

Death receptor pathways mediate targeted and non-targeted effects of ionizing radiations in breast cancer cells

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Delayed cell death by mitotic catastrophe is a frequent mode of solid tumor cell death after γ -irradiation, a widely used treatment of cancer. Whereas the mechanisms that underlie the early γ -irradiation-induced cell death are well documented, those that drive the delayed cell death are largely unknown. Here we show that the Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and tumor necrosis factor (TNF)- α death receptor pathways mediate the delayed cell death observed after γ -irradiation of breast cancer cells. Early after irradiation, we observe the increased expression of Fas, TRAIL-R and TNF-R that first sensitizes cells to apoptosis. Later, the increased expression of FasL, TRAIL and TNF- α permit the apoptosis engagement linked to mitotic catastrophe. Treatments with TNF- α , TRAIL or anti-Fas antibody, early after radiation exposure, induce apoptosis, whereas the neutralization of the three death receptors pathways impairs the delayed cell death. We also show for the first time that irradiated breast cancer cells excrete soluble forms of the three ligands that can induce the death of sensitive bystander cells. Overall, these results define the molecular basis of the delayed cell death of irradiated cancer cells and identify the death receptors pathways as crucial actors in apoptosis induced by targeted as well as non-targeted effects of ionizing radiation.

Introduction

While cells from breast cancer are generally refractory to early induction of apoptosis in response to ionizing radiation (IR) and chemotherapeutic agents (1,2), these treatments promote loss of proliferative capacity and/or late induction of cell death. Radiation-induced late cell death is often accounted for by a mitotic catastrophe, defined as a type of cell death occurring during mitosis or resulting from failed mitosis (3) and characterized by a prolonged G₂ arrest and subsequent cell death. Recently, apoptosis and mitotic catastrophe have been functionally linked and mitotic catastrophe is now defined as a subtype of caspase-mediated death resulting from a combination of deficient cell cycle checkpoints and persistence of DNA damage (4,5). During mitotic catastrophe, apoptosis could occur either during

Abbreviations: FADD, Fas-associated death domain; ICM, irradiated conditioned medium; IR, ionizing radiation; NICM, non-irradiated conditioned medium; PARP1, poly-ADP ribose polymerase-1; SM, standard medium; TNF, tumor necrosis factor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

or close to metaphase in a p53-independent manner or after asymmetric cell division and tetraploidy in a partially p53-dependent manner (6,7). This death pathway might account for the late apoptosis observed after IR or adriamycin treatment of breast cancer cells.

Numerous signaling pathways have been implicated in the regulation of apoptosis of breast cancer cells and several reports have suggested a link between radiation- or chemotherapy-induced apoptosis of breast cancer cells and activation of death receptor pathways (8–10). In breast cancer cells, chemotherapeutic agents and IR can induce the expression of Fas, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1 and -R2, tumor necrosis factor (TNF)-R1 and -R2, the receptors of the three main death receptors pathways: i.e., respectively, FasL, TRAIL and TNF- α signaling pathways (11–14). In addition, activation of the death receptors pathways has been shown to occur when exogenous death receptors ligands were added to breast cancer cells treated previously by antitumor agents or radiation and this addition leads to synergistic effects (12,15,16). Finally, irradiation can sensitize mammary cells to TRAIL-mediated apoptosis, both *in vivo* and *in vitro* (17,18). Addition of the ligands induces apoptosis through the formation of the death-inducing signaling complex, i.e. receptor aggregation, recruitment of the adaptor molecule Fas-associated death domain (FADD) and subsequent binding and activation of caspases 8 and 10 (19). All these results indicate that the three death receptors ligands, TNF- α , TRAIL and FasL, may act as major participants in apoptosis of solid tumors and might be used as potential anticancer agents (20). However, increased expression of death receptor ligands has been only observed after treatments with drugs, such as paclitaxel and sodium butyrate, which modulate Fas/FasL expression and induce early cell death of breast cancer cells (21,22). These data strongly suggest that death receptor signaling pathways might play an important role in the apoptosis of breast cancer cells, but no direct link has ever been established between reproductive cell death observed after irradiation of breast cancer cells and activation of death receptor signaling.

We have shown previously that γ -irradiation of breast cancer cell lines led to delayed cell death after growth arrest (23). We now showed that irradiated breast cancer cell lines died by apoptosis due to mitotic catastrophe. The FasL, TRAIL and TNF- α death-inducing signaling pathways mediated the delayed cell death of irradiated breast cancer cells through interactions of their ligands and receptors. Increased expression of the ligands occurred late and accounted for delayed radiation-induced apoptosis. Finally, we showed that these ligands were also produced as soluble forms whose secretion can induce the death of sensitive bystander cells.

Materials and methods

Cell cultures

T-47D, H-466B, ZR-75-1, BT-20, MDA-MB-231, HBL-100 and HEK 293 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cell line characteristics are summarized in supplementary Table I (available at *Carcinogenesis* Online). All cell lines, except BT-20, were maintained in Dulbecco's modified Eagle medium 4.5 g/l glucose, 0.11 g/l sodium pyruvate, glutamate (GlutaMAX 1TM) and pyridoxine, supplemented with 5% fetal calf serum, penicillin, streptomycin and amphotericin B (antibiotic-antimycotic mix) (all from Life Technologies, Cergy-Pontoise, France). BT-20 cells were grown in RPMI 1640 medium with GlutaMAX 1TM, 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid and 2.5 mM sodium pyruvate. All cells cultures were done in 5% CO₂ and 95% humidity.

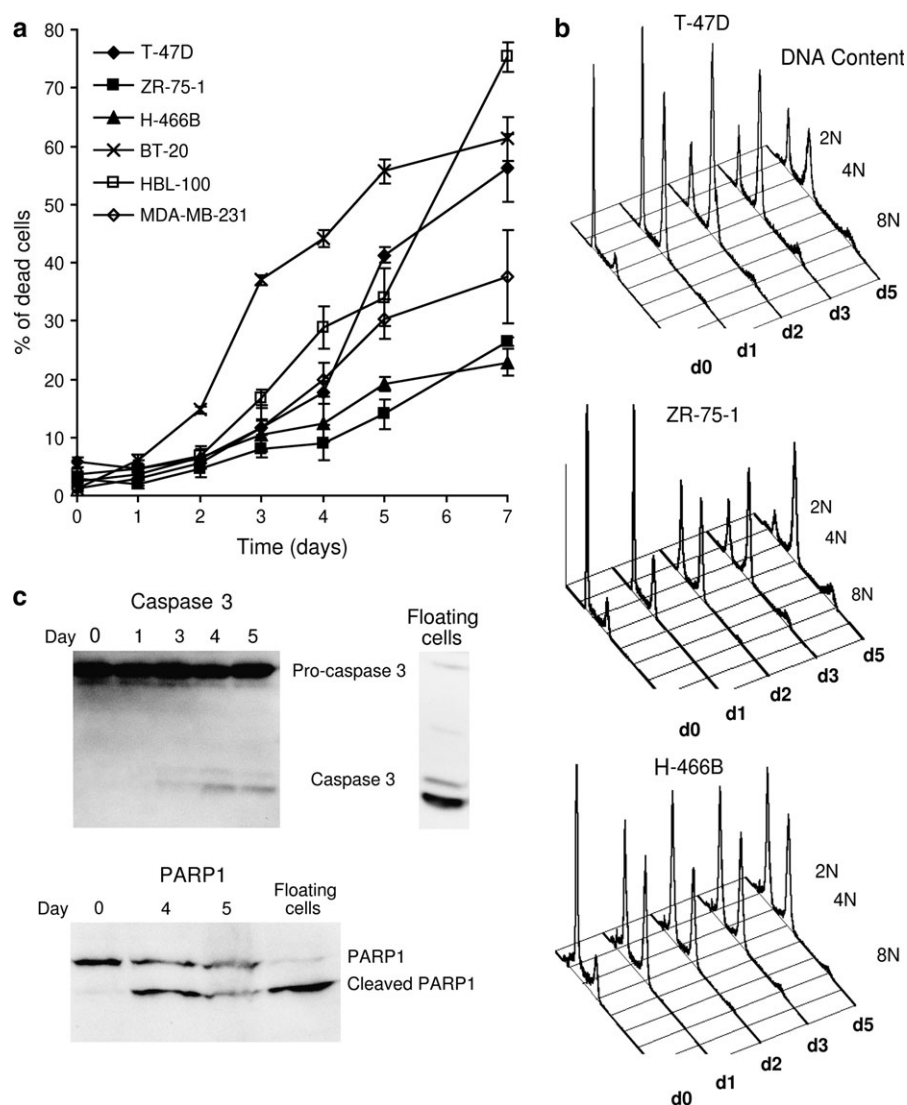


Fig. 1. Characterization of the delayed cell death of breast cancer cell lines after a 10 Gy irradiation. (a) Time course of cell death of six different breast cancer cell lines. Viable and dead cells were assessed by trypan blue staining. Results shown corresponded to the mean \pm SD of two to four independent experiments. (b) Cell cycle analysis of 10 Gy-irradiated cell lines over 5 days (d₀–d₅). Similar results were obtained in at least two different experiments. (c) Kinetics of cleavage of caspase 3 and PARP1 in T-47D cells at different times after 10 Gy irradiation. The western blot analysis shown is representative of three independent experiments.

Chemicals, reagents and antibodies

All biochemicals were from Sigma (Saint Quentin Fallavier, France) unless otherwise specified. Monoclonal anti-human Fas antibody (clone DX2), recombinant human TRAIL and TNF- α and soluble recombinant chimera (Fas/Fc, TRAIL-R1/Fc and TNF-R1/Fc) were from R&D Systems (Lille, France).

Irradiation

Cells were plated 48 h prior to irradiation. On day 0, cells were irradiated in a serum-free medium, using a ¹³⁷Cs irradiation unit at dose rate of \sim 2 Gy/min, and then incubated with fresh medium.

Proliferation, survival and apoptosis

Cell proliferation and survival analyses were performed in two or more independent experiments, by scoring at least 300 cells each time. Discrimination between viable and dead cells (including dead cells in the supernatant) was performed by trypan blue exclusion. Apoptosis was analyzed by *in situ* TUNEL assay using APO-BrdUTM kit (Becton Dickinson, Le Pont-de-Claix, France) according to the manufacturer's recommendation.

Flow cytometry

For cell cycle analysis, cells were trypsinized and harvested by centrifugation at 1400 r.p.m for 10 min, washed in phosphate-buffered saline, fixed in 70% cold ethanol and stored at -20°C . Before analysis, cells were washed in phosphate-buffered saline and stained in phosphate-buffered saline containing 25 $\mu\text{g}/\text{ml}$

propidium iodide (Sigma) and 50 $\mu\text{g}/\text{ml}$ RNase A (Roche Diagnostics, Meylan, France) for 30 min at 37°C . Samples were analyzed using a FACScalibur flow cytometer (Becton Dickinson) on at least 20 000 cells. Cellular debris, fixation artifacts and doublets were gated out with FL2-area and FL2-width parameters. The cell cycle was analyzed using Modfit software (Verity, Becton Dickinson).

RNA extraction and quantitative real-time RT-PCR

For the first-strand cDNA synthesis, 1 μg of total RNA, extracted with RNA Plus (Quantum Biotechnologies, Illkirch, France), was mixed with 1 μM of random primer p(dN)₆ (Roche Diagnostic) and 250 μM deoxynucleoside triphosphate (Life Technologies) in a 1 \times reverse buffer (6.7 mM MgCl₂, 67 mM Tris-HCl, pH 8.8, 16.6 mM (NH₄)₂SO₄), incubated for 5 min at 65°C , placed on ice before adding 200 U of mouse moloney murine leukemia virus reverse transcriptase (Life Technologies) in a final volume of 20 μl , further incubated for 30 min at 42°C and then incubated for 3 min at 72°C to inactivate reverse transcriptase (24). RT-PCR was performed with an ABI Prism 7300 detection apparatus (Applied Biosystems, Courtabeuf, France), using the SYBR Green PCR Core Reagent kit (Applied Biosystems) or the Taqman Universal Master Mix according to the recommendations of the manufacturer. The Ct value was determined with the Sequence Detection System software. For Taqman assays, the primers were from Applied Biosciences: Hs99999905_m1 (glyceraldehyde 3-phosphate dehydrogenase), Hs99999903_m1 (ACTG1/ β -actin), Hs00531110_m1 (FAS/TNFRSF6), Hs00181225_m1 (FASLG/FasL/TNFSF6), Hs00269492_m1 (TRAIL-R1/TNFRSF10A), Hs00366272_m1 (TRAIL-R2/TNFRSF10B), Hs00533560_m1

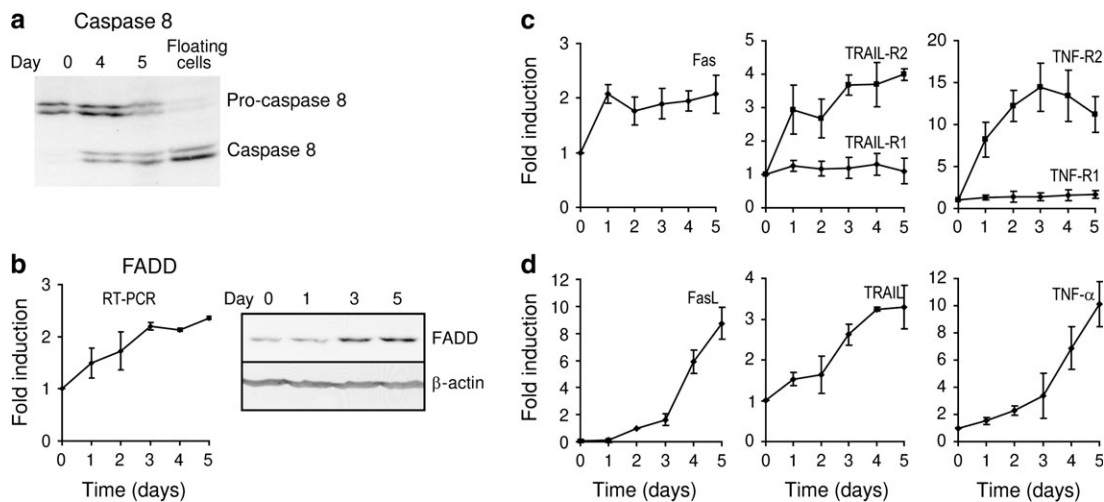


Fig. 2. The death receptor pathways are involved in delayed radiation-induced cell death. (a) Kinetics of cleavage of caspase 8 in T-47D cells after a 10 Gy irradiation. The western blot analysis shown is representative of three independent experiments. (b) Analysis of FADD expression by quantitative RT-PCR and western blot at different times after a 10 Gy irradiation of T-47D cells. The representative results from two independent experiments are shown. For all western blot analyses, β-actin was used as a loading control. (c) and (d) mRNA levels of death receptors and corresponding ligands after a 10 Gy irradiation of T-47D cells. These mRNA levels were determined by quantitative RT-PCR. Normalization was performed as indicated in Materials and Methods and the basal expression on day 0 was normalized to 1 (except for FasL, undetectable on day 0 and fixed at 1 on day 2). Each value corresponds to the mean value of at least two independent PCRs performed from three independent experiments. Error bars correspond to standard deviation.

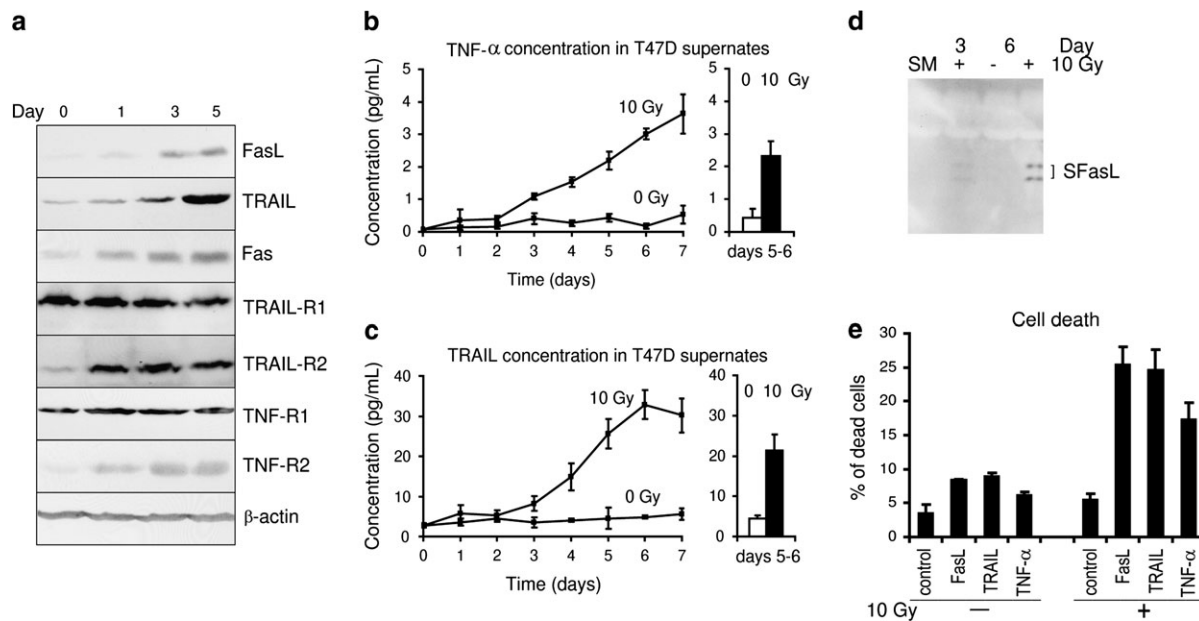


Fig. 3. Kinetics of expression of functional receptors and ligands of death receptors pathways after a 10 Gy irradiation of T-47D cells. (a) The kinetics of protein expression of receptors (Fas, TRAIL-R1, TRAIL-R2, TNF-R1 and TNF-R2) and ligands (FasL and TRAIL) of death receptors pathways were studied by western blot analysis using cellular extracts from 10 Gy-irradiated T-47D cells. β-Actin was used as loading control. (b), (c) and (d) Analysis of soluble forms of death receptors ligands in culture medium of 10 Gy-irradiated T-47D cells. TNF-α and TRAIL were detected by ELISA in supernatant of irradiated and non-irradiated cells each day for 7 days (b and c, left panel). To analyze ligand secretion over 24 h, medium was changed on day 5 and ELISA was performed on supernatant on day 6 (b and c, right panel). Each value corresponds to the mean of at least two independent measurements from two independent experiments. Error bars correspond to standard deviation. (d) Soluble FasL was detected by western blot after direct loading cell culture supernatants (see Materials and Methods). The representative results from three independent experiments are shown. (e) Irradiation sensitizes T-47D cells to TNF-α, TRAIL and anti-Fas agonist antibody. Cells were treated with anti-Fas agonist antibody (2 μg/ml), TRAIL (1 ng/ml) and TNF-α (0.5 ng/ml) 24 h after 10 Gy irradiation and 24 h later, viable and dead cells were counted after trypan blue staining. Results correspond to the mean ± SD of three independent experiments.

(TNF-R1/TNFRSF1A), Hs00153550_m1 (TNF-R2/TNFRSF1B), Hs00174128_m1 (TNF/TNF-alpha/TNFSF2) and Hs0000538709_m1 (FADD). For SYBR Green PCR assays, primers [forward (F) and reverse (R)] are for TRAIL/TNFSF10: GCAACTCCGTCAGCTCGTTAG (F) and GGCCCA-GAGCCTTTTCATTC (R). Levels of gene expression were normalized using GENORM software (25), which determines the most stable reference (housekeeping) genes from a set of tested candidate reference genes in a given sample panel. A gene expression normalization factor can be calculated for each sample based

on the geometric mean of a user-defined number of reference genes. In our experiments, levels of gene expression were normalized using glyceraldehyde 3-phosphate dehydrogenase and β-actin.

Western blot

Cell lysates (30 μg) or cell culture supernatants (12 μl) were subjected to Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and proteins were transferred and detected using a chemiluminescence procedure. Antibodies

directed against FasL (sc-957), TNF-R1 (sc-8436) and TNF-R2 (sc-8041) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies directed against FADD (# 2782), caspase 3 (# 9662) and caspase 8 (# 9746) were from Cell Signaling Technology (Ozyme, France). Anti-PARP1 antibody (AM30) and anti-TRAIL antibody (# 556468) was from BD Biosciences (Le Pont-De-Claix, France), anti- β -actin antibody (A-5316) was from Sigma, anti-TNF- α antibody (AF-210-NA) was from R&D Systems, anti-TRAIL-R1 antibody (# 06-744) was from Upstate (Millipore, France) and anti-TRAIL-R2 antibody (# 2019) was from ProSci (Coger S.A., France).

Enzyme-linked immunosorbent assay

The kinetics of TNF- α and TRAIL secretion into the media of irradiated and non-irradiated cells was quantified using Quantikine ELISA kits (R&D Systems) according to the manufacturer's recommendation.

Medium transfer experiments

Potential bystander effects were investigated by medium transfer experiments as described by Mothersill and Seymour (26,27). Breast cancer cells were grown to 20–30% confluence and then 10 Gy irradiated or not. After 6 days, conditioned medium was removed, filtered and transferred to flasks containing cells 10 Gy irradiated or not 24 h before. Conditioned medium was used pure or diluted with standard medium (SM). Cell death was measured 24 h later.

Results

IRs induce late mitotic catastrophe and apoptosis of breast cancer cell lines

Radiation-induced cell death was first studied over a period of 7 days on six human breast cancer cell lines. No significant cell death was observed during the first 24 h after a 10 Gy irradiation (Figure 1a). This observation reflects the lack of primary apoptotic response observed in breast tumors, indicating that these cell lines were good models to study the delayed cell death of breast cancer cells after γ -irradiation. Cell death started on day 2 after γ -irradiation for one cell line (BT-20), on days 3–5 for the other cell lines and increased with time indicating delayed cell death. Seven days after γ -irradiation, cell death was observed in all cell lines with a heterogeneous percentage of dead cells among the cell lines (Figure 1a). This delayed cell death seemed independent of the p53 status; since ZR-75-1 cells expressed a functional wild-type p53 protein, HBL-100 is a SV-40 transformed cell line whereas all the other cells expressed a mutant p53 protein.

As this delayed cell death may be accounted for by a mitotic catastrophe subsequent to growth arrest, we analyzed the cell cycle distribution and DNA content of the cells after irradiation of three representative cell lines. Twenty-four hours after irradiation, these three cell lines showed a G₂/M arrest that lasted several days and 2 or 3 days after irradiation, a fraction of cells became polyploid (Figure 1b). The effect of such prolonged G₂/M arrest has also been assessed by colony-forming assays. A plating efficiency between 2×10^{-4} and 4×10^{-4} was observed after a 10 Gy irradiation of T-47D, indicating an almost fully clonogenic inhibition. These results are fully in agreement with the definition of mitotic catastrophe since irradiation promote loss of proliferative capacity, and cell death appeared after a strong G₂/M blockade and took place in cells during mitosis and/or after a failed mitosis in polyploid cells.

We then investigated whether the γ -irradiation-induced cell death was due to apoptosis. Clear chromatin condensation and apoptotic bodies were detected in the γ -irradiated cells (data not shown), caspase 3 activation through procaspase 3 cleavage, a hallmark of apoptosis, started 3 days after γ -irradiation and increased on days 4 and 5, and PARP1, a target of caspase 3, was cleaved 4 days after radiation exposure (Figure 1c). When we studied the activation of these two proteins in dead floating cells, we observed that caspase 3 and PARP1 are almost fully cleaved, indicating that the majority of cells died by apoptosis (Figure 1c). Finally, *in situ* TUNEL assay indicated that late apoptosis occurred continuously during the last days of the kinetic (supplementary Figure S1 is available at *Carcinogenesis* Online).

Taken together, these results indicated that, late after γ -irradiation, breast cancer cells died of a caspase-dependent apoptosis associated with a mitotic catastrophe.

The death receptor pathways are activated after γ -irradiation of breast cancer cell lines

After γ -irradiation of T-47D cells, we found activation of the caspase 8, which is known to trigger apoptotic signals from the death receptors (Figure 2a). We also studied the expression of FADD, a downstream key element of the death receptor pathways implicated in the death-inducing signaling complex formation and potentially implicated in the apoptotic process (12) and showed a significant increase in FADD expression 3 days after γ -irradiation, both at the mRNA and protein levels (Figure 2b). These results strongly suggested an involvement of the death receptor pathways in the delayed cell death of the irradiated cells.

We, thus, studied the mRNA levels of receptors and ligands of these death receptors pathways. Fas, TRAIL-R2 and TNF-R2 mRNA levels were significantly increased 1 day after γ -irradiation, i.e. before any detectable cell death and this increase lasted throughout the time course of the experiment (Figure 2c). This increase was restricted to these three receptors as the mRNA levels of TRAIL-R1 and TNF-R1 remained constant (Figure 2c). We then studied the kinetics of mRNAs expression encoding the three ligands of the death receptors, FasL, TRAIL and TNF- α , and showed a delayed increased expression of these mRNAs that are highly expressed when apoptosis started (Figure 2d).

At the protein level, we found an early increased expression of Fas, TRAIL-R2 and TNF-R2 and a late increased expression of two of their ligands, FasL and TRAIL (Figure 3a). TNF- α is a soluble cytokine and was undetectable in cell lysate but enzyme-linked immunosorbent assay (ELISA) analysis of the γ -irradiated cells supernatants indicated a progressive increase of TNF- α concentration in these supernatants (Figure 3b, left panel). Even if the culture medium was renewed at day 5, a similar level of TNF- α was found 24 h later (at day 6), indicating an increase in TNF- α secretion after irradiation rather than a time-dependent accumulation of this cytokine (Figure 3b, right panel). Finally, we studied the expression of the truncated and soluble forms of FasL and TRAIL (sFasL and sTRAIL, respectively) and looked for sFasL and sTRAIL in the irradiated cell supernatants. As for TNF- α , ELISA analysis indicated a late increased secretion of sTRAIL (Figure 3c), and western blot analysis showed

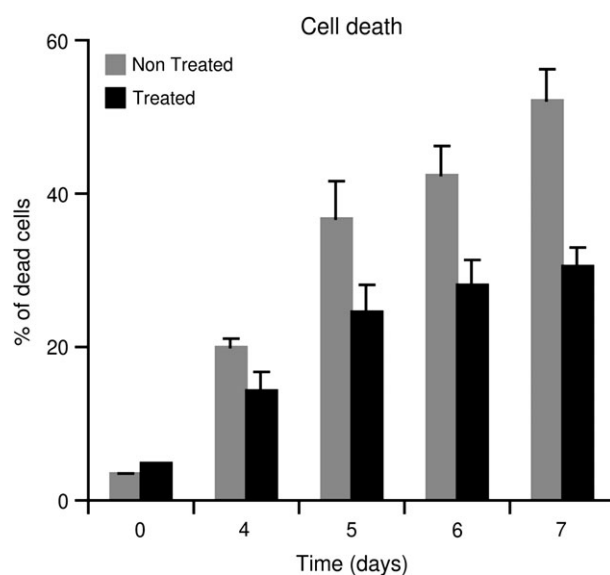


Fig. 4. Neutralization of the death receptor pathways reduces radiation-induced apoptosis. T-47D cells were 10 Gy-irradiated and treated or not with a combination of Fas/Fc, TRAIL-R1/Fc and TNF-R1/Fc chimera (250, 100 and 100 ng/ml, respectively). These chimeras, which are cytokines designed to neutralize apoptosis induced by the corresponding ligand, were added to the culture medium at day 0, 3 and 5. Viable and dead cells were counted after trypan blue staining. Results correspond to the mean \pm SD of three independent experiments.

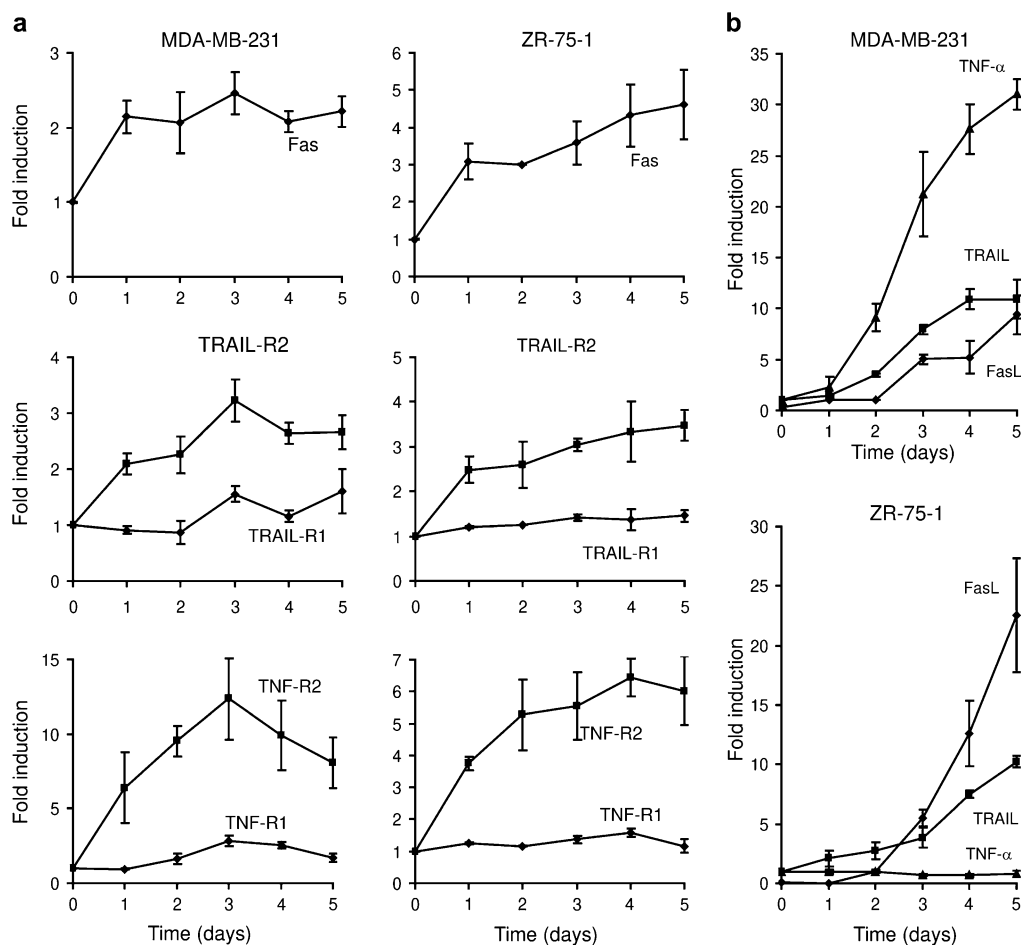


Fig. 5. Activation of death receptor pathways is found in all irradiated breast cancer cell lines studied. mRNA expression levels of (a) death receptors and (b) death receptors ligands were analyzed in ZR-75-1 that expresses a wild-type p53 and MDA-MB-231 after 10 Gy irradiation by quantitative RT-PCR as in Figure 2.

the late expression of two forms of sFasL (these two forms were described previously and correspond to distinct cleavage sites for matrix metalloproteinases (28)) (Figure 3d).

As the Fas, TRAIL-R2 and TNF-R2 death receptors were overexpressed early after γ -irradiation, we investigated whether irradiation could set up all the components of the associated death receptor pathways. Sham or irradiated T-47D cells were treated 24 h later with TNF- α , TRAIL or anti-Fas agonist antibody and cell death was analyzed 48 h after irradiation. Irradiation alone or TNF- α , TRAIL and anti-Fas agonist without irradiation induced a weak cell death but, when irradiated cells were treated with TNF- α , TRAIL or anti-Fas agonist antibody, a significant cell death was observed 48 h after irradiation (Figure 3e).

Altogether, these results showed that the sequential expression of different members of the death receptor pathways regulate the timing of radiation-induced apoptosis. The overexpression of the receptors and the set up of the death receptor pathways occurred first and, later, the delayed cell death paralleled the kinetics of expression of the three ligands, indicating that apoptosis occurred when the concentration of the ligands is sufficient to stimulate the death signaling pathways.

Neutralization of the death receptor pathways reduces radiation-induced apoptosis

To study if the activation of the death receptor pathways is a causal mechanism in the radiation-induced cell death of breast cancer cells, we used soluble recombinant chimera (Fas/Fc, TRAIL-R1/Fc and TNF-R1/Fc) that have been shown to inhibit the induced apoptosis of the corresponding ligand (soluble as well as surface ligands). Irradiated

T-47D cells were treated at day 0, 3 and 5 with the soluble recombinant chimera and cell death was analyzed 4–7 days after irradiation. Whereas each inhibitor alone could not impair the radiation-induced cell death (data not shown), the addition of the three inhibitors together significantly decreased the percentage of dead cells and diminished the slope of the percentage of dead cells over the time (Figure 4).

These results indicated that activation of death receptor pathways partly mediated radiation-induced cell death and showed that the late overexpression of death ligands is a causal key event in the late radiation-induced apoptosis in breast cancer cells.

γ -Irradiation-induced activation of death receptor pathways occurs in different human breast cancer cells

To study if activation of death receptor pathways in γ -irradiation-induced late cell death is restricted to the T-47D cell line or occurs in different human breast cancer cells, we studied the expression of the different components of the death receptor pathways in two other breast cancer cell lines, ZR-75-1 and MDA-MB-231, as these cell lines displayed different p53 status (wild-type and mutated, respectively) and different and lower radiosensitivity as compared with T-47D (Figure 1a). These two cell lines showed the same kinetics of expression of the death receptors—Fas, TRAIL-R2 and TNF-R2—than in T-47D cells (Figure 5a). The three ligands were also lately expressed in irradiated MDA-MB-231 cells, but only two ligands (FasL and TRAIL) were overexpressed in ZR-75-1 cells (Figure 5b).

In conclusion, early expression of death receptors and late expression of death ligands after γ -irradiation seemed to be a general

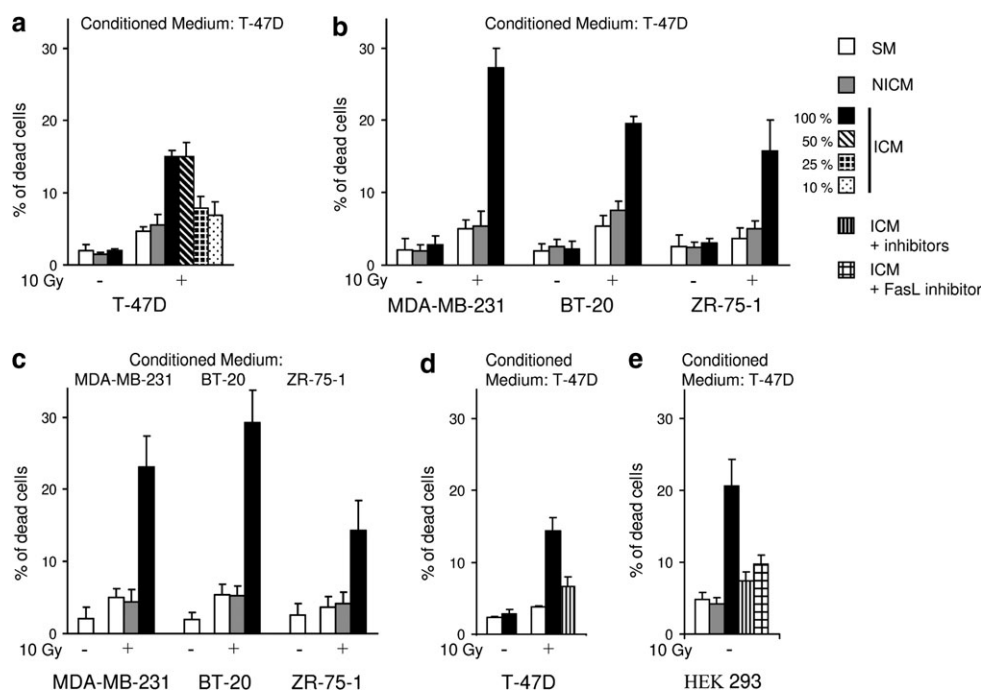


Fig. 6. Radiation-induced bystander effects by members of the death receptors ligands. Bystander effects were studied using media transfer from irradiated cells. (a) Media were conditioned for 6 days on 10 Gy-irradiated T-47D (irradiated conditioned medium: ICM) cells or non-irradiated T-47D (NICM) cells, filtered and transferred to T-47D cells which have been irradiated previously at 10 Gy or not irradiated. SM was used as control medium. ICM was used pure or 50%, 25%, and 10% diluted in SM; 24 h after media transfer, viable and dead cells were counted after trypan blue staining. (b) The effects of T-47D-conditioned media were studied on three other breast cancer cell lines: MDA-MB-231, BT-20 and ZR-75-1, using the same protocol. (c) The effects of conditioned media from MDA-MB-231, BT-20 and ZR-75-1 were studied on cells of the same type, using the same protocol. (d) ICM from T-47D were pretreated or not, for 2 h, with a combination of Fas/Fc, TRAIL-R1/Fc and TNF-R1/Fc chimeras (2 μ g/ml each). The effects of these inhibitors were studied on T-47D, using the same protocol. (e) The effects of T-47D-conditioned media were studied on the human embryonic kidney cell line HEK 293 that constitutively expresses the Fas receptor using the same protocol. A FasL-specific inhibitor or a combination of death receptors ligands inhibitors was used to study the mediation of HEK 293 cell death. For all these studies, results correspond to the mean \pm Sd of three independent measurements.

phenomenon in breast cancer cells, independent of p53 with, as expected, features specific to the cell line used.

Soluble death ligands mediate a radiation-induced bystander effects in breast cancer cells

As soluble death ligands were secreted by γ -irradiated breast cancer cells, we studied their potential-mediated bystander effects using a 6-day conditioned medium from either 10 Gy-irradiated conditioned medium (ICM) or non-irradiated conditioned medium (NICM) T-47D cells. Cell death was measured 24 h after transfer of SM, ICM and NICM to non-irradiated or irradiated T-47D cells. We did not observe any cell death after transfer of SM, ICM or NICM to non irradiated T-47D cells (Figure 6a). In contrast, when T-47D cells were irradiated 24 h before the transfer of conditioned medium, the rate of cell death was not significantly increased by NICM or MS treatments but was more than three times increased after ICM treatment. The increased cell death was similar after a 50% dilution of ICM with SM, but did not occur after 25% or 10% dilution indicating a threshold of death receptors ligands concentration for induced cell death (Figure 6a). We also showed similar effects of the T-47D ICM on irradiated MDA-MB-231, -BT-20 and -ZR-75-1 cell lines (Figure 6b). Finally, we studied the cell death induced by ICM of each cell line on the cell line itself and showed that all ICMs induced cell death but only on irradiated cells (Figure 6c). These results indicated that soluble factors present in ICM can induce cell death of breast cancer cells only if these cells have been sensitized by irradiation.

To show that the soluble factors, present in ICM, which can mediate cell death, were related to FasL, TRAIL and/or TNF- α , we neutralized the three ligands with the combined action of the three specific in-

hibitors used previously. ICM from irradiated T-47D was incubated for 2 h with the inhibitors and transferred to irradiated T-47D cells. As seen in Figure 6d, the addition of the inhibitors mixture decreased the percentage of dead cells to background. We finally studied T-47D ICM-induced cell death on the human embryonic kidney HEK 293 cell line known to constitutively express a high level of Fas and to undergo apoptosis in response to Fas stimulation. We showed that cell death occurred after addition of ICM, even when HEK 293 cells had not been irradiated previously, and this death-inducing effect could be similarly inhibited by FasL inhibitor or the mixture of the three inhibitors (Figure 6e).

Taken together, these results strongly suggest that late secretion of sFasL, sTRAIL and TNF- α by irradiated breast cancer cells participate to the induction of cell death of bystander cells if these cells are sensitive to external death inducing stimuli.

Discussion

In this paper, we showed that irradiation of breast cancer cells lead to a mitotic blockade followed by mitotic catastrophe associated with apoptosis. For all analyzed breast cancer cell lines, apoptosis occurs late after radiation exposure, is triggered by sequential overexpression of death receptors and death receptor ligands and is associated with characteristics of caspase-dependent apoptosis.

It has been suggested that prolonged growth arrest of epithelial cells after irradiation could be followed by apoptosis or necrosis (2). In our model, when studying floating dead cells after irradiation, we observed the almost total cleavage of caspase 3, 8 and PARP1, strongly suggesting that apoptosis but not necrosis is the most important modality of cell death after γ -irradiation. Apoptosis occurred after a G₂/M arrest and/or

in descendant cells having a 8 N DNA content indicating a mitotic catastrophe related to two deficient cell cycle checkpoints, i.e. spindle perturbation and/or mitotic slippage (7,29).

The delayed apoptosis observed after γ -irradiation of breast cancer cells is strongly related to triggering of the apoptosis through the death receptor pathways. Furthermore, the concomitant deregulation of expression of the members of the three pathways studied, their activation and the observation that their neutralization reduces significantly apoptosis, clearly indicated that all these pathways play a causal role, and the late apoptosis could be the result of the sum of the deregulation of these three pathways.

The triggering of apoptosis is mediated by early overexpression of members of death receptors and late overexpression of the death receptor ligands. The overexpression of the receptors have been already described after radiation exposure or therapeutics drugs treatment (11,12,14–18), but we now showed an early set up of all the components of the death receptor pathways after γ -irradiation. Among the death receptors, Fas, TRAIL-R2 and surprisingly TNF-R2 but not TNF-R1 were overexpressed after γ -irradiation. The two first receptors contain a death domain and transduce death signal by direct activation of procaspase 8, whereas TNF-R2 has no death domain. However, TNF-R1 is expressed in all studied cell lines and it was shown previously that TNF-R2 can induce apoptosis by strongly enhancing the activation of TNF-R1 by TNF- α and also by inhibiting nuclear factor kappa B-mediated protection (30), indicating that TNF-R2 overexpression may sensitize cells to TNF- α -mediated apoptotic signal after γ -irradiation. So, overexpression of the three receptors may lead all to activation of the death receptor pathways, directly for Fas and TRAIL-R2 and indirectly for TNF-R2, but always via an increase of procaspase 8 cleavage. The death receptors ligands started to be overexpressed 2 or 3 days after γ -irradiation and their kinetics of expression paralleled the appearance of apoptosis. Furthermore, neutralization of these ligands inhibited cell death. We also showed that the ligand's concentration is critical for cell death induction, dilution experiments of conditioned media indicating a threshold effect rather than a dose–response effect. To our knowledge, this is the first time that late and concomitant overexpression of death ligands is involved in radiation-induced death of epithelial tumor cells in relation to mitotic catastrophe. Finally, our results indicate that death receptors overexpression might be directly induced by γ -irradiation, whereas the death receptors ligands overexpression might be the consequence of the mitotic catastrophe in response to blockage of cell cycle checkpoints or abnormal mitosis (29). Indeed, many regulatory elements that control mitotic catastrophe, i.e. cell cycle-specific kinases (Cdk1, Plks, Aurora), cell cycle checkpoint proteins, survivin, p53 and p21^{waf1/cip1} could also be implicated in the regulation of death ligand expression (7,29).

We can detect soluble forms of the three death ligands TNF- α , sFasL and sTRAIL in culture medium 5 days after radiation exposure, and by medium transfer experiments, we showed that these ligands can induce the death of bystander cells. TNF- α is essentially described as a soluble cytokine, whereas FasL and TRAIL are mainly described as membrane-bound proteins. The cleavage of FasL and TRAIL by specific proteases to produce soluble forms seemed to be induced by γ -irradiation and/or mitotic catastrophe and might be related to the production of soluble forms of sFasL and sTRAIL in breast and colon adenocarcinomas and in the immune system (31–33). So, secretion of the three soluble ligands enhances the death signal induced by radiation exposure and cause the death of sensitive bystander cells. These soluble death receptors ligands could mediated cell death of all breast cancer cells tested, because ICM from a cell line could induce cell death in the other cell lines. However, we showed a death-inducing effect on receiving breast cancer cells only if the cells were first irradiated or in HEK 293 cells, in which Fas is known to be constitutively overexpressed. Taken together, all these results show that death receptor signaling pathways play an important role in medium-mediated death effect through soluble forms of death ligands.

Many non-targeted effects associated with exposure to IR have been described and reactive oxygen species or cytokines have been suggested as potential mediators of bystander effects such as genomic

instability or cell death of receiving cells (26,34–39). To date, only one recent report characterizes extracellular factors implicated in the bystander death-inducing effect and shows the release of sTRAIL and TNF- α after X-ray irradiation of lung carcinoma cell lines (40). However, in this study, sTRAIL and TNF- α were produced early and transiently after irradiation and were not implicated in delayed cell death. In the study reported in this paper, we have unambiguously characterized, on receiving sensitive breast cancer cells, a death-inducing bystander effect which was mediated by soluble forms of death ligands and showed that excreted ligands of death receptor pathways amplify the direct response to radiation by enhancing the rate of cell death through a bystander effect. Our results were obtained on breast cancer cell lines and we will investigate in the future if the phenomena observed are also found in primary breast cancer tumors and other types of tumors sensible to γ -irradiation.

In conclusion, we have shown that three death receptor pathways—Fas, TRAIL-R and TNF-R—play a crucial role in the apoptosis and bystander effect that occurred after γ -irradiation of breast cancer cells. The sequential expression of the receptors and ligands indicated why a synergy is observed between γ -irradiation and activation of the death receptor signaling pathways and why apoptosis is a late process after radiation exposure of tumor derived from epithelial cells. The presence of soluble ligands in culture media indicated that cell death induced by γ -irradiation might not be restricted to initially damaged cells but also, by bystander effect, to surrounding cells sensitive to these diffusible factors. This bystander effect indicated that the total response of the tumor cells and the tumor microenvironment to γ -irradiation may be broader than originally thought and ultimately may have implications for radiation risk assessment.

Supplementary materials

Supplementary Table I and Figure S1 can be found at <http://carcin.oxfordjournals.org/>

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