Mitochondria-dependent and -independent mechanisms in tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis are both regulated by interferon-γ in human breast tumour cells

Carmen RUIZ-RUIZ and Abelardo LÓPEZ-RIVAS[1]

Instituto de Parasitología y Biomedicina, CSIC, calle Ventanilla 11, 18001 Granada, Spain

Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL/APO-2L) induces apoptosis in a variety of tumour cells upon binding to death receptors TRAIL-R1 and TRAIL-R2. Here we describe the sensitization by interferon (IFN)-γ to TRAIL-induced apoptosis in the breast tumour cell lines MCF-7 and MDA-MB231. IFN-γ promoted TRAIL-mediated activation of caspase-8, Bcl-2 interacting domain death agonist (Bid) degradation, Bcl-2-associated X protein (Bax) translocation to mitochondria, cytochrome *c* release to the cytosol and activation of caspase-9 in these cell lines. No changes in the expression of TRAIL receptors were observed upon IFN-γ treatment. Overexpression of Bcl-2 in MCF-7 cells completely inhibited IFN-γ-induced sensitization to TRAIL-mediated cell death. Interestingly, TRAIL-induced apoptosis was also clearly enhanced by IFN-γ in caspase-3-overexpressing MCF-7 cells, in the absence of Bax translocation to mitochondria and cytochrome *c* release to the cytosol. In summary, our results suggest that IFN-γ facilitates TRAIL-induced activation of mitochondria-regulated as well as mitochondria-independent apoptotic pathways in breast tumour cells.

Key words: Bcl-2, caspase-8, death receptor, sensitization.

INTRODUCTION

Members of the tumour necrosis factor (TNF) superfamily, such as CD95 ligand (CD95L or FasL/APO-1L) and TNF-related apoptosis-inducing ligand (TRAIL/APO-2L), are potent inducers of apoptosis upon binding to their respective death domain-containing receptors, also known as death receptors [1,2]. Cytoplasmic death domains serve to recruit intracellular adapter molecules that in turn engage and activate procaspase-8, thereby initiating a cascade of events which lead to apoptotic cell death [3–6]. TRAIL can bind to the death receptors TRAIL-R1 and TRAIL-R2 (also known as DR4 and DR5 respectively), as well as to the decoy receptors TRAIL-R3/DcR1 and TRAIL-R4/DcR2, which fail to signal cell death [7]. Unlike CD95L, TRAIL transcripts are detectable in many normal organs and tissues [2] suggesting that this ligand may be non-toxic to normal cells. Furthermore, it has been demonstrated that TRAIL can induce apoptosis in a wide variety of transformed cell lines [2,8].

Breast cancer is the most common neoplasia amongst women in the Western world and therefore progress in its treatment is of outstanding interest. Genotoxic drugs as well as radiation therapy have been widely used to induce apoptosis in breast tumour cells [9]. Recently, different combined strategies have been investigated in order to improve the effects of chemotherapy and radiotherapy. In this respect we, and others, have reported that DNA-damaging drugs and ionizing radiation can sensitize breast cancer cells to death receptor-induced apoptosis [10,11]. Positive results have also been obtained in breast cancer treatment by immunotherapy with natural interferons (IFNs) and interleukins, particularly in combination therapies [12]. IFNs are a family of cytokines that play a key role in antiviral, antiproliferative and immunomodulatory responses [13]. The antitumour activity of IFN has also been reported against a variety of tumour cells such as lymphomas, melanomas and multiple myeloma [14]. Moreover, it has been demonstrated that IFN-γ can enhance the antitumour activity of antimetabolite in cancer cells [15]. However, the mechanism of interferon-mediated cellular susceptibility to apoptosis has not been completely elucidated. It has been described that IFN-γ, as well as IFN-α can up-regulate the expression of different apoptosis-related proteins including death receptors and their respective ligands, caspases and several members of the Bcl-2 family in a variety of cells [16,17].

We, as well as other authors, have recently reported that IFN-γ enhances CD95-mediated apoptosis in breast cancer cells [18,19]. However, activation of the CD95 pathway may not be a useful therapeutic strategy as normal human cells are killed upon CD95 ligation and massive apoptosis is observed in the liver of experimental mice injected with CD95 antibody [20]. In contrast, TRAIL has no systemic toxicity in preclinical studies with mice and nonhuman primates when administered at doses that inhibit the growth of breast and colon cancer xenografts [8]. Recently, however, a recombinant form of human TRAIL has been shown to cause apoptosis in human hepatocytes [21]. Nevertheless, more recent data have demonstrated that different recombinant versions of TRAIL vary considerably in toxicity towards normal human cells, but all of them maintain their antitumour properties [22]. Altogether, these results emphasise the importance of finding treatments that enhance the antitumour action of TRAIL and

Abbreviations used: TNF, tumour necrosis factor; TRAIL, tumour necrosis factor-related apoptosis-inducing ligand; IFN, interferon; Bid, Bcl-2 interacting domain death agonist; Bax, Bcl-2-associated X protein; mAb, monoclonal antibody; CD95L, CD95 ligand; FADD, Fas-associated death domain protein; MORT1, mediator of receptor-induced toxicity 1; PARP, poly(ADP-ribose)polymerase; PS, phosphatidylserine; IRF, IFN regulatory factor; RT-PCR, reverse transcription–PCR.

[1] To whom correspondence should be addressed (e-mail alrivas@ipb.csic.es)

prompted us to study the effects of IFN-γ on TRAIL-induced apoptosis in breast cancer cells. In this report, we demonstrate that IFN-γ is able to increase apoptosis mediated by TRAIL in breast tumour cell lines without altering the level of death receptors for this ligand. We also show that IFN-γ facilitates the activation by TRAIL of the initiator caspase-8, as well as several mitochondria-regulated events that eventually lead to apoptosis in MCF-7 and MDA-MB231 breast cancer cell lines. Finally, our data indicate that IFN-γ can also sensitize caspase-3-overexpressing MCF-7 cells to a TRAIL-induced mitochondria-independent apoptotic pathway.

EXPERIMENTAL

Reagents and antibodies

RPMI 1640 medium and fetal-bovine serum were obtained from Life Technologies (Paisley, Renfrewshire, Scotland, U.K.) Human IFN-γ and recombinant human TRAIL were obtained from PreproTech EC LTD (London, U.K.). Rabbit polyclonal antiserum against poly(ADP-ribose)polymerase (PARP) was from Roche Molecular Biochemicals (Monza, Italy). Mouse antihuman caspase-8 monoclonal antibody (mAb) was purchased from Cell Diagnostica (Münster, Germany). Mouse anti-Bax (Bcl-2-associated X protein) mAb and mouse anti-cytochrome *c* mAb were obtained from Pharmingen (San Diego, CA, U.S.A.). Mouse anti-Bcl-2 mAb was purchased from DAKO (Copenhagen, Denmark). Rabbit anti-cleaved caspase-9 and caspase-3 polyclonal antibodies were from New England BioLabs (Beverly, MA, U.S.A.). Mouse anti-α-tubulin mAb was obtained from Sigma Immunochemicals (St. Louis, MO, U.S.A.). Rabbit anti-Bid (Bcl-2 interacting domain death agonist) polyclonal antibody was generously provided by Dr X. Wang (Howard Hughes Medical Institute, Dallas, TX, U.S.A.).

Cell lines

The human breast tumour cell lines MCF-7 and MDA-MB231 were kindly provided by Dr M. Ruiz de Almodovar (Department of Radiology, University of Granada, Granada, Spain). They were maintained in culture in RPMI 1640 medium containing 10% fetal-bovine serum, 1 mM L-glutamine and gentamycin, at 37 °C in a humidified 5% CO_2/95% air incubator. Stable MCF-7 cell lines overexpressing human Bcl-2 protein were generated as described previously [18]. MCF-7 cells stably transfected with caspase-3 were generously provided by Dr D. R. Green (San Diego, CA, U.S.A.).

Determination of apoptotic cells

Phosphatidylserine (PS) exposure on the surface of apoptotic cells was detected by flow cytometry after staining with Anexin-V-FLUOS (Roche Molecular Biochemicals). Flow cytometry was performed on a FACScan cytometer using the Cell Quest software (Becton Dickinson, Mountain View, CA, U.S.A.).

Immunoblot detection of proteins

Following detachment with RPMI 1640 medium/3 mM EDTA, cells (5×10^5) were washed with PBS and lysed in 20 μl of Laemmli buffer. Cell samples were sonicated and the proteins were resolved on SDS/polyacrylamide minigels and detected as described previously [10].

For measurements of cytochrome *c* and Bax, cells were washed with PBS and lysed for either 8 min (MCF-7 cells) or 4 min (MDA-MB cells) in 30 μl of ice cold lysis buffer [80 mM KCl, 250 mM sucrose, 500 μg/ml digitonin, 1 μg/ml of each of the protease inhibitors: leupeptin, aprotinin, pepstatin, and 0.1 mM PMSF in PBS]. Then, cell lysates were centrifugated for 5 min at 10000 ***g***. Proteins from the supernatant (cytosolic fraction) and pellet (membrane fraction) were mixed with Laemmli buffer and resolved on SDS/polyacrylamide (12%) minigels. Cyto-

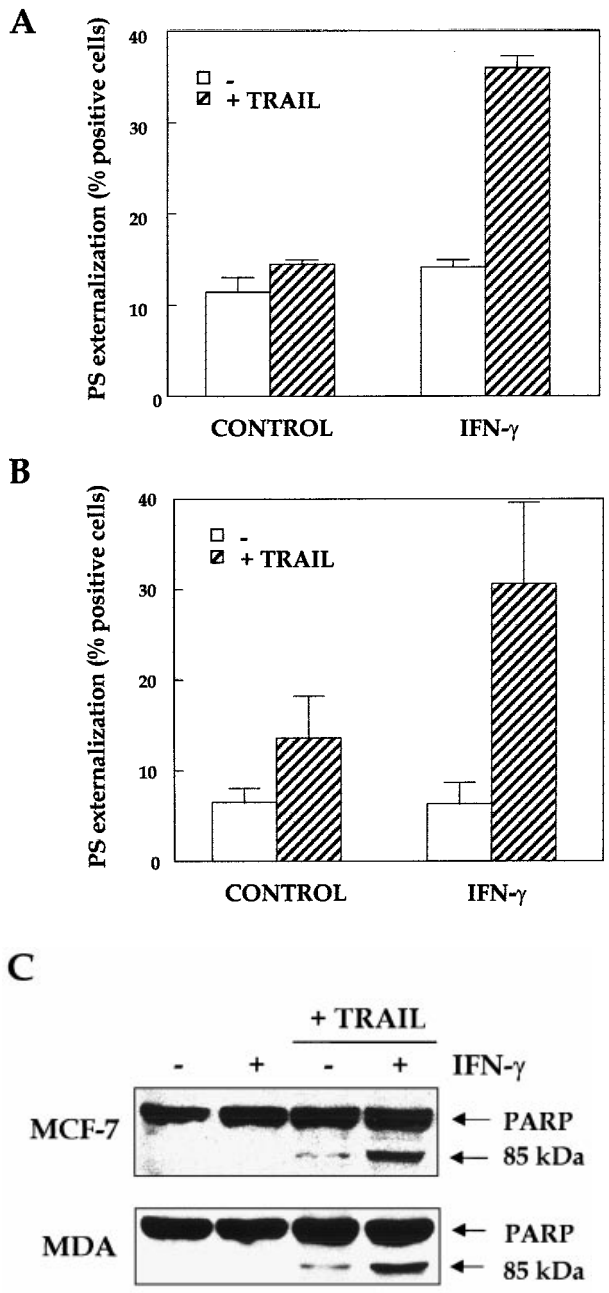

Figure 1 IFN-γ sensitizes breast tumour cells to TRAIL-induced apoptosis

PS externalization was determined in MCF-7 (**A**) and MDA-MB231 cells (**B**) incubated for 24 h in the presence (+) or absence (−) of 10 ng/ml IFN-γ and subsequently treated without or with 50 ng/ml soluble TRAIL for 15 h (**A**) or 24 h (**B**). Error bars represent S.D. from two independent experiments. (**C**) MCF-7 and MDA-MB231 cells were treated for 8 h and 24 h, respectively, without or with TRAIL (50 ng/ml) after preincubation in the presence (+) or absence (−) of IFN-γ (10 ng/ml) for 24 h. Cleavage of PARP was analysed by Western blot. Arrows show the proteolytic fragment of PARP (85 kDa).

chrome *c* and Bax were determined by Western-blot analysis as described above.

Reverse transcription (RT)–PCR

Total RNA was isolated from cells with Trizol reagent (Life Technologies, Inc., Grand Island, NY, U.S.A.) as recommended by the supplier. cDNAs were synthesized from 2 μg of total RNA using a RNA PCR kit (PerkinElmer, Indianapolis, IN, U.S.A.) with the supplied oligo d(T) primer under conditions described by the manufacturer. PCR reactions were performed using the following primers: human TRAIL-R1 sense 5′-CTGAGCAACGCAGACTCGCTGTCCAC-3′, and human TRAIL-R1 antisense 5′-TCCAAGGACACGGCAGAGCCTGTGCCAT-3′; human TRAIL-R2 sense 5′-GCCTCATGGACAATGAGATAAAGGTGGCT-3′, and human TRAIL-R2 antisense 5′-CCAAATCTCAAAGTACGCACAAACGG-3′; human β-actin sense 5′-TGACGGGGTCACCCACACTGTGCCCATCTA-3′, and human β-actin antisense 5′-CTAGAAGCATTTGCGGTGGACGATGGAGGG-3′; and human IFN regulatory factor (IRF)-1 sense 5′-CTTAAGAACCAGGCAACCTCTGCCTTC-3′, and human IRF-1 antisense 5′-GATATCTGGCAGGGAGTTCATG-3′, giving products of 506, 502, 661 and 406 bp, respectively. Cycle conditions for all the PCR reactions were 1 min at 95°, 1 min at 55° and 1 min at 72° for 30 cycles.

RESULTS

IFN-γ sensitizes breast tumour cells to TRAIL-mediated apoptosis

Two human breast cancer cell lines, MCF-7 and MDA-MB231, were used to examine the effects of IFN-γ on TRAIL-mediated cell death. Treatment of both cell lines with the combination of IFN-γ and soluble TRAIL caused an increased PS exposure in the extracellular side of the plasma membrane, which is a marker of apoptotic cell death (Figures 1A and 1B). In contrast, treatment with either IFN-γ or TRAIL had only a slight effect on PS externalization at the end of the incubation period. To further confirm that cells treated with IFN-γ and TRAIL were dying by apoptosis, we determined the proteolytic degradation of the caspases substrate PARP. As shown in Figure 1(C), PARP cleavage induced by TRAIL was clearly enhanced in both MCF-7 and MDA-MB231 cells pretreated with IFN-γ.

It has been reported that IFN-γ can modulate the expression of death receptors and their ligands in several cellular systems [16,17,23]. These data led us to analyse the expression of death receptors for TRAIL in response to IFN-γ in MCF-7 and MDA-MB231 cells. Results shown in Figure 2 indicate that IFN-γ treatment had no obvious effects on mRNA levels for TRAIL-R1 and TRAIL-R2 receptors. However, the same treatment caused an induction of mRNA for the transcriptional activator IRF-1, an IFN inducible gene that served as a control for IFN-γ action. We also determined by RT-PCR analysis the expression of two decoy receptors for TRAIL, TRAIL-R3 and TRAIL-R4, but we did not observe changes in the levels of these receptors (results not shown). These results suggest that sensitization of breast tumour cells by IFN-γ to TRAIL-induced apoptosis cannot be explained by the regulation of death receptor expression.

IFN-γ enhances TRAIL-induced activation of caspase-8 and a mitochondria-operated pathway in certain breast tumour cells

To understand the sensitization to TRAIL-mediated apoptosis by IFN-γ in breast cancer cells we decided to analyse different biochemical events that are known to occur upon ligation of TRAIL receptors at the cell surface. TRAIL initiates apoptosis by inducing the recruitment of the adapter molecule Fas-associated death domain protein (FADD)/ mediator of receptor-induced toxicity 1 (MORT1) to apoptotic TRAIL receptors and the subsequent engagement and activation of procaspase-8 [5,6,24]. We determined the activation of caspase-8 in MCF-7 and MDA-MB231 cells by analysing the processing of the procaspase into the 43–41 kDa intermediate proteolytic fragments corresponding to the cleavage of procaspase-8a and -8b. As shown in Figure 3, TRAIL-induced activation of caspase-8 was clearly enhanced by pretreatment with IFN-γ in both types of cells.

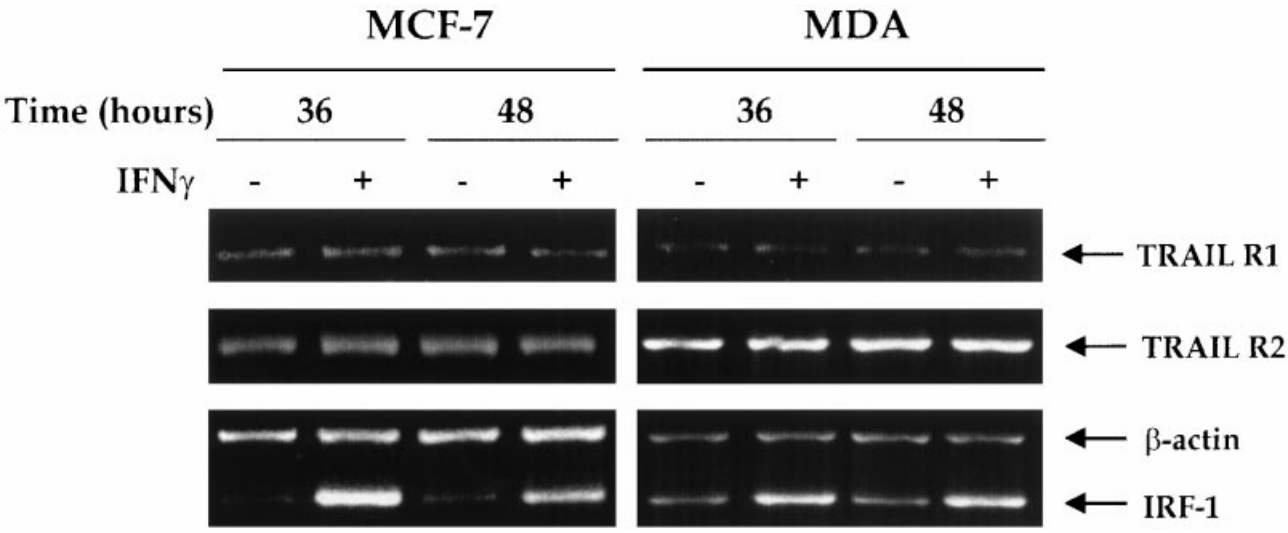

Figure 2 IFN-γ did not modulate the expression of TRAIL receptors in breast tumour cells

TRAIL-R1 and TRAIL-R2 mRNA levels were analysed by RT-PCR in MCF-7 and MDA-MB231 cells after incubation in the presence (+) or absence (−) of IFN-γ (10 ng/ml) for the indicated times. RT-PCR products of β-actin and IRF-1 were used as controls of RNA input and IFN-γ action respectively.

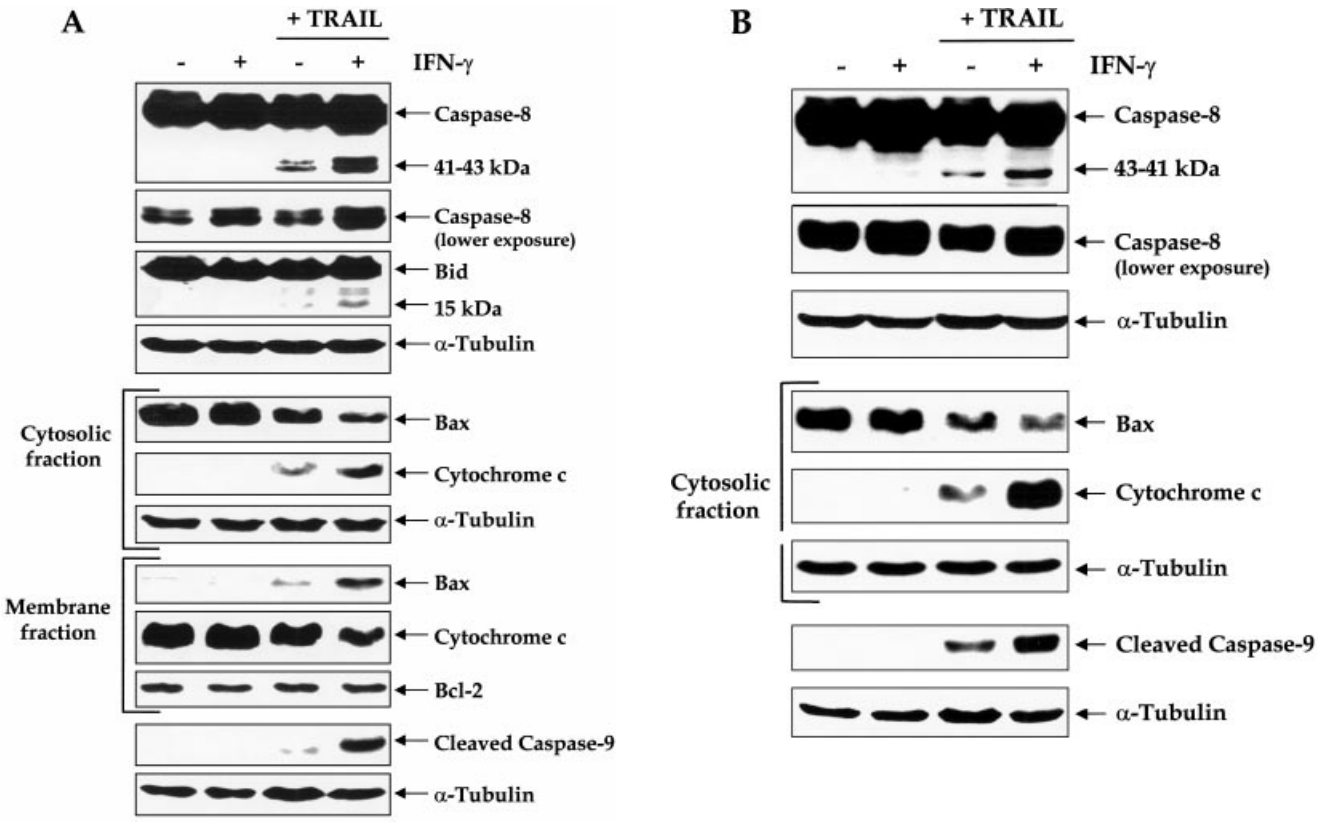

Figure 3 IFN-γ facilitates a TRAIL-induced mitochondria-operated apoptotic pathway in breast cancer cells

MCF-7 (**A**) and MDA-MB231 (**B**) cells were preincubated for 24 h in the presence (+) or absence (−) of 10 ng/ml IFN-γ before treatment without or with 50 ng/ml TRAIL for 8 h (**A**, caspase-8, Bid, Bax and cytochrome *c*), 12 h (**A**, caspase-9) or 24 h (**B**). Activation of caspases-8 and -9, translocation of cytosolic Bax, release of cytochrome *c* from mitochondria (**A**, **B**) and caspase-mediated cleavage of Bid (**A**) were determined by Western blot. Arrows show the cleaved intermediate forms of caspase-8 (41–43 kDa), Bid (15 kDa) and cleaved caspase-9 (37 kDa). α-Tubulin was used as control of loaded protein. In (**A**), Bcl-2 was used as a mitochondrial marker and control for protein loading. In (**A**) and (**B**), a lower exposure for caspase-8 zymogen is provided to show the up-regulation by IFN-γ of procaspase-8 levels.

Activation of caspase-8 causes the processing of substrate Bid to generate a fragment of 15 kDa which translocates to mitochondria and induces the release of cytochrome *c* thereby activating a mitochondrial apoptotic pathway [25]. Moreover, upon death receptor activation the cytoplasmic protein Bax migrates to the mitochondria where it co-operates with truncated Bid in the release of cytochrome *c* [26]. Furthermore, a role of mitochondria in TRAIL-induced apoptosis has been recently proposed [27,28]. Thus, we examined the activation of mitochondria-controlled apoptotic pathway by TRAIL in IFN-γ-treated breast tumour cells, by determining the proteolytic degradation of Bid, the translocation of cytosolic Bax to mitochondria and the release of cytochrome *c* from mitochondria. Results of Figure 3(A) indicate that Bid cleavage in response to TRAIL was clearly enhanced in MCF-7 cells preincubated with IFN-γ. In MDA-MB231 cells, we were not able to detect the proteolytic fragment of Bid upon incubation for 24 h with both TRAIL and IFN-γ in the experiment shown in Figure 3(B). However, we observed in this cell line an important decrease in the level of intact Bid after longer periods of incubation in the presence of IFN-γ and TRAIL (results not shown), which suggested its proteolytic processing. Next, we analysed the loss of Bax and the presence of cytochrome *c* in cytosolic extracts from MCF-7 and MDA-MB231 upon TRAIL receptor ligation. We observed that pretreatment with IFN-γ highly enhanced both events (Figure 3). These results were associated with the increase of Bax and the loss of cytochrome *c* in the mitochondria-containing membrane fraction of lysed MCF-7 cells (Figure 3A).

Once released from mitochondria, cytochrome *c*, together with apoptotic protease activating factor-1 and dATP as cofactors will form the apoptosome, a multimeric complex which activates caspase-9 [29]. We determined the activation of caspase-9 follwng treatment with TRAIL in MCF-7 and MDA-MB231 cells preincubated with or without IFN-γ by measuring the generation of the 37 kDa proteolytic fragment of caspase-9. We observed that IFN-γ clearly promoted the activation of this caspase (Figure 3). In IFN-γ-treated MCF-7 cells, we detected a slight processing of caspase-9 at 8 h after addition of TRAIL (results not shown), the time at which we had analysed the biochemical changes described above. However, we observed a greater activation of caspase-9 at 12 h after the addition of TRAIL (Figure 3A), suggesting an ordered relationship between all these events.

IFN-γ does not sensitize Bcl-2 overexpressing MCF-7 cells to TRAIL-mediated apoptosis

As indicated in the above section, several reports have recently described the involvement of mitochondria in TRAIL-induced apoptosis [27,28]. However, an important controversy exists

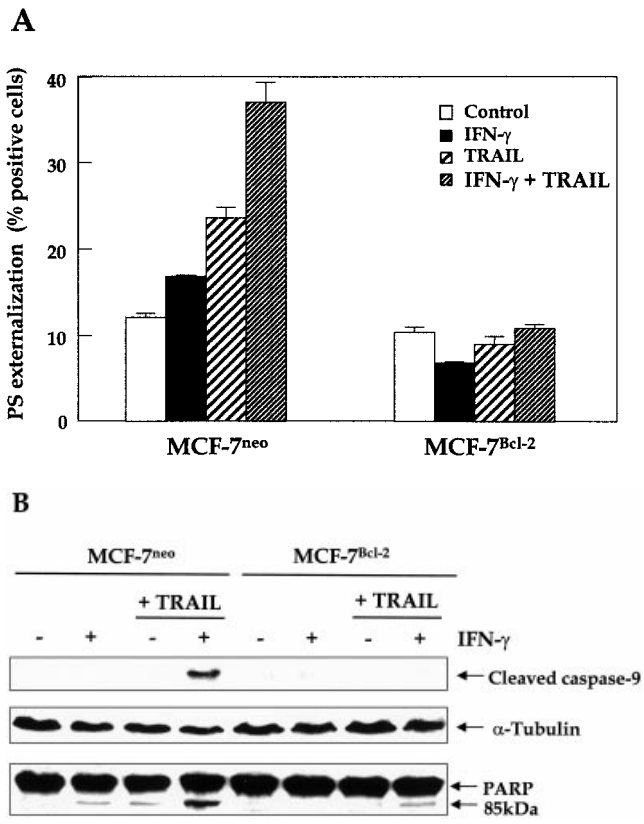

Figure 4 IFN-γ does not promote TRAIL-induced apoptosis in Bcl-2-overexpressing MCF-7 cells

MCF-7^{neo} and MCF-7^{Bcl-2} cells were preincubated for 24 h in the presence (+) or absence (−) of IFN-γ (10 ng/ml) and subsequently treated without or with TRAIL (50 ng/ml) for either 15 h (**A**) or 12 h (**B**). In (**A**), PS externalization was analysed as described above. Error bars represent S.D. from two independent experiments. In (**B**), caspase-9 activation and PARP cleavage were determined by Western-blot analysis. Arrows show the cleaved intermediate form of caspase-9 and the 85 kDa fragment of PARP. α-Tubulin was used as control for protein loading.

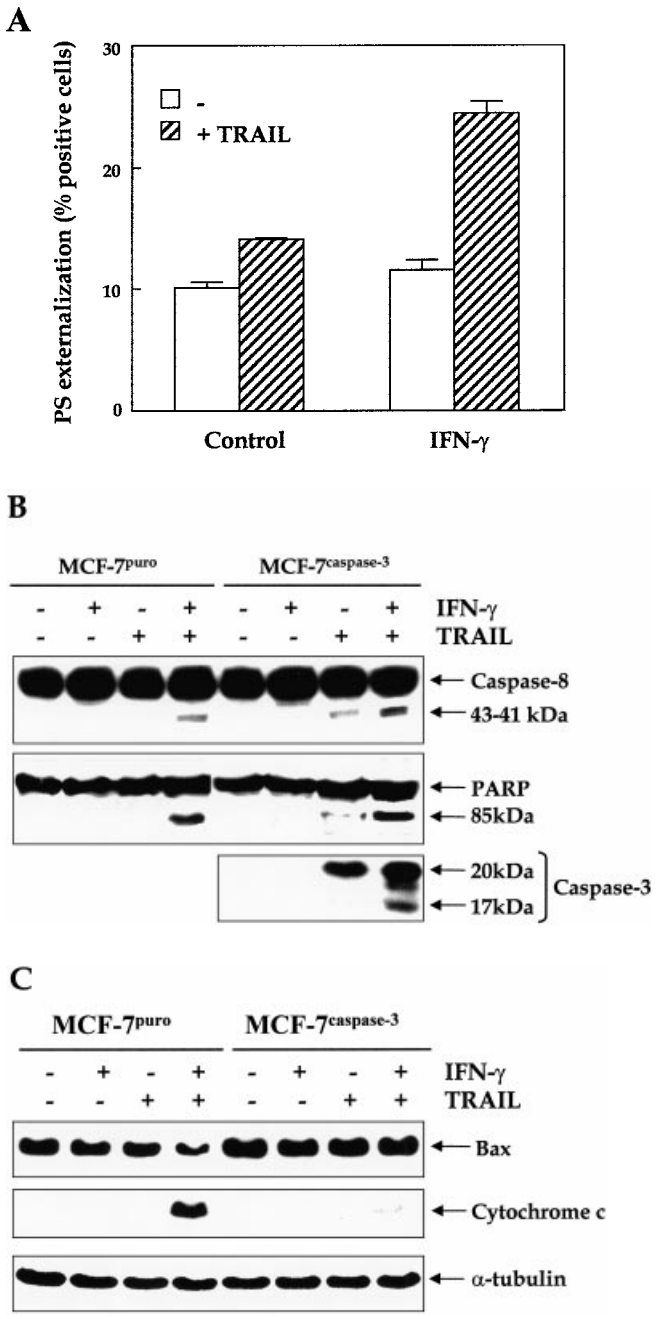

Figure 5 IFN-γ facilitates a TRAIL-induced mitochondria-independent apoptotic pathway in caspase-3-overexpressing MCF-7 cells

MCF-$7^{caspase-3}$ cells were preincubated for 24 h in the presence (+) or absence (−) of 10 ng/ml IFN-γ before treatment without or with 50 ng/ml TRAIL for 15 h. PS externalization (**A**) was determined by cytofluorimetric analysis. Error bars represent S.D. from two independent experiments. Activation of caspases-8 and -3 and PARP cleavage (**B**) and loss of cytosolic Bax and release of mitochondrial cytocrome *c* (**C**) were analysed by Western blot in MCF-7^{puro} and MCF-$7^{caspase-3}$ cells preincubated for 24 h in the presence (+) or absence (−) of IFN-γ (10 ng/ml) and subsequently treated with or without TRAIL (50 ng/ml) for 8 h (**B**, caspase-8 and **C**) or 12 h (**B**, caspase-3 and PARP). Arrows show the cleaved intermediate forms of caspase-8 (41–43 kDa), caspase-3 (17 and 20 kDa) and the 85 kDa fragment of PARP. α-Tubulin was used as control for protein loading.

about the role of this organelle in TRAIL-induced cell death. Thus, different authors have reported that cells overexpressing the anti-apoptotic protein Bcl-2, which inhibits cell death mainly at the mitochondrial level, remain sensitive to apoptosis induced by TRAIL [30,31]. However, we have recently described that resistance to TRAIL-mediated cell death in Bcl-2-overexpressing MCF-7 cells is determined by the level of overexpressed Bcl-2 protein [32]. To get further insight into the importance of mitochondria-mediated events in IFN-γ-induced sensitization to TRAIL, we studied whether TRAIL-induced apoptosis was facilitated by IFN-γ in a clone of MCF-7^{Bcl-2} cells (B6) which we had previously described as resistant to cell death upon TRAIL receptors ligation [32]. As shown in Figure 4(A), IFN-γ did not promote TRAIL-activated externalization of PS in MCF-7^{Bcl-2} cells, in contrast to what was observed in cultures of cells transfected with a control vector. Bcl-2 prevents the release of cytochrome *c* from mitochondria thus inhibiting the activation of the apoptosome and executioner caspases. Therefore, we determined the activation of caspase-9 processing and the cleavage of the caspases substrate PARP in MCF-7^{Bcl-2} cells incubated in the presence of both IFN-γ and TRAIL. We found that IFN-γ did not facilitate the activation of caspase-9 nor PARP cleavage in Bcl-2-overexpressing cells compared with control cells (Figure 4B). In contrast, we found no differences between mock transfected and Bcl-2-overexpressing MCF-7 cells in the early processing of procaspase-8 upon treatment with IFN-γ and TRAIL (results not shown). These results indicate that in Bcl-2-

overexpressing cells, sensitization by IFN-γ treatment is still observed at the earliest steps of TRAIL-induced apoptosis, e.g. death-inducing signalling complex formation, but the apoptotic pathway is blocked by Bcl-2 at a later step, possibly at the mitochondrial level. Taken together, our data indicate that IFN-γ does not sensitize Bcl-2 over-expressing cells to TRAIL-induced mitochondria-regulated cell death.

Sensitization by IFN-γ of MCF-7 cells overexpressing caspase-3 to a TRAIL-induced mitochondria-independent pathway of apoptosis

MCF-7 cells, which are deficient in caspase-3 expression [33], have been ascribed to the type II group of cells as they require the mitochondrial pathway for CD95-mediated apoptosis signalling [34]. Moreover, we have observed that overexpression of Bcl-2 in MCF-7 cells not only block apoptosis induced by CD95 antibody [18] but also TRAIL-mediated cell death ([32], and results above). However, it has been described that overexpression of caspase-3 converts MCF-7 cells to type I cells in which activation of executioner caspases is not regulated through the mitochondria and they are no longer protected from CD95-mediated apoptosis by Bcl-xL [34]. To ascertain whether IFN-γ also sensitized these types of cells to TRAIL-induced apoptosis and to get further insight into the mechanism of IFN-γ-induced sensitization, we decided to assess the effect of the combined treatment with TRAIL and IFN-γ in caspase-3-overexpressing MCF-7 cells. Results shown in Figure 5(A) indicate that pre-incubation with IFN-γ facilitated TRAIL-induced PS externalization in MCF-7$^{caspase\text{-}3}$ cells similarly to what occurred in cultures of caspase-3-deficient MCF-7 cells (Figure 1A). To better characterize the sensitization by IFN-γ of caspase-3-overexpressing MCF-7 cells we analysed TRAIL-mediated activation of caspases in cells pre-treated with or without IFN-γ. As shown in Figure 5(B), IFN-γ promoted the activation by TRAIL of caspase-8 and the cleavage of PARP in MCF-7$^{caspase\text{-}3}$ cells as well as in cells transfected with a control vector (puro). Furthermore, we observed that TRAIL-induced activation of caspase-3 was clearly enhanced in MCF-7$^{caspase\text{-}3}$ cells by pre-treatment with IFN-γ (Figure 5B). In these experiments we observed in IFN-γ-treated cells an increased processing of procaspase-3 to produce the final p17 subunit, a component of the active p17/p12 caspase-3. However, in contrast to what is observed in caspase-3-deficient MCF-7 cells (Figure 3A), IFN-γ did not promote the activation of a mitochondria-operated pathway of apoptosis in MCF-7$^{caspase\text{-}3}$ cells (Figure 5C). Thus, we did not observe the presence of mitochondrial cytochrome *c* and the loss of Bax in cytosolic extracts from TRAIL-treated MCF-7$^{caspase\text{-}3}$ cells at the time of the experiment (8 h), not even in the presence of IFN-γ (Figure 5C). We could only detect these TRAIL-induced mitochondrial events in MCF-7$^{caspase\text{-}3}$ cells after longer periods of incubation ($\geqslant$ 20 h, results not shown). These later results suggest that in MCF-7$^{caspase\text{-}3}$ cells, mitochondrial signalling is not involved in IFN-γ-promoted, TRAIL-mediated activation of the caspase cascade and apoptosis. This points to a more general role of IFN-γ as a sensitizing agent in death receptor mediated-apoptosis of breast tumour cells.

DISCUSSION

Induction of apoptosis in tumour cells by death receptor activation is a novel therapeutic strategy. However, in systemic anti-tumour treatments, severe toxic effects have been observed with TNF-α and CD95L. TNF-α causes a lethal inflammatory response [35] and CD95L produces lethal liver damage [20]. Preclinical studies in mice and nonhuman primates showed no systemic cytotoxicity upon injection of recombinant TRAIL at doses that effectively suppressed solid tumours such as colon and mammary carcinomas [8]. These data suggested that TRAIL could be a suitable approach in cancer therapy. Nevertheless, recent studies have questioned the potential utility and safety of systemic administration of TRAIL by revealing that, in culture, normal human hepatocytes are effectively killed by recombinant human TRAIL even though hepatocytes from rat, mouse and rhesus monkey remain resistant to this ligand [21]. However, it seems that different recombinant versions of TRAIL may vary in their potential for cellular and animal toxicity [22], despite maintaining their anti-tumour action. Moreover, it has been described that normal human liver cells can be protected from TRAIL-induced toxicity by co-exposure to the caspase-9 inhibitor Z-LEHD-FMK, while some cancer cells can still be killed despite the presence of this inhibitor [36]. Altogether, these results hint at a combination strategy to selectively kill tumour cells that need to be further explored.

In combination regimes, IFN-γ could be a promising anti-tumour therapeutic approach as it has been described to enhance cellular susceptibility to apoptosis in a variety of tumour cells. The mechanism by which IFN-γ promotes cell death seems to be the regulation of the expression of different proteins involved in apoptosis such as caspases, members of the Bcl-2 family and death receptors as well as their respective ligands [16,17,37]. On the other hand, IFN-γ induces the up-regulation of TRAIL in breast tumour cells (C. Ruiz de Almodovar, C. Ruiz-Ruiz and A. Lopez-Rivas, unpublished work) and a recent report has indicated the stimulation of the IFN pathway by TRAIL [38], suggesting the existence of cross-talk between the TRAIL and IFN signalling pathways. We had previously described the sensitization of breast tumour cells by IFN-γ to CD95-mediated apoptosis [18]. In the present report we have demonstrated that IFN-γ is also able to sensitize breast cancer cells to TRAIL-mediated cell death. In this respect, several mechanisms of resistance to TRAIL-mediated apoptosis have been described in tumour cells, such as the lack of TRAIL-death receptors, enhanced expression of TRAIL-decoy receptors and expression of apoptosis inhibitors [39,40]. We observed that MCF-7 and MDA-MB231 breast tumour cells expressed TRAIL-R1 and TRAIL-R2 death receptors as well as TRAIL-R3 and TRAIL-R4 decoy receptors for TRAIL, but IFN-γ treatment did not modify the mRNA level of any of these receptors. We have also analysed the expression, in MCF-7 cells, of the apoptosis inhibitors X-linked inhibitor of apoptosis protein, inhibitor of apoptosis proteins 1 and 2, as well as the recently described pro-apoptotic protein Smac/DIABLO [41] in response to IFN-γ, without finding significant changes in any of them (results not shown).

Although previous studies had yielded conflicting results about the role of adapter molecules and initiator caspases in the TRAIL signalling pathway, recruitment of FADD and caspase-8 to TRAIL-R1 and TRAIL-R2 receptors has been recently demonstrated as an essential step for apoptosis induction via TRAIL [5,6,24]. In this study, we describe that IFN-γ markedly enhances the activation by TRAIL of all the biochemical events that have been shown to take place following TRAIL receptor stimulation. Activation of caspase-8, cleavage of Bid, release of mitochondrial cytochrome *c*, activation of caspase-9 and cleavage of substrates for executioner caspases [28,42] were all enhanced in the presence of IFN-γ. We have also observed that IFN-γ facilitates TRAIL-induced translocation of cytosolic Bax to a mitochondria-containing membrane fraction, an event that has been described for other apoptotic stimuli [26,43]. The

mechanism by which Bax redistributes to mitochondria in response to the apoptotic stimuli is not completely understood but it seems that Bid associates with Bax on the surface of the mitochondrial outer membrane allowing Bax insertion in this membrane and its activation [44]. Furthermore, it has been described that Fas-induced Bax redistribution is caspase-dependent [26]. Thus, one could envisage IFN-γ promoting TRAIL-induced Bax translocation as it enhances caspase-8-mediated, Bid degradation and activation in response to TRAIL. Altogether, these results support the hypothesis that in certain breast tumour cells, IFN-γ favours the activation by TRAIL of a mitochondria-operated apoptotic pathway, which can be inhibited in Bcl-2-overexpressing cells. Sensitization of cells to death receptor mediated apoptosis can take place at different steps in the apoptotic caspase pathway. However, since IFN-γ enhanced early steps in TRAIL-activated caspase pathway, these results suggest that modulation of apical caspase-8 expression [18] might be the main mechanism for IFN-γ-induced sensitization of breast tumour cells.

In caspase-3-overexpressing MCF-7 cells we have described for the first time a TRAIL receptor-activated apoptotic pathway in which mitochondria seem not to be involved, since we observed activation of caspase-3 and PARP cleavage before any mitochondria-regulated events could be detected. Interestingly, IFN-γ was also able to enhance this TRAIL-induced mitochondria-independent apoptotic pathway, further supporting the idea that early activation of caspase-8 in IFN-γ-treated cells may be sufficient to mediate the sensitization to TRAIL-induced cell death. Moreover these data suggest that IFN-γ can be regarded as a general strategy to facilitate TRAIL-mediated apoptosis irrespective of the signalling pathway activated by TRAIL. Even though we have observed that Bcl-2-overexpressing MCF-7 cells are not sensitized by IFN-γ to TRAIL-induced apoptosis, we have to keep in mind that this is a special cell line because of its deficiency in caspase-3 expression [33]. Caspase-3 appears to be required to activate the mitochondria-independent pathway of apoptosis [34]. Thus, it is possible that IFN-γ can sensitize different human breast carcinomas expressing high levels of Bcl-2, which appears to be an important factor in inhibiting apoptosis [45], by facilitating the activation of apical caspase-8 and executioner caspases upon TRAIL receptors ligation. On the other hand, caspase-8 together with caspase-3, have been demonstrated to play a crucial role in apoptosis of colorectal adenocarcinoma induced by IFN-γ and/or 5-FU [46]. Moreover, in non-small cell lung cancer cell lines it has been recently described that a novel drug-inducible apoptotic pathway in which activation of caspase-8, in a mitochondria-dependent manner, mediates the activation of executioner caspases leading to apoptosis [47]. These results indicate that induction and activation of caspase-8 could facilitate not only death receptor-mediated apoptosis but also drug-induced cell death in tumour cells. Modulation of caspase-8 by IFN-γ has been described in other human cellular system such as HT-29 colon adenocarcinoma cells, erythroid colony-forming cells and U937 myeloid leukemic cells [16,48,49]. Therefore, IFN-γ-induced caspase-8 up-regulation might be envisaged as a more widespread mechanism for sensitization of different tumour cells to death receptor- and/or chemotherapy-induced apoptosis.

Interestingly, it has been reported that IFN-γ induces the expression of TRAIL in monocytes and dendritic cells suggesting a possible contribution of TRAIL in these cells to their anti-tumour activity *in vivo* [50,51]. Moreover, recent reports have described that in mouse liver, spleen and lung NK cells, endogenously produced IFN-γ regulates the expression of TRAIL, which is at least partly responsible for the anti-metastatic function of these NK cells [52]. Therefore these data, together with our present results, support a model in which IFN-γ can be a useful approach in tumour therapy, since it is able to modulate the sensitivity of tumour cells not only to other therapeutic strategies, but also to the action of the immune system.

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REFERENCES

1 Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993) Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell (Cambridge, Mass.) **75**, 1169–1178

2 Wiley, S. R., Schooley, K., Smolak, P. J., Din, W. S., Huang, C. P., Nicholl, J. K., Sutherland, G. R., Smith, T. D., Rauch, C., Smith, C. A. and Goodwin, R. G. (1995) Identification and characterization of a new member of the TNF family that induces apoptosis. Immunity **3**, 673–682

3 Chinnaiyan, A. M., O'Rourke, K., Tewari, M. and Dixit, V. M. (1995) FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell (Cambridge, Mass.) **81**, 505–512

4 Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O'Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Bretz, J. D., Zhang, M., Gentz, R., Mann, M. et al. (1996) FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. Cell (Cambridge, Mass.) **85**, 817–827

5 Kischkel, F. C., Lawrence, D. A., Chuntharapai, A., Schow, P., Kim, K. J. and Ashkenazi, A. (2000) Apo2L/TRAIL-dependent recruitment of endogenous FADD and caspase-8 to death receptors 4 and 5. Immunity **12**, 611–620

6 Sprick, M. R., Weigand, M. A., Rieser, E., Rauch, C. T., Juo, P., Blenis, J., Krammer, P. H. and Walczak, H. (2000) FADD/MORT1 and caspase-8 are recruited to TRAIL receptors 1 and 2 and are essential for apoptosis mediated by TRAIL receptor 2. Immunity **12**, 599–609

7 Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P. and Ashkenazi, A. (1997) Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. Science **277**, 818–821

8 Walczak, H., Miller, R. E., Ariail, K., Gliniak, B., Griffith, T. S., Kubin, M., Chin, W., Jones, J., Woodward, A., Le, T., Smith, C., Smolak, P. et al. (1999) Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand *in vivo*. Nat. Med. **5**, 157–163

9 Fisher, D. E. (1994) Apoptosis in cancer therapy: crossing the threshold. Cell (Cambridge, Mass.) **78**, 539–542

10 Ruiz-Ruiz, M. C. and López-Rivas, A. (1999) p53-mediated up-regulation of CD95 is not involved in genotoxic drug-induced apoptosis of human breast tumor cells. Cell Death Diff. **6**, 271–280

11 Chinnaiyan, A. M., Prasad, U., Shankar, S., Hamstra, D. A., Shanaiah, M., Chenevert, T. L., Ross, B. D. and Rehemtulla, A. (2000) Combined effect of tumor necrosis factor-related apoptosis-inducing ligand and ionizing radiation in breast cancer therapy. Proc. Natl. Acad. Sci. U.S.A. **97**, 1754–1759

12 Hadden, J. W. (1999) The immunology and immunotherapy of breast cancer: an update. Int. J. Immunopharmacol. **21**, 79–101

13 Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H. and Schreiber, R. D. (1998) How cells respond to interferons. Annu. Rev. Biochem. **67**, 227–264

14 Strander, H. (1986) Interferon treatment of human neoplasia. Adv. Cancer Res. **46**, 1–265

15 Wadler, S. and Schwartz, E. L. (1990) Antineoplastic activity of the combination of interferon and cytotoxic agents against experimental and human malignancies: a review. Cancer Res. **50**, 3473–3486

16 Ossina, N. K., Cannas, A., Powers, V. C., Fitzpatrick, P. A., Knight, J. D., Gilbert, J. R., Shekhtman, E. M., Tomei, L. D., Umansky, S. R. and Kiefer, M. C. (1997) Interferon-γ modulates a p53-independent apoptotic pathway and apoptosis-related gene expression. J. Biol. Chem. **272**, 16351–16357

17 Spanaus, K. S., Schlapbach, R. and Fontana, A. (1998) TNF-α and IFN-γ render microglia sensitive to Fas ligand-induced apoptosis by induction of Fas expression and down-regulation of Bcl-2 and Bcl-xL. Eur. J. Immunol. **28**, 4398–4408

18 Ruiz-Ruiz, C., Muñoz-Pinedo, C. and López-Rivas, A. (2000) Interferon-γ treatment elevates caspase-8 expression and sensitizes human breast tumor cells to a death receptor-induced mitochondria-operated apoptotic program. Cancer Res. **60**, 5673–5680

19 Keane, M. M., Ettenberg, S. A., Lowrey, G. A., Russell, E. K. and Lipkowitz, S. (1996) Fas expression and function in normal and malignant breast cell lines. Cancer Res. **56**, 4791–4798

20 Ogasawara, J., Watanabe, F. R., Adachi, M., Matsuzawa, A., Kasugai, T., Kitamura, Y., Itoh, N., Suda, T. and Nagata, S. (1993) Lethal effect of the anti-Fas antibody in mice. Nature (London) **364**, 806–809

21 Jo, M., Kim, T.-H., Seol, D.-W., Esplen, J. E., Dorko, K., Billiar, T. R. and Strom, S. C. (2000) Apoptosis induced in normal human hepatocytes by tumor necrosis factor-related apoptosis-inducing ligand. Nat. Med. **6**, 564–567

22 Lawrence, D., Shahrokh, Z., Marsters, S., Achilles, K., Shih, D., Mounho, B., Hillan, K., Totpal, K., DeForge, L., Schow, P. et al. (2001) Differential hepatocyte toxicity of recombinant Apo2L/TRAIL versions. Nat. Med. **7**, 383–385

23 Sedger, L. M., Shows, D. M., Blanton, R. A., Peschon, J. J., Goodwin, R. G., Cosman, D. and Wiley, S. R. (1999) IFN-γ mediates a novel antiviral activity through dynamic modulation of TRAIL and TRAIL receptor expression. J. Immunol. **163**, 920–926

24 Bodmer, J. L., Holler, N., Reynard, S., Vinciguerra, P., Schneider, P., Juo, P., Blenis, J. and Tschopp, J. (2000) TRAIL receptor-2 signals apoptosis through FADD and caspase-8. Nat. Cell Biol. **2**, 241–243

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

26 Murphy, K. M., Streips, U. N. and Lock, R. B. (1999) Bax membrane insertion during Fas(CD95)-induced apoptosis precedes cytochrome *c* release and is inhibited by Bcl-2. Oncogene **18**, 5991–5999

27 Yamada, H., Tada-Oikawa, S., Uchida, A. and Kawanishi, S. (1999) TRAIL causes cleavage of bid by caspase-8 and loss of mitochondrial membrane potential resulting in apoptosis in BJAB cells. Biochem. Biophys. Res. Commun. **265**, 130–133

28 Sarker, M., Ruiz-Ruiz, C. and López-Rivas, A. (2001) Activation of protein kinase C inhibits TRAIL-induced caspase activation, mitochondrial events and apoptosis in a human leukemic T cell line. Cell Death Diff. **8**, 172–181

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

30 Gazitt, Y., Shaughnessy, P. and Montgomery, W. (1999) Apoptosis-induced by TRAIL and TNF-alpha in human multiple myeloma cells is not block by Bcl-2. Cytokine **11**, 1010–1019

31 Walczak, H., Bouchon, A., Stahl, H. and Krammer, P. H. (2000) Tumor necrosis factor-related apoptosis-inducing ligand retains its apoptosis-inducing capacity on Bcl-2- or Bcl-xL-overexpressing chemotherapy-resistant tumor cells. Cancer Res. **60**, 3051–3057

32 de Almodovar, C. R., Ruiz-Ruiz, C., Munoz-Pinedo, C., Robledo, G. and Lopez-Rivas, A. (2001) The differential sensitivity of Bcl-2-overexpressing human breast tumor cells to TRAIL or doxorubicin-induced apoptosis is dependent on Bcl-2 protein levels. Oncogene **20**, 7128–7133

33 Jänicke, R. U., Sprengart, M. L., Wati, M. R. and Porter, A. G. (1998) Caspase-3 is required for DNA fragmentation and morphological changes associated with apoptosis. J. Biol. Chem. **273**, 9357–9360

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

35 Cerami, A. (1992) Inflammatory cytokines. Clin Immunol Immunopathol **62**, S3–10

36 Özören, N., Kim, K., Burns, T. F., Dicker, D. T., Moscioni, A. D. and El-Deiry, W. S. (2000) The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permiting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. Cancer Res. **60**, 6259–6265

37 Kayagaki, N., Yamaguchi, N., Nakayama, M., Eto, H., Okumura, K. and Yagita, H. (1999) Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: a novel mechanism for the antitumor effects of type I IFNs. J. Exp. Med. **189**, 1451–1460

38 Kumar-Sinha, C., Varambally, S., Sreekumar, A. and Chinnaiyan, A. M. (2002) Molecular cross-talk between the TRAIL and interferon signaling pathways. J. Biol. Chem. **277**, 575–585.

39 Zhang, X. D., Franco, A. V., Nguyen, T., Gray, C. P. and Hersey, P. (2000) Differential localization and regulation of death and decoy receptor for TNF-related apoptosis-inducing ligand (TRAIL) in human melanoma cells. J. Immunol. **164**, 3961–3970

40 Griffith, T. S., Chin, W. A., Jackson, G. C., Lynch, D. H. and Kubin, M. Z. (1998) Intracellular regulation of TRAIL-induced apoptosis in human melanoma cells. J. Immunol. **161**, 2833–2840

41 Verhagen, A. M., Ekert, P. G., Pakusch, M., Silke, J., Connolly, L. M., Reid, G. E., Moritz, R. L., Simpson, R. J. and Vaux, D. L. (2000) Identification of DIABLO, a mammalian protein that promotes apoptosis by binding to and antagonizing IAP proteins. Cell (Cambridge, Mass.) **102**, 43–53

42 MacFarlane, M., Merrison, W., Dinsdale, D. and Cohen, G. M. (2000) Active caspases and cleaved cytokeratins are sequestered into cytoplasmic inclusions in TRAIL-induced apoptosis. J. Cell Biol. **148**, 1239–1254

43 Wolter, K. G., Hsu, Y. T., Smith, C. L., Nechushtan, A., Xi, X. G. and Youle, R. J. (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. J. Cell Biol. **139**, 1281–1292

44 Desagher, S., Osend-Sand, A., Nichols, A., Eskes, R., Montessuit, S., Lauper, S., Maundrell, K., Antonsson, B. and Martinou, J. C. (1999) Bid induced conformational change of Bax is responsible for mitochondrial cytochrome *c* release during apoptosis. J. Cell Biol. **144**, 891–901

45 Rochaix, P., Krajewski, S., Reed, J. C., Bonnet, F., Voigt, J. J. and Brousset, P. (1999) In vivo patterns of Bcl-2 family protein expression in breast carcinomas in relation to apoptosis. J. Pathol. **187**, 410–415

46 Adachi, Y., Taketani, S., Oyaizu, H., Ikebukuro, K., Tokunaga, R. and Ikehara, S. (1999) Apoptosis of colorectal adenocarcinoma induced by 5-FU and/or IFN-γ through caspase 3 and caspase 8. Int. J. Oncol. **15**, 1191–1196

47 Ferreira, C. G., Span, S. W., Peters, G. J., Kruyt, F. A. E. and Giaccone, G. (2000) Chemotherapy triggers apoptosis in a caspase-8-dependent and mitochondria-controlled manner in the non-small cell lung cancer cell line NCI-H460. Cancer Res. **60**, 7133–7141

48 Dai, C. and Krantz, S. B. (1999) Interferon gamma induces upregulation and activation of caspases 1, 3, and 8 to produce apoptosis in human erythroid progenitor cells. Blood **93**, 3309–3316

49 Varela, N., Muñoz-Pinedo, C., Ruiz-Ruiz, C., Robledo, G., Pedroso, M. and López-Rivas, A. (2001) Interferon-γ sensitises human myeloid leukemia cells to death receptor-mediated apoptosis by a pleiotropic mechanism. J. Biol. Chem. **276**, 17779–17787

50 Fanger, N. A., Maliszewski, C. R., Schooley, K. and Griffith, T. S. (1999) Human dendritic cells mediate cellular apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). J. Exp. Med. **190**, 1155–1164

51 Griffith, T. S., Wiley, S. R., Kubin, M. Z., Sedger, L. M., Maliszewski, C. R. and Fanger, N. A. (1999) Monocyte-mediated tumoricidal activity via the tumor necrosis factor-related cytokine, TRAIL. J. Exp. Med. **189**, 1343–1353

52 Takeda, K., Hayakawa, Y., Smyth, M. J., Kayagaki, N., Yamaguchi, N., Kakuta, S., Iwakura, Y., Yagita, H. and Okumura, K. (2001) Involvement of tumor necrosis factor-related apoptosis-inducing ligand in surveillance of tumor metastasis by liver natural killer cells. Nat. Med. **7**, 94–10

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