Caspase-3 Is Essential for Procaspase-9 Processing and Cisplatin-induced Apoptosis of MCF-7 Breast Cancer Cells

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INTRODUCTION

Apoptosis, or programmed cell death, is essential for organogenesis during development, for proper function of the immune system, for elimination of genetically unstable cells, and for maintenance of tissue homeostasis in the adult (1). Apoptosis is also the main response of cells to chemotherapeutic agents (2). Apoptosis results from activation of members of the caspase family of aspartate-specific proteases (3, 4). Caspases form a proteolytic network within the cell whereby upstream initiator caspases are activated early in the apoptotic process (e.g., caspase-8 and caspase-9) and then activate other downstream caspases (e.g., caspase-3 and caspase-7). The downstream caspases are largely responsible for cleavage of many other cellular proteins, leading to the morphological manifestations of apoptosis.

In some cell types, the caspase-9 enzyme is found in the mitochondrial intermembrane space and is released into the cytosol together with cytochrome c after rupture of the outer mitochondrial membrane (5, 6). Once released, caspase-9 interacts with and is activated by the apoptosis-activating factor Apaf-1 in a cytochrome c- and dATP-regulated manner (7–10). Subsequently, procaspase-3 is recruited to the Apaf-1/caspase-9 complex and undergoes proteolysis and activation (10, 11). Interestingly, caspase-3 contains a caspase-3 cleavage site at position 330, and it has been shown that procaspase-9 is also a substrate of caspase-3 during apoptosis (12). Indeed, using a cell-free system of apoptosis, Slee et al. (13) showed that caspase-9 processing can be enhanced by caspase-3. Furthermore, these authors showed that cytochrome c mediates a hierarchical activation of numerous caspases in addition to caspase-9 and caspase-3, such as caspase-2, -6, -7, -8 and -10, in a complex proteolytic cascade. Once activated, caspase-9 translocates to the nucleus, where it may participate in nuclear dismantling during apoptosis (5). Studies investigating Apaf-1 and caspase-9-deficient animals show that the release of mitochondrial cytochrome c and the subsequent Apaf-1-dependent activation of caspase-9 and caspase-3 represent a common pathway used by many apoptosis-inducing stimuli that is important for tumor suppression by p53 (14–18).

Recent evidence suggests that caspase-3 plays an important role for several key events during apoptosis such as nuclear fragmentation, DNA fragmentation, and membrane blebbing in a cell type-specific and stimulus-specific manner (19). Furthermore, caspase-3 was reported to play a role as an amplifier of the apoptotic signals, i.e. by the cleavage of Bcl-2 (20, 21). The role of caspase-3 was studied extensively in caspase-3-deficient animals (22, 23). These animals exhibit massively impaired developmental apoptosis in the brain, whereas programmed cell death in other organs occurs normally. Caspase-3-deficient embryonic stem cells are resistant to UV- and sorbitol-induced cell death, whereas γ-irradiation-induced cell death occurs normally. Similar results were obtained in MCF-7 cells that harbor a spontaneous deletion of 47 bp within exon 3 of the CASP-3 gene (24). This mutation introduces a premature stop codon and leads to a complete absence of caspase-3 protein and activity. A comparison of MCF-7 cells and CASP-3-transfected MCF-7 cells revealed that DNA fragmentation and membrane blebbing were severely affected after TNFα or staurosporine treatment (24).

In this study, we extend our previous observations by showing that cisplatin-induced cytochrome c release, nuclear fragmentation, and fragmentation of genomic DNA were all strongly enhanced by restoring caspase-3 in MCF-7 cells. Cytochrome c- and caspase-8-mediated procaspase-9 processing were highly dependent on caspase-3, placing this caspase in a central position as a regulator and amplifier of essential apoptotic pathways in breast cancer cells.

MATERIALS AND METHODS

Cell Culture, Cisplatin Treatment, and Apoptosis Analysis. ZR-75-1 and MCF-7 are human breast cancer cell lines (derived by the American Type Culture Collection). MCF-7 cells lack functional caspase-3 (24, 25). MCF-7 CASP-3 and MCF-7 vc cells were obtained by stable transfection of caspase-3 cDNA or empty vector and selection with G418 as described previously (24, 25). All cells were maintained at a subconfluent stage in RPMI 1640 containing 10% FCS, 200 units/ml penicillin, and 200 μg/ml streptomycin in a humidified atmosphere of 5% CO2 at 37°C. For cisplatin treatment, MCF-7 cells were seeded at 60% confluence in 9-cm dishes. The next day, 10 μg/ml cisplatin (500 μg/ml stock solution; Bristol-Myers Squibb, Baar, Switzerland) was added, and culture was continued for 48 or 72 h. Cells were harvested by trypsinization and analyzed for either apoptotic morphology after DAPI staining or DNA fragmentation after isolation of total DNA. Experiments were performed three times and representative data are shown.

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The abbreviations used are: TNF, tumor necrosis factor; DAPI, 4′,6-diamidino-2-phenylindole; DEVD, Asp-Glu-Val-Asp; amc, amino-methyl-coumarin; HRP, horseradish peroxidase; HSP, heat shock protein.
repeated three to five times. DNA fragmentation was analyzed essentially as described previously (26). For DAPI staining (Roche Diagnostics, Rotkreuz, Switzerland), cells were fixed with 3% formalin and stained with 1 μg/ml DAPI in PBS for 10 min. Cells were mounted on glass slides, covered, and analyzed using fluorescence microscopy. For statistical analysis of each experiment, 5–10 fields (magnification, ×400) were counted per stimulation and cell type (between 400 and 700 cells in total). The mean ± SD was calculated and displayed as bar graph. t test analysis was performed, and P’s for each of the corresponding pairs were calculated using the Microsoft Excel program.

Preparation, Activation, and Reconstitution of Cell-free Extracts. Cell-free extracts and mitochondrial enriched fractions were prepared essentially as described previously (27, 28). Protein concentration was determined using the Pierce BCA protein assay kit. Extracts were activated either by the addition of 10 μM bovine heart cytochrome c (Sigma) in combination with 1 mM dATP (Sigma) or by the addition of purified recombinant active caspase-3 or caspase-8 as described previously (27). MCF-7 cell extracts were reconstituted by the addition of recombinant procaspase-3. For normalization, the amount of procaspase-3 that was added to extracts was checked by immunoblot analysis. Extracts were either activated directly as described above or preincubated for 30 min at 37°C before activation.

Immunoblot Analysis. Protein samples were separated by 12% SDS-PAGE and subjected to immunoblot analysis as described previously (28). Protein detection was performed using the Immunoblot Chemiluminescence Reagent Plus (New England Nuclear, Life Science Products, Boston, MA) following the instructions of the manufacturer.

Anti-Hsp60 (a gift from Dr. G. Schatz, Biocenter, Basel, Switzerland) was used at a dilution of 1:2000 in PBS containing 5% nonfat dry milk, anti-caspase-3 (polyclonal rabbit) and anti-cytochrome c (mouse monoclonal 7H8.2C12; PharMingen, San Diego, CA) antibodies were used at a dilution of 1:1000. Secondary antibodies (HRP-coupled goat anti-rabbit and goat antimouse antibodies; Bio-Rad Laboratories, Hercules, CA) were diluted 1:3000.

DEVD-amc Cleavage Assay. For fluorometric assays, equal amounts of cytosolic extracts (~40 μg of protein) were combined with 32 μl of caspase assay buffers (Promega, Madison, WI), 2 μl of DMSO, 1 μl of 1 mM DTT, 60 μl of H2O, and 1 μl of synthetic DEVD-amc caspase-3 substrate (100 μM stock solution of DEVD-amc in DMSO; Calbiochem, La Jolla, CA) and mixed briefly, and the emitted fluorescence at 30°C was kinetically measured over a 50-min period using a Spectramax Gemini Fluorometer (Molecular Devices, Sunnyvale, CA). The relative cleavage activity was determined by calculating the slope of the accumulation of amc fluorochrome during the linear portion of the reaction.

Analysis of Caspase Processing with In Vitro-generated Caspase-9 Proteins. One μg of pET21 plasmids encoding full-length procaspase-9 (29) was in vitro transcribed and translated in the presence of [35S]methionine or biotin-labeled L-methionine using the coupled transcription/translation TNT kit (Promega) according to the manufacturer’s instructions. Proteins were desalted in 30 mM MgCl2, 1 mM EDTA, 1 mM DTT, 100 μM phenylmethylsulfonyl fluoride with 1 μl of ZR-75-1 or MCF-7 extracts activated as described with cytochrome c, recombinant active caspase-8, or recombinant active caspase-3. For normalization, the amount of procaspase-3 that was added to extracts was checked by immunoblot analysis. Extracts were either activated directly as described above or preincubated for 30 min at 37°C before activation.

RESULTS

Caspase-3 is essential for procaspase-9 processing

Fig. 1. Cisplatin-induced apoptosis in breast cancer cells. A, ZR-75-1, MCF-7 vc, and MCF-7 CASP-3 cells were left untreated or were treated for 48 and 72 h with cisplatin. Cells were stained with DAPI and representative fields of one of three independent experiments are shown. B, quantification of the percentage of apoptotic nuclei before and after 48 and 72 h of cisplatin stimulation (mean ± SD; n = 3). C, ZR-75-1 (lanes 1–3), MCF-7 vc (lanes 4–6), and MCF-7 CASP-3 cells (lanes 7–9) were either left untreated or treated for 48 and 72 h with cisplatin. Total DNA was isolated and separated on a 1.5% agarose gel. A 100-bp DNA size marker was used as a reference (lane M). A representative example of three independent experiments is shown.
To determine in more detail the level at which caspase-3 deficiency interferes with the apoptotic process, release of cytochrome c from mitochondria was monitored. Fig. 2A demonstrates that cytochrome c is retained within the mitochondria for at least 48 h in MCF-7 vc cells (Lane 2) and is only released after 72 h (Lane 3). In MCF-7 CASP-3 cells, cytochrome c release was already complete 48 h after cisplatin stimulation (Lane 5). In contrast, levels of the mitochondrial matrix protein Hsp60 remain similar in mitochondria after cisplatin treatment (Fig. 2B). These results indicate that cisplatin-mediated cytochrome c release is accelerated in caspase-3-expressing cells.

**Processing and Activation of Caspase-3 by Cytochrome c in Breast Cancer Cell Extracts.** The data presented in Figs. 1 and 2 document a prominent role for caspase-3 during cisplatin-mediated apoptosis including an enhancement of the mitochondrial cytochrome c release. To further investigate the connection between cytochrome c and caspase-3, cell extracts from ZR-75-1 cells, untransfected MCF-7 cells, control transfected MCF-7 cells (MCF-7 vc), and CASP-3-transfected MCF-7 cells (MCF-7 CASP-3) were analyzed. Caspase-3 expression and processing were determined in extracts before and after stimulation with purified cytochrome c and dATP (Fig. 3A). As described previously for other cell lines (7, 27), caspase-3 was efficiently processed in ZR-75-1-derived cell extracts after stimulation with cytochrome c and dATP (Fig. 3A, Lanes 1–3). No caspase-3 protein was detected in MCF-7 cells (Fig. 3A, Lanes 5–8) and MCF-7 vc cells (Fig. 3A, Lanes 9 and 10). However, in MCF-7 CASP-3 cell extracts, expression of caspase-3 (Fig. 3A, Lane 11) and processing by the cytochrome-mediated pathway were fully restored (Fig. 3A, Lane 12).

Caspase-3 activity was measured from the same cell extracts by analyzing the cleavage of the synthetic caspase-3 peptide-substrate DEVD-amc (Fig. 3B). DEVD-based peptides have previously been shown to be specifically recognized and cleaved by active caspase-3 and, to a lesser extent, by active caspase-2 and -7 (31, 32). A significant induction of cytochrome c-mediated DEVD-amc cleavage activity was only detected in ZR-75-1 (Fig. 3B, Lanes 2 and 3) and MCF-7 CASP-3 cell extracts after cytochrome c stimulation (Fig. 3B, Lane 12), and it correlated well with caspase-3 expression and processing.

**Caspase-9 Processing after Stimulation with Cytochrome c and Caspase-8.** Major death signals are initiated within the cells by the release of mitochondrial cytochrome c, which initially activates procaspase-9, or by death receptors, which initially activate procaspase-8. Both pathways are likely to contribute to chemotherapy-induced cell death (33–35). To analyze the processing of procaspase-9 mediated by either cytochrome c or caspase-8, extracts derived from MCF-7 and ZR-75-1 cells were incubated with [35S]methionine-labeled in vitro-synthesized procaspase-9 and treated with cytochrome c (Fig. 4A) or active caspase-8 (Fig. 4B). This method has previously been shown to be very accurate for analyzing the fate of procaspase-9 (29). In ZR-75-1 cell extracts, both treatments resulted in an efficient and complete processing of [35S]methionine-labeled procaspase-9 (Fig. 4A, Lane 2 and Fig. 4B, Lane 2). In contrast, no processing of [35S]methionine-labeled procaspase-9 was observed when caspase-3-deficient MCF-7 cell extracts were treated with cytochrome c or caspase-8 (Fig. 4A, Lane 4 and Fig. 4B, Lane 4). As a control, incubation of [35S]methionine-labeled procaspase-9 with cytochrome c in the absence of cellular extracts did not result in any processing of in vitro-translated procaspase-9 (Fig. 4A, Lane 6). Similarly, incubation of [35S]methionine-labeled procaspase-9 with caspase-8 in the absence of extracts resulted in only a very minor processing of procaspase-9 (Fig. 4B, Lane 6).

**Reconstitution of MCF-7 Cell Extracts with Caspase-3 Restores Caspase-9 Processing.** To verify that the impaired procaspase-9 processing in MCF-7 cell extracts was due to the lack of caspase-3.
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This study documents the specific role of caspase-3 as an amplifier of mitochondrial cytochrome c release and of morphological changes of nuclei and DNA fragmentation during cisplatin-induced apoptosis in breast cancer cells. Moreover, cytochrome c- and caspase-8-mediated processing of procaspase-9 is strictly dependent on caspase-3 in these cells, suggesting that caspase-3 may be critical for the regulation of procaspase-9.

Our results with cisplatin confirm and extend previous results obtained with MCF-7 cells and CASP-3-transfected MCF-7 cells (24, 30). In these earlier studies, it was shown that during TNF- and staurosporine-induced apoptosis, DNA fragmentation and membrane blebbing were impaired due to the absence of caspase-3 (24). We found that cisplatin-mediated fragmentation of nuclei and the appearance of the DNA ladder are also largely dependent on caspase-3, further generalizing the contribution of caspase-3 to these events.

Cytochrome c release from mitochondria that occurs after cisplatin stimulation is controlled, at least in part, by caspase-3. This is evidenced by the fact that cytochrome c release is strongly delayed in the absence of caspase-3 in MCF-7 cells, and transfection of CASP-3 resulted in a release of cytochrome c. It may be that caspase-3 substrates influence the mitochondrial status. Bcl-2 and caspase-8 are possible candidates for such substrates. Bcl-2 can be converted to a proapoptotic protein by caspase-3 that may no longer protect mitochondria from cytochrome c release (20, 21). Caspase-3-mediated caspase-8 cleavage may result in cleavage of Bid, a proapoptotic protein that efficiently induces cytochrome c release (36–38). However, whether cleavage of Bcl-2 or Bid is a requirement for cytochrome c release after cisplatin treatment in breast cancer cells remains to be determined.

Cytochrome c release from mitochondria and caspase-8 activation bychrome c resulted in a processing of procaspase-9 only after reconstitution of extracts with inactive procaspase-3 (Fig. 6, Lane 6). Similarly, caspase-8-induced procaspase-9 processing only in the presence of exogenously added procaspase-3 (Fig. 6, Lane 7), and not in its absence (Fig. 6, Lane 3). In accordance with previous data, the addition of constitutively active recombinant caspase-3 was sufficient to completely process procaspase-9 (Fig. 6, Lane 4). A similar dependence of procaspase-9 processing on caspase-3 was obtained when extracts from MCF-7 vc and MCF-7 CASP-3 cells were compared (data not shown). The same MCF-7 cell extracts were also subjected to a DEVD cleavage assay. As shown in Fig. 6, DEVD cleavage activity was closely correlated with the presence of active caspase-3 and with the processing of procaspase-9. These results underscore the close relationship between the presence or absence of active caspase-3 and procaspase-9 processing.

**DISCUSSION**

In MCF-7 cells, caspase-3 reconstituted extracts were subjected to either cytochrome c or caspase-8 treatment. Fig. 5 shows that incubation of extracts with recombinant caspase-8 induced strong DEVD-amc cleavage activity (Fig. 5, Lane 3), whereas cytochrome c was rather inefficient in mediating a significant activation of procaspase-3 (Fig. 5, Lane 2).

Interestingly, when extracts were preincubated at 37°C for 20 min, sensitivity to cytochrome c was much improved (Fig. 5, Lanes 4 and 5). In contrast, caspase-8-mediated induction of caspase-3 activity was independent of the preincubation at 37°C (Fig. 5, Lane 6).

After having established the conditions for the reconstitution of MCF-7 cell extracts with recombinant procaspase-3, we analyzed cytochrome c-mediated procaspase-9 processing in these extracts. In vitro-synthesized biotinylated procaspase-9 was added to preincubated MCF-7 extracts in the absence (Fig. 6, Lanes 1–4) or presence of recombinant inactive procaspase-3 (Fig. 6, Lanes 5–7), and control cell extracts (Fig. 6, Lanes 1 and 5) or cell extracts stimulated with cytochrome c (Fig. 6, Lanes 2 and 6), recombinant active caspase-8 (Fig. 6, Lanes 3 and 7), or recombinant active caspase-3 (Fig. 6, Lane 4) were analyzed. Stimulation with cytochrome c resulted in a processing of procaspase-9 only after reconstitution of extracts with inactive procaspase-3 (Fig. 6, Lane 6). Similarly, caspase-8-induced procaspase-9 processing only in the presence of exogenously added procaspase-3 (Fig. 6, Lane 7), and not in its absence (Fig. 6, Lane 3). In accordance with previous data, the addition of constitutively active recombinant caspase-3 was sufficient to completely process procaspase-9 (Fig. 6, Lane 4). A similar dependence of procaspase-9 processing on caspase-3 was obtained when extracts from MCF-7 vc and MCF-7 CASP-3 cells were compared (data not shown). The same MCF-7 cell extracts were also subjected to a DEVD cleavage assay. As shown in Fig. 6, DEVD cleavage activity was closely correlated with the presence of active caspase-3 and with the processing of procaspase-9. These results underscore the close relationship between the presence or absence of active caspase-3 and procaspase-9 processing.

**FIG. 4.** In vitro processing of exogenously added procaspase-9.

**FIG. 5.** Sensitization of MCF-7 cell extracts for cytochrome c.

**FIG. 6.** Processing of procaspase-9 in caspase-3-reconstituted MCF-7 cell extracts.
Fas have both been implicated in chemotherapy-induced apoptosis (33–35). Our data obtained with cellular extracts indicate that both cytochrome c- and caspase-8-mediated processing of procaspase-9 are strongly dependent on the presence of caspase-3. These findings extend earlier studies that describe a dependence of procaspase-9 processing on caspase-3 during TNF- and granulocyte M-mediated apoptosis (39, 40). The dependence of procaspase-9 processing on caspase-3 after cytochrome c stimulation also sheds new light on the simple hierarchical relationship whereby caspase-3 is placed upstream of caspase-3 in the cytochrome c pathway. Our data indicate that processing of procaspase-9 requires caspase-3 activity, most likely in parallel with Apaf-1. This relationship now needs to be reexamined in cells derived from caspase-3- and caspase-9 knockout animals (15, 16, 22). A defect in caspase-3 leads to caspase-3 inactivation may generally promote tumorigenesis and may be the basis for this observation remains to be explored, but recently cortex-dependent activation may exist in MCF-7 cells. The molecular basis for this observation remains to be explored, but recently reported mechanisms of postmitochondrial apoptosis regulation provide a starting point for future investigations (41).

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