were used for each ra-
diation dose. MIA PaCa-2-Vector, MIA PaCa-2-Vector.P2, MIA PaCa-2/RII,
and MIA PaCa-2/RII.c9 cells were left untreated or exposed to a 1–5-Gy
dose of radiation. To block TGF-β1 signaling, a neutralizing antibody to
TGF-β1, anti-TGF-β1 antibody (AF-101-NA) (R & D Systems), was
added (500 ng/ml of medium) to the cultures prior to irradiation. For
control experiments, chicken immunoglobulin G (R & D Systems) was
added before irradiation. After incubation for 10 or more days, each dish
was stained with crystal violet, and the colonies containing more than
50 cells were counted. The surviving fraction (SF) was calculated as a
ratio of the number of colonies formed to the product of the number of
cells plated and the plating efficiency (44). The curve was plotted using
x-y log scatter (Delta Graph® 4.0) and fitted by a single-hit multitarget
model to obtain D50, n, and SF0 values. D50 is the dose required to reduce
the fraction of cells to 37%, indicative of single-event killing. The n
value of the curve is a measure of the width of the shoulder, indicative
of multiple event killing. SF0 is the survival fraction of exponentially
growing cells that were irradiated at the clinically relevant dose of 2 Gy.

Preparation of Cytosol and Mitochondrial Extract from MIA PaCa-2
Transfectants and Analysis of Cytochrome c Release—The mitochon-
drial fractions from MIA PaCa-2 transfectants were prepared as per
the protocol described previously (46, 47). Untreated and irradiated MIA
PaCa-2 transfectants (5 × 106/ml) were harvested by centrifuging at
1800 × g for 10 min at 4 °C. Pellet was washed twice with cold phos-
phate-buffered saline and resuspended in 100 μl of extraction buffer
(220 mM mannitol, 68 mM sucrose, 50 mM HEPES-KOH, pH 7.4, 50 mM
KCl, 2 mM MgCl2, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfo-
nyl fluoride) and incubated on ice for 15 min. The cells were broken by
passing 15 times through a G23 needle. The lysed cells were centrifuged
at 4000 rpm for 4 min. The supernatant was collected and centrifuged
at 50,000 rpm using an ultracentrifuge (Beckman). The resulting su-
pernatant was used as a cytosolic extract, and the pellet was used as
the mitochondrial fraction for cytochrome c expression analysis. The cyto-
sonic extract and mitochondrial fraction was subjected to Western blot
analysis using anti-cytochrome c monoclonal antibody (Biovision Inc.,
Palo Alto, CA).

Western Blot Analysis—Total proteins were extracted from un-
treated, irradiated, recombinant TGF-β1-exposed, anti-TGF-β1-neu-
tralizing antibody-exposed, or irradiated plus recombinant anti-
TGF-β1-neutralizing antibody-exposed cultures at various time
points after the treatments using Laemmli buffer. Total proteins were
electrophoresed in SDS-PAGE and transferred to membrane. After
blocking by milk, the membrane was incubated in a milk solution
containing anti-p21(1/2/3) monoclonal antibody, sc-817 or anti-Bcl-2
monoclonal antibody, sc-509 or anti-Bax monoclonal antibody, sc-7490
(Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-caspase-3
monoclonal antibody 2004, or anti-PARP monoclonal antibody 3001
(BioVision, Palo Alto, CA). The bound antigen-antibody complex was
detected by secondary antibody (either monoclonal or polyclonal,
depending upon primary antibody) and the chemiluminescence kit.
The same membrane was used for β-actin levels detected by anti-β-actin
antibody (Sigma) as an internal loading control.

32P-Reverse Transcriptase-Polymerase Chain Reaction (32P-RT-PCR)
of Bax mRNA Expression—Total RNA was isolated from untreated and
irradiated MIA PaCa-2-Vector and MIA PaCa-2/RII transfectant cells at
various time intervals using TRIZOL reagent (Invitrogen). One micro-
gram of total RNA was reverse transcribed into cDNA using oligo(dT)
primers and reverse transcriptase in a 40 μl reaction mix as described
previously (45). Radiation-induced Bax mRNA expression was analyzed
by PCR using the products of reverse transcription reaction and the
upstream and downstream primers flanking the human Bax gene (48).
The human glycerinaldehyde-3-phosphate dehydrogenase gene (49) was
used as an internal control.

Antisense Oligomer-mediated Inhibition of Bax and Effect on Re-
sponse to Ionizing Radiation—Two phosphodiester backbone Bax anti-
sense (AS) oligonucleotides and one nonsense (NS) oligonucleotide de-
scribed previously (50) were used in the experiments aimed at blocking
Bax expression. The AS oligomers or NS oligomer were incubated
with transferrin-polylysine conjugate for 1 h at 37 °C and added to the
cells cultures at a concentration of 10 μM (5 μM each of the two AS oligo-
er or 10 μM NS oligomer). Every 24 h, 5 μM oligomers (2.5 μM each of
the two AS oligomers or 5 μM NS oligomer), 50% of the initial concen-
tration, was added to compensate for degradation and also to maintain 10 μM
final concentration. After 5 days of oligomer treatment, the medium
was replaced with fresh medium containing 10 μM Bax AS or NS
oligomers and left untreated or irradiated at a 5-Gy dose. After 24 and
48 h, apoptosis was determined by TUNEL assays.

Immunoprecipitation—Cells were lysed with triple detergent lysis
buffer (50 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1%
SDS, 100 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1% Nonidet P-40, 0.5% sodium deoxycholate), and 100 μg of lysates were
incubated with antibodies (either monoclonal anti Bcl-xL antibody sc-
18392 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or rabbit poly-
clonal anti-Bax sc-493 antibody (Santa Cruz Biotechnology)) as indi-
cated. The antibody complexes were isolated using protein A/G-agarose
beads (Santa Cruz Biotechnology), washed three times with phosphate-
buffered saline. The immunoprecipitated proteins with beads were
boiled in SDS sample buffer, and the supernatants were analyzed on
SDS-PAGE and subjected to Western blot analysis using anti-Bcl-xL
antibody or anti-Bax antibody.

RESULTS

Transfection of MIA PaCa-2 Cells with RII cDNA Restores
TGF-β Signaling—MIA PaCa-2 cells lack RII expression, but
these cells express the other two TGF-β receptors (RI and RIII)
and DPC4 (4, 5). Thus, with the exception of RII expression, MIA
PaCa-2 cells appear to have the necessary repertoire of mole-
cules for TGF-β signaling. Therefore, we determined whether transfection of MIA PaCa-2 cells with RII cDNA might restore TGF-β signaling in these cells. To accomplish this, MIA
PaCa-2 cells were transfected with an expression vector con-
taining the complete RII cDNA sequence. Parental MIA
PaCa-2 cells were similarly transfected with an empty vector
lacking the RII cDNA. Neomycin-resistant cells were ring
cloned and then grown in an expansion culture and analyzed
for RII mRNA expression. RT-PCR was used as a screening
method to detect RII mRNA. Has578N, a nontransformed epi-
thelial cell line, was used as a control for RII mRNA expres-
sion (Fig. 1, lane 1). We previously showed that this cell line express-
ses RII (5). Three of the transfected clones tested showed
appreciable levels of RII mRNA as detected by RT-PCR (Fig. 1,
lanes 2, 4, and 6), and all three clones showed restoration of
TGF-β signaling as determined by p3TP-Lux assay (data shown
for only two clones). Vector transfected (MIA PaCa-2/ vector and
MIA PaCa-2/vector.P2) and the parental MIA
PaCa-2 cells showed a low basal p3TP-Lux activity, whereas
RII transfectant (MIA PaCa-2/RII and MIA PaCa-2/RII.C9)
cells showed a modest basal p3TP-Lux activity. The addition of exogenous TGF-β1 caused a significant increase of p3TP-Lux activity in RII transfectant cells compared with vector transfectant cells (p < 0.00014) (Fig. 2). This finding suggests that TGF-β signaling is restored in MIA PaCa-2/RII cells that express the RII cDNA.

Restoration of RII Receptor Enhances Recombinant TGF-β-induced Apoptosis in RII Transfectant Cells—We next determined whether restoration of RII expression and thus the TGF-β signaling had any effect on apoptosis in serum-starved RII transfectant cells, compared with vector transfectant cells, as determined by TUNEL staining. Upon serum deprivation, MIA PaCa-2/RII and RII.C9 clones showed a higher percentage of cell death than did vector transfectant cells (Fig. 3). The increase in apoptosis observed in serum-deprived MIA PaCa-2/RII cells was blocked when anti-TGF-β-neutralizing antibody was added to the medium (Fig. 3). Therefore, the enhanced cell death response to serum deprivation in RII transfectants is a result of restoration of a functional TGF-β signaling pathway.

Radiation Induces Activated TGF-β1 Protein in Vector and RII Transfectant Cells—The role of TGF-β signaling in radiosensitization is not well understood. Studies suggest that TGF-β signaling may increase sensitivity to radiation (51, 52). Treatment of cells with radiation has been reported to induce TGF-β1 expression (51–53). We determined whether TGF-β1 protein was inducible by ionizing radiation in vector and RII transfectants. Confluent cultures of vector and RII transfectants were left unexposed or irradiated at 5 Gy. Twenty-four hours later, activated TGF-β1 protein levels were determined from the medium of these cells by ELISA. RII transfectant cells showed a greater basal level of activated TGF-β1 protein in their cell culture medium than did vector transfectant cells (Fig. 4). Vector transfectant cells showed a significant induction of TGF-β1 protein in cell culture supernatants (p < 0.00001) after radiation (Fig. 4). For RII transfectant cells, the increase of activated TGF-β1 released in culture supernatant was 1.4-fold after radiation (Fig. 4). These results suggest that radiation induces TGF-β protein in both vector and RII transfectant cells. High basal levels of TGF-β in RII transfectants could be due to a positive feedback mechanism resulting from the restoration of the TGF-β signaling pathway.

Radiation Induces p21waf1/cip1 in MIA PaCa-2/RII Cells—One way to determine whether radiation-induced expression of TGF-β results in an increase in TGF-β signaling is to measure the expression of TGF-β target genes. p21waf1/cip1 has been thoroughly studied and is inducible by p53 (54). It is also known that TGF-β signaling through a Smad signaling complex activates the transcription of target genes such as...
data suggest that the lack of up-regulation of p21\textsuperscript{waf1/cip1} used to detect p21 Western blots were transfectant cells. Cells were left untreated (UT), treated with recombinant TGF-\(\beta\) neutralizing antibody (500 ng/ml). The blots were probed with an antibody for p21\textsuperscript{waf1/cip1} (sc-817) or \(\beta\)-actin.

**Fig. 5.** Radiation or recombinant TGF-\(\beta\) induces p21\textsuperscript{waf1/cip1} in RII transfectant cells. Western blots were used to detect p21\textsuperscript{waf1/cip1} in lysates of MIA PaCa-2 transfectants. Cells were left untreated (UT), treated with recombinant TGF-\(\beta\) (10 ng/ml), or exposed to a 5-Gy dose of radiation. TGF-\(\beta\) signaling was blocked in irradiated cells by anti-TGF-\(\beta\)/H9252 neutralizing antibody (500 ng/ml). The blots were probed with an antibody for p21\textsuperscript{waf1/cip1} (sc-817) or \(\beta\)-actin.

**Fig. 6.** Increased sensitivity of MIA PaCa-2/RII transfectant cells to irradiation. Cell survival curve of MIA PaCa-2 transfectants following irradiation as assayed by colony-forming ability and analyzed by single-hit multitarget model curve fit. Error bars represent the S.E. of two separate experiments.

p21\textsuperscript{waf1/cip1} and other cyclin-dependent kinase inhibitors, which are key effectors for TGF-\(\beta\)–mediated growth inhibition (14). MIA PaCa-2 cells contain one allele of p53 that is mutated at codon 248 (55). Western blot analysis showed a basal level of p21\textsuperscript{waf1/cip1} in both vector and RII transfectant cells (Fig. 5). p21\textsuperscript{waf1/cip1} was up-regulated in 6 h after radiation in RII transfectant cells but not in vector transfectant control cells (Fig. 5). In addition, recombinant TGF-\(\beta\) induced p21\textsuperscript{waf1/cip1} protein in RII transfectant cells but not in vector transfectant cells (Fig. 5). However, when these transfectants were exposed to anti-TGF-\(\beta\)-neutralizing antibody, radiation failed to up-regulate p21\textsuperscript{waf1/cip1} in RII transfectant cells (Fig. 5). These data suggest that the lack of up-regulation of p21\textsuperscript{waf1/cip1} protein in MIA PaCa-2/vector transfectants may be due to dysregulation in both the p53 and TGF-\(\beta\) pathways. However, in MIA PaCa-2/RII transfectant clones, the induction of TGF-\(\beta\) by radiation and the restoration of TGF-\(\beta\) signaling likely cause the up-regulation of p21\textsuperscript{waf1/cip1}, even in the absence of functional p53.

**Radiation Causes Enhanced Clonogenic Inhibition and Apoptosis in MIA PaCa-2/RII Cells**—The role of TGF-\(\beta\) signaling in restoring radiation sensitivity of cancer cells has not been well studied. One mechanism responsible for radiation sensitivity is the induction of apoptosis. To determine whether TGF-\(\beta\) signaling caused radiation-inducible clonogenic inhibition and apoptosis, we irradiated two clones of vector-transfected MIA PaCa-2 (MIA PaCa-2/Vector and MIA PaCa-2/Vector.P2) and two clones of MIA PaCa-2 that were transfected with RII cDNA (MIA PaCa-2/RII, and MIA PaCa-2/RII.C9). Table I shows the SF\(_2\) values for the SF\(_2\) survival fraction at 2 Gy. \(D_0\) dose required to reduce the fraction of cells to 37%; \(n\), a measure of the width of the shoulder, indicative of multiple-event killing; SHMT, single-hit multitarget.

| Radiation formalism | Cell lines |
|---------------------|------------|
| MIA PaCa-2/Vector | MIA PaCa-2/P2 | MIA PaCa-2/RII | MIA PaCa-2/RII.C9 |
| SF\(_2\) | 0.516 ± 0.01 | 0.582 ± 0.034 | 0.268 ± 0.101 | 0.258 ± 0.03 |
| \(n\) | 1.1 | 1.2 | 1.0001 | 1.3 |
| \(D_0\) | 293 cGy | 275 cGy | 165 cGy | 150 cGy |

Thus, compared with MIA PaCa-2/vector, the transfectants with the RII gene were significantly more sensitive (\(p < 0.0001\)) to ionizing radiation. Consistent with the observation on clonogenic inhibition, TUNEL analysis indicated that RII transfectant cells were more sensitive (\(p < 0.001\)) to ionizing radiation-induced apoptosis than were vector transfectant control cells (Fig. 7A). By flow cytometry assay using propidium iodide staining, the incidence of apoptosis after 24 and 48 h of radiation was 0.34 and 5.3%, respectively, in MIA PaCa-2/vector cells and 4.42 and 18.7%, respectively, in MIA PaCa-2/RII cells.
Similarly, for the other clones, the incidence of apoptosis after 24 and 48 h of radiation was 0.7 and 3.84%, respectively, in MIA PaCa-2/Vector.P2 cells and 4.42 and 19.78%, respectively, in MIA PaCa-2/RII.C9 cells (Fig. 7C). This study indicates that radiation induces TGF-β protein in both vector and RII transfectant cells. However, an increase
in cell death and inhibition of clonogenic growth was found only in MIA RII transfectant cells. To ascertain whether radiation-induced TGF-β protein is responsible for the increased sensitivity to radiation in RII transfectants, we performed clonogenic assays at a 2-Gy dose of radiation with and without neutralizing antibody to TGF-β. The SF₂ values of these treatments are shown in Table II. No changes in the values of SF₂ were observed in MIA PaCa-2/vector cells treated with or without neutralizing anti-TGF-β antibody. However, the clonogenic inhibition of MIA PaCa-2/RII and MIA PaCa-2/RII.C9 cells was decreased when neutralizing anti-TGF-β antibody was added to the medium (Table II). Together, these findings suggest that the increase in sensitivity to radiation in RII transfectant clones is mediated by the restoration of TGF-β signaling.

**Radiation Causes an Increase in Bax mRNA and Protein Expression in MIA PaCa-2/RII Cells**—TGF-β signaling has been implicated in the induction of apoptosis through a variety of mechanisms including the activation of caspases, the down-regulation of Bcl-2 and related family members, and the induction of Bax (56). We determined whether these factors might play a role in enhancing radiation-induced apoptosis of MIA PaCa-2/RII transfectants. 32P-RT-PCR analyses showed that radiation caused a significant induction of Bax mRNA in MIA PaCa-2/RII transfectants but not in vector transfectant cells. To further ascertain that radiation-induced TGF-β signaling caused the induction of Bax mRNA, we performed 32P-RT-PCR analyses of RNA samples from both vector- and RII-transfected clones, which were irradiated and exposed to neutralizing anti-TGF-β antibody. In MIA PaCa-2/RII and MIA PaCa-2/RII.C9 cells, exposure to neutralizing anti-TGF-β antibody partially inhibited radiation-induced Bax mRNA up-regulation. However, in the control MIA PaCa-2/Vector and MIA PaCa-2/Vector.P2 cells, combined treatment did not significantly change the levels of Bax mRNA (Fig. 8). Similarly, Western blot analysis using the lysates obtained from both vector- and RII-transfected cells, radiation caused no release of cytochrome c in the cytosol and was accompanied by elevation of inactive forms of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused no release of cytochrome c in the cytosol and was accompanied by elevation of inactive forms of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused an increase in the level of Bax protein, further characterized the downstream events of cell death by analyzing the activated form of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused no release of cytochrome c in the cytosol and was accompanied by elevation of inactive forms of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused an increased cytochrome c release in the cytosol with the activation of caspase-3 by reducing the inactive forms of caspase-3 and was associated with enhanced cleavage of 116-kDa PARP (Fig. 10, A and B). These observations suggest that TGF-β signaling is a positive effector of radiation-induced apoptosis through Bax, Cytochrome c release, and caspase activation.

**Antisense Bax or Overexpression Bcl-xL Blocked Radiation-induced Apoptosis in RII Transfectants**—Induction of Bax by ionizing radiation in RII transfectants was quite significant. To gain some insight into the role of Bax induction in radiation-induced TGF-β signaling-mediated apoptosis, we inhibited the

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**TABLE II**

| Treatments                          | Cell lines | Radiation  | Radiation + anti-TGF-β antibody |
|-------------------------------------|------------|------------|---------------------------------|
|                                     | MIA PaCa-2/Vector SF₂ | MIA PaCa-2/RII SF₂ | MIA PaCa-2/RII.C9 SF₂ |
| Radiation                           | 0.510 ± 0.081 | 0.271 ± 0.012 | 0.243 ± 0.0102 |
| Radiation + anti-TGF-β1 antibody    | 0.479 ± 0.098 | 0.367 ± 0.052 | 0.393 ± 0.02 |

*SF₂, surviving fraction at 2 Gy.*

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**FIG. 8.** 32P-RT-PCR analysis of Bax mRNA induction in vector and RII transfectants by radiation and radiation plus anti-TGF-β-neutralizing antibody. Total mRNA was isolated from MIA PaCa-2 transfectants that were left untreated (UT), exposed to a 5-Gy dose, or exposed to 500 ng/ml anti-TGF-β-neutralizing antibody plus a 5-Gy dose and incubated for the time interval indicated. Bax or glyceraldehyde-3-phosphate dehydrogenase (as an internal control) was amplified using specific radiolabeled primers, electrophoresed in PAGE, and autoradiographed.

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**Radiation Causes Release of Cytochrome c and Caspase-3 and Cleavage of Caspase-3 and PARP in MIA PaCa-2/RII Cells**—The up-regulation of Bax is believed to effect mitochondria permeability, favoring the release of cytochrome c, leading to caspase-9 activation, which subsequently induces caspase-3 activation (57). Since irradiated MIA PaCa-2/RII cells showed an increase in the level of Bax protein, we further characterized the downstream events of cell death by analyzing the activated form of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused no release of cytochrome c in the cytosol and was accompanied by elevation of inactive forms of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused no release of cytochrome c in the cytosol and was accompanied by elevation of inactive forms of caspase-3 and PARP cleavage. In MIA PaCa-2/Vector-transfected cells, radiation caused an increased cytochrome c release in the cytosol with the activation of caspase-3 by reducing the inactive forms of caspase-3 and was associated with enhanced cleavage of 116-kDa PARP (Fig. 10, A and B). These observations suggest that TGF-β signaling is a positive effector of radiation-induced apoptosis through Bax, Cytochrome c release, and caspase activation.
function of this gene using antisense oligodeoxynucleotides and Bcl-xL overexpression and analyzed the cell death response. The Bax antisense oligodeoxynucleotides efficiently inhibited radiation-induced Bax protein expression but not the nonsense oligodeoxynucleotides in the RII transfectants (Fig. 11A). Inhibition of radiation-induced Bax protein expression significantly reduced the induction of apoptosis ($p < 0.001$ for 24 h and $p < 0.00034$ for 48 h) in RII transfectants, as demonstrated by TUNEL assay (Fig. 11B). These results suggest that Bax induction is necessary to mediate cell death response through radiation-induced TGF-β signaling.

The Bcl-2 related protein, Bcl-xL has been shown to be a stronger protector of apoptosis than Bcl-2 in certain circumstances. It was demonstrated previously that heterodimerization between Bcl-xL and Bax is essential for the antiapoptotic activity of Bcl-xL (58). In this part of the study, we overexpressed Bcl-xL in vector and RII transfectants and analyzed the cell death response to radiation. Immunoprecipitation studies showed intense bound forms of Bax and Bcl-xL in Bcl-xL-overexpressed RII transfectants when compared with Bcl-xL-overexpressed vector transfectants (Fig. 11C). TUNEL analysis showed reduced incidence of apoptosis in RII transfectants overexpressing Bcl-xL protein when compared with RII transfectants expressing the empty vector alone (Fig. 11D). These observations indicate that radiation-induced Bax-mediated cell death in RII transfectants can be inhibited by strong anti-apoptotic protein Bcl-xL.

Together, these results demonstrate that induction of Bax may be central to the apoptogenic effects of restored RII expression in cell lines lacking TGF-β signaling due to the absence of RII expression.

**DISCUSSION**

We previously found that the TGF-β type II receptor is down-regulated in a number of pancreatic cancer cells and tissues (5). A separate study by Goggins et al. (59) indicated that the RI and RII genes are selective targets of genetic inactivation in pancreatic and biliary cancers. The highly tumorigenic pancreatic cancer cell line MIA PaCa-2 does not respond to TGF-β and lacks expression of RII (4, 5). Expression of a functional RII was...
shown to restore TGF-β-mediated growth inhibition in a variety of cancers including breast cancer (18), prostate cancer (60), and colon cancer (21). The purpose of the present study was to determine whether restoration of the expression of a functional RII gene in MIA PaCa-2 would render these cells sensitive to irradiation.

Serum deprivation or radiation caused an increase in cell death of MIA PaCa-2/RII cells as compared with MIA PaCa-2/vector cells. This increase in apoptosis was blocked by neutralizing antibodies to TGF-β, indicating that TGF-β signaling was directly responsible for the increase in cell death during serum deprivation in MIA PaCa-2/RII cells. This is in agreement with a recent finding that the restoration of TGF-β signaling suppresses tumorigenicity of human prostate cancer cells by inducing apoptosis (61).

It is known that radiation induces TGF-β in various cell types (51, 53). Thus, it can be hypothesized that radiation-induced endogenous TGF-β will exert clonogenic inhibition and cause apoptosis in cells with intact TGF-β signaling. In this study, we found a significant increase in sensitivity to radiation of MIA PaCa-2/RII cells both by long term clonogenic inhibition assays and by a short term apoptosis assay. The increase in sensitivity to irradiation of MIA PaCa-2/RII was mediated by TGF-β signaling, since neutralizing anti-TGF-β antibodies caused these cells to increase their radiation resistance, similar to vector-transfected cells (Table II). Interestingly, disruption of RII function in normal MEF cells inhibited radiation-induced TGF-β signaling with increased resistance to apoptosis (Fig. 11). Together, these data support the concept that TGF-β signaling is a vital mediator of radiation-induced clonogenic inhibition and apoptosis.

The interaction between ionizing radiation and TGF-β was previously investigated in a panel of eight human colorectal cancer cell lines (51). These cell lines showed differences in p53 mutational status and sensitivity to TGF-β (51). The investigators found that radiation-induced modulation of TGF-β sensitivity was transitory, and it was postulated that radiation-induced sensitization to TGF-β occurs in TGF-β-sensitive cells expressing wild-type p53 (51). Our study, however, demonstrates that MIA PaCa-2/RII cells with restored TGF-β signaling are sensitive to radiation despite the fact that these cells harbor a mutated p53 gene. In this context, we also observed that radiation induced the expression of the TGF-β protein in the culture media of both MIA PaCa-2/vector and MIA PaCa-2/RII cells. However, the basal level of TGF-β was greater in the RII transfectants than in vector controls. It is possible that the restoration of TGF-β signaling causes a positive feedback loop, thus increasing the basal expression of TGF-β.

It is well known that p21wafl/cip1 is transactivated by p53 following DNA damage, leading to G1 cell cycle arrest (54, 62). It is also known that TGF-β signaling transactivates p21wafl/cip1 through the Smad pathway (37). A recent study indicated that DPC4 (Smad4) overexpression led to p21wafl/cip1 protein elevation via direct transactivation of the p21wafl/cip1 promoter (63). Radiation did not induce an up-regulation of p21wafl/cip1 protein in MIA PaCa-2/vector cells, probably because the p53 and TGF-β pathways are not functional. This is in agreement with a previous report that exogenous TGF-β failed to induce p21wafl/cip1 mRNA in MIA PaCa-2 cells (38). There was a low basal level of p21wafl/cip1 expression in MIA PaCa-2/vector cells. The expression could be independent of TGF-β signaling and p53 or reflect a low level of TGF-β signaling. However, restoration of TGF-β signaling in MIA PaCa-2/RII cells led to p21wafl/cip1 protein elevation after radiation or the addition of exogenous TGF-β, despite a nonfunctional p53 pathway. Interestingly, we were able to abrogate the radiation-induction of p21wafl/cip1 in MIA PaCa-2/RII cells when they were exposed to anti-TGF-β neutralizing antibody. These observations suggest that the induction of p21wafl/cip1 is caused through radiation-induced TGF-β signaling after the restoration of RII expression in MIA PaCa-2/RII cells. Thus,
FIG. 11. Inhibition of Bax expression in MIA PaCa-2 vector and RII transfectants. A, vector or RII transfectants were exposed to antisense Bax or nonsense Bax oligodeoxynucleotides and left untreated or irradiated at 5 Gy. Immunocytochemistry was performed to determine Bax expression using anti-Bax antibody. B, antisense Bax- or nonsense Bax-treated cells were exposed to a 5-Gy dose of radiation, and TUNEL was performed after 24 or 48 h of exposure. The bar graph shows the percentage of TUNEL-positive cells. Data represent a mean of two experiments. Error bars represent the S.D. C, Bcl-xL overexpression in MIA PaCa-2 transfectants. Cell lysates from untreated or irradiated MIA PaCa-2 transfectants with or without overexpression of Bcl-xL were immunoprecipitated with anti-Bax antibody and were subjected to Western blot analysis using anti-Bcl-xL antibody. The same cell lysates were subjected to Western blot analysis of Bax expression. D, decreased apoptosis in RII transfectants overexpressing Bcl-xL protein. Transiently transfected Bcl-xL MIA PaCa-2 transfectants were exposed to a 5-Gy dose of radiation, and TUNEL was performed after 24 h of exposure. The bar graph shows the percentage of TUNEL-positive cells. Data represent a mean of two experiments. Error bars represent the S.D.
p21\textsuperscript{waf1/cip1} can be a marker of functional TGF-\(\beta\) signaling in the present study.

Several investigators have studied the role of TGF-\(\beta\)/H9252 signaling in apoptosis. Ohmori \textit{et al.} (64) reported that a blockade of TGF-\(\beta\) signaling increased the sensitivity of MDA-231 cells to cell death by treatment with cis-platinum. TGF-\(\beta\)/H9252 signaling has also been reported to protect hemopoietic cells from apoptosis by up-regulation of Bcl-XL (65). In contrast with the studies reported above, TGF-\(\beta\)/H9252 signaling has also been shown to induce apoptosis in a variety of cell lines (56, 61, 66–68). The reported mechanisms by which TGF-\(\beta\) induces apoptosis are varied and include induction of caspases (67), activation of Cdc2 and Cdk2 (68), down-regulation of Bcl-XL (69) and Bcl-2 (70), and induction and activation of Bax (56, 66). The study presented here supports the concept that TGF-\(\beta\) signaling can up-regulate Bax and activate caspases. The radiation induction of Bax protein in MIA PaCa-2/RII cells was abrogated when these cells were exposed to anti-TGF-\(\beta\)-neutralizing antibody, suggesting that the Bax induction is solely due to radiation-induced TGF-\(\beta\) signaling. The induction of Bax must be followed by proximal events close to programmed cell death such an increase in caspase-3 active forms and PARP cleavage. Bax is induced both by wild-type p53 (71, 72) and by TGF-\(\beta\) protein (66). Radiation failed to increase the expression of Bax protein in MIA PaCa-2/vector cells because the p53 and TGF-\(\beta\) pathways are not functional. However, restoration of TGF-\(\beta\) signaling in MIA PaCa-2/RII cells led to Bax protein elevation after irradiation, despite a nonfunctional p53 pathway. This elevation of Bax was followed by cytochrome \(c\) release and reduced inactive forms of caspase-3 with enhanced cleavage of PARP, suggesting a clear induction of programmed cell death. Induction of Bax by ionizing radiation in RII transfectants was quite significant. To gain some insight into the role of Bax induction in radiation-induced TGF-\(\beta\) signaling mediated apoptosis, we inhibited the function of this gene using antisense oligodeoxynucleotides and Bcl-\(x\textsubscript{L}\) overexpression and analyzed the cell death response.

![Fig. 12. Radiation-induced TGF-\(\beta\)-responsive promoter activity and apoptosis in normal primary MEFs transfected with vector alone or RII dominant negative mutant (LRII). A, MEF cells transfected with vector alone or RII dominant negative mutant and co-transfected with pRL-TK and p3TP luciferase constructs. TGF-\(\beta\)-responsive promoter activity (3TP-luc) is expressed as the ratio of firefly luciferase activity to \textit{Renilla} luciferase activity. The \textit{error bar} represents a mean of three experiments. B, radiation-induced apoptosis in normal primary MEFs transiently transfected with vector alone or RII dominant negative mutant. TUNEL staining was performed 24 or 48 h after radiation. Basal TUNEL-positive cells in untreated transfectants were normalized with the 24- or 48-h groups. The \textit{error bar} represents a mean of two experiments.](http://www.jbc.org/ ... }
Role of TGF-β Type II Receptor in Radiation Sensitivity

Bcl-2, or treatment with antisense oligodeoxynucleotides targeted against Bax significantly inhibited radiation-induced apoptosis in MIA PaCa-2/R1I but not in MIA PaCa-2/Vector cells, suggesting that Bax induction is necessary for radiation-induced TGF-β signaling mediated apoptosis. Altogether, induction of Bax may be central to the apoptogenic effects of restored RII expression in cell lines lacking TGF-β signaling due to the absence of RII expression. Thus, the restoration of TGF-β signaling is a novel strategy to overcome the effects of a nonfunctional p53 pathway and can be the mechanistic basis of the radiation sensitivity of these cells.

In summary, pancreatic tumors often lack response to TGF-β because of mutations of the DPC4 gene (3) or lack of RII expression (5) and often contain a mutation of the p53 gene (35, 36). This leads to the loss of function for both the TGF-β and p53 pathways. These two pathways have some overlapping function, since both can induce the expression of the cyclin-dependent kinase inhibitors and both can modulate the expression of components of the apoptotic pathway. Loss of function of p53 has been shown to play a role in radiation resistance in some cell types (31); however, the role of TGF-β signaling in radiation sensitivity has not been well studied. Here we show that restoration of TGF-β signaling in the p53 mutant, MIA PaCa-2 cells can increase their sensitivity to radiation. Furthermore, this study indicates that the increase in radiation sensitivity is mediated by TGF-β induction of proapoptotic Bax protein. Thus, restoration of the TGF-β pathway may be critical in enhancing radiation sensitivity in pancreatic cancer cells.

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Restoration of Transforming Growth Factor-β Signaling Enhances Radiosensitivity by Altering the Bcl-2/Bax Ratio in the p53 Mutant Pancreatic Cancer Cell Line MIA PaCa-2

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