Protein Kinase C Inhibitor and Irradiation-induced Apoptosis: Relevance of the Cytochrome *c*-mediated Caspase-9 Death Pathway[1]

Sonia Rocha, Maria S. Soengas, Scott W. Lowe, Christoph Glanzmann, Doriano Fabbro, Kaspar Winterhalter, Stephan Bodis, and Martin Pruschy[2]

Department of Radiation Oncology, University Hospital Zurich, CH-8091 Zurich, Switzerland [S. R., C. G., S. B., M. P.]; Laboratory for Biochemistry, Federal Institute of Technology, 8091 Zurich, Switzerland [S. R., K. W.]; Novartis Pharma Inc., 4002 Basel, Switzerland [D. F.]; and Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724 [M. S. S., S. W. L.]

Abstract

Caspases are a family of cysteine proteases that constitute the apoptotic cell death machinery. We report the importance of the cytochrome *c*-mediated caspase-9 death pathway for radiosensitization by the protein kinase C (PKC) inhibitors staurosporine (STP) and PKC-412. In our genetically defined tumor cells, treatment with low doses of STP or the conventional PKC-specific inhibitor PKC-412 in combination with irradiation (5 Gy) potently reduced viability, enhanced mitochondrial cytochrome *c* release into the cytosol, and specifically stimulated the initiator caspase-9. Whereas treatment with each agent alone had a minimal effect, combined treatment resulted in enhanced caspase-3 activation. This was prevented by broad-range and specific caspase-9 inhibitors and absent in caspase-9-deficient cells. The tumor suppressor p53 was required for apoptosis induction by combined treatment but was dispensable for dose-dependent STP-induced caspase activation. These results demonstrate the requirement for an intact caspase-9 pathway for apoptosis-based radiosensitization by PKC inhibitors and show that STP induces apoptosis independent of p53.

Introduction

Depending on the stimulus that initiates a death program, different caspase cascades, the core of the apoptotic program, are activated (1). Whereas death ligand-mediated receptor activation facilitates the clustering and autoprocessing of caspases (initiator caspase-8) at the plasma membrane, other stress stimuli activate caspases at intracellular sites (2). Mitochondrial cytochrome *c* is released into the cytosol on cellular stress, where, in the presence of ATP/dATP, it associates with Apaf-1, which processes procaspase-9 to its active form (3, 4). This, in turn, activates the effector caspase-3, leading to cellular apoptotic morphology.

Induction of DNA double-strand breaks was considered the major mechanism of IR[3]-induced cell death. However, more recent studies focus on multiple signal transduction cascades generated at the site of DNA damage and at the plasma membrane, which trigger cells to undergo apoptosis (5). Apoptosis by IR has been demonstrated in many cell types. Cellular radiation resistance may be linked to the overexpression of antiapoptotic proteins or the loss of apoptosis-inducing proteins like the tumor suppressor protein p53. Radioresistance of tumor cells lacking p53 may be a consequence of a diminished ability to undergo apoptosis *in vitro* and *in vivo* (6). Cellular stresses such as growth factor deprivation, DNA-damaging agents, and irradiation rapidly activate p53 as a transcriptional activator. Various p53-inducible genes are known, but the specific apoptotic signaling network induced by p53 is only now emerging (7–9).

To overcome treatment resistance of tumor cells is a formidable task for radiobiology. The use of chemical modifiers as radiosensitizers in combination with low-dose irradiation may increase the therapeutic effect by overcoming a high apoptotic threshold. Here, growth-promoting PKC represents an interesting target. The multigene family of PKC codes for serine/threonine kinases that act as transducers for various lipid second messengers in the regulation, transduction, and propagation of cell-proliferative stimuli. Previous studies have indicated the PKC family to be an interesting target not only for a single treatment modality but also in combination with additional chemotherapeutic agents and IR. The cellular response to stress comprises both proapoptotic and antiapoptotic pathways, and concomitant inhibition of growth-promoting or antiapoptotic pathways promotes cell death. PKC inhibitors such as STP and UCN-01 are potent inducers of apoptosis but also sensitize tumor cells to antimetabolites or cytotoxic agents. On the other hand, PKC stimulation by phorbol esters can rescue different cell types from cell death induced by glucocorticoid and growth factor withdrawal (10, 11). Different PKC inhibitors

Received 6/15/00; revised 7/25/00; accepted 8/3/00.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
[1] S. R. was supported by a predoctoral fellowship by the Baugarten Stiftung, and S. B. and M. P. were supported by a grant from the Swiss and Zurich Cancer Leagues.
[2] To whom requests for reprints should be addressed, at Department of Radiation Oncology, University Hospital Zurich, Raemistrasse 100, CH-8091 Zurich, Switzerland. Phone: 41-12558549; Fax: 41-12554435; E-mail: pum@dmr.usz.ch.

[3] The abbreviations used are: IR, ionizing radiation; PKC, protein kinase C; STP, staurosporine; MEF, mouse embryo fibroblast; PARP, poly(ADP-ribose) polymerase; pNA, *p*-nitroanilide; FMK, fluoromethyl ketone; cPKC, conventional PKC; DEVDase activity, protease activity for caspase-3 substrate Ac-DEVD-pNA.

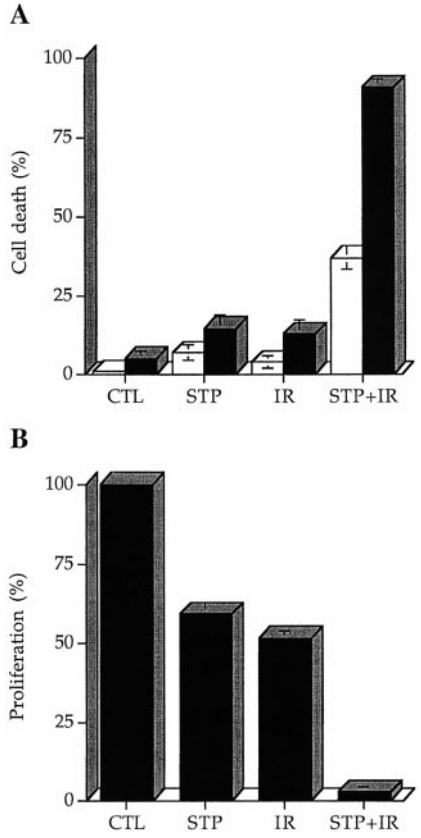

Fig. 1. Cell death induction by combination treatment with a low-dose of STP and IR. *A*, cell viability in E1A/ras-transformed MEFs was determined by trypan blue exclusion 6 (□) and 24 h (■) after treatment with STP (20 nM) and IR (5 Gy) alone or in combination. *B*, proliferation was assessed with the alamar blue assay 24 h after treatment with STP (20 nM) and IR (5 Gy) alone or in combination.

sensitize tumor cells for irradiation (12, 13), but mechanistic studies with STP or other PKC inhibitors as inducers of apoptosis in combination with irradiation are scarce.

Here we analyze the execution of apoptosis with the PKC inhibitor STP in combination with IR in genetically defined tumor cells that mimic the genetic conditions of developing tumors. Biochemical analysis of these processes complement the cell biological approach to demonstrate the importance of an intact cytochrome *c*-dependent caspase-9/-3 pathway for STP-induced radiosensitization and provide a model of treatment resistance.

Results

STP and IR Cooperatively Induce Apoptosis. We used E1A/ras-transformed MEFs to determine the cytotoxic effect of agents STP and IR. Quantitative analysis of dead cells was performed using the trypan blue exclusion assay. Treatment with a low dose of STP (20 nM) or 5 Gy of irradiation induced minimal cell death at an early time point (6 h), and even 24 h after treatment, only 15% of the cells were trypan blue positive. On the other hand, combined treatment with STP and IR induced cell death in 30% of the cell population at the 6 h time point, and 24 h after treatment, up to 90% of the total cell population was no longer viable (Fig. 1*A*). This massive induction of cell death correlated with a drastic reduction in metabolic activity of the treated cell population after combined treatment (more than 90% loss of metabolic activity; Fig. 1*B*). Metabolic activity was assessed with the alamar blue assay, a proliferation assay comparable to the tetrazolium-based [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay] quantification of cell metabolism. The comparative results of cell death and reduced metabolic activity on combined treatment indicate that combined treatment not only inhibited the proliferative activity but induced massive cell killing. STP or IR as a single agent alone only inhibited metabolic activity to 59% and 51%, respectively, in comparison with untreated cells. The more pronounced reduction of proliferative activity by STP or IR as a single treatment in comparison with the minimal effect on cell viability suggests a possible contribution of cell cycle arrest induced by each of the two agents (Fig. 1*B*).

To avoid clonal variations, follow-up experiments were also performed with E1A/ras-transformed cells obtained by uncloned mass culture.

Characteristic morphological changes indicative of apoptosis appeared after combined treatment. Cells started to round up 6 h after treatment and detached as single cells from the surface (data not shown). To assess the mode of cell death, the effector protease activity (caspase-3-like/DEVDase activity) induced as part of an apoptotic process was measured in the cytosolic S-100 fraction 6 h after the different treatments. Using Ac-DEVD-pNA as a colorimetric caspase-3 substrate, only minimal DEVDase activity was observed after treatment with STP (20 nM) or IR (5 Gy) alone, and the DEVDase activity was not further enhanced at later time points (data not shown). On the other hand, combined treatment increased caspase-3-like activity at least 10-fold (Fig. 2*A*). Because an appropriate α-murine caspase-3 antibody that recognizes both the zymogen and activated form of caspase-3 was not available, immunoblotting was carried out with an antibody that only recognizes active caspase-3. Combined treatment resulted in an increase of the active large p17 fragment and p19 fragment, corresponding to enhanced caspase activation. (Fig. 2*B*). In parallel, caspase activation was detected by cleavage of endogenous PARP. Partial cleavage of PARP could be detected only after combined treatment with STP and irradiation (Fig. 2*C*).

Using both exogenous and endogenous inhibitors of apoptosis, the relevance of apoptosis for the cooperative antiproliferative effect was tested. Preincubation of cells with increasing concentrations of the broad-range caspase inhibitor Z-VAD-FMK (10–100 μM) reversed the antiproliferative effect of combined treatment with STP and irradiation to the level induced by each treatment alone (Fig. 3*A*, compare with Fig. 1*A*). Members of the Bcl-2 family prevent activation of the cytochrome *c*-dependent caspase cascade (reviewed in Ref. 14), thereby increasing the endogenous apoptotic threshold. Using a retroviral construct, Bcl-2-overexpressing cells were generated, and the cytotoxic effect of combined treatment and DEVDase activation were tested with the proliferation assay. Bcl-2-overexpressing cells were up to 4 times more resistant than control cells (cells infected with an

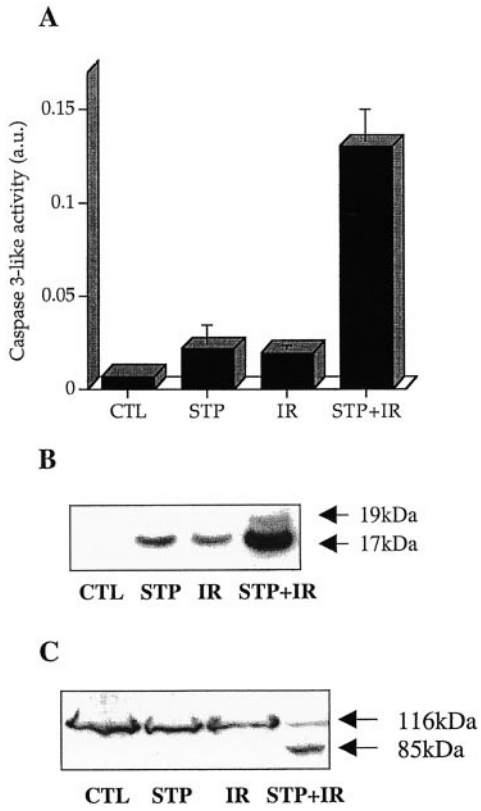

Fig. 2. Apoptosis induction in E1A/ras-transformed MEFs by a combination of low-dose STP and IR. *A*, caspase 3-like activity was determined 6 h after treatment in cytosolic extracts from control cells (*CTL*) using Ac-DEVD-pNA as a colorimetric caspase-3 substrate or cells treated with STP, IR, or a combination of both. *B*, formation of active caspase-3 was determined after the different treatments using an antibody recognizing the cleaved caspase-3 subunit. *C*, the corresponding samples were probed for endogenous PARP cleavage by Western blot using an anti-PARP antibody.

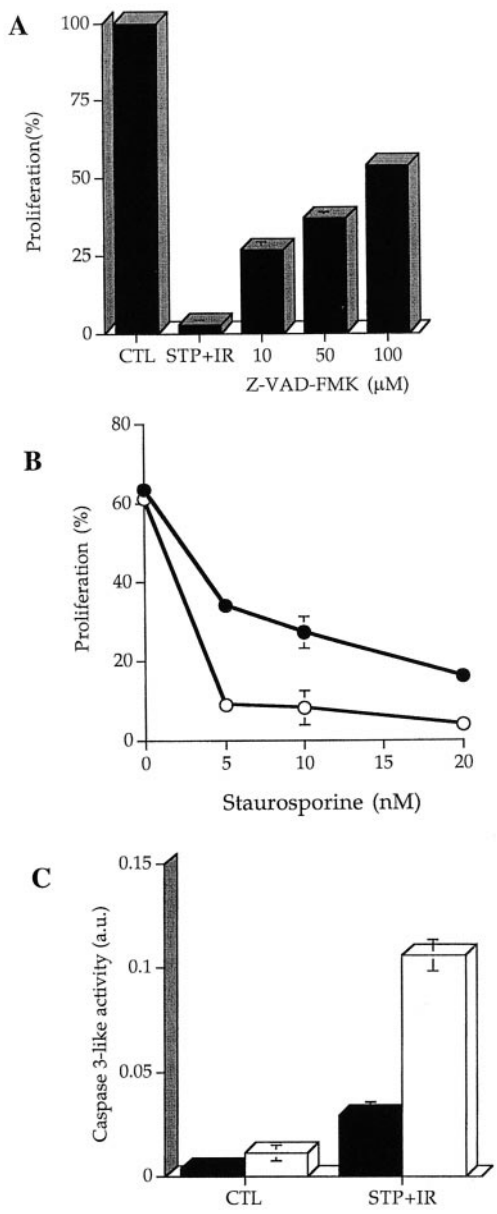

Fig. 3. Rescue of apoptosis using exogenous and endogenous inhibitors. *A*, proliferation of E1A/ras-transformed cells was assessed 24 h after combined treatment with STP (20 nM) and IR (5 Gy), with or without preincubation with increasing concentrations of Z-VAD-FMK. *B*, cell proliferation of E1A/ras-transformed MEFs overexpressing bcl-2 (●) and cells carrying only a control vector (○) was measured 24 h after combined treatment with IR (5 Gy) in the presence of increasing doses of STP (5–20 nM). *C*, caspase-3-like activities were measured in Bcl-2-overexpressing cells (■) and control cells (□) as described in the legend to Fig. 2.

empty vector), and caspase-3-like activity was drastically reduced compared with control cells (Fig. 3, *B* and *C*).

Combined Treatment with STP and IR Specifically Activates the Cytochrome *c*-mediated Caspase-9 Apoptotic Pathway. Two major apoptotic initiator pathways can transduce a death signal to the downstream effector caspase-3. To determine which specific apoptotic pathway is activated on combined treatment, the activity of the initiator caspases-8 and -9 and the unrelated caspase-1 was determined on combined treatment using caspase-specific peptide-based substrates. Caspase-9 was activated on treatment with 5 Gy of irradiation or with a low dose of STP (20 nM) alone and was further stimulated on combined treatment. No increase in caspase-1 and -8 activity was observed after each treatment alone or after combined treatment (Fig. 4*A–C*). These results suggested that the cytochrome *c*-activated caspase-9 pathway may be a key element for the cooperative induction of apoptosis by combined treatment.

In the presence of cytochrome *c* and Apaf-1, procaspase-9 is processed into a large subunit and a small subunit that heterodimerize to form the active enzyme. The amount of cytochrome *c* released into the cytosol was detected in cytosolic lysates of treated cells that were always fractionated in the presence of high sucrose concentrations to avoid unspecific mitochondrial cytochrome *c* leakage (3, 4). The level of cytosolic cytochrome *c* was enhanced in cells after combined treatment with STP and IR compared with that in cells treated with one modality alone (Fig. 5*A*). Similarly, immunoblotting demonstrated that processing of caspase-9 into the cleaved form (M_r 35,000–37,000) oc-

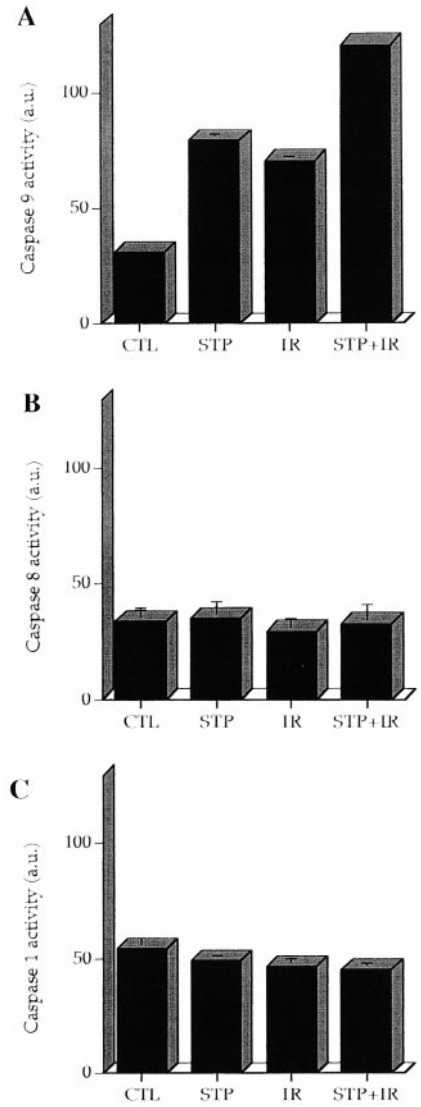

Fig. 4. Enhanced activity of caspase-9 after treatment with a low dose of STP combined with IR. Caspase-9 (*A*), caspase-8 (*B*), and caspase-1 (*C*) activities were determined 6 h after treatment using caspase-specific peptide substrates (Ac-LEHD-pNA, Ac-lETD-pNA, and Ac-YVAD-pNA, respectively) in cellular extracts derived from control cells (*CTL*) or from cells treated with STP (20 nM), IR (5 Gy), or both.

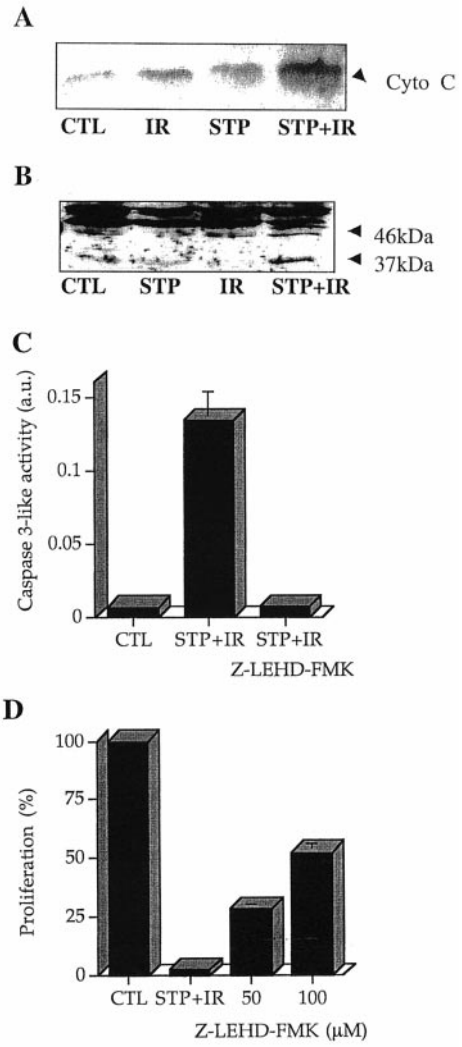

Fig. 5. Combined treatment with a low dose of STP and IR specifically activates the cytochrome *c*-dependent caspase-9 pathway. Cytosolic extracts from untreated cells (*CTL*) or from cells treated for 6 h with STP (20 nM), IR (5 Gy), or the combination (*STP+IR*) were probed using (*A*) monoclonal anti-cytochrome *c* antibody 7H8.2C12 and (*B*) a rabbit polyclonal anti-caspase-9 antibody. *C*, caspase-3-like activity 6 h after combined treatment in cytosolic extracts from cells preincubated with or without the specific caspase-9 inhibitor Z-LEHD-FMK. *D*, proliferation was assessed 24 h after combined treatment with STP (20 nM) and IR (5 Gy) preincubated with increasing concentrations (0, 50, and 100 μM) of the specific caspase-9 inhibitor Z-LEHD-FMK.

curred on combined exposure (Fig. 5*B*), whereas only the proform (M_r 46,000) was detectable on single treatment.

Preincubation of cells with the specific caspase-9 inhibitor Z-LEHD-FMK before treatment with STP in combination with IR abolished caspase-3 activation, and cytotoxicity was prevented almost to the level obtained after treatment with the single agents alone. These results demonstrate the importance of this specific apoptotic pathway for the cytotoxic effect of combined treatment (Fig. 5, *C* and *D*). Furthermore, overexpression of Bcl-2 also abrogated caspase-9 activation on treatment with STP and IR (data not shown), similar to the effect of Bcl-2 on DEVDase activity (see above).

To further explore the relevance of the cytochrome *c*-dependent caspase-9 pathway for the cooperative induction of apoptosis, E1A/ras-transformed MEFs derived from caspase-9 knockout were treated with STP and irradiation. Again, experiments were performed with transformed cells obtained from uncloned mass culture to avoid clonal variations. In the caspase-9-deficient cells, activation of the effector caspase-3 was assessed by immunoblotting. The processed large fragment of activated caspase-3 (M_r 17,000–19,000) was not detectable after treatment with STP and IR alone or in combination, indicating the requirement for an intact caspase-9 pathway (Fig. 6*A*, compare with Fig. 2*B*). Furthermore, in comparison to E1A/ras-transformed caspase-9 wild-type cells, the cytotoxic effect of combined

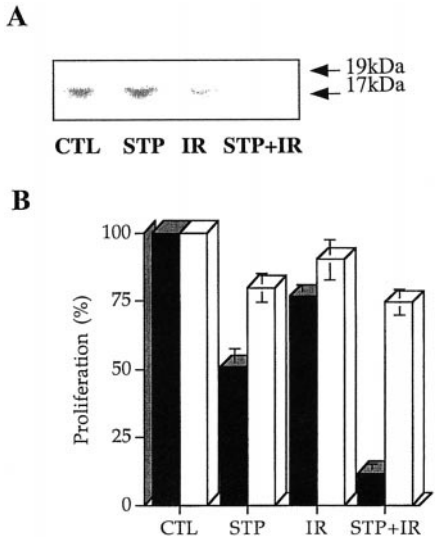

Fig. 6. Strict requirement of caspase-9 for STP-induced radiosensitization. *A*, formation of active caspase-3 in E1A/ras-transformed caspase 9-deficient cells was determined after the different treatments using an antibody recognizing the cleaved caspase-3 subunit. Compare with Fig. 2*B*. *B*, proliferation in E1A/ras-transformed caspase-9 wild-type cells (■) and caspase-9-deficient cells (□) was assessed 24 h after treatment with STP (20 nM), IR (5 Gy), or both in combination.

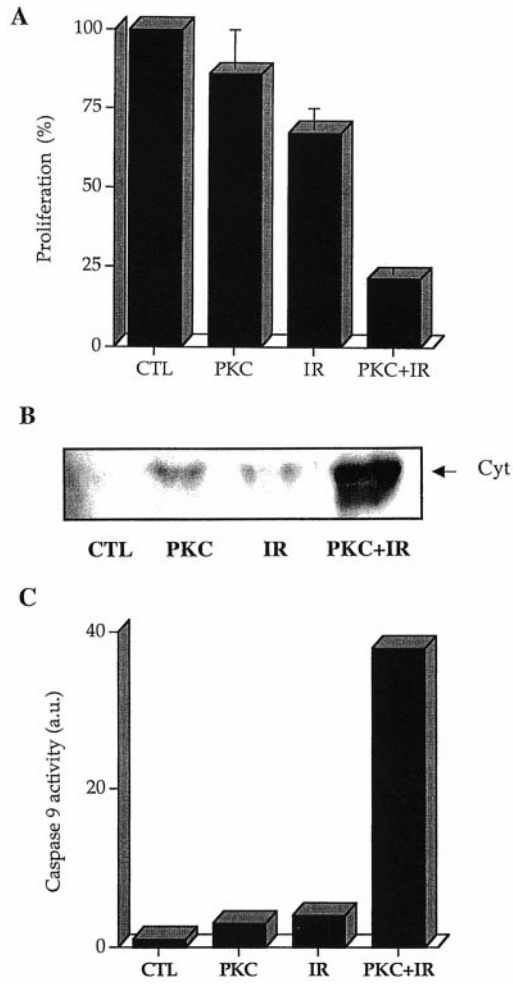

Fig. 7. Cooperative cytotoxicity of IR in combination with the cPKC-specific inhibitor PKC-412. *A*, proliferation was assessed 48 h after treatment with PKC-412 (200 nM) and IR (2 Gy) or both in combination. *B*, cytosolic extracts from untreated cells (*CTL*) or from cells treated for 18 h with PKC-412 (200 nM), IR (5 Gy), or both in combination (*PKC-412+IR*) were probed using the monoclonal anti-cytochrome *c* antibody. *C*, caspase-9 activity was determined 18 h after treatment using the caspase-9-specific peptide substrates (Ac-LEHD-pNA) in cellular extracts derived from control cells (*CTL*) or from cells treated with PKC-412 (200 nM), IR (5 Gy), or both in combination.

treatment with STP and IR was abolished up to 75% in these caspase-9-deficient cells (Fig. 6*B*).

The alkaloid STP is used as a standard cytotoxic agent to investigate apoptosis. It displays a potent inhibitory effect on PKC activity; however, STP reveals unspecific inhibition of other protein kinases. Therefore, combined treatment with irradiation was also performed with the *N*-benzoylated STP derivative PKC-412, which is very selective against the conventional α, β, and γ PKC subtypes but has an IC_{50} that is up to 10× higher than that for STP (15). This clinically relevant STP derivative is presently being tested as an antitumor agent in clinical Phase I/II studies. When used as a single agent, PKC-412 (200 nM) only slightly decreased the proliferative activity of this tumor cell population but also sensitized the cells for combined treatment with IR. Although the antiproliferative effect of PKC-412 was present at a later time point than that seen with STP (48 h *versus* 24 h), the same initial mechanism of apoptosis induction was observed but was shifted to a later time point (18 h *versus* 6 h). The level of cytochrome *c* released into the cytosol was drastically increased on combined treatment with IR and PKC-412, and caspase-9 was cooperatively activated (Fig. 7*A–C*). These results support the observation that inhibition of PKC is responsible for STP-based radiosensitization and is mediated via the mitochondrial death pathway.

p53 Is Dispensable for STP-mediated Apoptosis but Is Required for IR- and Combined Treatment-induced Apoptosis. The tumor suppressor protein p53 is a major upstream determinant for stress-induced apoptosis via the mitochondrial cytochrome *c*/caspase-9 pathway (8, 9). To assess the role of p53 in response to the different treatment modalities, proliferation was determined in p53-deficient E1A/ras-transformed cells after treatment with STP and IR alone or in combination. Treatment with STP (20 nM) alone reduced the proliferative activity to 61% in the treated cell population, whereas irradiation did not affect proliferation. Combined treatment did not induce any enhanced cytotoxicity in these cells, down-regulating the amount of proliferation only to the level achieved by STP alone (Fig. 8*A*). Likewise, only a small increase in DEVDase activity was observed after treatment with STP (20 nM) alone, and no cooperative effect on the level of DEVDase activity was observed after combined treatment in the p53-deficient cells (data not shown). Due to the different response to STP and IR in the p53-deficient cells, the amount of proliferation and DEVDase activity was assessed after treatment with increasing concentrations of IR and STP alone. High doses of irradiation did

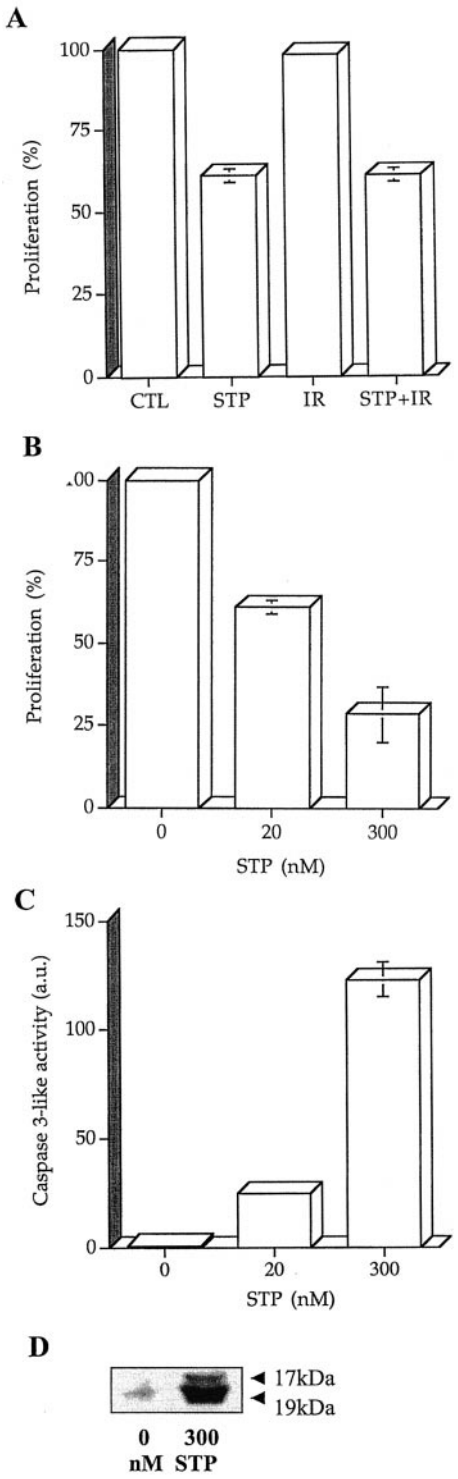

Fig. 8. p53-dependent radiosensitization but p53-independent cell kill by STP. *A*, proliferation of E1A/ras-transformed p53-deficient cells was assessed 24 h after treatment with STP (20 nM) and IR (5 Gy) or both in combination. *B*, proliferation of the transformed p53-deficient cells after treatment with increasing doses of STP (0, 20, and 300 nM). *C*, caspase-3-like activity was determined 6 h after treatment in cytosolic extracts from p53-deficient cells using Ac-DEVD-pNA as a colorimetric caspase-3 substrate (*CTL*) 6 h after treatment with STP. *D*, formation of active caspase-3 was determined after STP treatment (300 nM) using an antibody recognizing the cleaved caspase-3 subunit.

not alter proliferation 24 h after treatment or DEVDase activity (data not shown). However, a dose-dependent reduction in proliferative activity was observed in the p53-deficient cell population concomitant with increasing DEVDase activity and processing of the executioner caspase-3 upon treatment with increasing STP concentrations (Fig. 8, *B–D*). These results suggest that the tumor suppressor p53 is dispensable for STP-induced apoptosis but is required for IR-induced apoptosis and the enhanced effect of STP and IR.

Discussion

We describe the importance of the cytochrome *c*-mediated caspase-9 death pathway for radiosensitization by PKC inhibitors. Several lines of evidence demonstrate that specific activation of the cytochrome *c*-regulated caspase-9 cascade is required for the cooperative cytotoxic effect of combined treatment with IR and a low dose of STP or the cPKC-specific derivative *N*-benzoyl-STP. Combined treatment resulted in mitochondrial release of cytochrome *c* into the cytosol, enhanced caspase-9 and caspase-3 activation, and cleavage of the endogenous substrate PARP. Treatment with each single agent alone only minimally activated this apoptotic pathway and resulted in only a partial reduction of cell proliferation. The relevance of apoptosis as a mode of cell death induced by combined treatment is corroborated by the specific requirement for an intact caspase-9 pathway and by the fact that pretreatment with broad-range and caspase-9-specific caspase inhibitors reversed the combined cytotoxic effect.

STP is a well known inducer of apoptosis, but at doses 25–100 times higher when applied as a single agent (16). Irradiation alone can also induce apoptosis in cell lines like thymocytes at this dose level (17), although we observed only minimal biochemical and morphological signs of apoptosis in the tumor cells used here when irradiation was used as a single agent. However, we cannot exclude that the partial reduction of proliferative activity observed is secondary to low activation of the apoptotic machinery. Thus, combined treatment with a low dose of STP (or PKC-412) and irradiation can cooperatively activate the apoptotic machinery, leading to enhanced cell death. These experiments were performed in E1/ras-transformed MEFs, exploiting genetically defined conditions. Similar results were obtained with rat embryo fibroblasts transformed with the human-related myc/ras oncogenes (data not shown).

Currently, two death pathways are considered to transduce a stress response to the apoptotic machinery. Whereas the clustering of plasma membrane death receptors activates initiator caspase-8, death receptor-independent apoptotic stimuli induce the release of mitochondrial cytochrome *c* to facilitate activation of initiator caspase-9 in the presence of dATP and Apaf-1 (1, 2). Combined treatment specifically activates the cytochrome *c*-mediated caspase cascade involving initiator caspase-9 and effector caspase-3. Combined treatment with IR and STP, and even more prominent with the cPKC-specific inhibitor *N*-benzoyl-STP induced already at the level of mitochondrial cytochrome *c*-release, a supra-additive response, leads to the enhanced activation of

this pathway. This cytotoxic effect on combined treatment was reversed by pretreatment with a caspase-9-specific inhibitor or was even absent in caspase-9-deficient cells. Activated caspase-8 can directly stimulate the effector caspases, or it can induce the caspase 9-pathway via cleavage of Bid to induce the release of cytochrome *c* (18–20). Neither initiator caspase-8 nor caspase-1 was activated after combined treatment. Thus, the lack of any caspase-8 stimulation indicates that the death receptor pathway is not involved in this stress response and suggests that an intact caspase-9 pathway is both necessary and sufficient for the apoptotic response to STP in combination with IR. The strict requirement for an intact caspase-9 pathway was confirmed by the lack of response in caspase-9-deficient cells.

Gene knockout studies directed against Apaf-1 and caspase-9 demonstrated the importance of these proteins for cell type- and tissue-specific apoptosis induced by serum depletion, γ-irradiation, etoposide, or hypoxia. Furthermore, these studies put Apaf-1 and caspase-9 downstream of p53 and cytochrome *c* release in this apoptotic signal transduction cascade (9, 21, 22), suggesting that cytochrome *c* release is not always a lethal event (9). p53 induces apoptosis through mitochondrial cytochrome *c* release, and a lack of p53 is known to result in resistance to IR-induced apoptosis (8, 12). In our otherwise isogenic but p53-deficient E1A/ras-transformed tumor cell line, no cytochrome *c* was released into the cytosol, and neither caspase-9 nor caspase-3 was activated on combined treatment with PKC inhibitors and IR. Likewise cytochrome *c* was only released after treatment with high doses of IR in wild-type p53 transformed cells (data not shown). Thus, these treatment modalities require intact p53 to initiate the apoptotic machinery induced at the site of the mitochondria. On the other hand, treatment with a high dose of STP induced cytochrome *c* release and activated downstream caspases even in p53-deficient cells, clearly demonstrating a p53-independent and dose-dependent mechanism for PKC inhibitor-induced cytochrome *c* release. These results suggest that STP might act upstream of cytochrome *c* release but downstream of p53. Thus, mechanistically, a specific combined treatment modality does not simply correspond to a dose increase of either single agent alone. PKC inhibitors sensitize tumor cells for IR-induced apoptosis by overcoming an apoptotic threshold that is too high for a low dose of IR alone. However, apoptosis induction by combined treatment requires both an intact apoptotic pathway and wild-type p53, whereas high doses of PKC inhibitors do not depend on the latter.

PKC-regulated signal transduction cascades cooperate with IR-induced pathways to overcome the apoptotic threshold, leading to cytochrome *c*-mediated caspase activation. Thus far, we do not understand at which level upstream or at the site of the mitochondria PKC-regulated processes interact with IR-induced signal transduction, leading to synergistic cytochrome *c* release and caspase-9 activation. Antiapoptotic members of the Bcl-2-family might prevent the release of cytochrome *c* from mitochondria, whereas some proapoptotic family members such as Bax and Bid induce cytochrome *c* release (reviewed in Ref. 14). Both Bax-dependent and -independent induction of apoptosis by p53 have been demonstrated *in vitro* (14, 23). We could not find any difference in the protein levels of Bax, Bcl-2, or Bcl-x_L on treatment in our cells (data not shown), but we cannot exclude that conformational changes of these proteins or of other family members not investigated here modulate the treatment sensitivity of our cells.

Activation of PKC is known to promote cell survival and to protect against cell death. However, the exact role of the multiple PKC isoforms responsible for the growth-promoting effect is far from clear. For example, overexpression of PKC can abrogate stress-induced apoptosis by enhanced activation of the phosphatidylinositol 3′-kinase/Akt pathway (24) and concomitant phosphorylation of the proapoptotic Bcl-2 family member Bad (25, 26). Thus, PKC inhibitors might result in decreased Akt activity, thereby affecting the equilibrium of proapoptotic and antiapoptotic members of the Bcl-2-family and down-regulating an apoptotic threshold in tumor cells (12, 26, 27). PKC inhibitor-mediated radiosensitization might also be mechanistically linked to the hydrolysis of sphingomyelin and the generation of the apoptotic second messenger ceramide. Ceramide is increased on irradiation but prevented by PKC activators such as 12-*O*-tetradecanoylphorbol-13-acetate (5, 28–30). Ceramide can also directly induce the release of cytochrome *c* from mitochondria (31). Due to the numerous downstream targets of PKC, it will be important to elucidate which of the major growth-promoting and/or antiapoptotic cellular signal transduction cascades contribute to the STP-induced and the more specific PKC-412-induced radiosensitizing effect. We are currently investigating the level on which IR-induced signal transduction cascades merge with PKC-mediated pathways. This merge can be either upstream or at the level of the mitochondria to cooperatively induce the release of cytochrome *c*.

Treatment response often depends on the individual oncogenic background, but a disrupted apoptotic pathway could also interfere with treatment success. Combination of different antitumoral treatment modalities is often advantageous to limit unspecific toxicities frequently observed by an exceedingly high single treatment regimen. Here we demonstrate that PKC-mediated radiosensitization may overcome an apoptotic threshold but requires an intact caspase-9 pathway. A detailed molecular understanding of how single and combined treatment modalities induce and execute apoptosis will advance our knowledge of the apoptotic signaling network and will be important for successful treatment.

Materials and Methods

Cell Cultures, Irradiation, and STP/PKC-412 Treatment. Clonal selected MEFs transformed with the two oncogenes E1A and T24 H-ras (32) were used at low passage numbers and cultured in a 5% CO_2 atmosphere in DMEM containing 10% FCS and 10% bovine calf serum (Hyclone Laboratories) supplemented with penicillin and streptomycin. Uncloned mass cultures of E1A/ras-transformed wild-type, p53−/−, and caspase-9−/− MEFs were prepared as described previously (33) and cultured in DMEM containing 10% FCS supplemented with penicillin and streptomycin. Irradiation was carried out at room temperature using a Pantak Therapax 300 kV X-ray unit at 0.7 Gy/min. STP (Sigma) and PKC-412 (Novartis Pharma AG) were dissolved in DMSO and diluted in DMEM containing 10% or 20% FCS-412. For combined treatments, cells were preincubated with STP or PKC for 1 h before irradiation.

Bcl-2-overexpressing Cell Lines. BOSC23 cell were transfected with a total of 30 μg of pBabe(puro) plasmid DNA or its derivative (containing mouse bcl-2 cDNA; Ref.34) by using the calcium phosphate coprecipitation method as described previously (35). Medium containing the retrovirus was harvested 24–30 h after removal of the precipitate and used to infect the E1A/ras-transformed MEFs at 40% confluence. Puromycin selection (2 μg/ml medium) to obtain stably transfected cells was initiated 30 h after infection, and pools of puromycin-resistant cells (uncloned mass cultures) were analyzed for bcl-2 overexpression with α-bcl-2 antibodies (Upstate Biotechnology).

Trypan Blue Viability Assay. Floating and adherent cells were collected at the indicated times, spun down, and resuspended in PBS. The cells were then diluted 1:5 with 0.4% trypan blue solution (Sigma) and scored under a light microscope. The results presented represent the mean plus SD of two independent experiments, with a minimum of 500 cells scored per treatment.

Proliferation Assay. Tumor cell proliferation was assessed 24 or 48 h after treatment with the colorimetric alamar blue assay based on the detection of metabolic activity (Biosource International, Camarillo, CA). Absorption was measured at 570 and 600 nm using a Dynatech MR5000 spectrophotometer. Results are expressed as the mean plus SD of at least two experiments in triplicate.

Caspase Inhibitor Assay. The broad-range caspase inhibitor Z-VAD-FMK (10–100 μM; Calbiochem) and the caspase-9-specific inhibitor Z-LEHD-FMK (100–200 μM; Calbiochem) dissolved in culture medium were added to cells 1 h before treatment. Results are expressed as the mean plus SD of at least two experiments in triplicate.

Cell Fractionation. Cells were harvested by centrifugation at 1,800 × *g* for 10 min at 4°C and washed with ice-cold PBS. The cell pellet was suspended in 5 volumes of ice-cold buffer A [20 mM HEPES-KOH (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM DTT, 250 mM sucrose, and 0.1 mM phenylmethylsulfonyl fluoride] supplemented with protease inhibitors (5 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 μg/ml aprotinin). After sitting on ice for 15 min, the cells were disrupted by douncing 15 times in a Dounce homogenizer. Cell lysates were centrifuged at 1,000 × *g* for 10 min at 4°C (crude nuclear pellet), and the supernatant was further centrifuged at 100,000 × *g* for 1 h. The resulting supernatant (S-100 fraction) and pellet (mitochondrial fraction) were stored at −80°C.

***In Vitro* Caspase Assay.** To determine caspase-3-like activity, 50–80 μg of protein from the S-100 fraction were incubated at 37°C with the colorimetric caspase-3 substrate Ac-DEVD-pNA (100 μM; Calbiochem) and 1 mM dATP in a final volume of 120 μl. Measurements in the presence of the caspase-3 inhibitor Ac-DEVD-CHO (20 μM; Calbiochem) served to correct for unspecific background activity. To determine caspase-1 and -8 activity, the S-100 fraction was incubated at 37°C with the colorimetric caspase-1 substrate Ac-YVAD-pNA (100 μM; Calbiochem) or the colorimetric caspase-8 substrate Ac-IETD-pNA (200 μM; Calbiochem), in a final volume of 120 μl. Caspase-9 activity was measured with the R&D Systems colorimetric caspase-9 assay kit using Ac-LEHD-pNA as a substrate. Cleavage of the caspase substrates was monitored at 405 nm using a Dynatech MR5000 spectrophotometer. Results are expressed as the mean plus SD of at least three experiments performed in duplicate.

Antibodies and Western Blots. Mouse monoclonal antibody clone 7H8.2C12 recognizing murine cytochrome *c* was purchased from Research Diagnostics, Inc. or PharMingen. Rabbit polyclonal anti-PARP antibody was obtained from Upstate Biotechnology, rabbit polyclonal anti-caspase-9 antibody was obtained from PharMingen, and rabbit polyclonal anti-cleaved caspase-3 antibody was obtained from New England Biolabs. Cellular proteins (100–200 μg) from the different fractions were precipitated, resolved by SDS-PAGE, and blotted onto polyvinylidene difluoride membranes. Antibody detection was achieved using enhanced chemiluminescence (Amersham) and a horseradish peroxidase-conjugated secondary antibody according to the manufacturer's protocol.

Acknowledgments

We thank E. Peterhans for providing us with the Bcl-2 cDNA, W. Krek for preparation of the retroviral constructs, and A. Marti for stimulating discussions.

References

1. Sun, X-M., MacFarlane, M., Zhuang, J., Wolf, B. B., Green, D. R., and Cohen, G. M. Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. J. Biol. Chem., *274:* 5053–5060, 1999.

2. Tepper, A. D., de Vries, E., van Blitterswijk, W. J., and Borst, J. Ordering of ceramide formation, caspase activation, and mitochondrial changes during CD95- and DNA damage-induced apoptosis. J. Clin. Invest., *103:* 971–978, 1999.

3. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell, *91:* 479–489, 1997.

4. Liu, X., Kim, C. N., Yang, J., Jemmerson, R., and Wang, X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell, *86:* 147–157, 1996.

5. Haimovitz-Friedman, A. Radiation-induced signal transduction and stress response. Radiat. Res., *150:* 102–108, 1998.

6. Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. p53 status and the efficacy of cancer therapy *in vivo*. Science (Washington DC), *266:* 807–810, 1994.

7. Giaccia, A. J., and Kastan, M. B. The complexity of p53 modulation: emerging patterns from divergent signals. Genes Dev., *12:* 2973–2983, 1998.

8. Schuler, M., Bossy-Wetzel, E., Goldstein, J. C., Fitzgerald, P., and Green, D. R. p53 induces apoptosis by caspase activation through mitochondrial cytochrome *c* release. J. Biol. Chem., *275:* 7337–7342, 2000.

9. Soengas, M. S., Alarcón, R. M., Yoshida, H., Giaccia, A. J., Hakem, R., Mak, T. W., and Lowe, S. W. Apaf-1 and caspase-9 in p53-dependent apoptosis and tumor inhibition. Science (Washington DC), *284:* 156–159, 1999.

10. Tan, Y., Ruan, H., Demeter, M. R., and Comb, M. J. p90RSK blocks Bad-mediated cell death via a protein kinase C-dependent pathway. J. Biol. Chem., *274*: 34859–34867, 1999.

11. Whitman, S. P., Civoli, F., and Daniel, L. W. Protein kinase Cβ II activation by 1-β-D-arabinofuranosylcytosine is antagonistic to stimulation of apoptosis and Bcl-2α down-regulation. J. Biol. Chem., *272:* 23481–23484, 1997.

12. Chmura, S. J., Nodzenski, E., Crane, M. A., Virudachalam, S., Hallahan, D. E., Weichselbaum, R. R., and Quintans, J. Cross-talk between ceramide and PKC activity in the control of apoptosis in WEHI-231. Adv. Exp. Med. Biol., *406:* 39–45, 1996.

13. Hallahan, D. E., Virudachalam, S., Schwartz, J. L., Panje, N., Mustafi, R., and Weichselbaum, R. R. Inhibition of protein kinases sensitizes human tumor cells to ionizing radiation. Radiat. Res., *129:* 345–350, 1992.

14. Gross, A., MacDonnell, J. M., and Korsmeyer, S. J. BCL-2 family members and the mitochondria in apoptosis. Genes Dev., *13:* 1899–1911, 1999.

15. Gescher, A. Analogs of staurosporine: potential anticancer drugs? Gen. Pharmacol., *31:* 721–728, 1998.

16. Bossy-Wetzel, E., Newmeyer, D. D., and Green, D. R. Mitochondrial cytochrome *c* release in apoptosis occurs upstream of DEVD-specific caspase activation and independently of mitochondrial transmembrane depolarization. EMBO J., *17:* 37–49, 1998.

17. Lowe, S. W., Schmitt, E. M., Smith, S. W., Osborne, B. A., and Jacks, T. p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature (Lond.), *362:* 847–849, 1993.

18. Luo, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. Bid, a Bcl2-interacting protein, mediates cytochrome *c* release from mitochondria in response to activation of cell surface death receptors. Cell, *94:* 481–490, 1998.

19. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomaselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. Two CD95 (APO-1/Fas) signaling pathways. EMBO J., *17:* 1675–1687, 1998.

20. Stennicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q., Wolf, B. B., Yang, X., Zhou, Q., Ellerby, H. M., Ellerby, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. Procaspase-3 is a major physiologic target of caspase-8. J. Biol. Chem., *273:* 27084–27090, 1998.

21. Ding, H. F., McGill, G., Rowan, S., Schmaltz, C., Shimamura, A., and Fisher, D. E. Oncogene-dependent regulation of caspase activation by p53 protein in a cell-free system. J. Biol. Chem., *273:* 28378–28383, 1998.

22. Kuida, K., Haydar, T. F., Kuan, C-Y., Gu, Y., Taya, C., Karasuyama, H., Su, M. S., Rakic, P., and Flavell, R. A. Reduced apoptosis and cytochrome *c*-mediated caspase activation in mice lacking caspase 9. Cell, *94:* 325–337, 1998.

23. Juin, P., Hueber, A., Littlewood, T., and Evan, G. C-Myc-induced sensitization to apoptosis is mediated through cytochrome *c* release. Genes Dev., *13:* 1367–1381, 1999.

24. Li, W., Zhang, J., Flechner, L., Hyun, T., Yam, A., Franke, T. F., and Pierce, J. H. Protein kinase C-α overexpression stimulates Akt activity and suppresses apoptosis induced by interleukin 3 withdrawal. Oncogene, *18:* 6564–6572, 1999.

25. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. Akt phosphorylation of Bad couples survival signals to the cell-intrinsic death machinery. Cell, *91:* 231–241, 1997.

26. Zundel, W., and Giaccia, A. Inhibition of the anti-apoptotic PI(3)K/Akt/Bad pathway by stress. Genes Dev., *12:* 1941–1946, 1998.

27. Jones, B. E., Lo, C. R., Srinivasan, A., Valentino, K. L., and Czaja, M. J. Ceramide induces caspase-independent apoptosis in rat hepatocytes sensitized by inhibition of RNA synthesis. Hepatology, *30:* 215–222, 1999.

28. Fuks, Z., Haimovitz-Friedman, A., and Kolesnick, R. N. The role of the sphingomyelin pathway and protein kinase C in radiation-induced cell kill. *In:* V. T. DeVita, S. Hellman, S. A. Rosenberg (eds.), Important Advances in Oncology, pp. 19–31. Philadelphia, PA: J. B. Lippincott Company, 1995.

29. Haimovitz-Friedman, A., Kann, C. C., Ehleiter, D., Persaud, R. S., McLoughin, M., Fuks, Z., and Kolesnick, R. N. Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis. J. Exp. Med., *180:* 525–535, 1994.

30. Pruschy, M., Resch, H., Shi, Y-Q., Aalame, N., Glanzmann, C., and Bodis, S. Ceramide triggers p53-dependent apoptosis in genetically defined fibrosarcoma tumour cells. Br. J. Cancer, *80:* 693–698, 1999.

31. Ghafourifar, P., Klein, S. D., Schucht, O., Schenk, U., Pruschy, M., Rocha, S., and Richter, C. Ceramide induces cytochrome *c* release from isolated mitochondria. Importance of mitochondrial redox state. J. Biol. Chem., *274:* 2225–2233, 1999.

32. Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell, *74:* 957–967, 1993.

33. Hakem, R., Hakem, A., Duncan, G. S., Henderson, J. T., Woo, M., Soengas, M. S., Elia, A., de la Pompa, J. L., Kagi, D., Khoo, W., Potter, J., Yoshida, R., Kaufman, S. A., Lowe, S. W., Penniger, J. M., and Mak, T. W. Differential requirement for caspase 9 in apoptotic pathways *in vivo*. Cell, *94:* 339–352, 1998.

34. Hennet, T., Bertoni, G., Richter C., and Peterhans, E. Expression of BCL-2 protein enhances the survival of mouse fibrosarcoid cells in tumor necrosis factor-mediated cytotoxicity. Cancer Res., *53:* 1456–1460, 1993.

35. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. Production of high-titer helper-free retroviruses by transient transfection. Proc. Natl. Acad. Sci. USA, *90:* 8392–8396, 1993.