Antisense Oligonucleotide Inhibition of Tumor Necrosis Factor Receptor 1 Protects the Liver from Radiation-Induced Apoptosis

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Abstract

Purpose: Liver damage by radiation limits its efficacy in cancer treatment. As radiation can generate apoptotic signals, we wished to examine the potential to protect the liver by inhibiting apoptosis through two key mediators, FAS and tumor necrosis factor receptor 1 (TNFR1).

Experimental Design: Radiation-induced liver damage was assessed by serum aspartate aminotransferase and alanine aminotransferase, hepatocyte micronucleus formation, and apoptosis assays (terminal nucleotidyl transferase–mediated nick end labeling and caspase-3 cleavage) in mice. Protection was evaluated by pretreating mice with antisense oligonucleotides (ASO) for FAS or TNFR1 prior to radiation. TNF-α production in liver and in Kupffer cells were determined by ELISA.

Results: Radiation increased liver FAS and TNFR1 transcription in a dose- and time-dependent manner (maximized at 25 Gy and 8 hours postirradiation). Pretreatment with ASOs for FAS and TNFR1 resulted in the inhibition of liver FAS and TNFR1 by 78% and 59%, respectively. Inductions of serum aspartate aminotransferase and alanine aminotransferase were observed at 2 hours after radiation and could be reduced by pretreating mice with ASO for TNFR1 but not FAS or control oligonucleotide. Radiation-induced liver apoptosis (terminal nucleotidyl transferase–mediated nick end labeling staining and caspase-3 activation on Western blot) and hepatocyte micronucleus formation were reduced by pretreatment with ASO for TNFR1. In addition, radiation stimulated TNF-α production both in irradiated liver and in cultured Kupffer cells by 50% and 100%, respectively.

Conclusion: This study suggests that ionizing radiation activates apoptotic signaling through TNFR1 in the liver, and thus provides a rationale for anti-TNFR1 apoptotic treatment to prevent radiation-induced liver injury.

The major limitation to the use of radiation in the treatment of intrahepatic cancers is radiation-induced liver toxicity. Exposure of the whole liver to a radiation dose of greater than ~30 Gy, which is insufficient to control gross tumor, may result in radiation-induced liver injury. This injury is characterized by early veno-occlusive diseases (2 weeks to 4 months) and by late-onset of radiation fibrosis (6 months; ref. 1). Although apoptosis has been linked to liver injury resulting from radiation treatment in mice (2), it is unclear what cells initiate the apoptotic signal in response to radiation or which apoptotic signaling pathways are involved in radiation injury. Specifically, endothelial cell apoptosis may serve as the primary lesion initiating radiation damage in lung (3), brain (4), and intestine (5). Radiation also induces various proinflammatory cytokines including tumor necrosis factor-α (TNF-α), transforming growth factor-β1, interleukin-1, interleukin-6, platelet-derived growth factor, and insulin-like growth factor-I which contribute to pathologic changes for pulmonary fibrosis (6, 7), acute intestinal inflammation (8), and hepatic microvascular pathogenesis that lead to apoptosis in the liver (9). In particular, an increased TNF-α production has been associated with the progression of hepatic veno-occlusive diseases in stem cell transplant patients (10), suggesting the potential role of cytokines in radiation-induced liver apoptosis.

Fas (CD95) and TNF receptor 1 (TNFR1), both members of TNF receptor families, are known to be major mediators of apoptosis when bound by their ligands, Fas-L and TNF-α. Aberrant signaling through Fas and TNFR1 has been implicated in the pathogenesis of a wide spectrum of human diseases including hepatitis (11, 12), pulmonary fibrosis (13), and multiple sclerosis (14, 15). It has been shown that death by acetaminophen-induced fulminant hepatitis in mice can be suppressed by specific antisense oligonucleotide (ASO) blockage...
of Fas expression (16). Ionizing radiation can activate similar pathways. For instance, radiation can generate ceramide through sphingomyelin hydrolysis by a sphingomyelinase, a process initiated by TNF receptor activation on the cellular membrane, and lead to apoptosis in endothelial cells in vitro (17). Taken together, these data suggest that the study of Fas and TNFR1 activation following radiation treatment may provide insight into the molecular mechanism of radiation-induced liver damage and suggest strategies for liver protection against radiation.

Recent advances in ASO technology using the 2′-O-(2-methoxy)ethyl modification of oligonucleotide have resulted in increased potency and enhanced nuclease resistance for specific inhibition of target gene expression both in vitro and in vivo (16, 18). In this study, we used 2′-O-(2-methoxy)ethyl–modified ASOs to specifically reduce Fas and TNFR1 gene expression in liver. Radiation-induced liver damage and apoptosis were then assessed using liver enzyme activities, terminal nucleotidyl transferase–mediated nick end labeling (TUNEL), caspase-3 cleavage, and hepatocyte micronucleus formation. After finding that ASO against TNFR1 was protective in vivo (16, 18), we used 2′-O-(2-methoxy)ethyl–modified ASO specific for TNFR1, Fas, or nonspecific control (25 mg/kg, i.p., every other day for a total of four times; kind gifts from Isis Pharmaceuticals, Carlsbad, CA). Twenty-four hours after the last dose of ASO treatment, mice received ionizing radiation to the upper abdomen including the liver and duodenum at appropriate doses using an Orthovoltage Unit Pantak DXT 300 machine producing 300 kV X-rays (East Haven, CT). Blood was drawn at various time points after radiation, followed by portal venous perfusion with saline (0.9% NaCl), and collection of liver and duodenal tissue for various assays described below. The use of animals was in compliance with the regulations of the University of Michigan and with NIH guidelines.

**RT-PCR measurement for gene transcription.** Fifty nanograms of total RNAs isolated from liver tissue by Trizol reagent (Promega, Madison, WI) were applied in real-time reverse transcription-PCR assay (Opticon, MJ Research, Inc., San Francisco, CA) using QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA) and specific primers according to the manufacturer’s suggested conditions. Primers used were: Fas 5′ CTACTGCGATTCTCCTGCTGTGA, Fas 3′ CATAGCGCAGTTCTCGGACCTTT; TNFR1 5′ CCTCTCCGCGATAAAGCCAACC, TNFR1 3′ CCTTGCCACCTTTCACCCAGG; α-actin 5′ CGAGATCCTCTCCAAAATCAA, and α-actin 3′ TGTGGTCATGAGTCTTCACA. The levels of mRNA for FAS and TNFR1 were normalized to each α-actin level and were expressed as fold increases in comparison with nonirradiated saline controls.

**Liver enzyme activities.** Blood samples were collected at termination of the experiments. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assessed using a commercial kit (Biotron Diagnostics, Inc., Hemet, CA). Twenty-four hours after the last dose of ASO treatment, mice were given 2′-(2-methoxy)ethyl-modified ASOs to specifically reduce Fas and TNFR1 gene expression in liver. Radiation-induced liver damage and apoptosis were then assessed using liver enzyme activities, terminal nucleotidyl transferase–mediated nick end labeling (TUNEL), caspase-3 cleavage, and hepatocyte micronucleus formation. After finding that ASO against TNFR1 was protective, we initiated additional studies to determine whether the source of TNF-α production in response to radiation was Kupffer cells versus the hepatic parenchyma. Our data suggest that specific inhibition of TNFR1 expression is likely to protect the liver during radiotherapy for intrahepatic cancer.

### Materials and Methods

**Animal model, oligonucleotide, and radiation treatment.** Male BALB/c mice, 6 to 8 weeks of age (Charles River Laboratories, Wilmington, MA), were given 2′-O-(2-methoxy)ethyl–modified ASO specific for TNFR1, Fas, or nonspecific control (25 mg/kg, i.p., every other day for a total of four times; kind gifts from Isis Pharmaceuticals, Carlsbad, CA). Twenty-four hours after the last dose of ASO treatment, mice received ionizing radiation to the upper abdomen including the liver and duodenum at appropriate doses using an Orthovoltage Unit Pantak DXT 300 machine producing 300 kV X-rays (East Haven, CT). Blood was drawn at various time points after radiation, followed by portal venous perfusion with saline (0.9% NaCl), and collection of liver and duodenal tissue for various assays described below. The use of animals was in compliance with the regulations of the University of Michigan and with NIH guidelines.

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**Liver enzyme activities.** Blood samples were collected at termination of the experiments. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were assessed using a commercial kit (Biotron Diagnostics, Inc., Hemet, CA). Ten microliters (μL) of TUNEL reaction

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**Figure 1.** Radiation induces TNFR1 and Fas gene transcriptions that can be inhibited by ASOs. BALB/c mice (6-8 weeks), pretreated with or without ASOs (25 mg/kg, i.p., every other day for a total of four times) for Fas (z-Fas) or TNFR1 (z-TNFR1), were irradiated in the upper abdomen for 0.5, 2, 8, or 24 hours followed by total RNA isolation from liver tissue. Levels of mRNA for TNFR1 and Fas were quantified by real-time reverse transcription-PCR assay using specific primers as described in Materials and Methods. The levels for TNFR1 or Fas from unirradiated liver were set as 1. A, liver at various time points (0, 0.5, 2, 8, and 24 hours) after irradiation of 10 Gy of radiation. B, liver at 2 hours after various doses of radiation (5, 10, 15, and 25 Gy). Line (A and B), mean of mRNA induction from four mice; bars, s.e. C, liver received pretreatment with ASOs for TNFR1 or Fas prior to radiation of 10 Gy (0, 2, and 24 hours). Columns, mean of mRNA induction from four mice; bars, s.e. *P < 0.05, significantly reduced in comparison with saline control (n = 4).
mixture containing 1:2 dilution of enzyme for 2 hours at 37°C in a humidified chamber. Signals were then converted into horseradish peroxidase using antifuluorescein antibody and were visualized by 3,3′-diaminobenzidine coloration (Roche Applied Science) as recommended by the manufacturer. The tissues were counterstained with methyl green and were scored relative to the number of cells positive for TUNEL staining from five randomly selected areas under the microscope (×20) for comparison between each experiment group.

Liver apoptosis was also determined by caspase-3 cleavage seen using immunoblotting. Tissues were homogenized in PBS containing protease inhibitors using a Dounce homogenizer. Twenty microliters of lysates were fractionated on 10% acrylamide gels followed by protein transfer onto polyvinylidene difluoride membrane (Millipore Corp., Billerica, MA). Cleaved caspase-3 was detected by a rabbit anti-mouse caspase-3 antibody (R&D Systems, Minneapolis, MN) and was quantified by densitometry scanning of specific bands (17 and 19 kDa as total) that were adjusted for loading using β-actin expression level detected by specific antibody (Sigma, St. Louis, MO).

**Hepatocyte micronucleus assay.** Saline-perfused liver was minced and digested in Liver Digest Media (Promega) in a 37°C water bath for 1 hour before filtering through a nylon membrane (100 μm). After washing in PBS, cells were resuspended in 1 mL PBS with 1% bovine serum albumin and subjected to Percoll gradient centrifugation (Amersham Pharmacia Biotech, Piscataway, NJ). Viable hepatocytes (5 × 10⁵), verified by trypan blue staining, were cultured on collagen-coated dishes in the presence of insulin (10⁻⁶ mol/L) and epidermal growth factor (40 ng/mL) for 48 hours followed by 4′,6-diamidino-2-phenylindole nucleus staining, and were then scored for the presence of micronuclei by fluorescence microscopy as described previously (19). Approximately 500 cells were counted for each experiment, and the percentage of cells with micronuclei was determined.

**Isolation and culture of Kupffer cell and hepatocytes, and ELISA assay for TNF-α.** For measurement of TNF-α, livers were first perfused with 20 mL of saline through the portal vein, followed by tissue collection and homogenization. For isolation of hepatocytes and Kupffer cells, saline-perfused livers were digested by Pronase followed by differential centrifugation using the Percoll gradient method (20). Isolation and culture of hepatocytes were done as described for the micronuclei assay. Purified Kupffer cells were cultured in a 96-well plate at 1 × 10⁵ cells/well in Williams E medium containing 100,000 units/L penicillin, 100 mg/L streptomycin, 15 mmol/L HEPES, and 10⁻⁶ mol/L insulin, and were verified by staining cells with antibody against macrophage (F4/80; Abcam, Cambridge, United Kingdom) or endothelial cells (anti-CD31; BD Sciences PharmMingen, San Diego, CA) using a standard immunohistochemistry staining protocol. Cells were cultured overnight and were placed in fresh medium prior to radiation treatment. Cells received 10 Gy of ionizing radiation, and culture media was collected at various time points after treatment. TNF-α secretion into the media of irradiated cells was quantified by an ELISA kit containing antibody against mouse TNF-α (R&D Systems) according to the manufacturer’s recommendation and was compared with TNF-α secretion from nonirradiated cells.

**Data analysis and statistics.** Animal and cell culture experiments were carried out four times. Scoring for TUNEL staining and hepatocyte micronucleus formation was done by two individuals. Values are expressed as mean ± SE and were compared by ANOVA analysis. Data were considered significantly different when P < 0.05.

### Results

**Radiation induced TNFR1 and FAS gene transcriptions.** Because Fas and TNFR1 are apoptosis receptors, we first determined whether ionizing radiation increases Fas and TNFR1 expression using real-time reverse transcription-PCR. Liver irradiation (10 Gy) significantly increased mRNA for FAS at 2 hours after radiation as compared with nonirradiated liver (1.26 ± 0.05-fold). Fas mRNA induction was even greater at 8 hours (1.60 ±

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**Fig. 2.** Radiation-induced liver enzyme activities and protection by ASO for TNFR1. Mice were treated with ASOs for TNFR1 (anti-TNFR1), Fas (anti-Fas), control oligo (Ctrl ASO), or saline control (Saline) prior to 10 Gy of radiation in liver or no radiation control (Ctrl). Blood was collected at 2 hours (2h) after irradiation followed by serum AST (A) and serum ALT (B) measurements using commercial kits described in Materials and Methods. Columns, mean of serum AST/ALT levels; bars, s.e. *P < 0.05, significantly increased in comparison with each unirradiated control (n = 4).

**Fig. 3.** ASO for TNFR1 inhibits hepatocyte micronucleus formation. Mice were treated with ASOs for TNFR1 (anti-TNFR1), Fas (anti-Fas), control oligo (Ctrl ASO), or saline (Saline) prior to 10 Gy of radiation. Whole livers were harvested before irradiation [(−) RT] or at 2 hours after irradiation [(+) RT] and were subjected to hepatocyte isolation using Percoll gradient centrifugation. Hepatocytes were cultured for 72 hours followed by 4′,6-diamidino-2-phenylindole staining for nuclei. Hepatocytes containing micronucleus, represented in saline-treated liver (A), were counted under fluorescence microscope (×20) as a percentage from a total of 500 cells. Columns, mean of percentage for hepatocytes with micronucleus; bars, s.e. *P < 0.05, significantly decreased in comparison with other treatments (n = 4).
0.13-fold) and 24 hours after radiation (1.65 ± 0.10-fold). Similarly, TNFR1 mRNA levels were increased significantly at 2 hours (1.15 ± 0.05-fold) and reached a plateau at 8 and 24 hours after irradiation (1.25 ± 0.07-fold; Fig. 1A). Moreover, induction for both FAS and TNFR1 was radiation dose-dependent, increasing at 10 Gy and maximized at 25 Gy (Fig. 1B). This dose-response relationship suggested that radiation was the cause for FAS and TNFR1 up-regulation in liver.

**ASOs blocked TNFR1 and FAS gene expression in liver.** To test whether the activation of FAS or TNFR1 transcription plays a role in radiation-induced liver apoptosis, we developed a method to effectively inhibit gene expression using TNFR1 and Fas ASOs. Four doses of ASO significantly reduced TNFR1 and Fas in the liver to 41% and 22% of nonirradiated controls, respectively, as measured by quantitative RT-PCR (Fig. 1C). In contrast to the radiation induction for TNFR1 (1.15 ± 0.05-fold) and FAS (1.26 ± 0.05-fold) in irradiated saline controls (Saline RT24h, 10 Gy), both liver TNFR1 and Fas transcripts in ASO-treated mice were inhibited at 2 hours postirradiation. Inhibition was sustained 24 hours after irradiation (~50% and ~60% reduction, respectively; Fig. 1C) and remained repressed for up to 72 hours after irradiation (data not shown). Pretreatment with ASO for TNFR1 or Fas had no effect on α-actin gene expression (data not shown), whereas pretreatment with nonspecific control ASO failed to inhibit TNFR1 or Fas transcription regardless of radiation treatment in liver (data not shown). These data suggested that reduction of TNFR1 and FAS gene expression could be achieved by using specific ASOs and could be sustained during the course of radiation treatment of the liver.

**Treatment with ASO for TNFR1 protected liver from radiation-induced injury.** We assessed liver injury by serum AST and ALT activities after irradiation in mice to determine whether inhibition of TNFR1 or FAS has a protective effect. Radiation (10 Gy) alone significantly increased enzyme activities for AST from 40 ± 4 units/L (control) to 110 ± 10 units/L (2 hours postirradiation; Fig. 2A), and ALT activity from 22 ± 3 units/L (control) to 44 ± 4 units/L (2 hours postirradiation; Fig. 2B). Similarly, radiation impaired liver function in anti-Fas ASO-treated mice as shown by a transient increase of serum AST and ALT at 2 hours postirradiation (340 ± 25 and 59 ± 6 units/L, respectively). The same pattern was also observed in nonspecific ASO-treated mice (185 ± 15 and 43 ± 4 units/L, respectively), as compared with their nonirradiated controls. Notably, pretreatment of mice with anti-TNFR1 ASO completely blocked radiation induction of enzymes at 2 hours postirradiation in comparison with unirradiated control for AST (48 ± 4 versus 49 ± 5 units/L; Fig. 2A) and for ALT (29 ± 5 versus 25 ± 5 units/L; Fig. 2B). These data suggested that pretreatment with ASO for TNFR1 prevented radiation-induced liver injury.

Based on this initial finding, we further assessed the liver protection of ASO by measuring micronucleus formation in primary hepatocytes in vitro. Ten gray of radiation to the liver resulted in a significant increase in micronucleus formation in...
cultured hepatocytes isolated from saline-treated mice that received radiation (12.8 ± 1.1%) compared with unirradiation control mice (4.2 ± 0.8%; Fig. 3A and B). Similar inductions were observed in hepatocytes isolated from mice pretreated with ASO for FAS or control ASO. However, pretreatment of mice with ASO for TNFR1 significantly reduced radiation-induced micro-nucleus formation in hepatocytes (7.7 ± 0.6%) as compared with that observed after treatment with other ASOs or in the saline controls (Fig. 3). These findings are consistent with the reduction of serum AST and ALT, suggesting that TNFR1-mediated signaling played a role in radiation-induced injury.

ASO inhibition of TNFR1 reduced radiation-induced liver apoptosis. To identify apoptosis induced by radiation, and the role of TNFR1 in mediation of liver injury, we first examined DNA fragmentation due to apoptosis using TUNEL staining. TUNEL staining was significantly increased in both duodenum (positive control due to its higher sensitivity to radiation, data not shown) and liver at 2 hours after 10 Gy (Fig. 4A and B). In contrast to the induction observed in irradiated liver from mice pretreated with anti-FAS ASO or control ASO (Fig. 4A), pretreatment of mice with anti-TNFR1 ASO resulted in a significant reduction of TUNEL staining. Quantitatively, the relative numbers of TUNEL-positive cells in irradiated liver were increased by ~4.1-, 6.4-, or 5.3-fold in mice receiving pretreatment of saline, anti-FAS ASO, or control ASO, respectively. In contrast, animals treated with anti-TNFR1 ASO showed only a slight decrease in TUNEL-positive cells compared with untreated animals (Fig. 4B). These data suggested that inhibitory ASO for TNFR1, but not FAS or control, had a radioprotective effect.

We next used immunoblotting to assess radiation-induced apoptosis by measuring caspase-3 cleavage as a marker for apoptosis. Consistent with the TUNEL staining results, radiation (10 Gy) increased caspase-3 cleavage by ~2.9, 2.4, and 2.5-fold in saline treated, control ASO–treated, or anti-Fas ASO–treated mice, respectively. Saline-treated duodenal tissue was used as a positive control with a 7.9-fold increase in irradiated mice versus nonirradiated mice (Fig. 5A). This induction was abolished by pretreatment of mice with TNFR1 ASO (0.8-fold; Fig. 5A and B).

Radiation increased TNF-α production in liver and in Kupffer cells. We showed above that TNFR1-mediated radiation induced apoptosis. One possibility is that the activation of TNFR1 is caused by an elevation of TNF-α production in liver. To test this mechanism, we measured TNF-α production in liver, in primary hepatocytes, and Kupffer cells in response to radiation. We found that 10 Gy of radiation markedly increased liver TNF-α levels at 2 hours postirradiation (58.9 ± 6.7 pg/mg) as compared with the level in control liver (37.6 ± 5.8 pg/mg; Fig. 6A). This induction (~57%) was not detectable in serum or in liver at 24 hours postirradiation, suggesting that radiation induction of TNF-α was a transient and local event. To further identify the source of induced TNF-α levels, primary hepatocytes and Kupffer cells were isolated for in vitro culture. Purified Kupffer cells were verified by positive staining of antibody for the macrophage marker F4/80 and negative staining of CD31 (an endothelial cell marker). TNF-α secretion in Kupffer cells was detectable at 24 hours postplating and gradually increased with culture time (15 ± 2, 21 ± 3, and 27 ± 3 pg/mL at 24, 48, and 72 hours, respectively; Fig. 6B). Significantly, after radiation treatment (10 Gy), TNF-α secretion in Kupffer cells was increased at 24, 48, and 72 hours (22 ± 1.5, 40 ± 4, and 45 ± 5 pg/mL, respectively; Fig. 6B). By contrast, primary hepatocytes did not produce any detectable levels of TNF-α under any conditions regardless of radiation (data not shown). These data suggested that Kupffer cells in irradiated liver could be the primary source for increased TNF-α to activate the apoptotic signaling pathway by binding to TNFR1, thus leading to liver injury.

Discussion

In this study, we showed that radiation caused rapid liver injury, evidenced by elevated liver enzymes and hepatocyte

Fig. 5. Inhibition of radiation-induced caspase-3 cleavage by ASO for TNFR1. Mice were treated with saline (Saline Ctrl) or ASOs for TNFR1 (Anti-TNFR1), Fas (Anti-Fas), or control oligo (Ctrl ASO) prior to 10 Gy of radiation or no radiation as control. Duodenal tissues from saline-treated mice and liver from ASO–treated mice were homogenized and were subjected to Western blotting using rabbit anti-mouse caspase-3 (cleaved form) antibody. A, cleaved caspase-3 on Western blotting. B, densitometry scanning of caspase-3 bands (17 and 19 kDa) from Western blotting analysis was adjusted by each β-actin level. Columns, mean of band density; bars, s.e. *, P < 0.05, significantly increased in comparison with unirradiated controls (n = 4).
micronucleus formation. This early injury seemed to be the result of apoptosis, as evidenced by both increased TUNEL-positive staining and caspase-3 activation. By using ASOs, specific suppression of TNFR1, but not FAS, significantly inhibited apoptosis and protected the liver from radiation injury. Furthermore, radiation treatment increased TNF-α secretion by Kupffer cells, but not liver parenchymal cells, suggesting that macrophage-derived TNF-α and the subsequent activation of TNFR1 might be the mediator for early stage radiation-induced liver injury.

Programmed cell death, or apoptosis, is controlled by many interactive signaling cascades initiated by various intracellular and extracellular stimuli to maintain normal cell turnover or, more importantly for this study, in response to pathophysiological conditions. Initiation of apoptosis can be detrimental to normal cell function; specifically, aberrant activation of FAS and TNFR1 signaling has been implicated in many human diseases. For instance, elevated local TNF-α secretion from the activation of immunocompetent cells is well recognized as a major cause in the development of rheumatoid arthritis in which apoptosis and destruction of synovial cartilage and bone occur (21). FAS and TNF-α are both implicated in the pathogenesis of fulminant hepatic failure (22) and ischemic liver apoptosis (23). Furthermore, the data for this present study suggested that TNFR1 signaling is actively involved in radiation-induced liver apoptosis and subsequent injury, and that the expression of both FAS and TNFR1 mRNA in liver are up-regulated in response to irradiation.

The TUNEL data show a slightly greater fold induction between the control ASO and saline-treated group (Fig. 4B), whereas our caspase-3 data indicate similar induction between groups (Fig. 5B). This slight discrepancy in the magnitude of induction could be due to differences in assay sensitivity and possible involvement of other apoptotic intermediates during radiation. However, our current data indicated that radiation-induced liver injury correlated closely with the activation of liver apoptosis and was mediated at least partially through the TNFR1, but not the FAS, signaling pathway. An unexpected result from this study is that inhibition of Fas expression by ASO prior to radiation treatment actually enhanced radiation-induced liver apoptosis (represented by additional increases of AST/ALT activities and caspase-3 cleavage in Figs. 2 and 5) compared with saline control mice. In addition, inhibition of caspase-8, a major component of the Fas signaling pathway, increased radiation damage in a similar fashion (data not shown). It might be possible that there is inhibitory cross-talk between the Fas and TNFR1 signaling pathways, both of which lead to the activation of caspase-3 and apoptosis. Because TNFR1 can elicit polyphyletic pathways through different cytoplasmic adaptor proteins (such as TRADD, RIP, and FAN), it is of interest to further investigate which of the downstream intracellular signaling pathways is responsible for the apoptotic injury from irradiation, a FAN-mediated sphingomyelin pathway or a TRADD-caspase-8 pathway.

The hallmark of chemotherapy or radiation therapy-induced liver damage is veno-occlusive disease, which is initiated by endothelial cell damage, followed by a cascade of events leading to occlusion of the central veins and, ultimately, fibrosis (24). As there is no animal model for veno-occlusive diseases, we decided to study the initial damage and focus on events that may affect the endothelium. In this context, we raise the possibility that endothelial damage could be the result of radiation-induced overproduction of TNF-α in Kupffer cells, leading to further cytokine signaling, a mechanism found in bone marrow transplantation–mediated veno-occlusive diseases (25). However, this moderate increase of TNF-α may contribute only partly to the observed apoptosis in vivo. Increased macrophage release of TNF-α could either be the result of direct radiation damage to macrophages or the outcome of macrophage phagocytosis of apoptotic cells in vivo, both of which have been shown in other systems (26, 27). It does seem clear that human beings express unique downstream pathways after the initial radiation insult. It will be important to discover an experimental model that reproduces the full clinical syndromes.

A number of therapeutic strategies have been developed to protect the liver from chemotherapeutic and radiation-induced toxicity. For instance, blocking early damage at the organ and tissue levels following radiation can lead to diminished late effects, fulminant hepatitis or encephalomyelitis (16, 28),
indicating that prophylaxis treatment could be an effective strategy. Ursodeoxycholic acid, an antithrombotic agent, has been shown to protect endothelial cells and hepatocytes by inhibiting TNF-α and interleukin-1α production, or by increasing glutathione, and thus inhibiting apoptosis (29). Amifostine (WR-2721) is another clinically investigated radioprotector which decreases radiation toxicity in the parotid gland, and, possibly, the lung and esophagus (30, 31). We have previously reported that amifostine achieved a radiation dose modification factor of 2.4 to 3.3 in liver with minimal tumor protection (19), and a clinical trial has been initiated based on this concept. The recent development of manganese superoxide gene therapy resulted in significant improvement in protection of various organs including the lung (32), esophagus (33), and oral cavity (34) against radiation, and, was effective in protecting the liver against chemical-induced acute injury. This approach is thought to block the late apoptotic pathway by stabilizing the mitochondrial membrane (35). Because 70% to 90% of patients with liver cancer have preexisting liver disease, preventing liver toxicity is crucial in maintaining patient health during radiation or chemotherapy treatment.

Biologically active ASOs are potent, nontoxic, target-specific drugs that are potentially useful in improving the outcome of radiotherapy for patients with intrahepatic tumors by protecting the normal liver. Specifically, the 2′-O-(2-methoxy)ethyl–modified ASO as used in this study has been shown to be pharmacologically more potent than phosphorothioate oligonucleotides (~5-fold) for blocking gene expression in liver (16). In this study, we showed target-specific liver protection through the use of ASOs for specific blockade of FAS and TNFR1 gene expression. Increasingly advanced ASO technology will provide a future for “molecularly targeted radioprotection” and further insight into radioprotection mechanisms.

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References

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