Apoptosis Resistance of MCF-7 Breast Carcinoma Cells to Ionizing Radiation Is Independent of p53 and Cell Cycle Control but Caused by the Lack of Caspase-3 and a Caffeine-Inhibitable Event

Frank Essmann, Ingo H. Engels, Gudrun Totzke, Klaus Schulze-Osthoff, and Reiner U. Jänicke

University of Düsseldorf, Institute of Molecular Medicine, Düsseldorf, Germany

ABSTRACT

We have shown previously that ionizing radiation (IR) induces a persistent G2-M arrest but not cell death in MCF-7 breast carcinoma cells that harbor functional p53 but lack caspase-3. In the present study, we investigated the mechanisms of apoptosis resistance and the roles of p53, caspase-3, and cell cycle arrest in IR-induced apoptosis. The methylxanthine caffeine and the staurosporine analog UCN-01, which can inhibit ATM and Chk kinases, efficiently abrogated the IR-induced G2-M arrest and induced mitochondrial activation as judged by the loss of the mitochondrial membrane potential and the release of cytochrome c and Smac/Diablo. However, despite these proapoptotic alterations, cell death and activation of the initiator caspase-9 were not induced in MCF-7 cells but were interestingly only observed after reexpression of caspase-3. Sensitization to IR-induced apoptosis by caffeine or UCN-01 was abrogated neither by cycloheximide nor by pifithrin-α, an inhibitor of the transcriptional activity of p53. Furthermore, suppression of p53 by RNA interference could not prevent caffeine- and IR-induced mitochondrial alterations and apoptosis but resulted in an even more pronounced G2-M arrest. Collectively, our results clearly show that the resistance of MCF-7 cells to IR-induced apoptosis is caused by two independent events; one of them is a caffeine- or UCN-01-inhibitable event that does not depend on p53 or a release of the G2-M arrest. The second event is the loss of caspase-3 that surprisingly seems essential for a fully functional caspase-9 pathway, even despite the previous release of mitochondrial proapoptotic proteins.

INTRODUCTION

Intrinsic or acquired resistance of tumor cells to chemotherapy or radiotherapy remains a major obstacle to successful cancer management. Mechanisms causing resistance are diverse and poorly defined; however, recent evidence suggests that aberrant apoptosis contributes to this phenomenon. DNA-damaging agents, such as chemotherapy drugs or ionizing radiation (IR), are known to induce apoptosis via the intrinsic mitochondrial death pathway, in which a class of cysteine proteases, called caspases, plays a crucial role (1, 2). This pathway is initiated at the mitochondrion by the release of cytochrome c, which, together with dATP and apoptotic protease-activating factor 1, binds to procaspase-9 to form the apoptosisom (3). On formation of the apoptosome, procaspase-9 is autoproteolytically processed, resulting in the activation of downstream caspases such as caspase-3, -6, and -7 (4). Hence, caspase-9 constitutes a crucial component of the intrinsic death pathway. The finding that cells from caspase-9 knock-out mice are resistant to various death stimuli, including IR, further emphasizes this (5). Conversely, caspase-3, although required for some of the typical hallmarks of apoptosis (6, 7), seems to be dispensable for cell death induced by a variety of stimuli, such as tumor necrosis factor, CD95 ligand, or anticancer drugs (6–8). This is consistent with the observations that other effector caspases, such as caspase-6 or -7, can compensate for the lack of caspase-3, at least to some extent, in a stimulus- and cell type-dependent manner (9).

Whereas the pathways leading to caspase activation and cell death are mainly resolved, the sequence of events that take place upstream of the mitochondria following DNA damage is less well defined. Depending on the stimulus and cell type, DNA-damaged cells arrest in various phases of the cell cycle to ensure proper repair of damaged DNA (10). Whereas the G1 arrest is mediated by the transcription factor p53 via induction of the cyclin-dependent kinase inhibitor p21, the G2 arrest seems to be mainly controlled by the phosphoinositide 3-kinases ATM and ATR (11). Through a series of phosphorylation events involving the Chk1 and Chk2 kinases, ATM and ATR prevent activation of the cyclin B/Cdc2 complex that is essential for the cells to enter mitosis. Although not required for the initialization of this process, p53 seems to be necessary for the maintenance of the IR-induced G2-M arrest (12). This is achieved via the p33-dependent induction of 14-3-3 ο and GADD45, which specifically interfere with the ATM/ATR-controlled pathway. Together with its multiple activities that result in apoptosis induction, these functions make p53 a powerful tumor suppressor (13). The loss or functional inactivation of p53 that is observed in >50% of all of the human tumors correlates in many cases with apoptosis resistance.

However, apoptosis susceptibility does not always correlate with the status of p53 expression (14–16). We also have reported recently that MCF-7 breast carcinoma cells, regardless of whether they express caspase-3, are especially resistant to IR-induced apoptosis, although they harbor a functional p53 gene (8). Because the radiosensitive phenotype of these cells was accompanied by a persistent arrest in the G2 phase of the cell cycle, we postulated that this event prevents the generation of an as-yet unknown apoptotic signal. Several reports showed that abrogation of the G2 checkpoint by either the methylxanthine caffeine or the staurosporine analog UCN-01 correlated with a marked increase in the sensitivity of various tumor cells to ionizing radiation and certain chemotherapeutic agents (17–20). Although both compounds probably target several proteins, the release of the G2 block by caffeine and UCN-01 is most likely mediated by inhibition of the ATM/ATR and Chk1 kinases, respectively (21, 22). In further studies it was reported that the sensitizing effect of caffeine and UCN-01 is based on their ability to release cells from the IR-induced G2-M arrest and was preferentially observed in cells lacking functional p53 (23–26). In contrast, other reports suggested that caffeine overrides the G2-M block independently of the p53 status of the cell; however, these cell cycle control modifications by caffeine were not associated with enhancement of radiation-induced apoptosis or reduction of clonogenic growth (27, 28). Thus, these conflicting results suggest that the relationship between the radiosensitizing effect of caffeine, the G2-M checkpoint, and p53 is far from being elucidated.

On the basis of our previous findings that revealed a persistent irradiation-induced G2-M arrest in MCF-7 cells (8), we further inves-
tigated the role of p53 and the G2-M block with regard to the apoptosis-resistant phenotype of these cells following exposure to IR. To this end, the effects of caffeine and UCN-01 were studied in irradiated MCF-7 and MCF-7/CASP-3 cells in combination with agents that interfere with p53 function and expression. Using this approach, we found that the sensitizing effect of caffeine and UCN-01 to IR-induced apoptosis is mediated independently of p53 and their ability to release cells from the IR-induced G2-M arrest. We also show that sensitization by caffeine or UCN-01 requires the presence of caspase-3 because only caspase-3–expressing MCF-7 cells, but not MCF-7 cells lacking caspase-3, were killed by this treatment. Therefore, our results indicate that two separate events, the loss of caspase-3 and a caffeine- or UCN-01–sensitive process that does not require p53 or the release of the G2-M block, contribute to the apoptosis resistance of MCF-7 breast carcinoma cells to IR.

### MATERIALS AND METHODS

**Cells, Reagents, and Antibodies.** MCF-7 and MCF-7/CASP-3 breast carcinoma cells (7) were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 10 mmol/L glutamine, and 50 µg/mL each of streptomycin and penicillin. The monoclonal actin antibody, caffeine, cycloheximide, propidium iodide, and the protease inhibitors phenylmethylsulfonyl fluoride, aprotinin, leupeptin, and pepstatin were from Sigma (St. Louis, MO). Pifithrin-α and the fluorogenic caspase-3 and -9 substrates N-acetyl-Asp-Glu-Val-Asp-aminomethyl-coumarin (DEVD-AMC) and N-acetyl-Leu-Glu-His-Asp-aminomethyl-coumarin (LEHD-AMC), respectively, were from BIOMOL International (Plymouth Meeting, PA). The monoclonal p53 antibody (Ab-6) was from Calbiochem (San Diego, CA); the monoclonal p21, cycloheximide, and poly(ADP-ribose) polymerase (PARP) antibodies were from PharMingen, Inc (San Diego, CA). The polyclonal goat and rabbit antibodies recognizing caspase-3 and Smac/Diablo, respectively, were from R&D Systems (Minneapolis, MN), and the polyclonal caspase-9 antibody was from New England BioLabs, Inc (Beverly, MA). UCN-01 was a kind gift from R. Schultz (National Cancer Institute, Bethesda, MD).

**Treatment and Transfection of Cells.** Cells were exposed to IR (usually 20 Gy) using a Philips gamma chamber (Philips Medical Systems, Andover, MA) with a cobalt-60 source (XK 5105–11) in the absence or presence of either caffeine (1 mmol/L), UCN-01 (100 mmol/L), pifithrin-α (15 mmol/L), or cycloheximide (Chx; 15 ng/mL). None of these compounds applied alone elicited an apoptotic response at the indicated concentrations.

MCF-7/CASP-3 cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) with the pSilencer vector containing the specific p53 small interfering RNA (siRNA) sequence (29) according to the protocol supplied by the manufacturer (Ambion Inc., Austin, TX). After 48 hours, cells were trypsinized and reseeded in medium containing 400 µg/mL hygromycin (for selection of p53 siRNA-expressing clones) and 400 µg/mL G418 (for coselection of caspase-3–expressing clones).

**Preparation of Cell Extracts, Western Blotting, and DNA Fragmentation Analysis.** Cell extracts were prepared as described previously (8). Equal amounts of the proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amer sham Pharmacia, Piscataway, NJ). Proteins were visualized by enhanced chemiluminescence staining using ECL reagents (Amer sham Pharmacia). For DNA fragmentation analysis, cellular DNA was prepared using the Blood and Cell Culture Mini DNA kit (Qiagen, Valencia, CA). Purified DNA was incubated for 2 hours at 37°C with 200 µg/mL RNase and analyzed on 1.6% agarose gels. DNA was visualized by ethidium bromide staining.

**Measurement of Cytochrome c and Smac/Diablo Release.** Approximately 4 × 106 cells were permeabilized for 5 minutes at 4°C in a buffer containing 50 µg/mL digitonin, 250 µmol/L sucrose, 20 mmol/L HEPES (pH 7.4), 1.5 mmol/L MgCl2, 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, and 2 µg/mL of each of the protease inhibitors aprotinin, pepstatin, and leupeptin. Cells were centrifuged at 1000 × g for 5 minutes at 4°C to remove cell nuclei. The supernatant was transferred to a fresh tube and centrifuged at 10,000 × g for 15 minutes at 4°C. The resulting supernatants containing the cytosolic fractions were loaded onto a 0.1% SDS and 15% polyacrylamide gel. Cytochrome c and Smac/Diablo release were analyzed by immunoblot analysis.

**Determination of the Mitochondrial Transmembrane Potential.** The mitochondrial transmembrane potential (ΔΨm) was analyzed using the ΔΨm-specific stain TMRE (Molecular Probes, Eugene, OR). Briefly, 106 cells were stained in a solution containing 25 nmol/L TMRE for 30 minutes. Staining was quantified by FL2 and scatter characteristics using a flow cytometer.

**Cell Death Assessment, Cell Cycle Analysis, and Fluorometric Determination of Caspase-3 and Caspase-9 Activities.** Cell death was assessed by the uptake of propidium iodide (2 µg/mL) into nonfixed cells and subsequent flow cytometric analyses with the FSC/FL2 profile. For cell cycle analyses, nuclei were prepared by lysing cells in a hypotonic buffer (0.1% sodium citrate, 0.1% Triton X-100, and 50 µg/mL propidium iodide) and subsequently analyzed by flow cytometry with the FCS/FL2 profile. The proportion of cells in the G1 versus G2 phase is indicated. All of the flow cytometry analyses were performed on a FACScalibur (Becton Dickinson, Franklin Lakes, NJ) using CellQuest analysis software. For each determination, a minimum of 10,000 cells was analyzed. Caspase-3 and -9 activities were determined as described previously (8).

**Clonogenic Survival Assay.** Equal cell numbers were irradiated and seeded onto 96-well plates at 3000 cells per well. After 11 days, viable cells were stained for 20 minutes with 0.5% crystal violet in 20% methanol, washed extensively, and solubilized in 33% acetic acid followed by measurement of the A560.

### RESULTS

**Caffeine Abrogates the Ionizing Radiation-Induced G2-M Arrest in Both MCF-7 Lines but Induces Apoptosis only in MCF-7/CASP-3 Cells.** MCF-7 cells are a widely used cellular system for breast cancer. We showed previously that MCF-7 cells have lost caspase-3 expression because of a 47-bp deletion within exon 3 of the CASP-3 gene (7). Restoration of caspase-3 expression induced some hallmarks of apoptosis in response to tumor necrosis factor and anticancer drug treatment. However, regardless of the status of caspase-3 expression, MCF-7 cells remained resistant to apoptosis induction by IR. Instead, irradiated MCF-7 and MCF-7/CASP-3 cells displayed a persistent G2-M arrest (8) that correlated with the induction of cellular senescence as measured by staining of the senescence-associated β-Galactosidase activity (data not shown).

To test our hypothesis that the IR-induced G2-M arrest prevents the generation of an as yet unknown apoptotic signal, we exposed both cell lines to a single dose of 20 Gy in the absence or presence of the methylxanthine caffeine. Caffeine is a widely used radiosensitizing agent that was shown to override DNA damage-induced cell cycle arrest in a variety of cell lines (19, 20, 23–26). Despite the presence of wild-type p53, 1 mmol/L caffeine, a commonly used concentration, completely abrogated the IR-induced G2-M arrest in both MCF-7 cell lines (Fig. 1 A, top). Interestingly, induction of cell death as measured by the uptake of propidium iodide was only observed in caspase-3–expressing, but not in caspase-3–deficient, MCF-7 cells (Fig. 1 A, bottom). MCF-7/CASP-3 cells exposed to IR and caffeine detached from the plastic surface and showed the morphologic changes typical of cells undergoing apoptosis, such as shrinkage and blebbing (data not shown). Consistent with the cell death data, cleavage of the caspase substrate PARP (Fig. 1 B) and DNA fragmentation (Fig. 1 C) also were predominantly evident in caspase-3–expressing MCF-7 cells exposed to IR and caffeine. These results indicated that in contrast to apoptosis induction by various death stimuli including anticancer drugs, caspase-3 is essential for IR- and caffeine-induced death of MCF-7 cells. Similar results were obtained when the experiments were performed with the staurosporine analog UCN-01 (data not shown), which, like caffeine, sensitizes cells to radiation-induced...
apoptosis (22). Although MCF-7 cells do not undergo radiation-induced apoptosis even in the presence of caffeine, increasing radiation doses dramatically reduced their reproductive capacity as determined in a clonogenic survival assay (Fig. 1D). This effect was even more pronounced when MCF-7/CASP-3 cells were used, although the accelerated reproductive death rate was observed predominantly in the presence of caffeine, which is consistent with the apoptosis data. Collectively, these results indicate that caspase-3 radiosensitizes MCF-7 cells also in terms of their reproductive capacity in the presence of caffeine.

Caspase-9 Is Only Activated in MCF-7/CASP-3 Cells, Although Exposure to Caffeine and Ionizing Radiation Activates the Mitochondria in Both MCF-7 Cell Lines. These results suggested that the resistant phenotype of MCF-7 cells toward IR-induced apoptosis is caused by two independent events: one of them seems to be inhibitable by caffeine or UCN-01, and the other is most likely because of the absence of caspase-3. In an attempt to understand the latter, we analyzed characteristic apoptotic events that take place at the level or downstream of mitochondria. As expected, in contrast to either treatment alone, the combined treatment of MCF-7/CASP-3 cells with IR and caffeine resulted in the loss of the mitochondrial membrane potential (Fig. 2A), a process that is closely associated with the intrinsic death pathway (30). This treatment also induced the release of cytochrome c and Smac/Diablo, which represent two mitochondria-derived proapoptotic factors required for the activation of the intrinsic death pathway (Fig. 2B). Interestingly, all of these events also were induced to a similar extent by the combined treatment in MCF-7 cells, a cell line that does not undergo IR-induced apoptosis even in the presence of caffeine (Fig. 1).

Remarkably, despite our finding that IR, together with caffeine, induced cytochrome c and Smac/Diablo release in both cell lines, activation of the initiator caspase-9 was only observed in MCF-7/CASP-3 cells, in which this treatment resulted also in the activation of caspase-3 (Fig. 3). This could be readily shown by the specific fluorometric caspase substrate cleavage assays (Fig. 3A) and by Western blot analysis showing the processed and thereby activated caspase-3 (Fig. 3B). Whereas neither treatment alone was able to activate these caspases above background levels, the combination of IR and caffeine resulted in an efficient activation of caspase-3 and -9 only in MCF-7/CASP-3 cells. Collectively, these results suggest that
Caspase-3 is essential for the activation of caspase-9 and subsequently also for a fully functional mitochondrial death pathway induced by IR.

Caffeine Sensitizes MCF-7/CASP-3 Cells to Ionizing Radiation-Induced Apoptosis Independently of the Transcriptional Activity of p53 and Independently of Cell Cycle Control. Caffeine and UCN-01 specifically target and inhibit the ATM/ATR and the Chk1 kinases, respectively, which were both shown to phosphorylate p53 (11). To investigate a possible role of p53 in this process, we compared cell cycle progression and cell death induction in both MCF-7 cell lines exposed to a combination of IR and caffeine in the absence or presence of pifithrin-α/H9251, a potent inhibitor of the transcriptional activity of p53 (31). Similar to caffeine, treatment of MCF-7/CASP-3 and MCF-7 cells with pifithrin-α/H9251 completely abrogated the IR-induced p53-dependent expression of p21 without affecting p53 levels (Fig. 4B and data not shown), showing that pifithrin-α efficiently blocked the transcriptional activity of p53. However, in contrast to caffeine that completely released the IR-induced G2-M arrest in both cell lines, cotreatment of the cells with pifithrin-α resulted in an even more pronounced IR-induced cell cycle arrest that could not be overcome by caffeine (Fig. 4A, top). Treatment of the cells with pifithrin-α alone had no effect on cell cycle progression (data not shown).

However, although MCF-7/CASP-3 cells simultaneously exposed to the three treatments were arrested in the G2-M phase to a similar extent as cells that were only irradiated, they were not protected from apoptosis (Fig. 4A, bottom). Pifithrin-α also had no effect on apoptosis of MCF-7 cells because they remained radiation resistant in the absence or presence of either compound. Similar results were obtained when the experiments were performed with UCN-01 (data not shown). Thus, these results clearly show that the radiosensitizing effect of caffeine is mediated independently of the transcriptional activity of p53. They also provide evidence that caffeine sensitizes MCF-7/CASP-3 cells to IR-induced apoptosis independent of its ability to release the irradiation-induced G2-M arrest.

p53 Is Not Required for Caffeine-Mediated Radiosensitization of MCF-7/CASP-3 Cells. Next we asked whether p53 protein expression or new protein biosynthesis is required for the radiosensitizing effect of caffeine. To this end, we first performed similar experiments as described previously in the absence or presence of Chx. Although Chx almost completely blocked the IR-induced p21 expres-

Fig. 3. IR and caffeine induce the activation of caspase-3 and -9 only in MCF-7/CASP-3 cells. A, caspase-3 (DEVDase; top) and caspase-9 (LEHDase; bottom) activities in cytoplasmic extracts of cells exposed to a single dose of 20 Gy in the absence (○) or presence of 1 mmol/L caffeine (■). Caffeine alone had no effect on caspase activities (data not shown). B, Western blot analysis showing that IR and caffeine induce caspase-9 (top) and caspase-3 (middle and bottom) activation only in MCF-7/CASP-3 cells. The caspase proforms and their active subunits are indicated. One representative experiment of five is shown.

Fig. 4. Pifithrin-α reverses the caffeine-mediated release of the radiation-induced G2-M arrest but has no effect on apoptosis induction. A, Cells were either left untreated or exposed to a single dose of 20 Gy in the absence (○) or presence of 1 mmol/L caffeine (■), 15 μmol/L pifithrin-α (○), or to a combination of both (■). After the indicated times, the G2 to G1 ratio and the percentage of apoptotic cells were determined by FACS analyses. Data represent the mean of three independent experiments. B, Pifithrin-α inhibits the transcriptional activity of p53. Western blot analysis of the status of p53 and p21 expression in untreated MCF-7/CASP-3 cells and in cells that were either exposed to a single dose of 20 Gy, 1 mmol/L caffeine, 15 μmol/L pifithrin-α alone, or to the indicated combinations. Cell extracts were harvested at the indicated times. One representative experiment of two is shown.
Cell extracts were harvested after the indicated days. Untreated or exposed to a single dose of 20 Gy in the absence or presence of 15 ng/mL Chx.

MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy in the absence or presence of 15 ng/mL Chx.

Western blot analysis for the status of p21 in MCF-7 and MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy in the presence or absence of 15 ng/mL Chx. Cell extracts were harvested after the indicated days. C and D. Cell death determination of untreated MCF-7 and MCF-7/CASP-3 cells or cells that were exposed to a single dose of 20 Gy in the absence (○) or presence of 1 mmol/L caffeine (△), 15 ng/mL Chx (●), or to a combination of both (□). Data represent the mean of three independent experiments ± SD.

Caffeine-mediated sensitization toward IR-induced apoptosis does not require new protein synthesis. A. Western blot analysis for the status of p21 in MCF-7 and MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy or 15 ng/mL Chx or to a combination of both. Cell extracts were harvested after 3 days. B. Western blot analysis for the status of p53 in MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy in the absence or presence of 15 ng/mL Chx. Cell extracts were harvested after the indicated days. C and D. Cell death determination of untreated MCF-7 and MCF-7/CASP-3 cells or cells that were exposed to a single dose of 20 Gy in the absence (○) or presence of 1 mmol/L caffeine (△), 15 ng/mL Chx (●), or to a combination of both (□). Data represent the mean of three independent experiments ± SD.

Fig. 5. Caffeine-mediated sensitization toward IR-induced apoptosis does not require new protein synthesis. A. Western blot analysis for the status of p21 in MCF-7 and MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy or 15 ng/mL Chx or to a combination of both. Cell extracts were harvested after 3 days. B. Western blot analysis for the status of p53 in MCF-7/CASP-3 cells that were either left untreated or exposed to a single dose of 20 Gy in the absence or presence of 15 ng/mL Chx. Cell extracts were harvested after the indicated days. C and D. Cell death determination of untreated MCF-7 and MCF-7/CASP-3 cells or cells that were exposed to a single dose of 20 Gy in the absence (○) or presence of 1 mmol/L caffeine (△), 15 ng/mL Chx (●), or to a combination of both (□). Data represent the mean of three independent experiments ± SD.

To further investigate the role of p53 in radiosensitization by caffeine, we analyzed an MCF-7 clone (MCF-7/p53si) in which p53 expression was efficiently suppressed because of stable transfection of a p53 siRNA (Fig. 6A; ref. 29). Because MCF-7 cells do not undergo IR- and caffeine-induced apoptosis because of the lack of caspase-3 (Fig. 1), we first examined whether this treatment requires p53 for the mitochondrial membrane depolarization, which we have shown to proceed also in the absence of caspase-3 (Fig. 2). We found that exposure of these cells to IR in the presence of caffeine substantially reduced the mitochondrial membrane potential, indicating that this event occurs independently of p53 expression (Fig. 6B). However, IR had an even more dramatic effect on the cell cycle distribution of MCF-7/p53si cells when compared with parental MCF-7 cells because almost the entire population of p53-deficient cells was arrested in the G2-M phase (Fig. 6C). Furthermore, in contrast to p53-expressing MCF-7 cells, caffeine could not overcome this G2-M arrest in MCF-7/p53si cells (Fig. 6C), although the activation of the mitochondria was achieved in both cell lines to a similar extent regardless of the p53 status. These data are consistent with our observation that suppression of the transcriptional activity of p53 by pifithrin-α resulted in an even more pronounced IR-induced G2-M arrest that could not be overcome by caffeine (Fig. 4). Collectively, these results suggest that although p53 seems to be required for the caffeine-mediated release of the IR-induced G2-M arrest, this event is not a prerequisite for sensitization achieved by caffeine.

Finally, we generated MCF-7/CASP-3 cells stably expressing the p53 siRNA described previously in which apoptosis induction could be more easily addressed than in caspase-3-deficient MCF-7 cells. Several clones were obtained that displayed reduced p53 levels following IR, and three clones with the highest suppression efficiencies were analyzed in more detail (Fig. 7A). As shown in Fig. 7B, the inability of these clones to up-regulate p53 expression following IR did not impair apoptosis susceptibility as judged by caspase-3 activity assays. Exposure to IR and caffeine induced comparable caspase-3 activities in the four cell lines regardless of the p53 status, indicating

Fig. 6. Effect of the p53 siRNA on cell cycle progression and mitochondrial membrane depolarization in MCF-7 cells. A. Western blot analyses of the status of p53 in MCF-7 cells and cells stably expressing the p53 siRNA (29) that were either left untreated or exposed to 20 Gy. Cell extracts were prepared after the indicated times. Probing the membrane with actin served as loading control. One representative experiment of three is shown. B. Measurement of the mitochondrial membrane potential (ΔΨm) in untreated (control) MCF-7 and MCF-7/p53siRNA cells and in cells exposed to a single dose of 20 Gy, 1 mmol/L caffeine, or to a combination of both. Cells were analyzed after 2 days. The arrows indicate the decrease of the mitochondrial membrane potential induced by IR and caffeine. One representative experiment of three is shown. C. Cell cycle analyses of untreated MCF-7 and MCF-7/p53siRNA cells (control) or cells exposed for 1 day to 20 Gy in the absence or presence of caffeine. The arrows indicate cells in the G1 and G2-M phase, respectively. One representative experiment of three is shown.

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that p53 is dispensable for this event. This was further confirmed by analyzing the mitochondrial membrane potential, which also dramatically decreased in the three MCF-7/CASP-3/p53si clones following IR and caffeine treatment (data not shown). As expected and consistent with these data, PARP cleavage induced by IR and caffeine also was observed independently of p53 expression in these cell lines (Fig. 7C). Thus, these data provide further evidence that the caffeine-inhibitable event upstream of the mitochondria does not require p53.

DISCUSSION

Role of Caspase-3 in Resistance to Ionizing Radiation-Induced Apoptosis. Chemotherapy and radiotherapy are important treatment modalities for many cancers, but the frequent occurrence of drug- and radiation-resistant tumors is a common clinical problem. Many different mechanisms can account for poor patient prognosis and treatment failure, including the loss or mutation of proapoptotic genes that regulate the intrinsic death pathway, such as p53 or apoptotic protease-activating factor 1 (13, 32, 33). Caspase-3 also was proposed to play a crucial role in DNA damage-induced apoptosis because it is frequently activated during apoptosis induced by various death stimuli, including DNA-damaging agents. However, the mere activation of a caspase during apoptosis does not necessarily imply its requirement for this process. Although caspase-3 is known to cleave a multitude of cellular substrates and is absolutely required for some classical alterations associated with apoptosis, it was shown to be dispensable for cell death induced by various death stimuli, including anticancer drugs (6–8, 34).

In the present study, we show that caspase-3 is required for IR- and caffeine-induced apoptosis of MCF-7 breast carcinoma cells and postulate that one reason for the radiosensitizing phenotype of these cells is the functional deletion of the CASP-3 gene. Although IR did not evoke an apoptotic response even in MCF-7/CASP-3 cells, cotreatment with the methylxanthine caffeine or the staurosporine analog UCN-01 resulted in massive apoptosis in a caspase-3–dependent manner. Interestingly, exposure to IR in combination with caffeine triggered mitochondrial activation in both MCF-7 cell lines as judged by the loss of the mitochondrial membrane potential and the release of cytochrome c and Smac/Diablo. However, consistent with an obligatory role for caspase-9 in IR-induced apoptosis (5), activation of caspase-9 was only achieved in IR- and caffeine-treated MCF-7/CASP-3 cells, showing that caspase-3 also is required for this event. Because caspase-9 is believed to act upstream of caspase-3 in the mitochondrial death pathway, this scenario seems paradoxical. Nevertheless, similar observations also were reported previously in tumor necrosis factor- and cisplatin-induced apoptosis of MCF-7 cells in which caspase-9 was only activated in MCF-7/CASP-3 cells (35, 36). This is because a complete processing of caspase-9 requires two cleavage events in its proform, one of which is mediated at Asp-315 by the autoproteolytic activity of caspase-9 itself and the other by caspase-3 at Asp-330 (37, 38). However, caspase-9 activation differs greatly from that of other caspases because it does not necessarily require proteolytic processing. In this respect, it was shown that procaspase-9 contains only 10% of the activity of cleaved caspase-9, which increased 2000-fold following its integration into the apoptosome (39, 40). In our study, caspase-9 activity and processing as assessed by the cleavage of the fluorogenic substrate LEHD-AMC and by immunoblot analyses, respectively, were only detected in MCF-7/CASP-3 cells exposed to IR and caffeine but not in similarly treated MCF-7 cells lacking caspase-3. Therefore, our results suggest that caspase-3 is required for the activation of caspase-9 in IR- and caffeine-induced apoptosis of MCF-7 cells. These data also give rise to the speculation that caspase-3 may be an essential component of a fully active apoptosome. This is consistent with the finding that apoptosis induction by microinjected cytochrome c also requires caspase-3 in MCF-7 cells (41). Because the presence of caspase-3 in the apoptosome was documented in some reports (42, 43), but not in others (39), a possible obligatory role for caspase-3 in the apoptosome might be stimulus- and cell type-specific and remains to be further elucidated.

As mentioned, we have shown previously that the anticancer drugs etoposide and doxorubicin, but not IR, elicit a caspase-dependent apoptosis pathway even in the absence of caspase-3 (PARP cleavage and death of MCF-7 cells were indetectable by z-Val-Ala-Asp-fluoromethyl ketone; ref. 8). Furthermore, in contrast to IR, exposure of MCF-7/CASP-3 cells to these anticancer drugs resulted in the activation of the mitochondrial death pathway, including cytochrome c release and activation of caspase-9 and -3. Therefore, we postulated that anticancer drug-induced apoptosis, but not apoptosis induced by
IR, uses, in addition to the classical mitochondrial pathway, an alternative route leading to caspase activation and cell death. Our present results strengthen and further extend this hypothesis because they show that IR- and caffeine-induced apoptosis of MCF-7 cells largely depends on the mitochondrial death pathway that requires the presence of caspase-3. The possible requirement of caspase-3 for a fully functional mitochondrial pathway, at least in MCF-7 cells, further implies that the alternative caspase-3-independent apoptotic pathway used by anticancer drugs also may be independent of the apoptosome. Although the existence of such a pathway(s) was documented recently in several reports (44–46), further investigations, preferentially in the MCF-7 cell system, are required to decipher anticancer drug- and IR-induced apoptotic pathways.

**Potential Mechanisms Involved in Caffeine-Mediated Sensitization to Ionizing Radiation-Induced Apoptosis.** In addition to the lack of caspase-3, our study also shows that the resistant phenotype of MCF-7 cells toward IR-induced apoptosis is caused by a caffeine- or UCN-01–inhibitable event that takes place upstream of the mitochondria. Thus far, numerous reports showed that caffeine and UCN-01 sensitize various tumor cell lines to IR-induced apoptosis, and most of them attributed this effect to the ability of these compounds to override the IR-induced G2-M arrest (19–22) preferentially in cells lacking functional p53 (14, 23–26). However, our data show that both agents sensitize MCF-7/CASP-3 cells to IR-induced apoptosis not only independently of checkpoint inhibition but also independently of the p53 status. We did not observe a significant difference with regard to the rate of apoptosis induced by IR and caffeine in MCF-7/CASP-3 cells expressing a functionally active p53 protein and cells in which p53 was rendered inactive by either pifithrin-α or expression of a p53 siRNA. Abrogation of p53 function and/or expression by pifithrin-α and the p53 siRNA, respectively, clearly had the opposite effect on cell cycle distribution. Exposing MCF-7 or MCF-7/CASP-3 cells to these compounds significantly increased the IR-induced G2-M arrest, which can be explained by the fact that cells defective or deficient for p53 bypass the G1 checkpoint to accumulate in the G2-M phase (47). Interestingly, however, was the observation that this profoundly increased G2-M arrest could not be overcome by caffeine, although these cells were as efficiently killed by this treatment as p53-expressing MCF-7/CASP-3 cells. Even though a direct causal link between checkpoint abrogation and sensitization toward IR-induced apoptosis is difficult to prove because of the complexity of the system, our results show that caffeine also mediates its radiosensitizing effect in p53-expressing cells and independently of its ability to release the IR-induced G2-M arrest. Therefore, our results are, at least partially, consistent with more recent studies describing that caffeine releases the IR-induced cell cycle block in a p53-independent manner (27, 28). However, the reason why caffeine did not induce apoptosis in these studies is unknown but could be because of the lack of relevant mitochondrial apoptosis components in the used cells, a possibility that was not explored.

What are the mechanisms involved in radiosensitization by caffeine and UCN-01? Caffeine inhibits the nucleotide exchange activity of RCC1, alkaline phosphatase activity, phosphodiesterase activity, and the ATM and ATR kinases at least in vitro. Because ATM controls a pathway leading to G2-M arrest involving inhibition of Cdc2 and cyclin B1 and because ATM-deficient cells are hypersensitive to IR, it seemed logical to assume that especially inhibition of ATM and its downstream target Chk1 by caffeine and UCN-01, respectively, is responsible for the sensitizing activity of these compounds. However, whether caffeine also can inhibit ATM in vivo seems to be controversial (48). In this context, we did not detect any caffeine-mediated adverse changes in the expression levels of various ATM-controlled cell cycle regulators, including Cdc2 and cyclin B1 (data not shown), that could account for the radiosensitizing activity of caffeine as was reported previously (26). Caffeine also did neither inhibit p53 expression or ATM-mediated phosphorylation of p53 on serine 15, nor did it block the rate of DNA repair in irradiated MCF-7 or MCF-7/CASP-3 cells (data not shown).

Caffeine was shown previously to induce apoptosis via p53 and Bax (49, 50). Although transcriptionally independent activities of p53 are much less understood than its function as a transcription factor (13), recent evidence suggests that cytoplasmic or mitochondrial p53 may directly interact with proapoptotic and antiapoptotic members of the Bcl-2 family, respectively, thereby inducing apoptosis (51, 52). However, suppression of endogenous p53 using a p53 siRNA did affect neither apoptosis of IR- and caffeine-treated MCF-7/CASP-3 cells nor the loss of the mitochondrial membrane potential regardless of caspase-3 expression. Hence, we clearly favor a p53- and cell cycle-independent radiosensitization pathway mediated by caffeine.

The BH3-only proapoptotic BID protein might be involved in such a pathway because it was shown recently that cleavage of BID by an as yet unknown aspartate is required for apoptosis induced by DNA-damaging agents, including IR (53). With regard to this, we noticed that caffeine significantly reduced phosphorylation of BID, whereas expression of other members of the Bcl-2 family, such as Bcl-2, Bcl-xL, Bad, Bax, and Bak, was not affected by this treatment. Phosphorylated BID is uncleavable by caspases and hence is unable to induce cytochrome c release from mitochondria (54). Thus, our observation suggests that caffeine may radiosensitize cells via interfering with the phosphorylation status of BID perhaps by inhibiting caspase kinases I and II, which were shown to phosphorylate BID. Both kinases are efficiently inhibited in vitro by staurosporine (54), making it highly likely that the staurosporine analog UCN-01 also acts in a similar fashion. However, further, more detailed studies are required to verify this hypothesis.

In summary, we have shown that the resistant phenotype of MCF-7 cells toward IR-induced apoptosis is caused by two independent events. One is a caffeine- and UCN-01–inhibitable event that is independent of cell cycle progression and p53 status, and the other is the functional deletion of the CAPS-3 gene. Especially in view of our observation that reexpression of caspase-3 also accelerates IR-induced reproductive death, these results provide evidence that the inactivation of caspase-3 may promote tumorigenesis and may have contributed to the development of the breast cancer from which MCF-7 cells are derived. Therefore, it now will be of particular interest to study whether IR-induced apoptosis of other tumor cell lines also depends on a functional caspase-3 because such findings may help to develop improved therapies for the management of radioresistant tumors.

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