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Ionizing radiation and nitric oxide donor sensitize Fas-induced apoptosis via up-regulation of Fas in human cervical cancer cells

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Abstract

Fas/CD95/Apo1 is a transmembrane receptor known to trigger apoptotic cell death in several cell types. In the present study, we showed that ionizing radiation (IR) and NO donor, S-nitroso-N-acetyl-penicillamine (SNAP), sensitized Fas-induced apoptotic cell death of HeLa human cervical cancers. Suboptimal dose of IR and SNAP up-regulated cell-surface Fas antigen, detected by FACScan using FITC-anti-Fas antibody. When combined with IR or SNAP, agonistic anti-Fas antibody CH-11 resulted in marked enhancement of apoptosis. This sensitization was completely abrogated by anti-Fas neutralizing antibody ZB4. During the IR and SNAP sensitized Fas-induced apoptosis, mitochondria permeabilization, cytochrome c release, and DNA fragmentation were detected. Furthermore, combined treatment of IR and SNAP additively up-regulated the surface Fas protein expression and sensitized Fas-induced apoptosis. Our finding demonstrate that sensitization of HeLa cervical cells to Fas-mediated apoptosis by IR and NO donor is most likely due to the up-regulation of Fas expression and also provides a means with which to sensitize tumors to the killing effects of cancer therapy via the Fas receptor.

1. Introduction

Fas (Apo-1/CD95), a receptor for Fas ligand, belongs to the tumor necrosis factor receptor family and mediates apoptosis in Fas-expressing cells (1-3). Fas-mediated apoptosis plays an important role in cytotoxic T cell-mediated and natural killer cell-mediated cytotoxicity and apoptosis against cancer cells (4, 5). Activated cytotoxic T cells and natural killer cells up-regulate Fas ligand on their surfaces and induce apoptosis in cancer cells expressing Fas. Cross-linking of Fas with anti-Fas mAb has been shown to cause apoptosis in Fas-bearing cells (6, 7).

Radiotherapy is one of the most commonly used treatments of cancer patients. The level of cell death incurred after ionizing irradiation correlates with the induction and amount of double-strand breakage (8). However, given the prevalence of clinical irradiation, pathways leading to cell death remain largely unclarified.

Nitric oxide (NO), which is produced in various cell types under both physiological and pathophysiological conditions, contributes to a variety of regulatory biological processes. These include the regulation of vascular tone, the modulation of synaptic transmission in the brain as well as antimicrobial and antitumor activities (9). Nevertheless, NO appears to have both toxic and protective effect (10), and may have a role in the pathogenesis of some diseases such as inflammation (11) and cancer (12).

Not all the Fas expressing cells undergo death after ligation of Fas receptor. For example, human cervical carcinoma HeLa cells express Fas, however they are normally insensitive to cross-linking with anti-Fas antibody. Even though much is known about the Fas-mediated signaling transduction, mechanisms by which the Fas sensitivity of human cervical cancer cells is determined are poorly understood. In the present study, we demonstrated that ionizing radiation and NO donor sensitized the resistance of human carcinoma HeLa cells to Fas-mediated apoptotic cell death and, sensitization was accompanied with up-regulation of Fas gene expression. Furthermore, combined treatment of IR and SNAP additively up-regulated the surface Fas protein expression and sensitized Fas-induced apoptosis. Our findings demonstrate that IR and NO plays a role in the sensitization of tumor cells to Fas-mediated apoptosis, by regulation of Fas gene expression and/or signaling toward apoptosis. The present results also raise a possibility that IR and NO generation (NO-based therapies) can be clinically used for the treatment of tumor patients.

2. Materials and Methods

Mitochondrial transmembrane potential assay

Loss of mitochondrial membrane potential was measured by ApoAlert^{TM} mitochondrial membrane sensor kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. Thus, 1 X 10^5 cells were treated with drugs and Mitosensor was directly added to the cell media. Cells were then incubated for 15 min under 5% CO_2, while protected from light. After washing with working-diluted wash buffer, samples were analyzed by flow cytometry.

Fas expression assay

Surface Fas antigen expression on HeLa cells was determined by flow cytometry. Breifly, harvested cells were washed with cold buffer and incubated with anti-Fas monoclonal antibody (DAKO) for 15 min at room temperature. The cells were then washed twice and analyzed by FACScan flow cytometry (Beckton-Dikinson).

Evaluation of apoptosis

Apoptosis was determined by staining cells with both annexin V-FITC and propidium iodide (PI); annexin V can identify externalization of phosphatidylserine during the apoptotic progression and detect cells in early apoptotic process. To quantitate apoptosis, cells were washed with cold PBS and then resuspended in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, 2.5 mM $CaCl_2$] at a concentration of 1 X 10^6 cells/ml. Five μl of annexin V-FITC (PharMingen, SanDiego, CA) and 10 μl of PI (PharMingen) were added to these cells, which were then analyzed with FACScan flow cytometer (Becton Dickinson, San Jose, CA).

3. Results

To demonstrate that ionizing radiation (IR) and NO donor SNAP treatment would affect Fas-induced apoptosis, we pretreated HeLa cells with IR and SNAP before the addition of agonistic anti-Fas antibody CH-11 for 48 h. Normally, these cells were not sensitive to stimulation of the Fas, since the control cells and cells incubated only with CH-11 antibody rarely showed apoptosis, detected by FITC-annexinV and PI staining. In contrast, the cells pretreated with IR and SNAP before the addition of CH-11 antibody showed significantly increased apoptotic cell death. Treatment of the cells with IR and SNAP alone marginally induced apoptosis. Fas neutralizing antibody blocked apoptotic cell death induced by concomitant treatment with IR or SNAP together with CH-11 (Fig 1).

To elucidate the mechanisms by which IR and SNAP sensitized HeLa cells to Fas-mediated apoptosis, we employed Western blotting analysis to examine the expression

of anti-apoptotic molecules such as survivin, c-IAP-1/2, bcl-2, and FADD, and found that these proteins were constitutively expressed, however, a suboptimal dose of IR and SNAP did not affect their expression (Fig. 2A). Since it was possible that either IR or SNAP sensitized HeLa cells to CH-11 by increasing expression of Fas ligands or receptors, the expression of Fas ligands and receptors was analyzed after treatment of IR and SNAP. After exposure to either 3-10 Gy irradiation or 0.2 mM SNAP, Fas receptor expression in HeLa cells was increased at 48 h, and combined treatment with IR and SNAP additively generated more Fas receptor than either treatment alone (Fig. 2B), whereas the expression of Fas ligand was not detected in these cells (data not shown).

Since combined treatment with IR and SNAP additively generated more Fas receptor than either treatment alone, we determined apoptotic cell death of the cells by treating them with IR and SNAP together with CH-11, and found that apoptosis was additively increased (Fig. 3). Furthermore, the cells treated with SNAP plus IR revealed much more increased apoptosis than those treated with either IR or SNAP singulary.

4. Discussion

In the present study, we showed that ionizing radiation (IR) and exogenous sources of NO-related species might influence the apoptotic pathway elicited by Fas-induced apoptosis. Indeed, we found that IR and NO donor SNAP significantly sensitized Fas-induced apoptotic cell death in a dose or concentration-dependent manner by up-regulation of surface Fas protein. Moreover, combined treatment of IR and SNAP additively up-regulated surface Fas protein expression and sensitized Fas-induced apoptosis. In addition, NO releasing compounds were able to enhance susceptibility to apoptosis induced by combination of IR and anti-Fas antibody. Recent studies, on the involvement of Fas/FasL system and signal transduction pathway in NO-induced apoptosis in human neoplastic lymphoid cells demonstrated that NO triggers the death receptor system by regulating the expression of ligands involved in apoptosis like FasL and TRAIL without altering Fas expression (13). In contrast to the above, our present results showed that Fas-induced apoptosis of cervical carcinoma cells synergized with the Fas agonist antibody CH-11 via upregulation of Fas when treated with NO donor, SNAP.

It has already been established that Fas expression is induced by both T (14) and B (15) lymphocyte activation, and it has been proposed that the increase in Fas expression may provide a means by which activated cells may be eliminated once they have served their function in the immune response (16). Our observation suggests an additional

function of inducible Fas: Surface expression of this receptor increased after both irradiation and NO donor treatment. Therefore, multiple sources of cellular injury seem to increase Fas expression, and increased Fas may serve as a marker which targets injured cells for destruction by FasL-bearing T lymphocytes. In effect, induction of Fas expression may serve as a means, by which the immune system can perform immunological surveillance to identify and eliminate not only activated cells, but also cells that have undergone injury and require apoptosis and subsequent elimination. This mechanism would supplement the direct apoptosis-inducing effects of radiation, nitrosative stress, and other forms of physical injury.

In summary, surface levels of Fas were enhanced following IR and NO donor treatment. The signaling pathway for IR and NO donor-induced Fas up-regulation appeared to be uncoupled from the pathway leading to constitutive expression in HeLa cell, implying that more than one mechanism may exist for therapeutic manipulation of Fas levels on target tissues. Mechanisms involved in modulating of Fas expression by IR and NO donor treatment are not clearly understood and further studies are needed.

5. Acknowledgements

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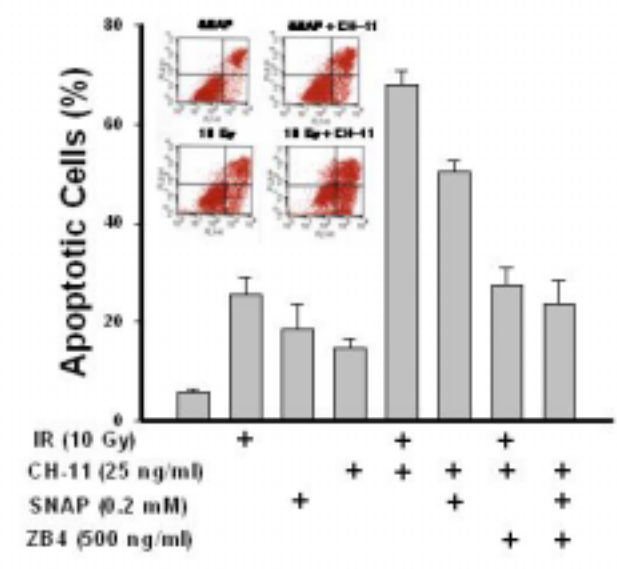
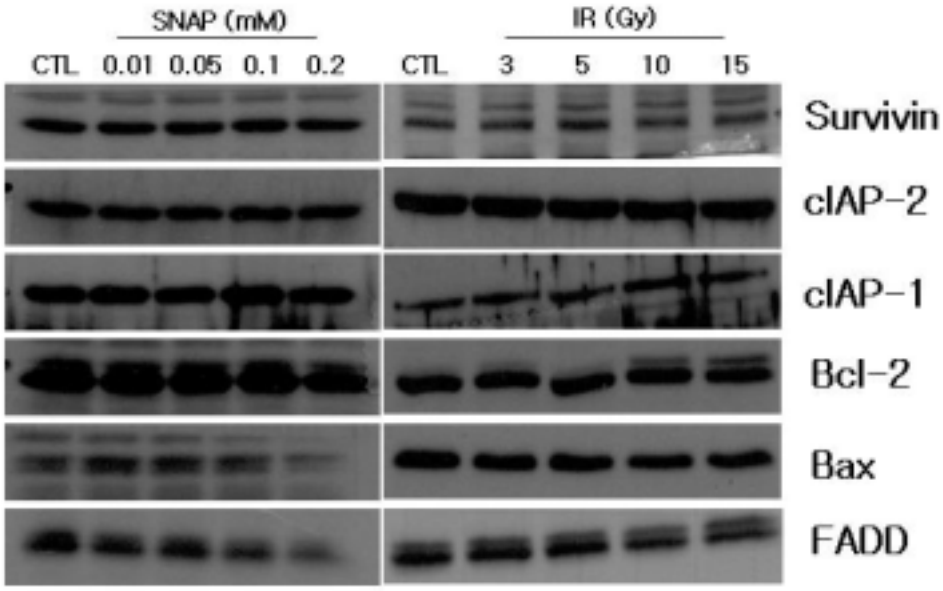


Figure 1. IR and NO donor SNAP sensitize cervical carcinoma cells to Fas-mediated

apoptotic cell death

A



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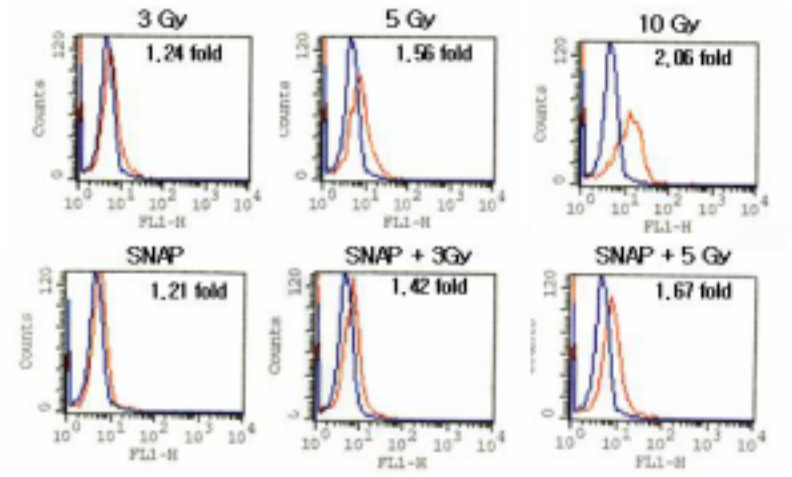

Figure 2. Up-regulation of Fas expression of HeLa cells by IR and NO donor SNAP. (A) Effects of sub-lethal doses of IR and SNAP on the Survivin, cIAP1/2, Bcl-2, Bax, and FADD protein expressions, examined by Western blotting. Cell lysates were extracted at 48 h after the treatment with varying doses of IR and SNAP. (B)

Surface Fas levels on HeLa cells were measured by flow cytometry. A representative fluorescence staining for Fas expression after treatment with IR and/or SNAP is shown. Cells were stained with FITC-anti-Fas Mab (gray line) or irrelevant antibody (solid line). Numerals indicate increased mean value of fluorescence intensity of treated cells.

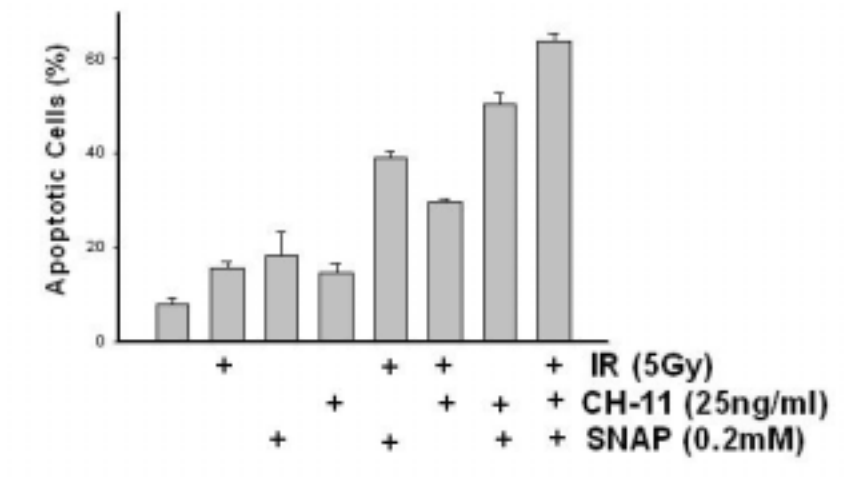

Figure 3. IR and SNAP additively sensitize Fas-mediated apoptosis. Apoptotic cell death was assessed by FITC-annexinV/PI staining at 48 h after treatment of the cells with indicated agents.

6. References

1. Oehm A, Behrmann I, Falk W, Pawlita M, Maier G, Klas C, Li-Weber M, Richards S, Dhien J, Trauth BC, Ponsting H and Krammer PH: Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor superfamily. Sequence identity with the Fas antigen. J Bio Chem 267: 10709-10715, 1992.
2. Nagata S and Golstein P: The Fas death factor. Science (Washington DC), 267: 1449-1456, 1995.
3. Suda T, Takahashi T, Golstein P and Nagata S: Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell, 75: 1169-1178, 1993.
4. Kagi D, Vignaux F, Ledermann B, Burki K, Depaetere V, Nagata S, Hengartner H and Golstein P: Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity. Science (Washington DC), 265: 528-530, 1994.

5. Arase H, Arase N and Saito T: Fas-mediated cytotoxicity by freshly isolated natural killer cells. J Exd Med 181: 1235-1238, 1995.
6. Ogsawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Ito N, Suda T and Nagata S: Lethal effect of the anti-Fas antibody in mice. Nature (Lond), 364: 806-809, 1993.
7. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima SI, Sameshima M, Hase A, Seto Y and Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell, 66: 233-243, 1991.
8. Radford I: The level of induced DNA-double-strand breakage correlates with cell killing after X-irradiation. Int J Radiat Biol 48: 45-54, 1986.
9. Moncada S and Higgs A: Mechanisms of disease. N Engl J Med 329: 2002-2012, 1993.
10. Kim YM, De Vera ME, Watkins SC and Billiar TR: Nitric oxide protect cultured rat hepatocytes from tumor necrosis factor-α-induced apoptosis by inducing heat shock protein 70 expression. J Biol Chem 272: 1402-1411, 1997.
11. Ioannidis I, Batz M, Paul T, Korth HG, Sustmann R and Groot H: Enhanced release of nitric oxide causes increased cytotoxicity of S-nitro-N-acetyl-DL-penicillamine and sodium nitroprusside under hypoxic conditions. Biochem J, 318: 789-795, 1996.
12. Bruch-Gerharz D, Ruzicka T and Kolb-Bachofen V: Nitric oxide in human skin: current status and future prospect. J Invest Dermatol, 110: 1-7, 1998.
13. Chlichlia K, Peter ME, Rocha M, Scaffidi C, Bucur M, Krammer PH, Schirrmacher V and Umansky V: Caspase activation is required for nitric oxide-mediated, CD95(APO-1/Fas)-dependent and independent apoptosis in human neoplastic lymphoid cells. Blood, 91: 4311-4320, 1998.
14. Drappa J, Brot N and Elkon KB: The Fas protein is expressed at high levels on CD4+CD8+ thymocytes and activated mature lymphocytes in normal mice but not in the lupus-prone strain, MRL lpr/lpr. Proc Natl Acad Sci USA 90:10340-10346, 1993.
15. Onel KB, Tucek-Szabo CL, Ashany D, Lacy, E, Nikolic-Zugic J and Eisenberg KB: Expression and function of the murine CD95/FasR/APO-1 receptor in relation to B cell ontogeny. Eur J Immunol 25: 2940-2947, 1995.
16. Cohen PL and Eisenberg RA: Apoptosis and the immune response, ed. Gregory C (Wiley, New York), pp 169-186.