Cell-permeable intrinsic cellular inhibitors of apoptosis protect and rescue intestinal epithelial cells from radiation-induced cell death

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(Received 15 May 2014; revised 21 August 2014; accepted 13 September 2014)

One of the important mechanisms for gastrointestinal (GI) injury following high-dose radiation exposure is apoptosis of epithelial cells. X-linked inhibitor of apoptosis (XIAP) and cellular IAP2 (cIAP2) are intrinsic cellular inhibitors of apoptosis. In order to study the effects of exogenously added IAPs on apoptosis in intestinal epithelial cells, we constructed bacterial expression plasmids containing genes of XIAP (full-length, BIR2 domain and BIR3-RING domain with and without mutations of auto-ubiquitylation sites) and cIAP2 proteins fused to a protein-transduction domain (PTD) derived from HIV-1 Tat protein (TAT) and purified these cell-permeable recombinant proteins. When the TAT-conjugated IAPs were added to rat intestinal epithelial cells IEC6, these proteins were effectively delivered into the cells and inhibited apoptosis, even when added after irradiation. Our results suggest that PTD-mediated delivery of IAPs may have clinical potential, not only for radioprotection but also for rescuing the GI system from radiation injuries.

INTRODUCTION

Exposure to high-dose ionizing radiation results in severe radiation injuries [1]. Protection of normal tissue from the toxic effects of radiation is clinically important in radiation therapy for cancer, and treatments are also sought for tissue damage resulting from radiation accidents. The gastrointestinal (GI) tract is one of the most sensitive organs to radiation, and lethal damage to the GI tract causes acute radiation syndrome (ARS). Experiences with accidents involving whole-body exposure have revealed that GI syndrome is the primary limiting factor affecting a patient’s survival or mortality, since exposure to high-dose radiation leads to the involvement of multiple organs [2]. With increasing irradiation doses, apoptosis occurs in the intestinal crypt stem cells, and they cannot produce enough new cells to repopulate the villi, resulting in blunting and diminution of villus height and eventual functional incapacity [3, 4]. There is still debate as to whether vascular endothelial cells also have primary involvement in GI syndrome caused by high-dose irradiation [5, 6, 7]. It has been demonstrated, however, that increased apoptosis and reduced cell proliferation in the intestinal epithelium play a crucial role in critical illness of both infectious and non-infectious origins [3, 8, 9]. Thus, it is very important to find effective and useful substances for the protection and/or rescue of GI cells from radiation-induced cell death. This is further complicated by the fact that the mechanism for radiation-induced GI syndrome remains unclear.

Apoptotic responses are mediated by the sequential activation of caspases, a family of cysteine proteases [10, 11]. Caspase is activated by the proteolytic processing of caspase itself. When initiator caspases, such as caspase-8, -9 and -10, are activated, they in turn activate effector caspases, such as caspase-3 and -7. Once caspase-3 is activated, it proteolytically inactivates inhibitor of CAD (ICAD), thereby activating caspase-activated DNase (CAD), which is responsible for nuclear DNA fragmentation during apoptosis [12]. Activation of caspase-8 and -10 is required for a cell death ligand to bind to its cell surface receptor. TNF-α is a one of the ligands, the production of which is induced upon DNA...
damage. On the other hand, caspase-9 is activated when cytochrome c is released from mitochondria, and then apoptosisome complex (containing caspase-9, cytochrome c and Apaf-1) is formed. Pro-apoptotic proteins, such as p53 upregulated modulator of apoptosis (PUMA) and Bax, facilitate the release of cytochrome c from mitochondria. Some of the pro-apoptotic genes are transcriptionally activated by p53 upon DNA damage, including that caused by radiation. Knock-out of the PUMA gene has been shown to lead to resistance to intestinal epithelial apoptosis caused by radiation, thereby suppressing GI syndrome in experimental animals [13]. These results suggest the involvement of DNA damage-induced apoptosis in GI syndrome, implying that inhibition of apoptosis is useful for prevention of (or rescue from) the syndrome.

X-linked inhibitor of apoptosis (XIAP) and cellular IAP 1 and 2 (cIAP1 and 2) are intrinsic cellular inhibitors of apoptosis [14]. IAPs directly or indirectly inhibit caspase activity. All IAPs contain the baculovirus IAP repeat (BIR) domain. XIAP is the best-characterized IAP in terms of both its structure and biochemical mechanism. XIAP contains three N-terminal BIR domains (BIR1, BIR2, and BIR3) and a C-terminal Really Interesting New Gene (RING) finger domain. The BIR1 domain of XIAP is involved in NF-κB activation, a signaling event that promotes cell survival [15]. The linker region between BIR1 and BIR2 inhibits caspase-3, whereas both the linker region and the BIR2 domain inhibit caspase-7. The linker region of XIAP binds the substrate-binding active site of caspase-3 and -7, thereby inhibiting substrate entry [16]. On the other hand, the BIR3 domain of XIAP inhibits caspase-9 by sequestering caspase-9 in a catalytically inactive monomeric state [17]. The RING domain of XIAP acts as an E3 enzyme in the ubiquitination pathway and is required for the ubiquitin-mediated degradation of both XIAP itself and the target proteins, such as caspase-3 [18, 19]. Self-ubiquitylation sites of human XIAP, Lys 322 and Lys 328, are identified in the BIR3 domain [20]. The XIAP mutants that have replaced the ubiquitylation sites by arginine or deleted the RING domain still possess anti-apoptotic activity. cIAP1 and cIAP2 also contain the RING domain and act as E3 enzymes. cIAP1 and cIAP2 associate with TNF receptor 1 (TNF-R1) via interaction with TNF-R-associated factor 1 and 2 (TRAF1 and TRAF2). Upon binding of TNF-α to the receptor, cIAP1 and cIAP2 induce ubiquitylation of receptor-interacting protein 1 (RIP1), which also associates with TNF-R1. Ubiquitylation of RIP1 blocks the complex formation to induce activation of caspase-8, and instead induces NF-κB activation [21]. In addition, cIAPs have been shown to induce ubiquitylation of an apoptosis inducer, Smac, and also caspase-3 and -7, suggesting that cIAPs inhibit apoptosis through ubiquitin-mediated degradation of the death inducers [22, 23]. However, the exact molecular mechanism of the anti-apoptotic activity of cIAP1 and cIAP2 is still not well understood.

Radiation-induced apoptosis is inhibited by the overexpression of XIAP, cIAP1 and cIAP2 in human cells [24, 25], suggesting the possibility that these IAPs may be useful for protection against radiation-induced GI cell death. Further studies will be needed to confirm this hypothesis. In addition, gene delivery systems into cells (especially irradiated cells) by introducing vehicles such as viral vectors presents several problems. Delivered exogenous sequences may induce unknown genetic changes by random integration into target cells. Moreover, time remains an issue: DNA transfection requires time until the protein is produced via a complicated mechanism including transcription and translation after transfection with the gene. The delayed production of underlined proteins by gene expression is not suitable, especially for the prevention or suppression of apoptosis after exposure to radiation, because there are cells that commit apoptosis within a few hours.

Recently, protein-transduction domains (PTDs) have been identified as possessing the ability to transduce protein into cells. PTD-linked peptides and proteins can be transduced into cells simply by adding them to tissue-culture medium [26]. The constructs are internalized in a rapid, concentration-dependent manner that achieves maximum intracellular concentration in 1 h. Among these, PTDs such as the 11 amino acids derived from the HIV-1 Tat protein (TAT) (Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Arg-Arg) appear to have the highest level of protein-transduction efficiency in a range of mammalian cells [26, 27]. In the present study, we purified several Tat-conjugated IAPs. We found that these PTD-linked proteins could be effectively transduced into irradiated rat small intestinal epithelial cells (IEC6) by adding them to the culture medium, inhibiting radiation-induced cell death. Moreover, the inhibitory effects also occurred when the proteins were added to cell culture after irradiation.

**MATERIALS AND METHODS**

**Cell culture**

IEC6 cells were maintained in the presence of Dulbecco’s modified Eagle’s medium (DMEM)-high glucose (Life Technologies, Carlsbad, CA) containing 5% fetal calf serum (FCS), 4 μg/ml insulin, 50 units/ml penicillin (Life Technologies) and 50 μg/ml streptomycin (Life Technologies). Cells were grown at 37°C under 5% CO2.

**Plasmids**

cDNAs of full-length, BIR2 domain (residues 150–250) and BIR3-RING domain (residues 240–496) of rat XIAP containing a BamHI site and HA tag at the N-terminal and a XhoI site and TAT sequence at the C-terminal were amplified by PCR of the cDNA library derived from IEC-6-cells with the following primers for full-length XIAP: (forward, 5′-CGGGATCCTACCCCATACGATTCCAGATCCGACTTTAACAGTTTGAAGGATCTAGAAC-3′; reverse,
5'-CCGCTCGAGCTAGCAGCGCCGCTGGCAGCTTTCTACCCATAGGACGAGAAGAACT-3' for the N-terminal and a TAT sequence at the C-terminal was named pGEX-BIR3-RING(2R)-TAT. The PCR products were amplified by PCR with the following primers: (forward, 5'-GGGACGAGGACCGCACTTTGTCACCGGGATCGTTCTCAGT TACAGCGTACGTTTCACTAGACGACGAGAAGAACT-3'; reverse, 5'-GGGGACCACTTTGTCACCGGGATCGTTCTCAGT TACAGCGTACGTTTCACTAGACGACGAGAAGAACT-3').

The constructed plasmids were named pGEX-XIAP-TAT, pGEX-BIR2-TAT and pGEX-BIR3-RING-TAT, respectively. The cDNA of full-length rat cIAP2 containing a FLAG tag at the N-terminal and a TAT sequence at the C-terminal was obtained with the following primers: (forward, 5'-CCGCTCGAGCTAGCAGCGCCGCTGGCAGCTTTCTACCCATAGGACGAGAAGAACT-3'; reverse, 5'-CCGCTCGAGCTAGCAGCGCCGCTGGCAGCTTTCTACCCATAGGACGAGAAGAACT-3'). The cDNA of full-length rat cIAP2 containing an HA tag at the N-terminal and a TAT sequence at the C-terminal was amplified by PCR with the following primers: (forward, 5'-GGGACGAGGACCGCACTTTGTCACCGGGATCGTTCTCAGT TACAGCGTACGTTTCACTAGACGACGAGAAGAACT-3'; reverse, 5'-GGGGACCACTTTGTCACCGGGATCGTTCTCAGT TACAGCGTACGTTTCACTAGACGACGAGAAGAACT-3').

All TAT-conjugated XIAP and GFP-TAT proteins were purified at 37°C with 0.1 mM IPTG overnight. GFP protein was expressed in Escherichia coli strain Origami B containing the plasmid pTF16. Bacteria was grown in L-broth containing 0.5 mg/ml of L-arabinose at 37°C. When the density reached ~1 × 10⁸ cells/ml, protein production was induced at 20°C with 0.1 mM IPTG overnight. GFP protein was expressed in Escherichia coli strain BL21(DE3). When the density reached ~1 × 10⁸ cells/ml, protein production was induced at 37°C with 0.1 mM IPTG for 5 h.

Expression and purification of recombinant proteins

All TAT-conjugated XIAP and GFP-TAT proteins were expressed in Escherichia coli strain BL21(DE3) containing the plasmid pATYCDuet-TrxA. Bacteria were grown at 30°C to an optical density of 0.6, and protein production was induced at 30°C with 0.4 mM isopropyl-b-D-thiogalactopyranoside (IPTG) for 2 h. cIAP2-TAT protein was expressed in Escherichia coli strain Origami B containing the plasmid pTF16. Bacteria was grown in L-broth containing 0.5 mg/ml of L-arabinose at 37°C. When the density reached ~1 × 10⁸ cells/ml, protein production was induced at 20°C with 0.1 mM IPTG overnight. GFP protein was expressed in Escherichia coli strain BL21(DE3). When the density reached ~1 × 10⁸ cells/ml, protein production was induced at 37°C with 0.1 mM IPTG for 5 h.

For purification of TAT-conjugated IAPs, the collected cells were resuspended in about 5 ml/g of PBS lysis buffer [140 mM NaCl, 2.7 mM KCl, 10% glycerol, 1 mM dithiothreitol (DTT), 10 mM NaHPO₄, 1.8 mM KH₂PO₄, pH 7.4] containing lysozyme (0.25 mg/ml), phenylmethylsulfonyl fluoride (PMSF, 1 mM), benzamidine (1 mM), and Complete EDTA-free Protease Inhibitor Cocktail (one tablet/ml; Roche Applied Science, Basel, Switzerland). After cell disruption by ultrasonication, the cell suspension was centrifuged at 14 000×g for 30 min and at 77 000×g for 60 min. The extract was loaded onto a GSTrap HP column (GE Healthcare) equilibrated with PBS lysis buffer. After washing with the same buffer, the column was equilibrated with PreScission buffer [50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% glycerol]. PreScission protease (800 units/ml column; GE Healthcare) was loaded onto the column, and the column was incubated at 4°C overnight.

For purification of GFP-TAT and GFP proteins, the collected cells were resuspended in ~5 ml/g of sonication buffer [50 mM sodium phosphate (pH 7.4), 250 mM NaCl, 10%...
glycerol] containing lysozyme (0.25 mg/ml), phenylmethylsulfonyl fluoride (PMSF, 1 mM), benzamidine (1 mM), and Complete EDTA-free Protease Inhibitor Cocktail (one tablet/ml). After cell disruption by ultrasonication, the cell suspension was centrifuged at 14,000g for 30 min and at 77,000g for 60 min. The extract was loaded onto a HisTrap HP column (GE HealthCare) equilibrated with HisTrap buffer [50 mM sodium phosphate (pH 7.4), 500 mM NaCl, 10% glycerol] containing 20 mM of imidazole. After washing with the same buffer, proteins were eluted with a 20–250 mM imidazole gradient in HisTrap buffer. The fractions containing GFP-TAT or GFP were identified by SDS-PAGE followed by CBB staining.

**In vitro ubiquitylation assay**

Ubiquitylation assay was carried out using an ubiquitylation reaction mixture (20 μl) contained the following components: ubiquitin conjugation buffer, 20 U/ml inorganic pyrophosphate solution, 1 mM DTT, 5 mM Mg-ATP, 100 mM E1, 2.5 μM E2 (UBch5a, -b and -c), 2.5 μM biotinylated ubiquitin and 0.2 μM of the target proteins (IAPs). The reactions were incubated at 37°C for 1 h, stopped by adding an equal volume of 2× Laemmli sample buffer and boiling at 95°C, and subjected to SDS-PAGE followed by immunoblot analyses using anti-HA antibody or Streptavidin–Alkaline Phosphatase (AP) conjugate (Roche Applied Science).

**Cell death assay**

For the cell death assay, IEC6 cells (0.15 × 10⁵ cells per well) were seeded in a 4-well Lab-Tek chambered coverglass (Thermo Fisher Scientific, San Jose, CA) and cultured for 48 h, and then the cells were subjected to Cs137 gamma-ray irradiation. At 20 h after irradiation, apoptotic cell death was determined using a Cell Death Detection ELISA PLUS kit (Roche Applied Science) following the manufacturer’s protocol. In brief, floating cells were collected from the culture medium by centrifugation at 2400g for 5 min at 4°C. Both floating and attached cells were lysed in 500 μl of lysis buffer, and the supernatants (cytoplasmic fraction) were collected by centrifugation at 900g for 5 min at 25°C. After 1:4 dilution, the supernatants (20 μl) were added to a streptavidin–coated microplate. Then, an immunoreagent containing anti-histone–biotin and anti-DNA-POD was added to each well of the microplate, and the samples were incubated under shaking for 2 h at room temperature. The microplate was washed three times with incubation buffer (250μl/well), and 100 μl of 2,2′-azino-di-3-ethylbenzthiazoline sulfonate (ABTS) was added to each well for colour development. Optical density at 405 nm was read using a microplate-luminometer (Wallac 1420 ARVO SX; Perkin-Elmer, Waltham, MA). The assay is based on a quantitative sandwich–enzyme-immunoassay-principle using antibodies against DNA and histone, respectively. This allows the specific determination of cytoplasmic histone-associated DNA fragments after induced cell death. The absorbance value at 405 nm represents the cytoplasmic histone-associated DNA fragments of cell lysates. The extent of apoptosis was measured by the change in absorbance value at 405 nm at room temperature.

**Caspase-3/-7/-9 activity assay**

A caspase-3/-7 Assay kit (Ac-DEVD-AMC substrate) (Merck Millipore) was used to measure the caspase-3/-7 activity. In brief, 50 ng of activated caspase-3 (Merck Millipore) or 20 ng of activated caspase-7 (R&D Systems, Emeryville, CA) was mixed with the indicated amounts of GFP or IAPs in 50 μl of 1.5x caspase buffer (30 mM PIPES, pH7.2, 112.5 mM NaCl, 3.75 mM EDTA, 0.15% CHAPS, 11.25% sucrose, 15 mM DTT). After incubation at 37°C or at room temperature, 25 μl of 3x caspase substrate (a mixture of 75 μl of Ac-DEVD-AMC solution in the kit with 2.425 ml of 1.5X caspase buffer) was added to the reaction mixture. Following the addition of the caspase substrate, the fluorescence intensity resulting from the cleavage of Ac-DEVD-AMC in each sample was measured with excitation at 355 nm and emission at 460 nm with the Wallac 1420 ARVO SX microplate-luminometer (Perkin-Elmer) for 5–60 min. The caspase-9 activity was measured with IEC6 cell extracts supplemented with bovine heart cytochrome c (Sigma–Aldrich, St Louis, MO) and dATP (Sigma–Aldrich) for the activation of caspase-9. Preparation of cell extracts and activation of caspases in the cell extracts followed the protocol for the activity assay of recombinant human XIAP BIR3 domain (R&D Systems). Briefly, IEC6 cells were solubilized in ice-cold extraction buffer [50 mM Hepes (pH 7.5), 10 mM KCl, 5 mM EGTA, 1 mM MgCl2, 0.2% Chaps, 0.2 mM DTT] at a density of 2 × 10⁸ cells/ml after washing twice with phosphate-buffered saline (PBS). Cell extracts were centrifuged at 14,000g for 5 min at 4°C. Then, 10 μl of the supernatants was mixed with 5 μl of cytochrome c (1 mg/ml stock) and 5 μl of dATP (5.0 mM stock) in the presence or absence of IAPs or caspase-9 inhibitor peptide LEHDCCHO (Takara), and the sample mixtures were incubated at 37°C for 1 h. DEVD cleavage assay was carried out by adding 80 μl of assay buffer [10 mM Hepes (pH 7.5), 0.5 mM EGTA, 5 mM DTT, 10% glycerol] and 20 μl of Ac-DEVD-AMC substrate (1/15 diluted solution of the Ac-DEVD-AMC stock solution of the kit in the assay buffer) to the sample mixtures, and incubating the mixture at 37°C for 60 min. The fluorescence intensity of each sample was measured as described above.

**Fluorescence microscopy**

The transduction of TAT-conjugated IAPs into IEC6 cells was measured with immunofluorescent staining, for which cells were cultured in 35-mm glass-base dishes (IWAKI, Chiba, Japan). Following exposure to TAT-IAPs for 1 h, cells were fixed with 4% paraformaldehyde for 30 min,
After the growth medium was replaced by Hanks Salt Solution (HBSS; Life Technologies), the cells were exposed to 2.5 μg/ml of the proteins for 1 h. Because BIR2-TAT does not possess the RING domain that is required for the recruitment of E2 protein, ubiquitylation by an in vitro assay. Ubiquitin (Ub) reaction is mediated by ubiquitin-activating enzyme (E1), its carrier protein (E2), ligase (E3) and Ub itself. In this assay, we used biotinylated Ub instead of an unmodified one. As shown in Fig. 2A, we detected ubiquitylation in these recombinant proteins by western blot analysis using the Streptavidin-AP detection system as described in the Methods and Materials. In the absence of E1, ubiquitylation was not detected. When both E1 and E2 were incubated with Ub, Ub-E2 thioester intermediate was detected in the cells. On the other hand, when the full length XIAP-TAT or cIAP2-TAT was included in the reaction mixture, specific bands corresponding to the ubiquitylated XIAP-TAT and cIAP2-TAT were observed. These results indicate that purified TAT-conjugated rat XIAP and cIAP2 possess E3 ligase activities. We also used anti-HA antibody for detection of ubiquitylated proteins by western blotting, since all of the recombinant IAPs contain HA tag (Fig. 2B).

For the fluorescence microscopy of GFP-TAT and GFP, IEC6 cells were exposed to 2.5 μg/ml of Hoechst 33258 to counterstain nuclear DNA. All antibodies were diluted with PBS containing 1% BSA. Dishes were mounted in drops of Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence microscopy was performed using an Olympus IX70 instrument and Meta Morph software (Molecular Devices, Sunnyvale, CA).

For the fluorescence microscopy of GFP-TAT and GFP, IEC6 cells were exposed to 2.5 μg/ml of the proteins for 1 h. After the growth medium was replaced by Hanks’ Balanced Salt Solution (HBSS; Life Technologies), the cells were examined with the IX70 instrument.

**RESULTS**

**Purification and biochemical analysis of recombinant TAT-conjugated IAPs**

In order to study the effects of TAT-conjugated IAPs on radiation-induced apoptosis in IEC6 cells, we constructed bacterial expression plasmids containing full-length cIAP2, and a series of XIAP (full-length, BIR2 and BIR3-RING) with or without substitution of lysine to arginine at the putative ubiquitylation sites (Lys321 and Lys327)) (Fig. 1A). cIAP2 and XIAPs were expressed in the soluble fraction only when co-expressed with bacterial chaperon protein, either Tig or TrxA. We purified cIAP2-TAT, XIAP-TAT, BIR2-TAT, BIR3-RING-TAT and the BIR3-RING-TAT mutant (BIR3-RING (2R)-TAT) from the soluble fraction in native condition (Fig. 1B). As a control, we also purified the GFP protein with or without the TAT sequence (data not shown).

**Major ubiquitylation sites of human XIAP**

Amino acid sequence alignment shows that Lys321 and Lys327 of rat XIAP correspond to Lys322 and Lys328 of human XIAP, respectively. We confirmed whether Lys321 and Lys327 of rat XIAP were ubiquitylated by an in vitro assay. Ubiquitin (Ub) reaction is mediated by ubiquitin-activating enzyme (E1), its carrier protein (E2), ligase (E3) and Ub itself. In this assay, we used biotinylated Ub instead of an unmodified one. As shown in Fig. 2A, we detected ubiquitylation in these recombinant proteins by western blot analysis using the Streptavidin-AP detection system as described in the Methods and Materials. In the absence of E1, ubiquitylation was not detected. When both E1 and E2 were incubated with Ub, Ub-E2 thioester intermediate was detected in the cells. On the other hand, when the full length XIAP-TAT or cIAP2-TAT was included in the reaction mixture, specific bands corresponding to the ubiquitylated XIAP-TAT and cIAP2-TAT were observed. These results indicate that purified TAT-conjugated rat XIAP and cIAP2 possess E3 ligase activities. We also used anti-HA antibody for detection of ubiquitylated proteins by western blotting, since all of the recombinant IAPs contain HA tag (Fig. 2B).

The introduction of mutation at Lys321 and Lys327 resulted in reduced ubiquitylation of BIR3-RING-TAT. From these results, we conclude that rat BIR3-RING is mainly ubiquitylated at Lys321 and Lys327. Because BIR2-TAT does not possess the RING domain that is required for the recruitment of E2 protein, ubiquitylation of BIR2-TAT was not detected, as expected.

**Effects of TAT-conjugated IAPs on caspase activity**

We next examined whether TAT-conjugated recombinant IAPs inhibit caspase activity. For this purpose, we used in vitro assays for activated caspase-3- and -7-mediated cleavage of a peptide substrate containing the sequence DEVD, and conjugated a fluorescent compound, aminomethyl coumarin (AMC). The intact substrate Ac-DEVD-AMC is non-fluorescent. However, when the peptides are cleaved by the activated caspase, the resulting cleaved product emits fluorescence. Therefore, caspase activity can be measured by fluorescence of the cleaved product. Addition of the XIAP-TAT protein to the reaction mixture markedly reduced the caspase-3-mediated DEVD-cleavage in a dose-dependent manner; a significant reduction was observed with the addition of 0.8 μM of the protein, reaching a ~90% reduction level at >5 μM (Fig. 3A), whereas the addition of control GFP-TAT protein had no significant effect on the cleavage (Fig. 3B). BIR2-TAT or cIAP2-TAT did not inhibit the caspase-3-mediated DEVD-cleavage (Fig. 3C and D). Our purified BIR2-TAT did not contain the linker region, which is required for the inhibition of caspase-3. Because the BIR2 domain of XIAP is responsible for inhibition of caspase-7, we next examined the effect of BIR2-TAT on the caspase-7-mediated DEVD-cleavage. We found that caspase-7 mediated DEVD-cleavage was inhibited by BIR2-TAT (Fig. 3E).
Fig. 1. Structures and purification of TAT-conjugated recombinant IAPs. (A) Schematic representation of TAT-conjugated recombinant IAPs. The recombinant proteins contain an HA tag at the N-terminal and a TAT sequence at the C-terminal. The locations of the BIR domains, RING domain and caspase recruitment domain (CARD) are shown in rectangles. Mutation sites of BIR3-RING (2R)-TAT are shown as K321R or K327R. (B) Purification of TAT-conjugated recombinant IAPs. Purified proteins were subjected to SDS-PAGE (with 5–20% gradient gel) followed by Coomassie Blue staining. Lane 1, cIAP2-TAT; Lane 2, XIAP (FL)-TAT; Lane 3, BIR2-TAT; Lane 4, BIR3-RING-TAT; Lane 5, BIR3-RING (2R)-TAT; Lane M, molecular weight markers. The arrows indicate each purified protein.

Fig. 2. Cell-free ubiquitylation of IAPs. Cell-free ubiquitylation reactions were performed using the indicated sets of protein components. Aliquots of the reaction mixtures were subjected to immune-blot analyses using Streptavidin-AP conjugate (panel A) or anti-HA antibody (panel B). The arrows indicate the unmodified or ubiquitylated form of each protein. Molecular weight markers are indicated on the left.
Fig. 3. Effects of TAT-conjugated IAPs on caspase activity. Inhibitory effect of TAT-conjugated IAPs on caspase activity was measured using Ac-DEVD-AMC substrate as described in the Materials and Methods. (A–D) Caspase-3 activity in cells transduced proteins. The reactions were performed using 50 ng of active caspase-3, and the indicated amounts of TAT-conjugated XIAP (A), GFP (B), BIR2 (C), or cIAP2 (D) are shown. After 30 min incubation at 37°C, caspase-3 activity was determined by measuring Ac-DEVD-AMC hydrolysis. Values are presented as mean and standard deviation of the experiments in triplicate. (E)
XIAP-TAT also inhibited the caspase-7-mediated DEVD-cleavage much more strongly (~90% reduction) than BIR2-TAT (~40–50% reduction) (Fig. 3E). Because the BIR3 domain of XIAP inhibits caspase-9, we indirectly examined the effect of BIR3-RING-TAT on caspase-9 activity by measuring the activated caspase-3- and -7-mediated DEVD-cleavages following the activation of caspase-9. In this assay, we used cell extracts from IEC6 cells and added cytochrome c and dATP to them, which allowed formation of an apoptosis and caspase-9 activation, and then caspase-3 and -7 activation in the cell extracts. The addition of a caspase-9 inhibitor, LEHD peptide, blocked the DEVD-cleavage (~80% reduction). We also observed that BIR3-RING-TAT or BIR3-RING (2R)-TAT inhibited the DEVD-cleavage (~30% reduction), whereas GFP-TAT failed, indicating that both BIR3-RING-TAT and BIR3-RING (2R)-TAT proteins can specifically inhibit caspase-9 activity (Fig. 3F). Taken together, these results suggest that our purified TAT-conjugated IAPs possess biochemical activities, such as E3 ligase and/or caspase-inhibitory activities.

Transduction of TAT-conjugated IAPs into IEC6 cells

We examined whether our purified TAT-conjugated proteins were delivered into IEC6 cells. Recombinant proteins were added to cultures of IEC6 cells, and 1 h later, the protein transduction was analyzed by fluorescence microscopy. As shown in Fig. 4A, transduction of the GFP protein was observed when GFP protein was conjugated with TAT sequence, which was consistent with previous results [28]. We also observed the transduction of TAT-conjugated cIAP2 protein and a series of XIAP by fluorescence microscopy (Fig. 4B).

We next studied the protein transduction efficiency. Recombinant proteins were added to IEC6 cells and the cells were incubated for 1 or 2 h. After washing the cells, whole cell extracts were subjected to Western blot analysis. Since these recombinant proteins contain HA-Tag, we can compare the transduction efficiency of each protein in the same transfer membrane with the same HA antibody. As shown in Fig. 4C, transduction of BIR3-RING-TAT with or without substitutions of lysine to arginine was the most efficient among the TAT-conjugated IAPs. Although transduction of full-length XIAP-TAT was less efficient than that of BIR3-RING-TAT, full-length XIAP-TAT was delivered into the cells more efficiently than the other proteins BIR2-TAT and cIAP2-TAT. Transduction of cIAP2-TAT was the least efficient among these proteins. Prior reports have shown that the transduction efficiency of TAT-conjugated proteins was increased in a time-dependent manner within 1 h [29]. In the present study, we showed that the cellular concentration of cIAP2-TAT increased in a time-dependent manner until 2 h after addition of the protein to the culture. On the other hand, there was no difference in cellular concentrations of the other proteins between 1 and 2 h.

Stability of TAT-conjugated IAPs in IEC6 cells

Intracellular stability of protein is one of the important mechanisms for enzymatic activity of the protein in cells. Therefore, we next examined the stability of the transduced TAT-conjugated IAPs in IEC6 cells. IEC6 cells were treated with each of the TAT-conjugated IAPs for 1 h, cultured in new culture medium without the protein for the indicated durations, and then sequentially harvested (Fig. 5A and B). Transduced XIAP-TAT, BIR2-TAT and cIAP2-TAT proteins were degraded within 7 h. In contrast, the levels of the BIR3-RING-TAT and BIR3-RING (2R)-TAT proteins were decreased in a time-dependent manner; the remaining levels of the BIR3-RING-TAT and BIR3-RING (2R)-TAT proteins were 11.9% and 35.7% after 7 h of those at washing, respectively, and these proteins were still detected even at 23 h after the transduction. Thus, the BIR3-RING-TAT and BIR3-RING (2R)-TAT proteins were more stable than the other proteins, with BIR3-RING (2R)-TAT being the most stable. Thus, mutations at the ubiquitylation sites increased the intracellular protein stability.

In parallel, we also cultured cells in medium containing each of the TAT-conjugated IAPs without washing; cells were continuously cultured in medium with the protein. Then, the levels of these proteins were sequentially examined (Fig. 6A and B). Significantly increased levels of the TAT-conjugated IAPs were detected even 24 h after their addition to the cells as compared with levels without their addition. The levels of BIR3-RING-TAT and BIR3-RING (2R)-TAT were decreased by ~40% at 24 h; the levels of both proteins showed similar kinetics. On the other hand, those of the other proteins decreased to as low as 16–19%.

Inhibition of caspase-7 activity by XIAP-TAT and BIR2-TAT. The reactions were performed using the indicated amounts of TAT-conjugated proteins and 20 ng of activated caspase-7. After 5 min incubation at room temperature, caspase-7 activity was determined by measuring Ac-DEVD-AMC hydrolysis. Values are shown as mean and standard deviation of experiments in triplicate.

(F) Inhibition of caspase-9 activity by BIR3-RING-TAT and BIR3-RING (2R)-TAT. Caspases were activated by the addition of cytochrome c and dATP to IEC6 cell extracts. Caspase-9 inhibitor peptide (LEHD-CHO; 250 μM) or 2.5 μM of GFP-TAT, BIR3-RING-TAT or BIR3-RING (2R)-TAT was added. Values are presented as mean and standard deviation of experiments in triplicate.
Effect of TAT-conjugated IAPs on radiation-induced cell death

To examine whether TAT-conjugated IAPs can inhibit radiation-induced cell death, we studied apoptosis in cells transduced with each of these proteins by determining the levels of apoptosis-induced cytoplasmic histone-associated DNA fragments by ELISA. In this assay, the magnitude of apoptotic cell death is shown as the absorbance value at 405 nm. TAT-conjugated IAPs were added to IEC6 cells either 2 h before or 30 min after γ-irradiation. Cells were cultured in the presence of each protein for 16 h and then apoptosis was determined. The addition of GFP-TAT to IEC6 cells significantly increased the absorption in the cells not irradiated, indicating that transduction of TAT-conjugated protein itself has a certain toxic effect on these cells and induces apoptotic cell death (data not shown). As a control, therefore, we used the GFP-TAT-added cells instead of those with the buffer alone. Treatment of cells with each of these TAT-conjugated IAPs significantly reduced the absorbance in cells not exposed to irradiation (~30–50% reduction), and irradiation at 10 or 20 Gy significantly increased the absorbance in these cells (Fig. 7A, B and C). Pre-treatment with each of these TAT-conjugated IAPs significantly inhibited the radiation-induced apoptosis (~40–55% reduction) (Fig. 6A and C). Importantly, the addition of each of XIAP-TAT, BIR2-TAT, BIR3-RING-TAT, BIR3-RING(2R)-TAT or cIAP-TAT significantly inhibited radiation-induced apoptosis, even after irradiation (~40–50% reduction) (Fig. 7B). On the other hand, cIAP2-TAT failed to inhibit radiation-induced apoptosis when the protein was added to the cells after irradiation (Fig. 7D). Among the TAT-conjugated IAPs, BIR2-TAT, BIR3-RING-TAT and BIR3-RING (2R)-TAT inhibited radiation-induced apoptosis more efficiently than the other TAT-conjugated IAPs.
**DISCUSSION**

GI injury is one of the most serious problems in accidental exposure of the whole body to radiation as well as in cancer radiotherapy of abdominal and pelvic areas. However, no effective treatment has yet been established for radiation-induced GI injury. PTD is useful for the delivery of a range of biologically active recombinant proteins or peptides into various cells [30, 31, 32, 33]. Using this direct delivery system for getting proteins into cells, we determined whether XIAP and cIAP2, which are intrinsic cellular proteins, inhibit radiation-induced apoptosis in rat intestinal IEC-6 cells. Interestingly, these proteins were directly incorporated into cells within 1 h, and anti-apoptotic effects were then observed in these cells. In the present work, we showed that TAT-conjugated full-length proteins (and also the shorter constructs of XIAP) blocked radiation-induced cell death.

Furthermore, radiation-induced apoptosis was inhibited, even when cells were treated with these proteins after irradiation. A prior study reported that glutathione S-transferase (GST)-tagged full-length cIAP1 and cIAP2 proteins, as well as XIAP, prevented the proteolytic processing of pro-caspases-3, -6 and -7 by inhibiting the activation of pro-caspase-9 in vitro [34]. Later, Eckelman and Salvesen showed that cIAP1 and cIAP2 bound to caspases but did not inhibit their activities; the BIR2 domain of cIAP1 and cIAP2 did not inhibit caspase-3 and -7, and the BIR3 domain of the two cIAPs also did not inhibit caspase-9 in vitro [35]. Purifying the full-length cIAP1 protein, furthermore, they also demonstrated the poor (and physiologically insignificant) ability of this protein to inhibit caspase-3, -7 and -9 [35]. Our present results were consistent with those of Eckelman and Salvesen, indicating the inability of the full-length cIAP2-TAT protein to inhibit caspase-3. Taken together, these results suggest...
that the inhibitory mechanism of apoptosis by cIAP2 is exclusively mediated by ubiquitylating proteins involved in apoptosis, rather than by directly inhibiting the proteolytic activities of caspases. The reason for the discrepancy in the results between these studies is not clear. Recently, TAT-mediated transduction has been successful for a variety of protein sizes and biological functions [26]. However, there are problems (including fold of protein) to be resolved technically in the PTD system; the artifacts produced when transducing proteins into cells attributed to a large tag such as GST may affect conformation of the fused protein [7, 19].

Studies have shown that the stably-expressed XIAP lacking the RING domain cannot catalyze self-ubiquitylation in vitro, and that this protein was relatively resistant to protein degradation in cells [18]. Moreover, there are also reports that stably expressed XIAP lacking the RING domain inhibited apoptosis more efficiently than the full-length XIAP, suggesting that mutation of ubiquitylation sites within the BIR3 domain of BIR3-RING-TAT leads to protein stabilization, thereby enhancing the inhibitory effect of the protein on cell death. In our study, mutating the BIR3-RING-TAT protein reduced self-ubiquitylation of the protein in vitro and increased the protein stability as expected. However, no difference in activity for inhibition of radiation-induced apoptosis was observed between the BIR3-RING-TAT (wild type) and BIR3-RING (2R)-TAT (mutated type) proteins. Since the intracellular half-lives of these proteins were much shorter than 7 h, we determined the effects of these proteins on apoptosis in their presence; these proteins were continuously transduced into cells during the experiments. There was no difference in the levels of the two proteins, and the kinetics of the levels of both proteins incorporated into cells were similar. Accumulation of the transduced protein in cells is regulated by the rates of both incorporation and degradation. If the BIR3-RING (2R)-TAT protein is more stable but the rate of incorporation of BIR3-RING-TAT into cells is.

Fig. 6. Levels of transduced TAT-conjugated IAPs after addition to IEC6 cells. (A) IEC6 cells were incubated with 0.5 μM of each of the indicated TAT-conjugated IAPs at 37°C for the indicated durations. Whole cell extracts were then prepared and subjected to immunoblot analysis using anti-HA and anti-β-actin antibodies. (B) Levels of TAT-conjugated IAPs in each lane of panel A were quantified and normalized to the levels of β-actin. Data were plotted as relative levels to β-actin proteins; levels of these proteins in cells treated for 2 h were assumed to be 100%.
greater than that of BIR3-RING (2R)-TAT, there may be no significant difference in cellular levels or inhibitor effects between the two proteins. Further studies using various concentrations are required.

Apoptosis induced by TNF-α is known to play a pivotal role in the development of intestinal inflammation in Crohn’s disease [36]. Anti-TNF-α antibody has now been clinically applied in the treatment of Crohn’s disease, since the antibody blocks TNF-α-induced intestinal epithelial cell apoptosis in the disease [37]. TNF-α has been shown to activate the two opposite signal transductions: pro- and anti-apoptotic pathways. Inhibition of TNF-α by anti-TNF-α antibody suppresses the latter pathway for cell survival mediated via activation of NF-κB. On the other hand, cIAP1 and cIAP2 specifically inhibit the TNF-α-induced apoptotic pathway and promote TNF-α-induced NF-κB activation [21, 38]. We have shown that TAT-conjugated cIAP2 was incorporated into intestinal epithelial cells and inhibited apoptosis of the cells. Therefore, our results suggest the possibility that PTD-mediated delivery of the cIAP2 protein may also be useful for the treatment of Crohn’s disease.

Among the TAT-conjugated IAPs purified, post-treatment with the cIAP2-TAT protein failed to inhibit radiation-induced apoptosis in the present study. Analysis of cell death

Fig. 7. Effects of cell-permeable IAPs on radiation-induced cell death in IEC6 cells. Cells were irradiated with 10 or 20 Gy either 2 h after (A and C) or 30 min before (B and D) addition of 0.5 μM of each TAT-conjugated protein to the cell culture. As a control, GFP-TAT was used. At 16 h after irradiation, the cells were collected for cytotoxic assay. Values are presented as mean and standard deviation from four independent experiments for A and D, seven experiments for B, and six experiments for C. The statistically significant difference between the GFP-TAT-treated control cells and each of the TAT-conjugated IAP-treated cells is indicated by asterisks (*P < 0.05, **P < 0.01, and ***P < 0.001 by t-test).
revealed that the shorter constructs of XIAPs inhibited apoptosis more efficiently than the full-length protein of XIAP. In the present study, we determined the intracellular concentration of the protein 1 or 2 h after its addition to the culture at a concentration of 0.5 μM. Prior reports have shown that the transduction efficiency of TAT-conjugated proteins was increased in a time-dependent manner [29], and that the intracellular concentration can also be controlled by varying the amount added to the culture medium [26]. Our western blot analysis showed less transduction efficiency of cIAP2-TAT than for those of the other TAT-conjugated IAPs. More detailed studies using their higher concentrations and/or longer exposure to the cIAP2-TAT protein may lead to the rescue of cells from irradiation. Further studies are in progress. In the present study, the shorter constructs of XIAPs were expressed much more abundantly in E. coli than the full-length proteins (data not shown). Some proteins are known to be poorly expressed in bacteria or require posttranslational modifications. Therefore, some TAT-fusion proteins may have to be expressed in other systems, such as yeast or baculovirus.

In conclusion, we showed that the PTD-mediated delivery system of several IAPs into cells is useful for prevention and rescue of intestinal epithelial cells from radiation-induced cell death. There may also be other IAPs that are good candidates for PTD-mediated protein therapy for cell death. On the other hand, our results have raised a problem concerning the TAT-mediated delivery system of protein into intestinal epithelial cells, namely, the toxicity for cells. Future work is required to resolve this problem by using a less toxic PTD system for the transduction of protein into intestinal epithelial cells. How to specifically transduce these proteins into intestinal epithelial cells also remains to be established.

FUNDING

This work was supported by the Research Project for High-Dose Radiation Injuries at the National Institute of Radiological Sciences. This work was also partially supported by the Futaba Electronics Memorial Foundation (T.Y.). Funding to pay the Open Access publication charges for this article was provided by National Institute of Radiological Sciences.

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