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Silencing Daxx increases the anti-tumor activity of a TRAIL/shRNA Bcl-xL-expressing oncolytic adenovirus through enhanced viral replication and cellular arrest

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ABSTRACT

We previously showed that an increase of cellular Bcl-xL mediates acquired resistance to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and knockdown of Bcl-xL expression greatly sensitized TRAIL-induced cytotoxicity. Here, we show that Daxx downregulation increases the anti-tumorigenic activity through enhancement of viral replication and cellular arrest with combination of TRAIL/shBcl-xL-induced apoptosis. This study was conducted to determine the effect of Daxx downregulation on the anti-tumorigenesis induced by oncolytic adenovirus arming TRAIL or TRAIL/shRNA of Bcl-xL genes. Unlike the enhanced cancer cell death induced by exogenous TRAIL or TRAIL plus shRNA of Bcl-xL, oncolytic adenovirus expressing TRAIL or TRAIL plus shRNA of Bcl-xL did not show much enhanced cancer cell death compared to oncolytic adenovirus itself. On the other hand, enhanced cytotoxic cell death and viral replication was observed after infection with oncolytic adenovirus expressing TRAIL plus shRNA of Bcl-xL and shRNA of Daxx at the same construct. Then we realized that enhanced adenoviral replication through Daxx downregulation was caused by increased adenoviral E1A protein expression and Daxx downregulation also stimulated cellular arrest through p21/p53 accumulation. Taken all together, we have shown here that Daxx downregulation should be essentially needed for the increase of anti-tumor activity through enhancement of viral replication and cellular arrest with the combination of TRAIL/shBcl-xL-induced apoptosis and oncolytic adenovirus.

1. Introduction

The downregulation of Bcl-xL sensitizes resistant cells to TRAIL-induced apoptotic cell death [1,2], suggesting that Bcl-xL upregulation during acquisition is a critical factor in TRAIL-induced acquired resistance. Bcl-xL is an anti-apoptotic molecule that is critical in oncogenesis and tumor progression, and its expression is controlled by STAT, Rel/NF-κB, Ets, AP1, and activated Ras [3,4]. Further evidence that Bcl-xL is a critical anti-apoptotic molecule against TRAIL-induced apoptosis is that the downregulation of Bcl-xL by siRNA enhances the susceptibility to TRAIL [5], resulting in an increase in TRAIL-mediated apoptosis in human cancer cells with both acquired and intrinsic TRAIL resistance. [5,6]. However, because the apoptosis pathway is seriously compromised in many tumor cells by other compensatory signaling pathways [7], the sensitization to TRAIL-induced apoptosis by the downregulation of Bcl-xL is not always sufficient to cause cell death. One strategy to overcome this resistance to cell death in cancer cells is to exploit the existence of other innate, redundant cell death mechanisms [7,8].

Death domain-associated protein (Daxx) is a highly conserved and ubiquitously expressed nuclear protein. Daxx acts as a transcription factor and can also contribute to the stabilization of apoptosis signal-regulating kinase 1 (ASK1) to increase apoptosis [9,10]. However, there are also controversial reports indicating that Daxx can function as an anti-apoptotic protein. Chen et al. [11] reported that silencing Daxx sensitizes the apoptotic signaling pathway induced by Fas or stress. Zhang et al. [12] suggested that Daxx also regulates Mdm2 degradation by ubiquitination and p53-target ubiquitination functions of Mdm2. According to this report, disrupting Mdm2-Daxx-HAUSP induces Mdm2 degradation and leads to p53 stabilization and

Abbreviations: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; Daxx, death domain-associated protein; STAT, signal transducer and activator of transcription; PI, propidium iodide; FACS, fluorescence-activated cell sorter; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; FITC, fluorescein isothio-cyanate; Mdm2, mouse double minute 2 homolog; HDAC, histone deacetylases; PML, promyelocytic leukemia; PARP, poly(ADP-ribose) polymerase; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay.

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accumulation, whereas a tumorigenic mutant p53 disrupts the Daxx-ASK1-JNK/p38 loop to make cells more stress-tolerant [13]. Some p53 mutants, but not wild-type p53, interact with Daxx and activate stress-inducible kinase pathways to inhibit Daxx-dependent activation of the apoptosis signal-regulating kinase 1 stress-inducible kinases and Jun N-terminal kinase [14]. These data suggest that in the more than 50% of human cancers that contain p53 gene mutations [15,16], Daxx expression would not induce cell death. However, silencing Daxx often induces cellular apoptosis, suggesting that Daxx may play a protective role in preventing apoptosis [11]. Moreover, Daxx silencing would be indispensible when using an oncolytic adenovirus without E1B 55 KD as a gene delivery tool. This is because adenoviral replication can be effectively maintained only when Daxx is degraded due to the E1B 55 KD which can bind Daxx and induce its proteasome-dependent degradation [17,18].

2. Materials and methods

2.1. Cell culture

The human cancer cell lines DU145 (human prostate adenocarcinoma), A375 (human skin melanoma), and HeLa (human cervical adenocarcinoma) were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum (HyClone, Logan, UT, USA). HCT116 cells (p53 +/+, −/−) were purchased from Genetic Resources Core Facility at Johns Hopkins University. All cells were maintained in a 37 °C humidified atmosphere containing 5% CO_2.

2.2. Reagents

Antibodies to Bcl-xL and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to E1A, Daxx and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-adenovirus type 5 (Ad5) antibody was purchased from Abcam (Cambridge, UK), Trizol was purchased from Life Technologies (Carlsbad, CA, USA), and all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.3. Construction of TRAIL-expressing recombinant adenovirus

The adenoviral shuttle vector, pCA14 (Microbix, Mississauga, Ontario, Canada), which contains the IX gene of Ad5, was linearized by *Xmn*I digestion. The adenoviral vector dl324-*Bst*BI, in which nucleotides 340–4641 of Ad5 in the E1 region and nucleotides 28,592–30,470 of Ad5 in the E3 region were deleted, was linearized by *Bst*BI digestion. The linearized vectors were co-transformed into *Escherichia coli* BJ5183 for homologous recombination. The resultant homologous recombinant adenoviral plasmid was named dl324-IX.

To construct the defective adenoviral plasmid expressing TRAIL, pEGFP-TRAIL (Addgene #10953) was cleaved by *Eco*RI–*Sma*I, and the resultant full-length TRAIL gene was subcloned into pCA14, which was digested with *Hin*dIII-blunt-*Eco*RI. Defective dl324-TRAIL adenovirus was produced after homologous recombination (Ad-TRAIL). To construct the oncolytic adenoviral plasmid expressing TRAIL, the pCA14-3484-CMV-ΔE1B55K plasmid, previously described in detail [19], was used to insert the TRAIL gene from pCA14-TRAIL. To insert TRAIL, PCR was performed using a sense primer flanked at the 5′ end with an *Mfe*I site (5′-CCGCAATTGCTAATTCCCTGGCATTATGCCC-3′) and an antisense primer flanked at the 5′ end with a *Bgl*II site (5′-GGAAGATCTTCG ATGCTAGACG-3′). The resultant TRAIL PCR product was digested with *Mfe*I–*Bgl*II, subcloned into pCA14-3484-CMV- ΔE1B55K, digested with *Eco*RI–*Bgl*II, and the oncolytic dl324-3484-TRAIL (Ad-3484-TRAIL) was produced after homologous recombination. For the efficient adenoviral amplification expressing TRAIL, 293A-CrmA stable cell was used after stable transfection of pcDNA3.1 hygro-CrmA. CrmA gene was originated from pcDNA-CrmA (Addgene plasmid 11832) and subcloned into pcDNA3.1 hygro after *Hin*dIII–*Xho*I cleavage to make pcDNA3.1 hygro-CrmA.

2.4. Construction of Bcl-xL and Daxx shRNAs

For the expression of the shRNA targeting human Bcl-xL, the target sequence was 5′-AGGATACAGCTGGAGTCAG-3′, which was previously demonstrated to be effective in achieving reduction of Bcl-xL [20]. The top strand sequence was 5′-GATCCAGGATACAGCTGGAGTCAGTTCAAG AGACTGACTCCAGCTGTATCCTTTTTTTGGAAA-3′, and the bottom strand sequence was 5′-AGCTTTTCCAAAAAAAGGATACAGCTGGAGTCAGTCTC TTGAACTGACTCCAGCTGTATCCT-3′. The annealed Bcl-xL shRNA was subcloned into the pSP72ΔE3-U6, E3 shuttle vector. The adenoviral shuttle vector, pSP72ΔE3-U6-sh-Bcl-xL, was linearized by *Xmn*I digestion. The adenoviral vector dl324-IX was linearized by *Spe*I digestion, and the two linearized vectors were co-transformed into *E. coli* BJ5183 cells for homologous recombination. The homologously recombined adenoviral plasmids, Ad-NC and Ad-shBcl-xL, were then digested with *Pac*I and transfected into HEK-293 cells to generate the replication-incompetent adenovirus. The infectious titer of the adenovirus was determined by a limiting dilution assay in 293A cells.

To construct human Daxx shRNA, we screened five candidate sequences. Target selection was performed using an algorithm developed by Genolution Pharmaceuticals Inc. (Seoul, South Korea). The selected target sequence was 5′- GCTACAAGCTGGAGAATGAGAAGCT-3′ and the loop sequence was 5′-TCTC-3′. To express hDaxx shRNA in adenovirus, the top strand sequence (5′-GATCCGCTACAAGCTGGAGAAT GAGAAGCTTCTCAGCTTCTCATTCTCCAGCTTGTAGCTTTTTTA-3′) and the bottom strand sequence (5′-AGCTTAAAAAA GCTACAAGCTGGAGAATG AGAAGCTGAGAAGCTTCTCATTCTCCAGCTTGTAGC G-3′) were annealed and subcloned into *Bam*HI–*Hin*dIII-digested pSP72ΔE3-H1 sh hTGFβ2, which was constructed by substitution of U6 in pSP72ΔE3/U6-shTGFβ2 with H1 promoter.

2.5. Generation of recombinant adenovirus expressing TRAIL and Bcl-xL shRNA

To produce the defective adenovirus expressing both TRAIL and Bcl-xL shRNA, the adenovirus vector dl324-*Bst*BI-ΔE3-U6-hshBcl-xL was linearized with Bsp1191 and the adenoviral shuttle vector pCA14-Full TRAIL was linearized with *Fsp*I. The two linearized vectors were co-transformed into *E. coli* BJ5183 cells for homologous recombination. To produce the oncolytic adenovirus expressing both TRAIL and Bcl-xL shRNA, dl324-*Bst*BI-ΔE3-U6-shBcl-xL was linearized with Bsp1191, whereas pCA14-3484-ΔE1B55-TRAIL was linearized with *Drd*I.

2.6. Recombinant oncolytic adenovirus expressing TRAIL and shRNAs of Bcl-xL + Daxx

For the construction of the adenoviral shuttle vector expressing Bcl-xL and Daxx shRNAs, pSP72ΔE3-H1-shDaxx was digested with *Sph*I-blunt-*Kpn*I to make an insert with H1 promoter-shDaxx-SV40 poly A to ligate into *Hin*dIII-blunt-*Kpn*I-digested pSP72ΔE3-U6-shBCL-xL, which became the plasmid called pSP72ΔE3-U6-shBcl-xL-H1-shDaxx. This shuttle vector plasmid was co-transformed with dl324-*Bst*BI to generate the homologous recombinant, dl324-BstBI-ΔE3-U6-shBcl-xL-H1-shDaxx. Finally, the Bsp1191 linearized dl324-*Bst*BI-ΔE3-U6-shBcl-xL-H1-shDaxx construct was co-transformed with the *Drd*I–*Fsp*I linearized pCA14-3484–ΔE1B55-CMV-TRAIL construct to generate the homologous recombinant, Ad-3484-TRAIL-shBcl-xL-shDaxx. The schematic diagram of defective or oncolytic adenoviral vector expressing TRAIL and shRNAs of Bcl-xL + Daxx is shown in Fig. 1A. All viruses were amplified for purification, according