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Radiation-induced apoptosis of tumor cells is facilitated by inhibition of the interaction between Survivin and Smac/DIABLO

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Abstract

To investigate the mechanism of radioresistance of solid tumor cells, we created two expression vectors encoding Survivin mutants, T34A and D53A. When T34A and D53A were overexpressed in NIH3T3, A549 and HeLa cells, radiation-induced apoptosis was significantly enhanced. Furthermore, we examined the binding capability of Survivin with Smac/DIABLO in the cells that overexpressed these mutants. Coimmunoprecipitation analysis revealed that mutant form of Survivin, D53A and T34A could bind to Smac/DIABLO, but with much less affinity compared to the authentic form. These results suggest that radiation-induced apoptosis of tumor cells is increased by inhibition of the interaction between Survivin and Smac/DIABLO through overexpression of T34A and D53A.

Keywords: Survivin; Radiation; Apoptosis; Dominant-negative mutant; Adenoviral vector

1. Introduction

Ionizing radiation is a useful tool for cancer therapy. In solid tumor cells derived from adenocarcinoma, squamous cell carcinoma or melanoma, it is generally considered that radiation-induced cell death mainly occurs through reproductive cell death but not apoptotic cell death. However, in hematopoietic cell lines, malignant lymphoma and leukemia cells [1–3], etc., apoptotic cell death is prone to be induced by genotoxic agents, including ionizing radiation. In the treatment combined with some anti-tumor drugs, radiation-induced cell death is sometimes enhanced through an increase of apoptosis even in radioresistant solid tumor cell lines [4–6]. In fact, we have reported that a novel anti-cancer drug, 1-(3-C-ethynyl-β-D-ribo-pentofutanosyl)cytosine (ECyd), enhances radiation-induced apoptosis in human gastric adenocarcinoma MKN45 (p53 wild-type), MKN28 (p53 mutation) and murine

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rectum adenocarcinoma Colon26 (p53 status unknown) cells. The decrease in the radiation-induced expression of Survivin, Bcl-2, cyclin B1 and Wee1 was partly responsible for the enhancement of radiation-induced apoptosis by ECyd regardless of p53 status and cell type *in vitro* [7] and *in vivo* [8]. Furthermore, in MKN45 and MKN28 cells, purvalanol A, a cyclin-dependent kinase inhibitor, also enhances radiation-induced cell killing through an increase of apoptosis by downregulation of inhibitor of apoptosis (IAP) family members (Survivin and XIAP), Bcl-2 family members (Bcl-X_L and Bcl-2), cyclin B1 and Wee1 [9]. Since it is generally considered that ionizing radiation promotes apoptotic signaling such as cytochrome *c* release from mitochondria, followed by activation of caspase-9, -3 or the caspase-activated deoxyribonuclease (DNase) (CAD) pathway [10,11], the constitutive or inducible anti-apoptotic proteins seem to inhibit this apoptotic signaling in the radioresistance of solid tumor cells.

Survivin is a member of the inhibitor of apoptosis protein (IAP) family protein [12], which is intensively expressed in the G_2/M phase and potentially involved in both inhibition of apoptosis and control of cell division [13–16]. Its expression is prominently upregulated in most human cancer cells, but is undetectable or very low in normal tissues [17,18]. The high expression of Survivin in cancer cells is a marker of negative prognostic significance and a cause of tumor resistance to therapies [19–22]. The phosphorylation at threonine 34 of Survivin by Cdc2 is accepted to be essential for the anti-apoptotic activity of Survivin [23]; however, the anti-apoptotic mechanisms of Survivin have remained controversial. Early studies suggested that the Survivin directly bound to caspase-3 and suppressed apoptosis [24,25]. However, others failed to demonstrate direct effects on this protease [26–28]. As another possible mechanism, Song et al. [29] recently showed that Survivin bound to Smac/DIABLO, which is known as a proapoptotic protein that binds to IAPs such as XIAP, and prevents apoptosis by suppressing caspase-9 and -3. The replacement to alanine at aspartic acid 53 (D53A) of Survivin has been demonstrated to fail to associate with Smac/DIABLO and the disruption of this binding activity enhances TRAIL-, doxorubicin- and RIP3-induced apoptosis in HeLa cells [30]. However, the relationship between the phosphorylation of Survivin and its binding activity with Smac/DIABLO is still unclear.

In the present study, we examined whether ionizing radiation promoted cytochrome *c* release from mitochondria in A549 cells and Survivin was involved in inhibition of apoptotic signaling downstream of mitochondria. For this purpose, two expression vectors encoding Survivin mutants, T34A (phosphorylation-defect mutant) and D53A (defective in binding activity with Smac/DIABLO) were created. Furthermore, binding activity of Survivin with Smac/DIABLO in the cells overexpressing these mutants was also studied.

2. Materials and methods

2.1. Reagents

Carbobenzoxy-Val-Ala-Asp-fluoromethane (Z-VAD-fmk), acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO), acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) and acetyl-Ile-Glu-His-Asp-aldehyde (Ac-IETD-CHO) were from Peptide Institute (Osaka, Japan). Propidium iodide (PI) and nocodazole were from Sigma Chemical Company (St. Louis, MO). ^{32}Pi (orthophosphoric acid) was from MP Biomedicals, Inc. (Irvine, CA). Protein G-Sepharose was from Amersham-Pharmacia Biotech (Buckinghamshire, UK). The following antibodies were used for Western blotting and immunoprecipitation: anti-Survivin and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-cytochrome *c* (BD PharMingen, Erebodegem, Belgium), anti-Smac/DIABLO (Calbiochem) and anti-cleaved caspase-3 (Cell Signaling Technology, Beverly, MA). The chemiluminescence detection kit, Western Lighting™ Chemiluminescence Reagent Plus, was from Perkin-Elmer (Boston, MA).

2.2. Cell culture

Mouse fibroblast cell line NIH3T3, embryonic kidney cell line 293A and human cervical carcinoma cell line HeLa were grown in DMEM containing 10% fetal calf serum at 37 °C in 5% CO_2. Human lung carcinoma A549 cells were maintained in RPMI 1640 medium containing 10% fetal calf serum at 37 °C in 5% CO_2.

2.3. PCR-mediated mutagenesis

The cDNA of Survivin was amplified by RT-PCR from total RNA of cell line HeLa and cloned into pCR2.1-TOPO (Invitrogen, Carlsbad, CA). To obtain the Survivin mutants, Survivin-T34A (T34A), -D53A (D53A), -T34E (T34E) and -T34D (T34D), we employed PCR-mediated mutagenesis. Mutations were further verified using the CEQ8000 DNA Analysis System (Beckman Coulter, Fullerton, CA).