Ionizing Radiation-induced Apoptosis in Ataxia-Telangiectasia Fibroblasts

ROLES OF CASPASE-9 AND CELLULAR INHIBITOR OF APOPTOSIS PROTEIN-1*

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Ionizing radiation (IR) has been shown to induce apoptosis to a greater extent in a fibroblast cell line AT5BIVA derived from an individual with ataxia-telangiectasia (AT) than in control fibroblasts. However, the signaling pathway that underlies IR-induced apoptosis in AT cells has remained unknown. The mechanism of apoptosis in response to γ-irradiation has now been examined in three AT fibroblast lines (AT3BIVA, AT4BIVA, and AT5BIVA) derived from different individuals with AT. The apoptotic indexes of these cell lines at 72 h after irradiation were 12, 31, and 35%, respectively, compared with a value of 2.3% for control fibroblasts. Immunoblot analysis and fluorometric assays revealed that the extents of IR-induced activation of caspase-3 and caspase-9 were markedly greater in AT4BIVA and AT5BIVA cells than in AT3BIVA and control cells. Furthermore, the basal abundance of the apoptotic inhibitor, a cellular inhibitor of apoptosis proteins (c-IAP-1), was markedly reduced in AT4BIVA and AT5BIVA cells compared with that in AT3BIVA and control cells. The overexpression of either caspase-9 mutant forms or recombinant c-IAP-1 in AT5BIVA cells inhibited the IR-induced activation of caspases-3 and 9 and reduced the apoptotic index of the irradiated cells. These results indicate that the extent of IR-induced apoptosis in different AT cell lines is inversely related to the abundance of c-IAP-1 and directly related to the extent of activation of caspases-3 and 9.

Apoptosis is a genetically regulated and highly conserved mechanism of cell death, impairments of which contribute to human diseases such as cancer and autoimmune disorders (1–4). The principal effectors of apoptosis in mammalian cells are members of a family of cysteine-containing aspartate-specific proteases known as caspases (5). Several caspases have been isolated from human cells and characterized as cell typespecific and stimulus-dependent (6). Each caspase is synthesized as an inactive precursor (zymogen) that is converted by proteolytic cleavage to an active heterodimer when cells are exposed to a variety of insults (7, 8). Once activated, caspases cleave a host of cellular substrates, resulting in the morphological hallmarks of apoptosis such as DNA fragmentation and condensation of cellular organelles (9).

The characterization of the regulation of various caspases in mammalian cells has led to the definition of two distinct apoptotic signaling pathways (10). In one of these pathways, procaspase-8 is recruited by the cytosolic domains of members of the tumor necrosis factor receptor family (11). Such recruitment results in the proteolytic cleavage of procaspase-8, and the activated enzyme then induces the activation of downstream effector caspases, such as caspases-3, 6, and 7 (7, 8). In the second pathway, cytochrome c is released from mitochondria into the cytosol (12–14), resulting in its high affinity interaction with apoptotic protease-activating factor-1 and subsequent activation of caspase-9 (15, 16). The active caspase-9 in turn activates downstream effector caspases that mediate the execution phase of apoptosis (6, 9, 14, 15).

The activation of various caspases is regulated by cellular inhibitor of apoptosis proteins (c-IAP)1 (10, 15, 17), which are conserved among various species including humans (18, 19). These proteins inhibit apoptosis induced by a variety of stimuli by interacting with and inhibiting the activity of caspases (15–18). For example, they block the cytochrome c-induced activation of caspase-9 (10). Thus, the basal concentration of these inhibitors appears to be an important determinant of cell survival (19, 20).

Cells from individuals with the genetic disease ataxia telangiectasia (AT) exhibit defects in cell cycle checkpoints, DNA repair, and signaling pathways associated with regulation of apoptosis in response to ionizing radiation (IR) (21–23). Both the tumor suppressor protein p53 and the protein ATM encoded by the gene mutated in individuals with AT contribute to IR-induced cell death (24–27). We have previously shown that the apoptotic index of AT5BIVA cells, a cell line derived from an individual with AT, is increased by γ-irradiation to a greater extent than that of control cells (23). With the use of additional AT cell lines each derived from a different individual with AT, we have now shown that IR-induced apoptosis in these cells is mediated by a series of events that include the activation of caspases-3 and 9. Furthermore, our data suggest that a deficiency of c-IAP-1 may contribute to IR-induced programmed cell death in AT fibroblasts.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—AT5BIVA fibroblasts were obtained from the Human Genetic Mutant Cell Repository (Bethesda, MD).

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1 The abbreviations used are: c-IAP, cellular inhibitor of apoptosis; AT, ataxia-telangiectasia; IR, ionizing radiation; PARP, poly(ADP-ribose) polymerase; MOCAc, 7-methoxycoumarin-4-yl)acetyl; DNP, 2,4-dinitrophenyl; MCA, 4-methylcoumaryl-7-amide; AFC, N-acetyl-S-farnesyl-L-cysteine; Gy, gray.
RESULTS

Effects of IR on the Apoptotic Index of AT Fibroblasts—We have previously shown that γ-radiation induces a markedly greater increase in the apoptotic index of AT5BIVA cells than it does in that of control MRC5CV1 cells (23). We, therefore, investigated the effects of IR on the apoptotic index of two additional AT fibroblast lines, AT3BIVA and AT4BIVA. Apoptotic cells were apparent within 24 h after irradiation of AT3BIVA, AT4BIVA, and AT5BIVA fibroblasts, although the apoptotic index varied. At 72 h after irradiation, the apoptotic indexes of AT4BIVA (31%) and AT5BIVA (35%) cells were similar to each other and markedly greater than that of AT3BIVA cells (12%) (Fig. 1a), suggesting differences in the cell death programs among these AT cell lines. The apoptotic index of MRC5CV1 cells 72 h after irradiation was only 2.3%.

Effects of IR on Caspase-3 Activity in AT Fibroblasts—Various proteins that contribute to the maintenance of cellular integrity are cleaved by caspases during apoptosis. For example, the nuclear enzyme PARP, which plays an important role in the repair of DNA damage and in the maintenance of genomic integrity, is specifically cleaved between its NH2-terminal DNA-binding domain and its multifunctional COOH-terminal domain by caspase-3 or caspase-3-like proteases early during the execution phase of apoptosis (9). Therefore, we monitored the amounts of PARP after exposure of AT and control cells to IR. Immunoblot analysis revealed the presence of intact PARP (116 kDa) in all untreated cells (Fig. 1b). A cleavage product of PARP (89 kDa) appeared within 24–48 h after irradiation in both AT4BIVA and AT5BIVA cells but not in AT3BIVA or control cells, correlating with the effects of IR on the apoptotic indexes of these cells. These observations suggest the importance of caspase-3 as a downstream protease responsible for the cleavage of PARP during IR-induced apoptosis in AT4BIVA and AT5BIVA cells. In addition, the abundance of intact PARP gradually increased after the exposure of AT3BIVA and AT4BIVA cell lines to IR.

We also measured the activities of caspases-1, 3, 6, and 8 in cell extracts prepared from AT and control cells at various times after irradiation. Fluorometric determination of the cleavage of specific fluorogenic peptide substrates revealed little activity of caspases-1, 6, and 8 (data not shown), but a marked increase in caspase-3 activity in AT5BIVA and AT4BIVA cells that was apparent at 24 h and maximal at 48 h after exposure to IR (Fig. 1c). In contrast, only a small increase in caspase-3 activity was apparent in AT3BIVA or MRC5CV1 cells by 72 h after irradiation. Immunoblot analysis also revealed that the time course of the conversion of procaspase-3 to the active protease in AT4BIVA and AT5BIVA cells was consistent with the results obtained with the fluorometric activity assay (Fig. 1d). Also consistent with the activity data, the cleavage of procaspase-3 was not detected in AT3BIVA and control cells up to 72 h after irradiation.

Effects of IR on Caspase-9 Activity—We next examined the effects of γ-irradiation on the activity of caspase-9 in AT and control cells. Fluorometric assay revealed that IR induced a marked time-dependent increase in caspase-9 activity that was apparent as early as 24 h after irradiation in both AT4BIVA and AT5BIVA cells (Fig. 2a). In contrast, the activity of caspase-9 was increased only slightly by IR in AT3BIVA and control cells up to 72 h after irradiation.

Effects of IR on Mitochondrial Membrane Potential—It has been recently demonstrated that the mitochondrial membrane potential is critical for maintaining the integrity of the plasma membrane (20). Mitochondria are a primary source of reactive oxygen species (ROS) (21) that contribute to cell death (22). We, therefore, investigated the effects of IR on mitochondrial membrane potential. Cells were stained with a fluorescent dye, rhodamine 123, which accumulates within the mitochondrial compartment (23). The potential was monitored using a flow cytometer equipped with a 488 nm laser and a 530 nm filter set. Changes in the fluorescence excitation and emission wavelengths of the dye were monitored at 488 nm and 500 nm, respectively. A decrease in the mitochondrial membrane potential was observed within 24 h after irradiation in both AT4BIVA and AT5BIVA cells (Fig. 2b). In contrast, the IR-induced decrease in the mitochondrial membrane potential was not observed in AT3BIVA cells.

Effects of IR on Mitochondrial Protein Expression—IR induced a marked increase in the expression of BAX and BAK, which are members of the proapoptotic BCL2 family of proteins (24). The expression of BAX was increased in both AT4BIVA and AT5BIVA cells at 12 h after IR, whereas the expression of BAK was increased in AT4BIVA cells only (Fig. 2c). In contrast, the expression of BCL2 was not affected by IR in any of the cell lines examined (data not shown). Taken together,
these data implicated caspases-3 and 9 as effectors of IR-induced apoptosis in AT4BIVA and AT5BIVA cells.

To determine a causal relationship between caspase-9 and 3 activities, AT5BIVA cells were transfected with plasmid DNA containing either a dominant negative form, which is an alternatively spliced isoform and lacks the central large subunit caspase domain (28), or the active site mutant (C287A) form of caspase-9 cDNA (29). At 24 h posttransfection, cells were exposed to IR and harvested at indicated intervals. As shown in Fig. 3, in the expression of either mutant form of caspase-9, the apoptotic index was significantly reduced (∼50%) within 48 h after irradiation (Fig. 3a). Both caspase-9 and 3 activities were also markedly inhibited as well (Fig. 3b and c); however, the vector alone did not modify the IR-induced apoptotic index and caspases-9 and 3 activities. These data show that the inhibition of caspase-9 activity attenuates a cellular apoptotic response and the activation of caspase-3, supporting caspase-9 as an upstream effector of caspase-3. In turn, caspase-9 inhibition reduced caspase-3 activity. This observation is consistent with recent reports suggesting that caspase-9 can initiate a caspase-cascade involving caspase-3 (28, 29, 34–36).

**Effects of IR on the Abundance of Bel-2 and Bax and on Cytochrome c Release**—The role of the mitochondrial pathway of apoptosis in the IR-induced death of AT cells was examined by immunoblot analysis of the abundance of Bel-2, an antiapoptotic protein, and of Bax, an effector of this pathway, in a mitochondrial fraction. These proteins regulate apoptosis by controlling the release of cytochrome c from mitochondria (13, 14).

The basal amounts of Bax and of Bel-2 did not differ markedly among the three AT cell lines, although the abundance of these proteins in the AT cells was greater than that in MRC5CV1 cells (Fig. 4). The abundance of Bax in the mitochondrial fraction of AT cells but not that in control cells was increased 48 h after irradiation (Fig. 4a). In contrast, the amount of Bel-2 in the AT cell lines but not that in control cells was decreased 48 h after irradiation (Fig. 4b).

We next examined whether the IR-induced activation of caspase-9 in AT4BIVA and AT5BIVA cells is mediated by the release of mitochondrial cytochrome c. A cytosolic fraction was prepared from irradiated cells under conditions that maintain the integrity of mitochondria (31). Immunoblot analysis of this fraction revealed that the basal amounts of cytochrome c in AT cells were similar to each other and greater than that in control
cells (data not shown). The amount of cytochrome c released from mitochondria into the cytosol was markedly increased within 24–48 h after irradiation of AT4BIVA or AT5BIVA cells (Fig. 5). In contrast, cytochrome oxidase (subunit II), which was assayed as a mitochondrial marker protein, was undetectable in the cytosolic fractions prepared from these cell lines before or after irradiation (data not shown) confirming that the presence of cytochrome c in the cytosol was not simply attributable to contamination by mitochondria. Together, these data suggest that IR-induced apoptosis in AT4BIVA and AT5BIVA cells is mediated, at least in part, by the mitochondrial-signaling pathway.

Effects of IR on c-IAP-1 Expression—The activation of caspase-9 in the mitochondrial-signaling pathway of apoptosis is inhibited by c-IAP proteins (10, 15, 17). To investigate the role of c-IAP-1 and c-IAP-2 in IR-induced apoptosis in AT cells, we determined the effects of γ-irradiation on the abundance of these proteins. The basal amounts of c-IAP-1 in AT4BIVA and AT5BIVA cells were substantially smaller than those in AT3BIVA or control cells (Fig. 6). In contrast, cytochrome c oxidase (subunit II), which was assayed as a mitochondrial marker protein, was undetectable in the cytosolic fractions prepared from these cell lines before or after irradiation (data not shown) confirming that the presence of cytochrome c in the cytosol was not simply attributable to contamination by mitochondria. Together, these data suggest that IR-induced apoptosis in AT4BIVA and AT5BIVA cells is mediated, at least in part, by the mitochondrial-signaling pathway.

Effects of IR on c-IAP-1 Expression—The activation of caspase-9 in the mitochondrial-signaling pathway of apoptosis
index in response to IR as well as the lowest abundance of c-IAP-1, we transfected these cells with a plasmid encoding an hemagglutinin-tagged fragment of human c-IAP (residues 1–342) under the control of the cytomegalovirus promoter. After G418 selection and subcloning, one G418-resistant clone (AT5BIVA-c-IAP-1/CL3) that overexpressed hemagglutinin-tagged c-IAP-1 (Fig. 6b) was subjected to further analysis.

The extent of IR-induced apoptosis (as reflected by the apoptotic index) in the transfected cells was reduced by 50% compared with that observed in the parental AT5BIVA cells (data not shown). The extent of IR-induced cleavage of PARP was also reduced in AT5BIVA-c-IAP-1/CL3 cells (Fig. 7a). Furthermore, fluorometric assays revealed that the IR-induced increases in the activities of caspase-3 (Fig. 7b) and caspase-9 (Fig. 7c) were substantially reduced in the transfected cells compared with those apparent in the parental cells. These data suggest that c-IAP-1 inhibits the activation of caspases-3 and 9, and that the abundance of this protein is a critical factor in determining the extent of IR-induced apoptosis in AT cells.

**DISCUSSION**

Apoptotic cell death is one response to the genotoxic effects of ionizing radiation. We previously showed that radiosensitive AT5BIVA cells undergo apoptosis in response to γ-irradiation (23). We have now shown that the apoptotic index differs among three AT cell lines after exposure to IR. Furthermore, IR-induced apoptotic signaling in AT4BIVA and AT5BIVA cells that showed the highest apoptotic indexes appears to be mediated by the release of mitochondrial cytochrome c and the activation of caspases-3 and 9. The IR-induced release of mitochondrial cytochrome c into the cytosol in these two AT cell lines coincided with a decrease in the abundance of Bcl-2. The basal intracellular concentration of c-IAP-1 in AT and control cell lines also appeared to be inversely related to the apoptotic index after exposure to IR, suggesting a role for c-IAP-1 in limiting the extent of caspase-9 activation and apoptosis in these cells. This conclusion was supported by the observation that overexpression of mutant caspase-9 and c-IAP-1 in AT5BIVA cells reduced the extent of apoptosis as well as that of caspase-9 activation in response to γ-irradiation.

The activation of caspase-9 is mediated by a mitochondrial signaling that involves the release of cytochrome c and the activation of caspase-3 (12–14). Using dominant negative and active site mutant forms of caspase-9, we demonstrate that caspase-9 is involved in IR-induced apoptotic signaling and activation of caspase-3. Furthermore, the amount of cytochrome c in the cytosol of AT4BIVA and AT5BIVA cells was...
markedly increased by exposure to IR. The basal abundance of cytochrome c in the cytosol of AT cells was greater than that apparent in control cells, which possibly contributes to cell death, results from the impairment in mechanisms that protect against oxidative stress in AT cells (37, 38). Taken together, our data suggest that IR-induced apoptosis in AT fibroblasts is mediated by this mitochondrial-signaling pathway involving cytochrome c release and the activation of caspases-9 and 3.

The ratio between the amounts of the proapoptotic Bax and the antiapoptotic Bcl-2 is an important determinant of cell survival versus cell death (39). Both proteins regulate the release of cytochrome c from mitochondria with Bax promoting this process and Bcl-2 preventing it (40–47). IR induced an increase in the amount of Bax and a decrease in the abundance of Bcl-2 in the AT cell lines examined, suggesting that this change in the Bax/Bcl-2 ratio contributes to the release of mitochondrial cytochrome c and apoptosis in these AT cells.

Furthermore, the basal abundance of c-IAP-1 in AT4BIVA and AT5BIVA cells was less than that apparent in AT3BIVA and control cells, consistent with the differences in the apoptotic indexes among these cells and the fact that the abundance of c-IAP is a critical factor in the apoptotic process (20). The overexpression of c-IAP-1 in AT5BIVA cells resulted in a marked inhibition of IR-induced apoptosis, PARP cleavage, and an activation of caspase-9, suggesting that the low concentration of c-IAP-1 in AT5BIVA cells contribute to their high sensitivity to IR. These data are consistent with observations by other laboratories demonstrating that IAPs directly or indirectly inhibit the activation of caspases-3, 7, and 9, and that the endogenous threshold level of the IAPs is correlated with cellular sensitivities to apoptosis inducing stimuli in various cell types (10, 18, 20, 48).

In summary, the apoptotic index was shown to differ markedly among three AT fibroblast lines after exposure to γ-radiation being greatest in the AT4BIVA and AT5BIVA lines. Apoptosis in these cells appeared to be mediated by the mitochondrial-signaling pathway involving the release of cytochrome c into the cytosol and the activation of caspases-9 and 3. A reduced abundance of c-IAP in these two AT cell lines appeared to be a critical factor in their increased susceptibility to IR-induced apoptosis. Different mutations in the atm gene thus may contribute to the variation in the response of the three AT fibroblast lines examined to IR.

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