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Pretreatment with A Small-Molecule Tumor Necrosis Factor-Alpha (TNF-α) Inhibitor, UTL-5g, Reduced Radiation-Induced Acute Liver Toxicity in Mice

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Abstract

Radiation-induced liver toxicity is a major limitation to the use of radiation in the treatment of intrahepatic cancers. The purpose of this study was to evaluate the potential radioprotective effect of a small-molecule tumor necrosis factor alpha (TNF-α) inhibitor, UTL-5g, against radiation-induced acute liver injury. Mice were pre-treated by *i.p.* injection with UTL-5g and control vehicle one hr prior to liver irradiation at 15 Gy. Blood and liver were collected 2 hr after irradiation and analyzed for levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities in serum and TNF-α in liver tissue extracts. Both AST and ALT in serum and TNF-α in liver induced by irradiation were significantly reduced by UTL-5g in a drug dose-dependent manner. The reductions of AST, ALT and TNF-α appeared to correlate with the reduction of liver apoptosis detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay. A radiation dose escalation study (5, 15 and 25 Gy) showed that UTL-5g at 60 mg/kg was effective as a radioprotective agent at 5 and 15 Gy; the protection was only modest at 25 Gy. In summary, our results suggest that the TNF-α inhibitor, UTL-5g, is potentially radioprotective against acute phase of radiation-induced liver injury.

Keywords: liver, radiation, radioprotective, TNF-α, UTL-5g.

1. Introduction

While radiotherapy is an important modality for malignant hepatoma, its use is limited by radiation-induced liver toxicity in which patients inevitably suffer from liver damage and function failure [1]. Radiation-induced toxicity results from a series of pathological changes at both cellular

and biochemical levels, which include DNA damage, early cell apoptosis, inflammation and, ultimately, radiation-induced latent fibrosis and loss of liver function. Many strategies and compounds have been studied for their protective effects against radiation [2-4]. Ethyol® (amifostine) is the only radioprotector approved by the FDA to reduce xerostomia associated with radiotherapy of head-and-neck cancer. Presumably, amifostine works as an antioxidant that scavenges free radicals [5], but other mechanisms such as DNA repair have also been proposed [6, 7]. While amifostine reduces normal organ damage caused by radiation [4, 6, 8], it does have serious adverse effects including nausea and vomiting, as well as transient hypotension [9, 10].

In general, radioprotective agents fall into the following classes: 1) thiol compounds [11-13]; 2) nitroxides [14, 15]; 3) polysaccharides [12]; 4) oligonucleotides, such as TNF receptor antisense oligonucleotides (ASO) [3, 16]; and 5) other potential radioprotective agents, such as melatonin, vitamins, and pharmacological inhibitors of cyclin-dependent kinases [11, 17, 18].

It is known that TNF-α and other inflammatory cytokines are up-regulated in various organs following radiation exposure [3, 19]. Numerous studies have shown that excessive amounts of TNF-α can cause cell apoptosis, resulting in injury of normal liver tissues [20, 21]. TNF receptor 1 (TNFR1) ASO was suggested to be a potential treatment to prevent radiation-induced liver injury. Huang et al. reported that 10 Gy irradiation transiently increased liver TNF-α levels by ~50% at 2 hr in mice as compared to non-irradiated control, and pre-treating mice with TNFR1 ASO was effective in preventing the liver damage [3]. In a similar study, Zhang et al. showed that inhibition of TNF-α pathway with TNFR1 ASO reduced radiation-induced lung injury without compromising tumor response [16].

Since down-regulation of TNFR1 with target-specific ASO showed positive results in liver radioprotection, we hypothesize that lowering the elevated TNF-α production induced by radiation may be a potential route of liver radioprotection. Previously, we have synthesized a series of small molecule carboxamidyl isoxazoles, UTL-series, which are inhibitory to TNF-α production [22-24]. Here, we screened three of these TNF-α inhibitors (Fig. 1) and found that one of them, UTL- 5g, was the most potent liver radioprotector against early phase liver toxicity.

2. Methods and materials

2.1 Animals

Male C57BL/6 mice (4-6 weeks, 20-25 g body weight) were obtained from the Jackson Laboratories (Bar Harbor, Maine). All animal experiments were conducted in full accordance with the Institutional Animal Care and Use Committee (IACUC) Guidelines for the care and management of laboratory animals.

2.2 Materials

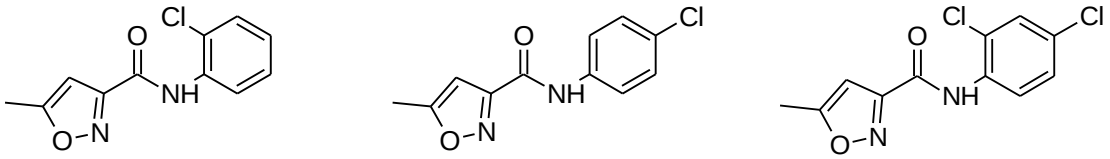
UTL-5b, -5d, and -5g (purity > 99%) (Fig. 1) were obtained from Unitech Pharmaceuticals, Inc. (Ann Arbor, MI). Individual compounds were prepared as stock solutions at 40 mg/mL in DMSO. Equal volume of the stock solution and a premixed solvent (cremophor EL:propylene glycol 4:6 v/v) or ethanol were mixed and then added with saline individually to make a suspension in DMSO/(cremophor:propylene glycol 6:4)/saline 5:5:90 v/v (vehicle I) or in DMSO/ethanol/saline 5:5:90 v/v (vehicle II) prior to injection. Alpha-MEM was obtained from MediaTech (Manassas, VA). TUNEL staining kit was obtained from Roche Applied Science (Indianapolis, IN).

2.3 Radiation protection study

To test the radioprotective effect, animals were treated by *i.p.* injection with 0.3 mL of control vehicle or selected compounds one hr prior to liver irradiation using a Philips RT 250 orthovoltage unit machine that produces 250 kV X-ray at 1 Gy/min. Conscious mice were placed in plastic restrainer and the liver portion was centered in a 2x4 cm exposure field with other abdominal structures and the body protected by a customized lead shielding. Control mice were subjected to similar procedures without receiving the radiation dose. Blood (by cardiac puncture) and liver were collected at 2 hr after radiation. Harvested serum and tissue were stored at -70°C until needed for biochemical and ELISA tests.

2.4 Liver enzyme activities

Radiation-induced liver damage was assessed by measuring blood liver enzyme activities. Serum AST and ALT activities were assessed using a commercial assay kit according to the manufacturer's instruction (Biotron Diagnostics, Inc., Hemet, CA). Ten μL of each serum sample were used for each test. Data were presented as units per liter (Unit/L) ± S.D.



UTL-5b UTL-5d UTL-5g

Figure 1. Structures of three small-molecule TNF-α inhibitors.

2.5 Determination of TNF-α levels in liver extracts

For measurement of liver TNF-α, livers were first perfused with 20 mL of saline through the portal vein, then minced into small chunks (8 mm^3) and homogenized in 150 μL of PBS containing protease inhibitors using a Dounce homogenizer. The tissue homogenates were then subjected to centrifugation at 6,000 rpm (3,500 x **g**) for 5 min to remove tissues debris. Supernatants from liver tissue extracts were further diluted with ELISA reagent diluent (1:50) and the diluted supernatants (100 μL/well) were assayed for TNF-α using a commercial ELISA kit according to manufacturer's recommendation (R & D Systems, Minneapolis, MN). Each sample was measured in triplicate and TNF-α concentration (pg/mg tissue protein) was calculated based on a standard curve and the total amount of tissue protein in the supernatant.

2.6 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Radiation-induced liver cell death was assessed by measuring DNA strand breaks using an *in situ* TUNEL staining approach (Roche Applied Science, Indianapolis, IN). Briefly, liver tissue were collected, cryopreserved and sectioned. The tissue slides were fixed in 4% paraformaldehyde for 10 min followed by washing in PBS. After permeabilization in a solution of 0.1% Triton-X-100 and 0.1% sodium citrate, liver tissue sections were labeled with 25 μL of TUNEL reaction mixture containing 1:2 dilution of enzyme at 37°C in a humidified chamber for 2 hr. Fluorescence signals positive for TUNEL staining were counted from 5 randomly selected areas under a fluorescence microscope (40 x).

2.7 Statistics

Power analysis was performed before each animal study and a sample size of 4 mice per group was determined to have a 80% power of detecting a mean difference of 11.70 with a significance level of 5% (two sided). Values are shown as the means ± standard deviation (S.D.)

3. Results

3.1 Radioprotective effects of UTL-5b, -5d, and -5g

In a preliminary study, three UTL compounds (5b, 5d and 5g) were examined for their radioprotective effect in C57BL/6 mice. One hr prior to liver irradiation (15 Gy), animals were treated with the test compounds by *i.p.* injection (30 mg/kg). Two hr later, the animals were sacrificed and serum AST and ALT enzyme activities were determined. As shown in Fig. 2, pretreatment of the animals with these compounds lowered serum AST and ALT levels induced by irradiation. Among them, UTL-5g was the most

effective in lowering both AST and ALT enzyme activities and was selected for further experiments.

During the preliminary study, it was found unexpectedly that the vehicle used (vehicle I), DMSO/(Cremophor/propylene glycol)/saline, was associated with some liver toxicity as indicated by the moderate increase of serum AST and ALT activities in animals treated with vehicle alone (Fig. 3). Therefore, a different vehicle (vehicle II), DMSO/EtOH/saline (5/5/90 v/v) was tested and compared to that of vehicle I. As shown in Fig. 3, injection with vehicle I did show some liver toxicity in mice. In contrast, treatment with DMSO/EtOH/saline (5/5/90 v/v) vehicle showed essentially no toxicity and was selected as the desired vehicle for preparing UTL-5g solution in subsequent studies.

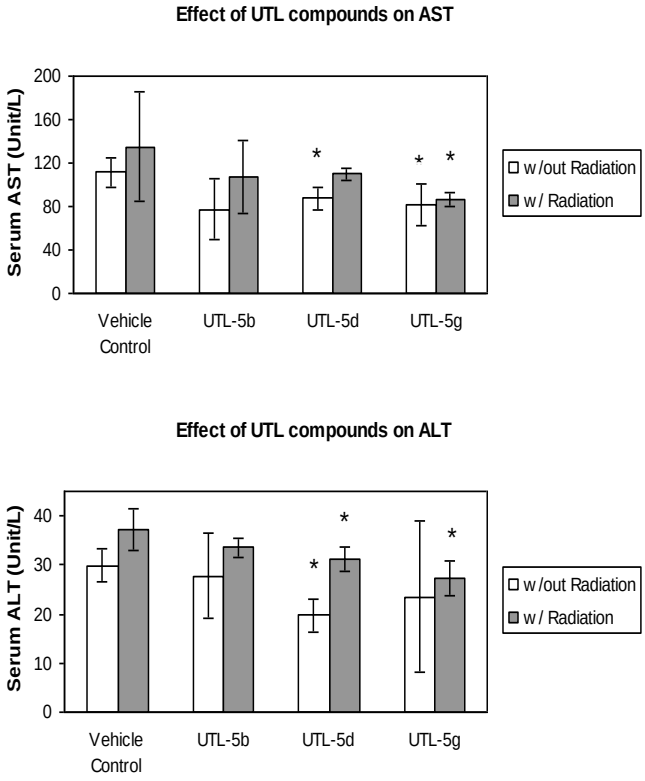


Figure 2. C57BL/6 male mice were treated with UTL-5b, -5d, and -5g each in 0.3 mL vehicle (Vehicle I) by *i.p.* injection (30 mg/kg) 1 hr prior to liver irradiation with 15 Gy. Control mice received vehicle only followed by irradiation. Serum was obtained at 2 hr after irradiation and assayed for AST and ALT activities. Data are means ± standard deviation (S.D.) * indicates $p < 0.05$ as compared to Vehicle I, n = 4.

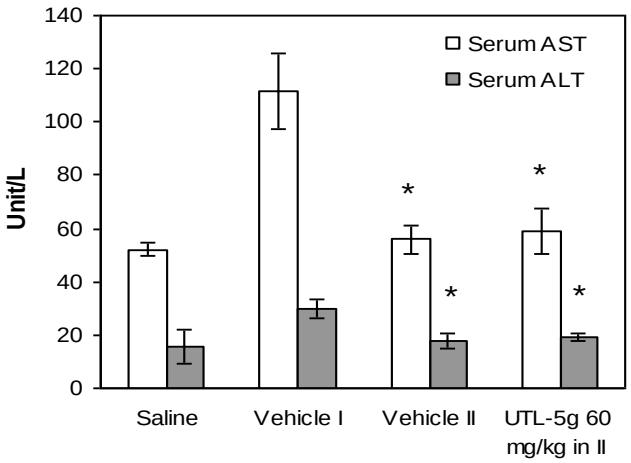


Figure 3. Comparison of the liver toxicity by two different vehicles without radiation. All treatments were made by *i.p.* injection. Vehicle I: DMSO/(Cremophor:PG)/saline 5/5/90 v/v. Vehicle II: DMSO/EtOH/saline 5/5/90 v/v. *Significantly reduced AST or ALT levels as compared to Vehicle I ($p<0.05$, n = 4).

3.2 Dose-dependent radioprotective effect of UTL-5g

Based on the preliminary studies, UTL-5g was selected for dose-dependent radioprotective studies. In this study, animals were treated with increasing doses of UTL-5g (from 0 to 60 mg/kg) one hr prior to liver irradiation (15 Gy). Two hr after irradiation, animals were sacrificed and serum AST/ALT enzyme activities and liver TNF-α levels were determined. As shown in Fig. 4, liver irradiation at 15 Gy significantly induced serum AST/ALT activities (increased from 52 to 135 Unit/L and 15 to 29 Unit/L, respectively). Pretreatment of UTL-5g one hr before irradiation significantly reduced serum AST/ALT activities in a drug dose-dependent manner ($p < 0.05$, paired t test). For animals pre-treated with UTL-5g at 30 mg/kg, AST/ALT activity levels were lowered from 135/29 to 89/22 Unit/L, and those pre-treated with UTL-5g at 60 mg/kg, AST/ALT levels were reduced from 135/29 to 74/19 Unit/L, respectively. However, treatment with high dose of UTL-5g (60 mg/kg) alone slightly increased the levels of both AST and ALT enzyme activities in animals without irradiation (Fig. 4).

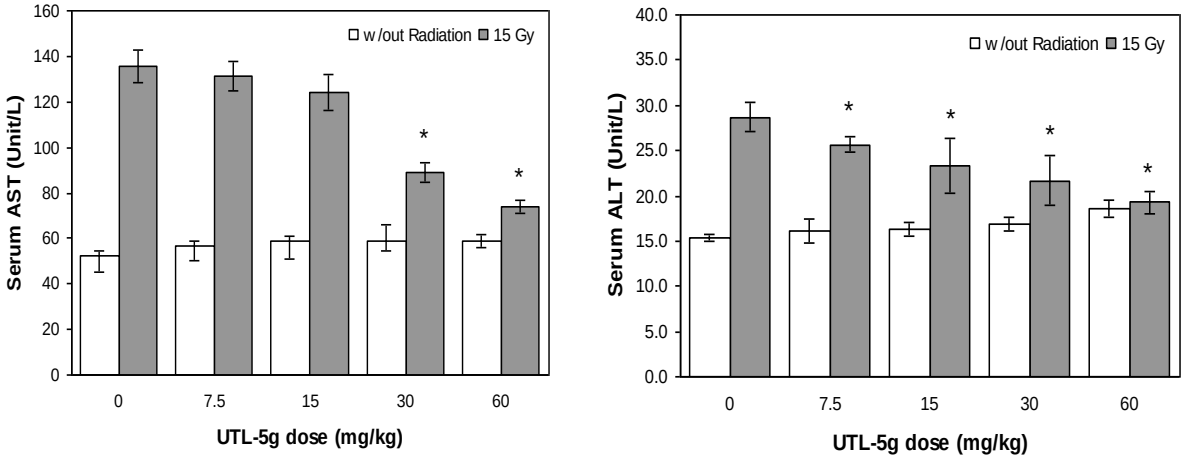


Figure 4. Drug dose dependent radioprotection of liver by UTL-5g. Mice were treated with various doses of UTL-5g (0, 7.5, 15, 30, and 60 mg/kg, *i.p.*) 1 hr prior to liver radiation with 15 Gy. Serum was obtained at 2 hr after irradiation and assayed for AST and ALT activities. Data shown are means ± standard deviation (S.D.) *Significantly reduced AST or ALT levels as compared to irradiated mice treated with vehicle only, $p<0.05$ (n = 4). Two-tailed paired t-Test showed $p< 0.05$.

As shown in Fig. 5, TNF-α levels in liver extracts from irradiated mice (15 Gy) were increased by 56% compared with non-irradiated control animals (from 37.6 to 58.9 pg/mg tissue protein). Likewise, pre-treatment of animals with UTL-5g significantly reduced liver TNF-α levels by 20% and 29% at 30 mg/kg and 60 mg/kg, respectively.

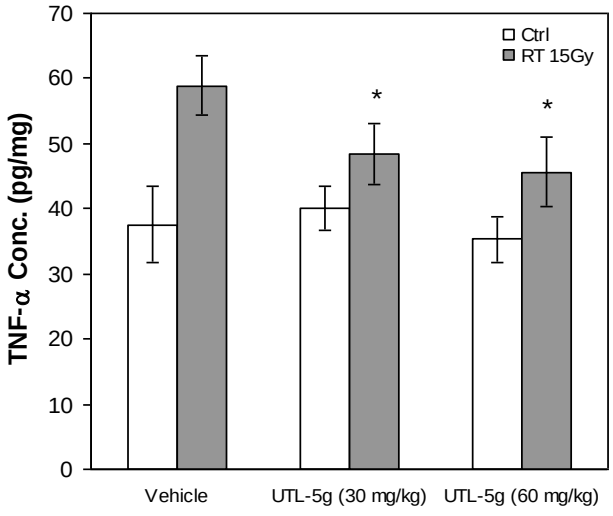


Figure 5. Drug dose-dependent reduction of TNF-α. Mice were treated individually with 0.3 mL each of UTL-5g preparations *i.p.* 1 hr prior to liver irradiation at 15 Gy. Liver TNF-α levels were analyzed by standard ELISA methodology. TNF-α levels in irradiated liver (at 15 Gy) were increased by 56% (from 37.6 to 58.9 pg/μg) and the pretreatment by UTL-5g significantly reduced TNF-α by 20% and 29% at 30 mg/kg and 60 mg/kg, respectively (∗ $p < 0.05$, 2-tailed paired t-Test comparing treated and untreated for both 30 mg/kg and 60 mg/kg groups, n = 4).

3.3 Radiation doses and radioprotective effect of UTL-5g

Next, we investigated the radioprotective effect of UTL-5g with increased irradiation doses. In this study, animals were pre-treated with UTL-5g at 60 mg/kg and randomly divided into 4 groups. One hr later, they were subjected to liver irradiation at 0, 5, 15 and 25 Gy. Consistent with the previous study, pretreatment with UTL-5g at 60 mg/kg was radioprotective against 15 Gy as evidenced by the reduction of serum AST activity (103.0 vs. 159.8 Unit/L in UTL-5g and in control vehicle groups, respectively) and ALT activity (17.8 vs. 32.3 Unit/L in UTL-5g and in control vehicle groups respectively) (Fig. 6). However, only moderate protection was observed in group of mice irradiated with 25 Gy. At lowest irradiation dose (5 Gy) used in this study, both AST and ALT levels were slightly elevated by

radiation and reduced by UTL-5g but not statistically significant.

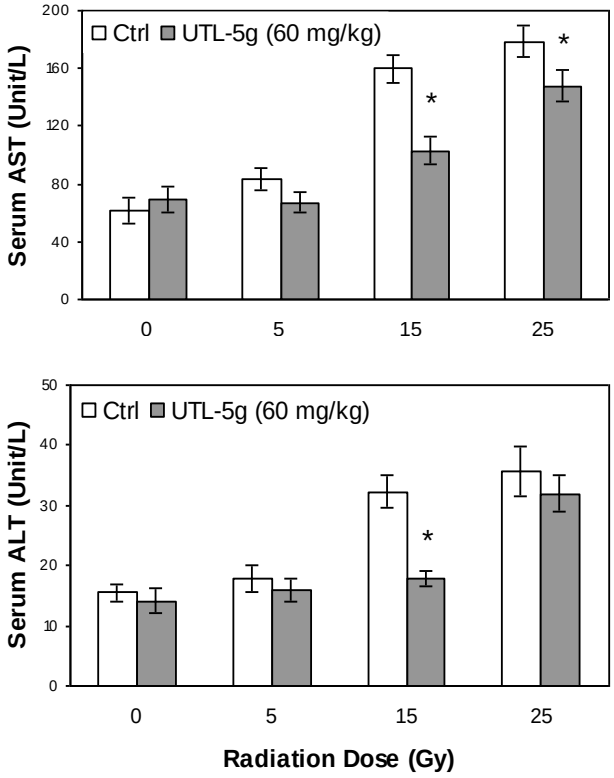
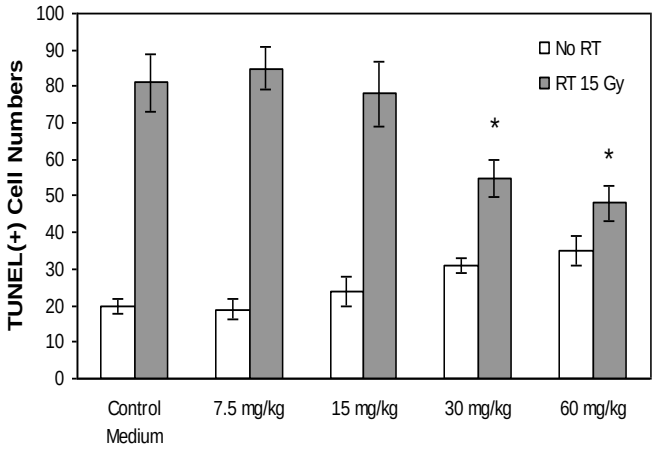


Figure 6. Radiation dose escalation and radioprotection by UTL-5g. Mice were pre-treated with UTL-5g (60mg/kg, *i.p.*) at 1 hr prior to radiation dose escalation (0, 5, 15, and 25 Gy). Serum was harvested at 2 hr after injections and was subjected to AST/ALT measurements as described in Methods. Results were compared to control mice received control vehicle. Data are means ± standard deviation (∗ $p < 0.05$, 2-tailed paired t-Test comparing treated and untreated groups, n = 4).

3.4 TUNEL assay for apoptotic liver cells

To further analyze the radioprotective effect of UTL-5g, we examined the number of liver apoptotic cells by *in situ* TUNEL staining. At the end of treatment, liver tissue sections were prepared, fixed and stained with TUNEL staining for apoptotic cells. There were "spontaneous" apoptotic liver cells in control animals as detected by TUNEL staining (20/five random fields), approximately 0.2% of total liver cells. The numbers of TUNEL positive cells in the tissue sections were markedly increased (82/five random fields) (> 4-fold) by 15 Gy of radiation as compared to non-irradiated controls (Fig. 7). The radioprotective effect of UTL-5g was drug dose-dependent. At the lowest dose of UTL-5g (7.5 mg/kg), there was no significant protective effect. However, for higher doses, the numbers of TUNEL positive cells induced by irradiation in the liver were significantly reduced from 4.1 to 3.3, 1.8, and 1.4-fold for animals treated with 15, 30, and 60 mg/kg UTL-5g, respectively. Unexpectedly, treatment with UTL-5g alone at the higher doses (30 and 60 mg/kg) also slightly increased the number of apoptotic cells in the liver tissue section from non-irradiated animals.



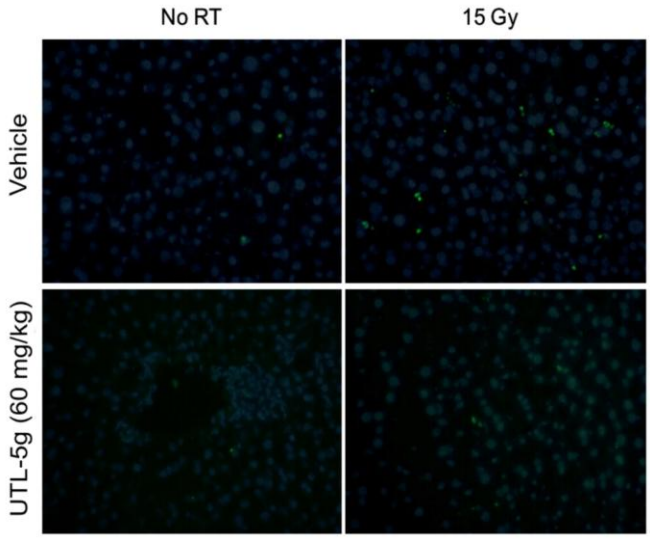


Figure 7. UTL-5g reduced radiation-induced liver apoptosis measured by TUNEL staining. Mice were pre-treated with various doses of UTL-5g (7.5, 15, 30, and 60 mg/kg, *i.p.*) or control vehicle at 1 hr prior to

liver radiation with 15 Gy. Liver tissues were harvested at 2 hr after radiation for cryosections and were subjected to TUNEL staining. Positive cells were counted under fluorescent microscope (40 x) from 5 randomly selected fields and were plotted as averages. ∗TUNEL(+) cell numbers were significantly reduced relative to vehicle ($p < 0.05$, $n = 4$) (top). Representative liver sections showing TUNEL positive cells (green) counter-stained with DIPA (4'-6-diamidino-2-phenylindole) for nuclei under fluorescent microscope (40x) (bottom). RT: radiation treatment.

4. Discussion

Earlier studies have pointed to a close relationship between the levels of TNF-α and radiation-induced apoptosis [3, 16]. To further investigate the mechanistic connections during these early events, we examined the levels of TNF-α and liver cell apoptosis in irradiated mice. Irradiation of the animals with 15 Gy markedly increased the number of apoptotic liver cells by more than four-fold within two hr. At the same irradiation dose, the levels of liver TNF-α production increased 56% compared to non-irradiated group (Fig. 5). It is not known why the % increase of liver TNF-α level (56%) is much lower than the % increase of liver apoptotic cells (> four-fold) following irradiation. Conceivably, other inflammatory cytokines and/or mechanisms such as reactive free radical are also operative in causing apoptosis in liver. Another possible explanation is that locally produced TNF-α following irradiation is rapidly removed and dissipated into circulation. Nonetheless, pretreatment with UTL-5g could effectively lower the levels of both TNF-α and number of apoptotic liver cells in a drug dose-dependent manner 2 hr after irradiation, indicating that TNF-α is indeed an important factor responsible for liver cell apoptosis. A quantitative relationship between liver apoptotic cells and radiation-induced liver acute toxicity as defined by liver enzyme activities, however, remains to be established.

The cell types responsible for TNF-α production following irradiation are not known at present. A recent study suggests that Kupffer cells (liver macrophages) may be the primary sources responsible for the production of TNF-α *in situ* [3]. We have shown that UV irradiation can trigger epithelial cells to release TNF-α *in vitro* [23]. Other cell types such as fibroblasts and epithelial cells in the liver can produce TNF-α in response to irradiation as had been shown previously in skin cells [25, 26]. Thus, it is likely that multiple cell types are responsible for TNF-α production in the liver.

Our data and others established the feasibility of radioprotection by inhibiting the TNF-α pathway. However, it remains to be determined if inhibition of TNF-α pathway by UTL-5g may cause adverse effects on the therapeutic efficacy by radiation. In a previous study, Zhang et al. showed that inhibition of TNFR by either genetic deficiency or ASO could protect lung function without compromising lung tumor sensitivity to radiation [16]. We recently reported that UTL-5g treatment did not attenuate the anti-tumor effect of cisplatin but enhanced the therapeutic efficacy of cisplatin in a SCID mouse model inoculated with human HCT-15 tumor cells [24]. Treatment with TNF-α inhibitors, such as UTL-5g, therefore is justified as a protective measure during either chemotherapy or radiation treatment. These findings also suggest that aberrant TNF-α production induced by radiation and anti-cancer agents is in fact clinically detrimental to the overall therapeutic efficacy.

Radiation dose escalation experiments showed that the radioprotection by UTL-5g is less effective when the radiation dose was increased to 25 Gy, suggesting that the transient liver damaged induced by high dose irradiation may have reached the maximal protection capability of UTL-5g as currently formulated and administered. Giving that multiple factors and mechanisms are likely involved in causing liver toxicity and pathologic changes, a combined treatment using various radioprotectors with different mechanisms of action may be essential to achieving optimal radioprotective effect. The current study focused on the early events of radiation-induced liver acute toxicity. Thus, radioprotective effect of UTL-5g for long-term hepatic damage is not known at present. Recent studies suggest that much of the delayed damage may be caused by aberrant and prolonged production of wound healing proinflammatory cytokines including TNF-α *in situ* [2, 16, 19]. Since the production of these

cytokines is regulated by identical and interrelated inflammatory signaling pathways, it is reasonable to speculate that UTL-5g may have long-term radioprotective effect as well, but further studies are needed to investigate the long-term effect of UTL-5g. UTL-5g is very tolerable in animal acute toxicity test and can be administered orally at higher dose for extended period. In a previous study, we showed that UTL-5g has a LD_{50} of >2,000 mg/kg [24]. Future studies on the effect of UTL-5g on the levels of wound healing cytokines such as platelet-derived growth factor, transforming growth factor-β and fibroblast growth factor in delayed liver damage may provide crucial insights into the potential mechanistic connections.

Acknowledgments

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Abbreviations: ASO, antisense oligonucleotide; AST, aminotransferase; ALT, alanine aminotransferase; TNF-α, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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