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Tipping the balance between necrosis and apoptosis in human and murine cells treated with interferon and dsRNA

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Abstract

Interferons enhance the cellular antiviral response by inducing expression of protective proteins. Many of these proteins are activated by dsRNA, a typical by-product of viral infection. Here we show that type-I and type-II interferons can sensitize cells to dsRNA-induced cytotoxicity. In caspase-8- or FADD-deficient Jurkat cells dsRNA induces necrosis, instead of apoptosis. In L929sA cells dsRNA-induced necrosis involves high reactive oxygen species production. The antioxidant butylated hydroxyanisole protects cells from necrosis, but shifts the response to apoptosis. Treatment with the caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone or overexpression of Bcl-2 prevent this shift and promote necrosis. Our results suggest that a single stimulus can initiate different death-signaling pathways, leading to either necrotic or apoptotic cell death. Inhibition of key events in these signaling pathways, such as caspase activation, cytochrome *c* release or mitochondrial reactive oxygen species production, tips the balance between necrosis and apoptosis, leading to dominance of one of these death programs.
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Keywords: necrosis; apoptosis; dsRNA; interferon; caspase; reactive oxygen species

Abbreviations: Ac-DEVD-amc, acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin; BHA, butylated hydroxyanisole; CHX, cycloheximide; CrmA, cytokine response modifier A; DD, death domain; DHR123, dihydrorhodamine 123; FADD, Fas-associated death domain; GA, geldanamycin; hsp, heat-shock protein; IFN, interferon; PI, propidium iodide; PKR, interferon-inducible protein kinase; R123, rhodamine 123; RIP, receptor-interacting protein; ROS, reactive oxygen species; TB, Trypan blue; TLR, Toll-like receptor; TNF, tumor necrosis factor; zDEVD-fmk, benzyloxycarbonyl-Asp-Glu-Val-Asp-fluoromethylketone; zVAD-fmk, benzyloxycarbonyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone.

Introduction

Besides efficient production and spread of progeny, successful viral replication requires evasion of host defense mechanisms that limit replication or kill infected cells. However, infection by many viruses triggers not only immune and inflammatory responses, but also results in death of their cellular host. Infected cells warn other cells to protect themselves against an invading virus, mainly through the cytokines of the interferon (IFN) family. IFN promotes resistance to viral infection by inducing expression of many protective proteins, such as Mx proteins, IFN-inducible protein kinase (PKR) and enzymes of the 2-5A synthetase/RNase L pathway.[1,2] The latter two systems mainly target viral RNA and its translation, and were shown to be important for the resistance and clearance of viruses. Although IFNs induce the production of proteins needed for cellular antiviral response, they usually do not activate them. The trigger activating many of these proteins, including PKR and 2-5A synthetases, seems to be the presence of viral dsRNA. Indeed, most viruses promote the synthesis of dsRNA at some time during their replication cycle.[3] Viral dsRNA, released from dying infected cells, may act as a signal warning for infection. dsRNA is a strong inducer of inflammation, promoting activation of the nuclear factor κB[4,5] and production of inflammatory cytokines.[3,6] A recent report demonstrated that the innate immune-recognition Toll-like receptor (TLR) 3 recognizes dsRNA and induces, after activation, cytokine production through an MyD88-dependent signaling pathway.[7] Both synthetic and viral dsRNA were shown to lead to cell death.[5,8,9] IFNs have been suggested to potentiate virus-induced apoptosis by initiating the Fas-associated death domain (FADD)/caspase-8 death-signaling pathway in a dsRNA-dependent way by activating PKR.[10–12] Overexpression and activation of either PKR or RNase L promote apoptosis.[10,13–15] Another mechanism implicated in protection against viral infections is the production of reactive oxygen species (ROS). Indeed, HIV and hepatitis B infections induce ROS production in infected cells.[16]

ROS production was reported to occur both in apoptosis and necrosis.[17–19] Apoptosis avoids proinflammatory spilling of the cellular content into the surrounding tissue. *In vivo*, apoptotic cells are rapidly engulfed in a process that is considered immunologically silent.[20] Apoptotic cells reduce their volume by pumping out ions and organic osmolytes, and contracting and reorganizing the cytoskeleton. The process involves nuclear condensation, DNA degradation and blebbing of the plasma membrane. Among the players best characterized in the apoptotic machinery are cysteine aspartate-specific proteases called caspases.[21] Necrosis is characterized by cellular swelling leading to burst of the plasma membrane and release of the cytosolic contents to the surrounding tissue, causing inflammation. The generation of an appropriate immune

response during apoptotic and necrotic damage may be critical for either antitumor or antiviral responses.[5,22–24] In apoptosis and necrosis, events occurring in the mitochondria were shown to play a major role in the cell-killing process.[18,25,26] Originally, necrosis was regarded as a disordered mode of cell death, resulting from severe and acute injuries under conditions where apoptosis could not take place for some reason. However, studies have demonstrated that necrosis can also occur in normal cell physiology and embryonic development[27] as well as in ischemia-reperfusion damage.[28] The fact that members of the tumor necrosis factor (TNF) family can induce necrotic cell death in certain cells and cellular conditions, further supports the idea that a necrotic-like cell death pathway, controlled by a particular intrinsic death program (distinct from apoptosis), really exists.[18,28–36]

In the present study we determined the type of death induced in human and murine cells by IFNs, dsRNA and their combination, compared death with that induced by TNF and anti-Fas treatment, and investigated whether ROS production and proteins, such as caspases, FADD and the death domain (DD)-containing receptor-interacting protein (RIP), play a role in the process. Our results show that IFNs can sensitize cells to dsRNA-induced apoptosis or necrosis. We demonstrate that a single stimulus, be it dsRNA or anti-Fas, can initiate different cell death pathways, leading either to necrosis or to apoptosis. We show that the availability of certain proteins, caspase activation and events in the mitochondria, such as elevated production of ROS or release of cytochrome *c*, can determine whether a cell will die by necrosis or by apoptosis. Our results suggest that IFN- and dsRNA-induced necrosis may play a role, besides apoptosis, in cellular antiviral responses and pathologies.

Results

dsRNA can induce caspase-8- and FADD-dependent, as well as caspase-8- and FADD-independent cell death in human Jurkat cells

Jurkat E cells respond to anti-Fas by rapid apoptosis. However, JB6 cells derived from Jurkat E (which lack caspase-8 and overexpress Bcl-2) are resistant to anti-Fas-induced apoptosis.[29,36] We tested the response of both cell lines to anti-Fas and to a 24 h pretreatment with either IFN-α or IFN-γ, followed by a challenge with dsRNA. Light microscopy revealed that Jurkat E cells died by anti-Fas-induced apoptosis, whereas JB6 did not die (Figure 1A). Both cell lines died in response to dsRNA (Figure 1A,B). However, the morphology of the dying cells differed. Jurkat E died with typical apoptotic appearance, whereas JB6 responded by necrosis. The cells rounded up and were swelling. The cytoplasm cleared and the content concentrated around the nucleus (Figure 1A). The response of JB6 cells was stronger and faster than that of Jurkat E (Figure 1B,C). IFNs were not cytotoxic by themselves and increased the sensitivity of cells to dsRNA only slightly, without changing the cell death morphology of either cell line (data not shown). To ensure that dsRNA-induced cell death occurred directly and did not require *de novo* protein synthesis, cells were untreated or pretreated with 10 μg/ml of the translation inhibitor cycloheximide (CHX) before a challenge with dsRNA (Figure 1B). Pretreatment with CHX did not protect cells from dsRNA-induced cytotoxicity and even increased the response of Jurkat E cells (Figure 1B). These results suggest that both dsRNA-induced apoptosis and necrosis do not require *de novo* protein synthesis.

Previous reports demonstrated that dsRNA-induced cell death occurs by caspase activation.[10–12] Therefore, we tested whether caspases were activated in Jurkat cells during dsRNA-induced cell death and whether treatment with caspase inhibitors protected these cells form death. An increase in DEVDase activity was detected only in Jurkat E cells treated with either dsRNA or anti-Fas (Figure 1C). Pretreatment of Jurkat E with the pan-caspase inhibitor benzyloxycarbonyl-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD-fmk) protected cells from anti-Fas-induced apoptosis, whereas the caspase-3 and caspase-7 inhibitor benzyloxycarbonyl-Asp(OMe)-Glu(OMe)-Val-DL-Asp(OMe)-fluoromethylketone (zDEVD-fmk) only slightly decreased the apoptotic response (Figure 1D). By contrast, the inhibitors did not at all affect the level of cell death observed with dsRNA in both cell lines (Figure 1D). These results suggest that, although dsRNA can induce apoptosis in certain cells and conditions, as observed with Jurkat E, it can also kill cells without caspase activation, as observed with Jurkat E+zVAD-fmk and the caspase-8-deficient Bcl-2-overexpressing JB6.

It has previously been reported that treatment with IFN-α/β greatly sensitizes fibroblasts to FADD-dependent apoptosis in response to a dsRNA treatment or influenza virus infection.[12] Many reports showed that the FADD/caspase-8 pathway is required for DD receptor-mediated apoptosis.[37–39] However, several recent reports demonstrated that necrosis could also be initiated by FADD.[29,33,36] To further explore the role of caspase-8 and FADD in dsRNA-induced apoptosis and necrosis we tested the response of caspase-8-deficient (J9.2) and FADD-deficient (J2.1) Jurkat cells to dsRNA and IFN-γ.[37,40] In contrast to their parental cells (JA3), caspase-8- and FADD-deficient cells were completely resistant to anti-Fas-induced cell death (Figure 2A). All three cell lines were resistant to dsRNA-induced cytotoxicity unless pretreated with IFN (Figure 2A). However, J9.2 and J2.1 cells were more sensitive to IFN+dsRNA-induced cell death than their parental JA3 cells. Light microscopy and DEVDase activity measurements showed that the parental cells responded by apoptosis, while the caspase-8- and FADD-deficient cells died by necrosis (Figure 2B). These results demonstrate that both caspase-8 and FADD are not needed for dsRNA-induced necrosis and suggest that their removal shifts the response from apoptosis to necrosis. Overexpression of dominant-negative FADD sensitizes cells to TNF-induced cytotoxicity.[32,33] FADD-deficient T cells have also been shown to be resistant to TRAIL and Fas ligand-induced caspase-independent death, but not to TNF-induced necrosis.[34] JA3 and caspase-8-deficient J9.2 cells failed to respond to TNF,

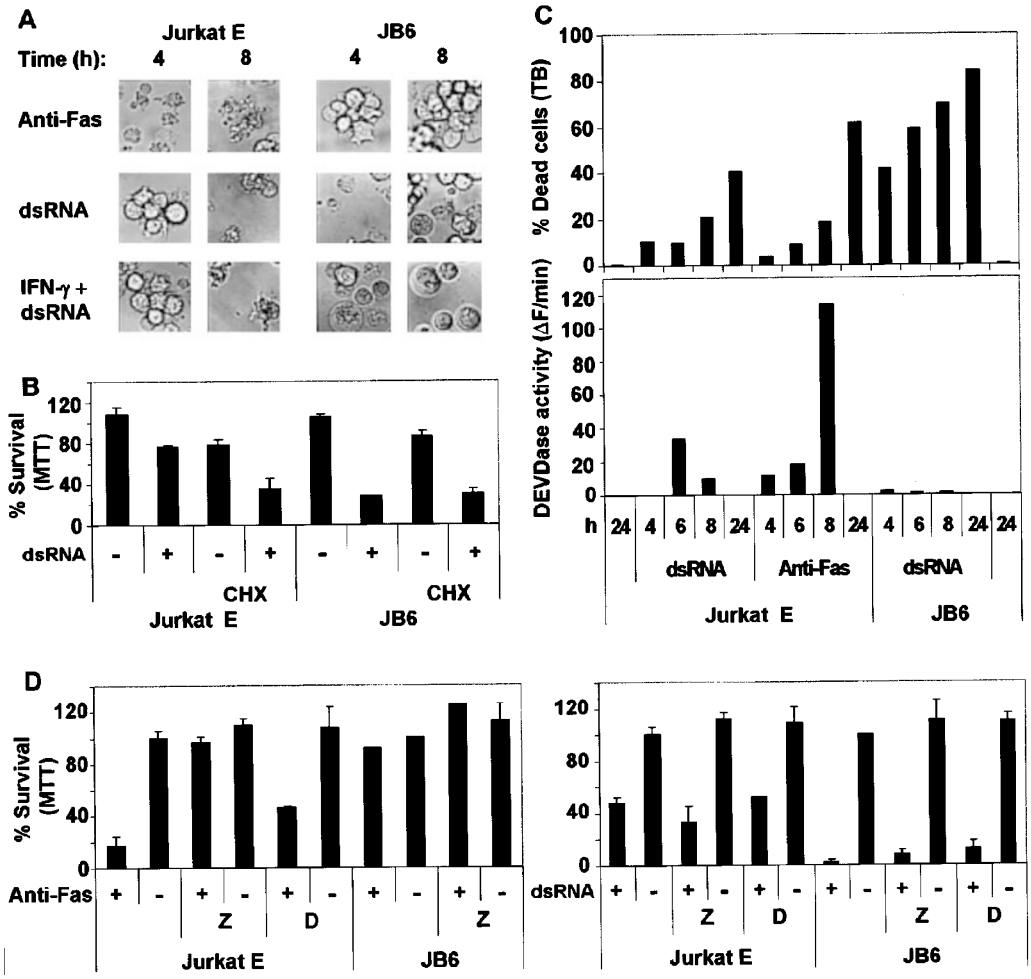

Figure 1 Treatment with dsRNA induces both caspase-dependent and caspase-independent cell death in human Jurkat cells. (**A**) Light microscopy of cells dying in response to dsRNA (100 μg/ml), with or without IFN-γ (1000 IU/ml). Death of Jurkat E cells resembles apoptosis induced with 100 ng/ml anti-Fas. JB6 cells are resistant to anti-Fas-induced apoptosis and respond to dsRNA by necrosis. (**B**) dsRNA-induced cell death does not depend on *de novo* protein synthesis. Cytotoxicity of 100 μg/ml dsRNA in the presence or absence of 10 μg/ml of the mRNA translation inhibitor CHX. (**C**) Caspase activation occurs in cell death induced with 30 ng/ml anti-Fas and 100 μg/ml dsRNA in Jurkat E, but not in JB6 cells. Nevertheless, the latter die more rapidly than the former in response to dsRNA. Loss of membrane integrity as a measure for cell death was determined as fractions stained by Trypan blue (TB). Caspase activity was measured by Ac-DEVD-amc cleavage. (**D**) Caspase inhibitors delay or inhibit Fas-mediated apoptosis, but not dsRNA-induced cell death. Cells were preincubated for 60 min with 50 μM of zVAD-fmk (Z) or zDEVD-fmk (D) before addition of anti-Fas (30 ng/ml) or dsRNA (100 μg/ml)

even at high concentrations, and FADD-negative J2.1 cells responded clearly by necrosis (data not shown). This again demonstrates that although necrosis can be initiated by FADD, the latter is not required for initiating the necrotic-signaling pathway by dsRNA or TNF.

It has also been shown that pretreatment with geldanamycin (GA), an anti-cancer drug and inhibitor of the chaperone heat–shock protein (hsp) 90, or RIP deficiency protect Jurkat cells from DD receptor-induced necrosis.[34] We tested the effect of pretreatment of JE and JB6 cells with GA on their response to dsRNA. Treatment with GA alone did not affect the viability of JB6 cells (Figure 3A), However, GA protected these cells from dsRNA-induced necrosis. Moreover, GA induced Jurkat E to die by apoptosis and intensified their apoptotic response to dsRNA (Figure 3A). GA was previously shown to decrease the concentration of RIP in Jurkat cells, and RIP deficiency was reported to protect these cells from necrosis mediated by TRAIL receptor-2, Fas and TNF receptor-1.[34,41] Therefore, we tested the response of RIP-deficient Jurkat cells[42] to dsRNA and IFN-γ. Only RIP-positive cells responded to dsRNA following pretreatment with IFN, though both cell lines failed to respond to dsRNA alone. Moreover, RIP-positive cells died in response to IFN+dsRNA also after pretreatment with zVAD-fmk, while RIP-negative cells remained resistant; this suggests that both the apoptotic and necrotic signaling pathways initiated by dsRNA are affected by the lack of RIP (Figure 3B). Both RIP-positive and RIP-negative cells responded by apoptosis to an anti-Fas treatment and were protected from death by zVAD-fmk (Figure 3B). This demonstrates that the lack of response to dsRNA in RIP-deficient cells did not arise from a dysfunction in their apoptotic machinery.

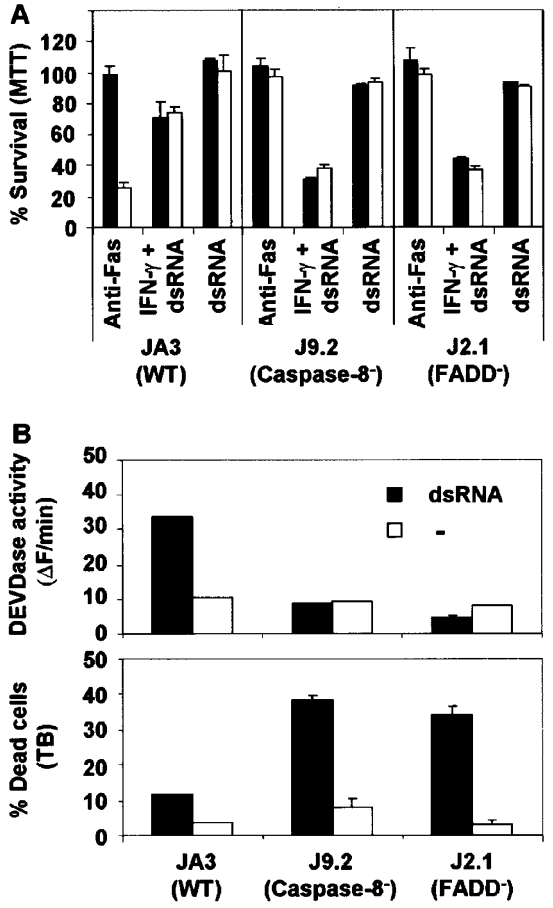

Figure 2 FADD and caspase-8-deficient Jurkat cells respond to IFN+dsRNA by necrosis, whereas their parental JA3 cells die by apoptosis. (**A**) Cytotoxicity with (filled boxes) and without (open boxes) zVAD-fmk (25 μM) of Anti-Fas (30 ng/ml), dsRNA (100 μg/ml) or dsRNA (100 μg/ml)+IFN-γ (1000 IU/ml). (**B**) Caspase activation occurs in parental JA3, but not in caspase-8-deficient (J9.2) or FADD-deficient (J2.1) cells pretreated with 1000 IU/ml IFN-γ and induced to die with 100 μg/ml dsRNA. Caspase activity was measured by Ac-DEVD-amc cleavage and loss of membrane integrity was determined by TB staining following 6 h of dsRNA treatment

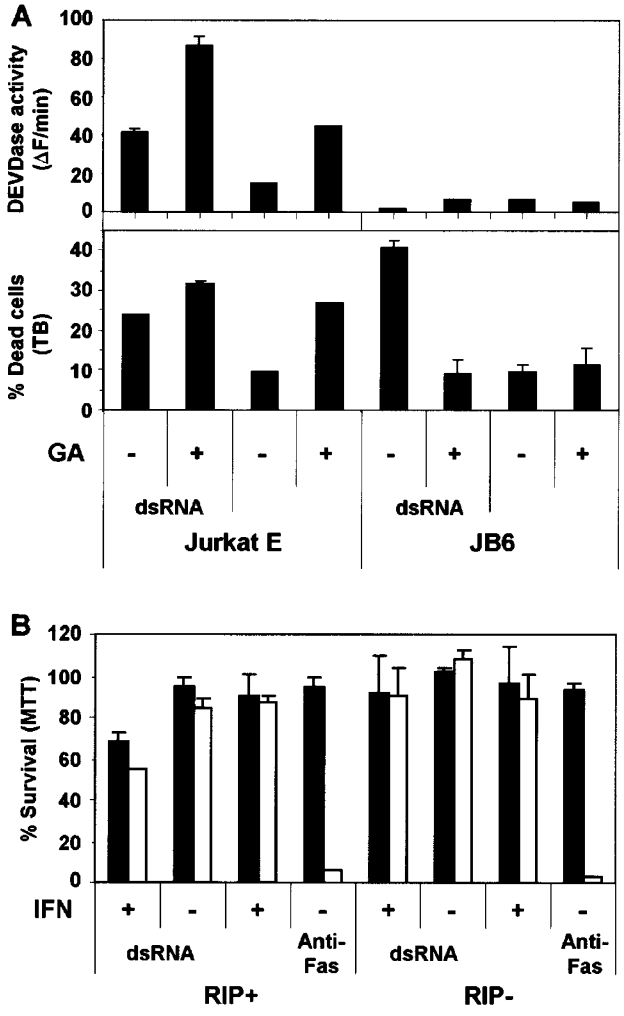

Figure 3 Effect of inhibition of hsp-90 or RIP deficiency on dsRNA-induced cell death. (**A**) Cells were treated for 24 h with the hsp-90 inhibitor GA (100 nM) and then induced to die for 6 h with dsRNA (100 μg/ml). (**B**) The cytotoxicity of dsRNA (100 μg/ml), 1000 IU/ml IFN-γ and their combination was tested on RIP-positive and RIP-negative Jurkat cells with (filled boxes) and without (open boxes) zVAD-fmk (25 μM). Treatment with anti-Fas (30 ng/ml) was used as a control

Pretreatment of murine L929sA cells with type-I or type-II IFNs, followed by a challenge with dsRNA, leads to necrosis in a TNF-independent manner

L929sAFas cells die by necrosis when treated with TNF, and respond by apoptosis to an anti-Fas treatment.[30,31] We tested the sensitivity of these cells to dsRNA killing with or without 24-h pretreatment with type-I or type-II IFNs. IFN or dsRNA alone were not cytotoxic, but a combination of IFN with dsRNA killed the cells efficiently in a synergistic manner. The level of cytotoxicity depended on the concentration of dsRNA and IFN used (Figure 4A). Light microscopy revealed that the dying cells had a necrotic morphology (Figure 4B). The same type of response was observed with all IFNs tested, though the response was the strongest with IFN-β (Figure 4A).

IFN and dsRNA may induce expression of TNF and Fas ligand, and of their receptors.[12,43,44] Therefore death may occur in an indirect way. Pretreatment of L929sAFas with IFNs had no effect on Fas-mediated apoptosis, whereas IFN-γ, but not IFN-β, increased their sensitivity to TNF-induced necrosis (data not shown). To ensure that the cell death observed with IFN+dsRNA did not arise from endogenously expressed TNF and TNF receptors we repeated the experiments in the presence of anti-TNF-neutralizing antiserum (Figure 4C). The antibodies blocked death induction by TNF, but failed to affect the cytotoxicity of type-I or type-II IFNs+dsRNA. Control serum had no effect on both. Similar results were obtained with TNF-resistant L929r1 cells (Figure 4C). Hence IFNs can sensitize cells to dsRNA-induced necrosis in a TNF-independent manner.

Butylated hydroxyanisole (BHA) shifts the response to a treatment with IFN-β+dsRNA from necrosis to apoptosis

Induction of ROS production occurs both in apoptosis and necrosis, and was suggested to play a crucial role in the

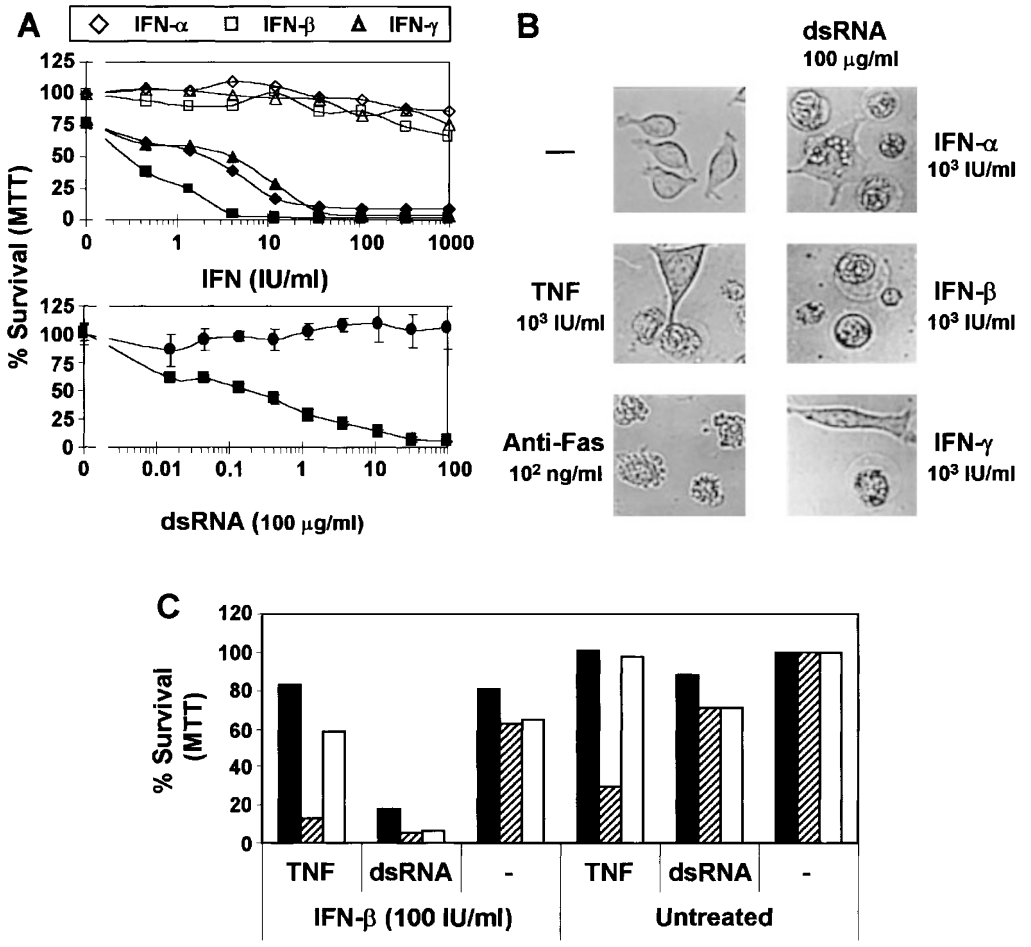

Figure 4 Type-I and type-II IFNs prime murine L929sA cells to dsRNA-induced necrosis. (**A**) IFN-α, IFN-β and IFN-γ prime cells to dsRNA toxicity. The effect depends on the concentration of dsRNA and IFN used. Top, with (filled boxes) and without (open boxes) dsRNA (100 μg/ml); bottom, with (squares) and without (circles) IFN-β (200 IU/ml). (**B**) Light microscopy of L929sAFas cells. Cell death induced by IFN+dsRNA resembles TNF-induced necrosis and not Fas-mediated apoptosis. (**C**) IFN- and dsRNA-induced necrosis is not mediated by endogenous p55 TNF receptor. TNF-resistant L929r1 cells (filled bars) were treated with TNF or dsRNA with or without IFN. TNF-sensitive L929sA cells were treated similarly, but with addition of control (dashed bars) or anti-TNF-neutralizing antisera (white bars). Results obtained with all three IFNs were similar (only those of IFN-β are shown)

cellular death process. Although several possible sources for this ROS production were reported in cell death signaling, the mitochondria were shown to be the main producers.[17–19,25] In L929sA cells, TNF induces oxidative stress and ROS production in the mitochondria. Antioxidants like BHA can inhibit ROS production by TNF and block its cytotoxicity. Hence, ROS are essential for TNF-induced necrosis.[17,30] We tested the effect of BHA on the cytotoxicity and ROS generation by treatment with IFN-β+dsRNA or TNF+LiCl, a treatment previously shown to increase the sensitivity of cells to TNF-induced cytotoxicity[45] (Figure 5). Both treatments induced ROS production in propidium iodide (PI)-negative cells that kept their membrane integrity, demonstrating that ROS preceded cell death (Figure 5A). However, BHA was more efficient in blocking ROS in response to TNF+LiCl than in response to IFN-β+dsRNA. Addition of BHA inhibited the cytotoxicity of TNF+LiCl, but did not affect that of anti-Fas (Figure 5B). Cell death rates obtained with IFN-β+dsRNA and anti-Fas were similar. Both occurred more rapidly than with TNF+LiCl (Figure 5B). BHA delayed death after IFN-β+dsRNA treatments, but the protective effect measured by the absence of PI staining disappeared completely within 6 h (Figure 5B). Controls of BHA, BHA+IFN-β, or BHA+dsRNA treatments were not cytotoxic.

Since addition of BHA did not block the death of cells treated with IFN-β+dsRNA, we checked if caspases were activated in the process. DEVDase activity was clearly apparent in cells treated with anti-Fas, with and without BHA (Figure 6A). However, a strong increase in DEVDase activity occurred in cells treated with IFN-β+dsRNA only if BHA was added (Figure 6A). Such BHA-dependent increase in activity was not observed with control cells or cells treated with TNF+LiCl, IFN or dsRNA. Western blot analysis showed that the extent of procaspase-9, procaspase-3 and procaspase-7 processing in cells treated with IFN-β+dsRNA+BHA was similar to that observed with anti-Fas (Figure 6B). Maximal caspase proteolysis in cells treated with IFN-β+dsRNA+BHA was observed 3 h after dsRNA administration and coincided with the peak in cytosolic DEVDase activity (Figure 6A,B).

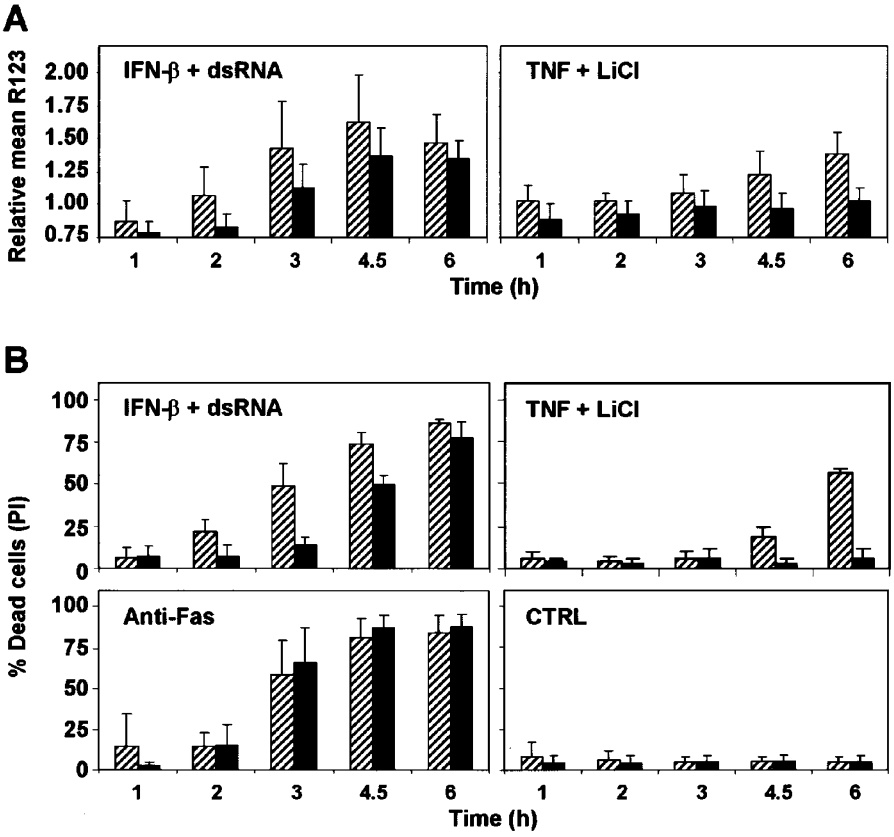

Figure 5 BHA decreases ROS production and delays loss of membrane integrity in cells treated with IFN-β+dsRNA, or TNF+LiCl. L929sAFas cells were treated (filled bars) or not (dashed bars) with 100 μM BHA and analyzed by FACS. (**A**) Activation of ROS production in viable cells measured by conversion of DHR123 to R123. (**B**) Loss of membrane integrity as a measure for cell death determined as fractions stained by PI. Note that BHA inhibited TNF-induced cell death, but caused only a delay in cell death induced by IFN+dsRNA

Light microscopy revealed that addition of BHA shifted the death appearance of cells treated with IFN-β+dsRNA from necrosis to apoptosis (Figure 7A). Side-scatter *vs* forward-scatter FACS analysis demonstrated that the different treatments resulted in a distinct shift in pattern of complete cell populations (Figure 7B). Necrosis correlated with an increase in side-scatter and a decrease in forward-scatter, whereas apoptosis (as observed after treatments with anti-Fas or IFN+dsRNA+BHA) led to a decrease in both parameters. Such an effect of BHA on cell morphology was not observed with control cells or cells treated with TNF, TNF+LiCl, IFN or dsRNA. These results demonstrate that the presence of BHA can shift the response to a treatment with IFN+dsRNA from necrosis to apoptosis.

Caspase inhibitors increase both ROS production and sensitivity to necrotic signals

Inhibition of caspases, either by synthetic peptide inhibitors like zVAD-fmk or by overexpression of cytokine response modifier A (CrmA), leads to protection from apoptotic cell death in many cellular systems.[46] In L929sA cells overexpression of CrmA leads to a dramatic increase in sensitivity to TNF-induced necrosis. Similarly, pretreatment with zVAD-fmk also sensitizes cells to TNF-induced necrosis and shifts the response to anti-Fas of L929sAFas cells from apoptosis to necrosis.[30,31]

We compared the effects of zVAD-fmk on cytotoxicity and ROS production in L929sAFas cells treated with anti-Fas, TNF or IFN-β+dsRNA. Light microscopy showed that zVAD-fmk shifted the appearance of cells treated with anti-Fas from apoptosis to necrosis, and accelerated necrosis after a treatment with IFN-β+dsRNA (Figure 7A). The whole cell population appeared to be affected, as clear from side-scatter *vs* forward-scatter FACS analysis (Figure 7B). The percentage of cells with necrotic appearance in populations exposed to TNF, IFN-β+dsRNA or anti-Fas increased dramatically with zVAD-fmk, but not in control cells treated with zVAD-fmk alone. Treatment with zVAD-fmk accelerated the response to TNF and IFN-β+dsRNA, but not the response to anti-Fas (Figure 8A). Moreover, the inhibitor strongly increased ROS production in response to TNF or IFN-β+dsRNA (Figure 8A).

DNA hypoploidy, a measure for degradation, was observed only in apoptosis mediated by Fas or induced by IFN-β+dsRNA+BHA, and was clearly inhibited by zVAD-fmk (Figure 8B). Light microscopy confirmed that the morphology of cells dying in the presence of zVAD-fmk was necrotic, even if BHA was added (data not shown). Therefore, in addition to its ability to overcome apoptosis,

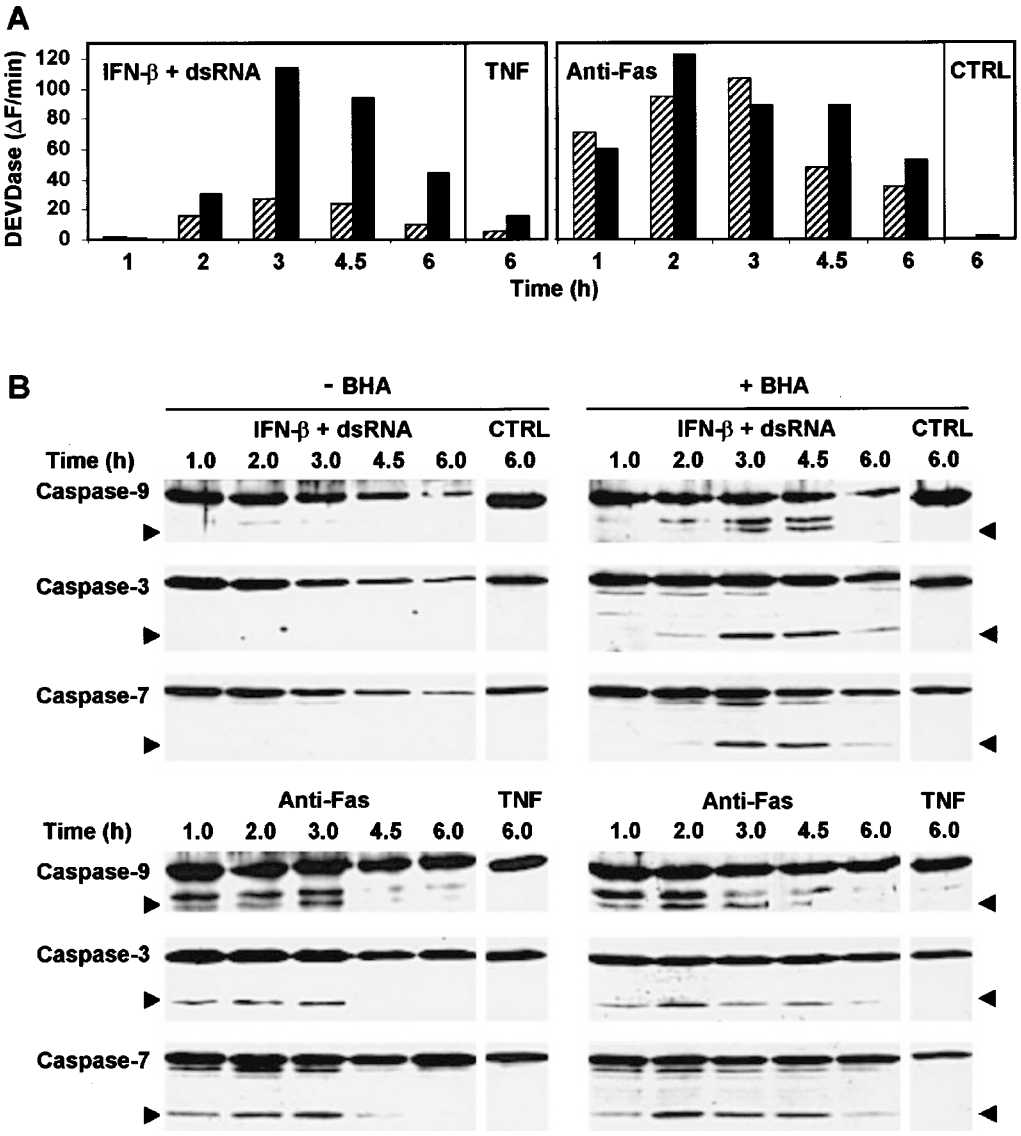

Figure 6 BHA shifts the response of L929sAFas cells to IFN-β+dsRNA from necrosis to apoptosis. Cells were treated without BHA (dashed bars) or with 100 μM BHA (filled bars). At different time points after treatment, cells were collected and lysed. The soluble fraction was tested for caspase activity and processing. (**A**) Caspase activity measured by Ac-DEVD-amc cleavage. Note that IFN-β+dsRNA induced caspase activation only when combined with BHA. (**B**) Western blots demonstrating that caspase-9, caspase-3 and caspase-7 are processed in cells treated with anti-Fas or IFN+dsRNA+BHA. Such caspase processing did not occur in cells treated with IFN+dsRNA without BHA, nor in cells treated with TNF+LiCl (*TNF*), nor in untreated controls (CTRL). Arrows indicate cleaved fragments

zVAD-fmk is capable of accelerating necrosis induced by IFN+dsRNA, suggesting that caspase activation is not required and on the contrary may be inhibitory for this cell death process.

Bcl-2 prevents BHA from shifting the response to IFN+dsRNA from necrosis to apoptosis

Bcl-2 overexpression was shown to protect cells from damage to the mitochondria and death induced by different cytotoxic compounds.[47] Part of the beneficial effect of Bcl-2 is correlated with the ability to prevent release of cytochrome *c*.[26,48,49] We tested the effect of Bcl-2 on the cytotoxicity of IFN and dsRNA. Although Bcl-2 overexpression delayed the response to anti-Fas and decreased the level of caspase activation, cells still died by apoptosis (Figure 9A,B). Bcl-2 overexpression did not delay the necrotic response to IFN-β+dsRNA (Figure 9A). However, Bcl-2 overexpression efficiently prevented activation of caspases in response to IFN-β+dsRNA+BHA (Figure 9B), the morphology of dying cells being clearly necrotic (data not shown). This demonstrates that the apoptotic pathway induced by the presence of BHA in cells treated with IFN+dsRNA is completely suppressed by Bcl-2 and suggests an involvement of mitochondrial cytochrome *c* release.

Treatment with IFN+dsRNA did not lead to cytochrome *c* release (Figure 9C). However, addition of BHA resulted in release of cytochrome *c* and proteolysis of procaspase-9,

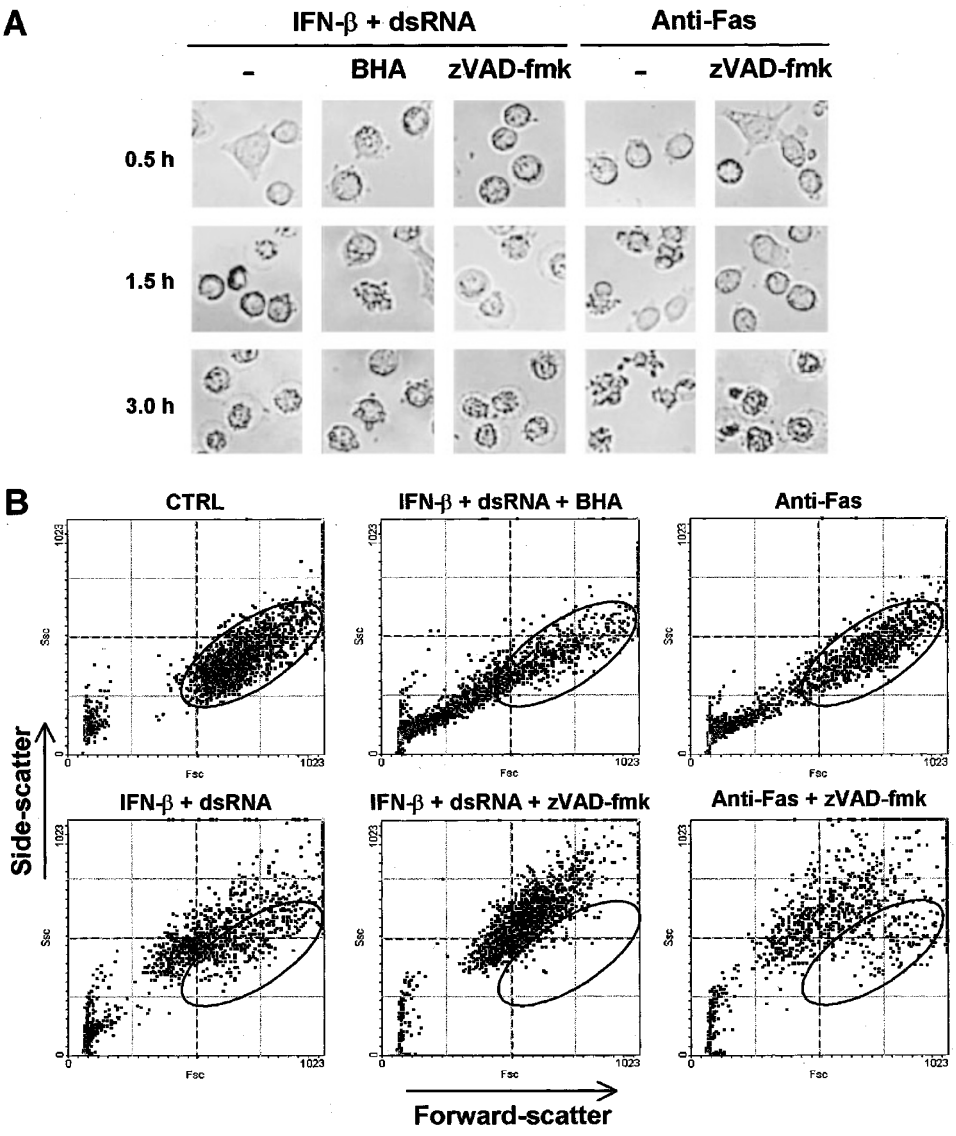

Figure 7 Effect of BHA and zVAD-fmk on the morphology of L929sAFas cell death. (**A**) Light microscopy of cells treated with IFN-β+dsRNA or anti-Fas, without or with 100 μM BHA, or 25 μM zVAD-fmk. Note that BHA shifted the cell death morphology observed with IFN+dsRNA from necrosis to apoptosis, whereas the caspase inhibitor shifted the cell death pattern with anti-Fas treatment from apoptosis to necrosis. (**B**) Changes in cellular morphology of whole-cell populations as detected by side-scatter *vs* forward-scatter FACS analysis. Necrosis correlates with a small decrease in forward-scatter and an increase in side-scatter, whereas apoptosis leads to a strong decrease in both. BHA and zVAD-fmk alone did not affect the cell morphology. Circled areas indicate the location of the control cell population

procaspase-3 and Bid. All of these events were prevented by Bcl-2 overexpression (Figure 9C), suggesting that the apoptotic process occurring in the presence of BHA is initiated by cytochrome *c*-dependent activation of caspase-9, which in turn activates caspase-3 and caspase-7. Cleavage of Bid probably occurs downstream of the point of Bcl-2 action and may be catalyzed by caspase-3. This results in a potential positive feedback loop, which amplifies release of cytochrome *c* and thus the apoptotic process. Such a feedback loop was described previously for apoptosis induced by cytotoxic drugs and UV radiation.[50–52] These results demonstrate that the shift induced from necrosis to apoptosis by BHA involves events in the mitochondria leading to release of cytochrome *c*.

Discussion

In this report we show that in addition to their known ability to protect cells from viral infections, type-I and type-II IFNs can sensitize cells to death induced by dsRNA. This cell death occurred rather rapidly, did not require *de novo* protein synthesis and hence was most probably not mediated by the induction of a DD receptor and its ligand. We show that treatment with dsRNA can lead to either apoptosis or necrosis, depending on the cellular context. In parental Jurkat cells dsRNA-induced death occurs by apoptosis. However, caspase-8 or FADD deficiency or inhibition of caspases with zVAD-fmk alter the response from apoptosis to necrosis. Nevertheless, cell death induced by IFN+dsRNA can be

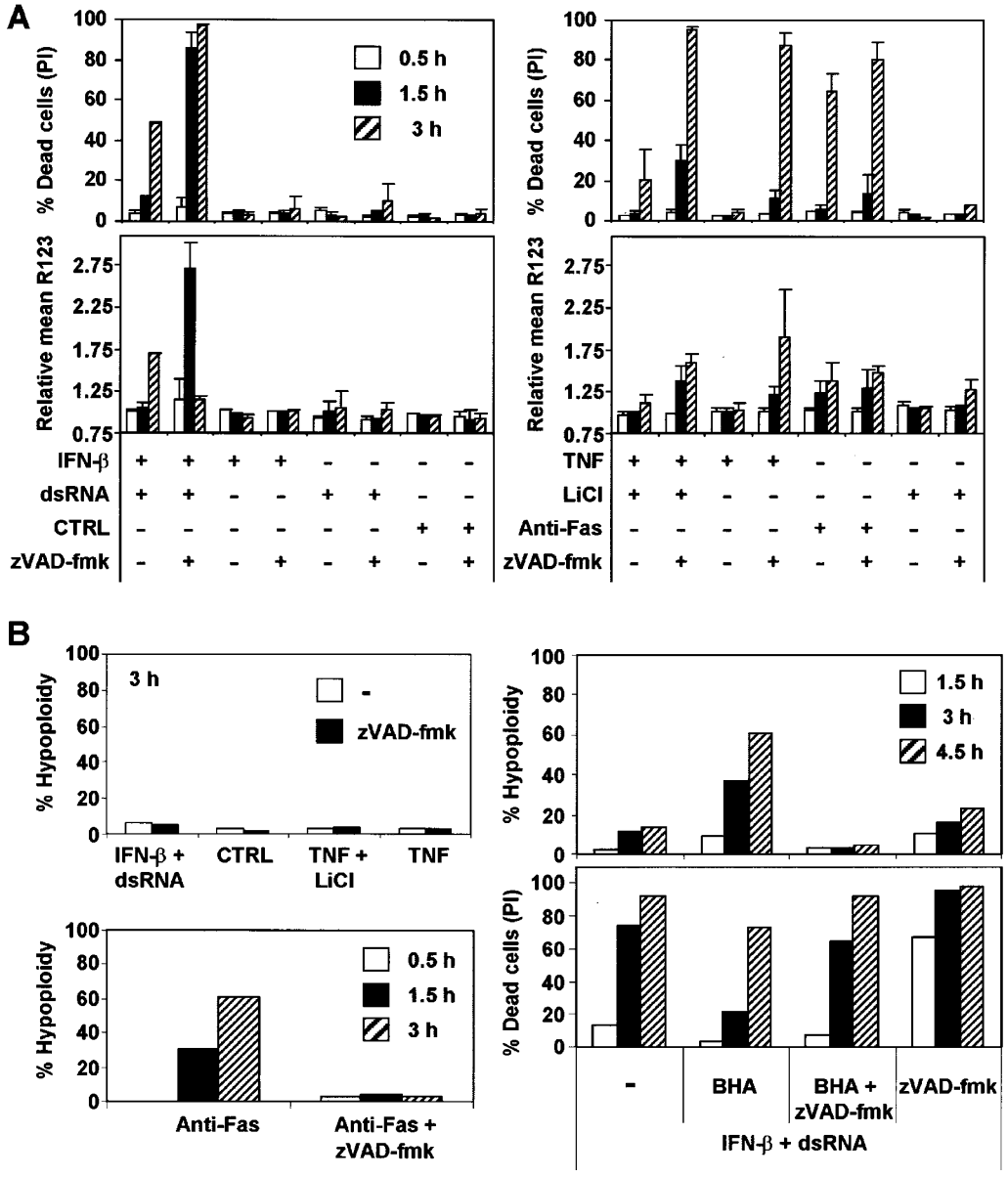

Figure 8 zVAD-fmk increases both ROS production and necrotic signaling in response to TNF and IFN+dsRNA, but blocks apoptosis. Cells were treated without or with 25 μM zVAD-fmk and analyzed by FACS. (**A**) Upper panels, fractions of PI-positive cells as a measure for loss of membrane integrity and cell death; bottom panels, activation of ROS production in viable cells as measured by conversion of DHR123 to R123. (**B**) DNA degradation expressed as percentage of cells with hypoploid DNA levels measured by PI staining following freezing and thawing. Note that hypoploidy occurs under apoptotic conditions and is inhibited by zVAD-fmk

necrosis, even when the apoptotic machinery is present and functional, as observed with L929sAFas cells which respond to anti-Fas by apoptosis. These results suggest that caspase-8 and FADD are not required for dsRNA-induced necrosis, although they may be needed for dsRNA-induced apoptosis.[12]

Inhibition of hsp-90 can inhibit the mediation of necrosis by several DD receptors.[34] Pretreatment with the hsp-90 inhibitor GA blocked dsRNA-induced necrosis, but not apoptosis. Inhibition of hsp-90 was reported to lead to the degradation of a large number of cellular proteins, many of which are enzymes playing a role in cell survival or death, such as Raf, MEK, FAK, PKR, nitric-oxide synthase, p53 and RIP.[53] Among these, PKR was suggested to be involved in dsRNA-induced apoptosis,[10,12] and nitric-oxide synthase in dsRNA-induced necrosis.[54,55] The DD-containing kinase RIP was shown to be required for apoptosis mediated by TNF receptor-2[42] and for DD receptor-mediated necrosis.[34] Here we show that RIP-deficient cells are also resistant to IFN+dsRNA cytotoxicity, suggesting that RIP is required for initiation of both dsRNA-induced apoptosis and necrosis.

ROS production occurs in apoptotic cells.[19,56] Its importance for death induction is, however, regarded to be higher in necrosis, since antioxidants such as BHA efficiently block this mode of cell death.[18] We show that dsRNA-induced necrotic cell death involves high ROS

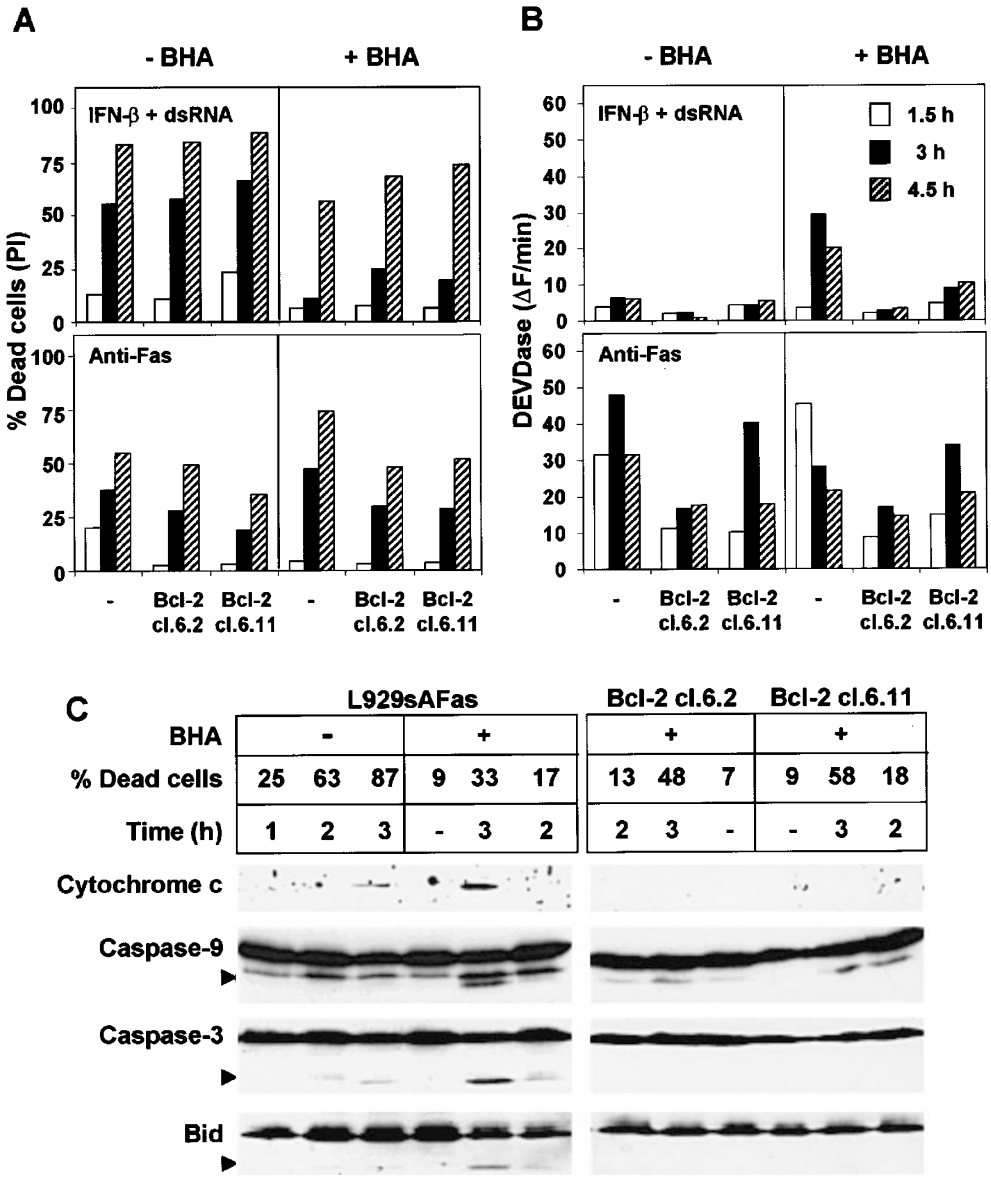

Figure 9 Bcl-2 prevents BHA from shifting the response to IFN+dsRNA from necrosis to apoptosis. Clones of L929sA cells were transfected with human Fas alone (−) or with human Bcl-2 (cl.6.2 and cl.6.11). Cells were treated without or with 100 μM BHA. (**A**) Loss of membrane integrity and death measured as PI-positive cells. (**B**) Caspase activity as measured by Ac-DEVD-amc cleavage. Note that Bcl-2 overexpression inhibits caspase activation after treatment with IFN+dsRNA+BHA. (**C**) Western blots showing that Bcl-2 overexpression blocks release of cytochrome *c*, caspase-9 and caspase-3 activation, as well as Bid cleavage in cells treated with IFN+dsRNA+BHA. Arrows indicate cleaved fragments

production and is independent of endogenously expressed TNF, TNF receptors and activation of apoptotic caspases. In L929sAFAS, BHA inhibited TNF-induced necrosis, but not Fas-mediated apoptosis.[30] BHA did not inhibit the cytotoxicity of IFN+dsRNA, but clearly shifted the response from necrosis to apoptosis. In addition, Bcl-2 overexpression itself delays the necrotic response of L929sAFAS cells to TNF,[57] but not induction of necrosis by IFN+dsRNA. These results suggest that the pathway leading from TNF receptor-1 to necrosis is different from that initiated by dsRNA. Moreover, in Bcl-2-overexpressing L929sAFas.Bcl-2 and JB6 cells, in which the mitochondrial apoptotic pathway is blocked, BHA did not block dsRNA-induced necrosis. Hence the necrotic stress was too high to overcome; possibly, mechanisms other than ROS production are leading to necrosis. On the other hand, inhibition of caspases by zVAD-fmk increased the production of ROS in response to either TNF or the combination of IFN and dsRNA, resulting in accelerated necrosis. Addition of zVAD-fmk blocked apoptosis induced by anti-Fas or the combination of IFN+dsRNA+BHA, and shifted the response from apoptosis to necrosis. Hence, caspases are not only needed for the apoptotic pathway, but they may also have a negative effect on necrosis and ROS production.

Mitochondria are a major target for Bcl-2 protein family members, some of which (Bid, Bax or Bad) promote cell

death, and others (Bcl-2 and Bcl-X_L) usually delay it.[26] Caspase-cleaved Bid targets mitochondria and leads to cytochrome *c* release. Several reports demonstrated that caspase-8 activation, Bid cleavage and apoptosis in response to UV radiation are abrogated in cells over-expressing Bcl-2 or Bcl-X_L, whereas the response to Fas stimulation is only partially attenuated.[50–52] These studies showed that cleavage of Bid during apoptosis induced by cytotoxic drugs and UV radiation occurs downstream of mitochondrial damage, is catalyzed by caspase-3 and represents a feedback loop for amplification of apoptosis-associated mitochondrial cytochrome *c* release. In our assays, Bcl-2 overexpression retarded anti-Fas-induced apoptosis significantly, suggesting that the mitochondrial apoptotic pathway amplified the cellular response. However, Bcl-2 overexpression completely blocked the shift from necrosis to apoptosis induced by the presence of BHA in cells treated with IFN+dsRNA, abolishing cytochrome *c* release, caspase-9 and caspase3 activation, as well as cleavage of Bid. These results demonstrate that the shift to apoptosis induced in the presence of BHA starts in the mitochondria, indicating that a switch between the two death pathways can occur either at the level of the receptor complex[30,32,34,35] or at the level of the mitochondria.

What is the physiological importance of dsRNA-induced apoptosis and necrosis? Both responses might play a role in innate and acquired immune responses. When a cell fails to overcome the propagation of a virus, it is to the benefit of the organism for the cell to die in the cleanest way, allowing destruction of all virus particles present. Apoptosis coupled to phagocytosis by macrophages was suggested to function in that way and to efficiently clear influenza A virus.[58] High cytoplasmic dsRNA contents may function as an internal signal initiating such a cell death program. However, many viruses encode for caspase inhibitors, such as baculovirus p35 or cowpoxvirus CrmA, which are exploited to prevent apoptotic suicide of the cellular host.[46,59] In such cases, the existence of another cell suicide program, either resistant to, or enhanced by, caspase inhibition, may be crucial for survival of the organism. The necrotic cell death pathway induced by IFNs and dsRNA, as the one described here, could fulfil such a function. Indeed, the explosion of the cell in what is called a lytic cycle, occurring in certain acute viral infections, might represent such a necrotic cell death. Moreover, in such cases the infected cell releases to the micro-environment infectious viral particles accompanied by immature unpacked viral material including dsRNA. Neighboring cells, which are also likely to become infected, may respond to this high load of dsRNA by either inflammatory cytokine production or cell death. In both cases the newly discovered dsRNA receptor TLR3 might be involved. Indeed, TLR3 signaling occurs through the DD-containing MyD88,[7] a protein reported to recruit caspases by binding of FADD in response to bacterial infection and TLR2 activation, a process that also induces ROS production.[60,61] Such a caspase activation pathway was also discovered in Drosophila, where Toll receptor activation can lead to recruitment of the caspase Dredd via binding of dFADD to dMyD88.[62]

The spilling out of the cellular contents in IFN- and dsRNA-induced necrosis might contribute to the development of autoimmunity and other pathologies. Insulin-dependent diabetes mellitus is a disease thought to result from autoimmune destruction of the insulin-producing β-cells in the pancreatic islets of Langerhans. Recently, several reports suggested that viral infection might induce diabetes even without direct infection of the pancreatic islets or induction of specific autoimmunity.[63] Such virus-induced diabetes is suggested to occur due to inflammatory cytokines, and not perforin from autoreactive (antiviral) cytotoxic T-lymphocytes.[64] The cytokines interleukin-1, TNF and IFN are cytotoxic to β-cells by inducing formation of oxygen-free radicals, nitric oxide, and peroxynitrite in the β-cells themselves.[64,65] The effect of IFNs is intensified by dsRNA that can inhibit β-cell function and induce islet damage by its own.[54,66] Interestingly, insulin-dependent diabetes mellitus was reported to occur also after treatment of viral hepatitis patients with exogenous IFNs.[67,68] The identification of pathways and molecules involved in dsRNA-induced apoptosis and necrosis will hopefully lead to a better understanding of the role of this mode of cell death in immune responses and pathologies.

Finally, our results suggest that necrosis is not a passive and slow cell death process occurring only when apoptosis fails. They show that a given stimulus, be it anti-Fas or the combination of IFN and dsRNA, can lead to apoptosis or necrosis. The final outcome is a result of the interplay between pathways leading to either of the two death programs. The rapidity and the intensity of the response may decide how the cell will finally die. Inhibition of caspase recruitment and activation, or prevention of mitochondrial cytochrome *c* release block apoptosis, and thus promote necrosis. On the other hand, inhibition of ROS production may block necrosis and allow apoptotic pathways to become apparent.

Materials and Methods

Cell culture

Caspase-8-deficient and Bcl2-overexpressing JB6 cells (donated by Dr. S Nagata and Dr. H Matumura) were derived from Jurkat E human T-cell lymphoma.[29] JA3, as well as caspase-8-deficient and FADD-deficient Jurkat clones were a gift from Dr. J Blenis.[37,40] RIP-negative Jurkat and parental cells were obtained from Dr. B Seed.[42] L929sA and L929r1 cells are derivatives of L929 murine fibrosarcoma cells. The first were selected for their sensitivity to TNF cytotoxicity, whereas the latter are negative for both TNF receptors and therefore TNF-resistant.[69,70] L929sA cells were transfected with the human Fas receptor cDNA with or without human Bcl-2 cDNA, resulting in L929sAFas and L929sAFas.Bcl-2 clones, respectively.[30,57]

Antibodies, cytokines and reagents

Recombinant murine TNF (specific biological activity of 2.2×10^8 IU/ml) was produced in *E. coli* and purified in our laboratory. Anti-human Fas antibody (anti-Fas, clone 2R2) was purchased from Cell Diagnostica (Munster, Germany). Recombinant murine IFN-α and recombinant human IFN-γ were supplied by BioSource International

(Camarillo, CA, USA). Recombinant human IFN-γ was obtained from Roche Molecular Biochemicals (Basel, Switzerland). Recombinant murine IFN-γ and IFN-β were produced in *E. coli* and purified in our laboratory. Poly(I)-poly(C) (synthetic dsRNA) was dissolved at 3.5 mg/ml in water (Amersham Pharmacia Biotech, Rainham, UK). GA (Sigma Chemical Co., St. Louis, MO, USA), dissolved at 100 mM in DMSO, was used at 100 μM. BHA (Sigma Chemical Co.), dissolved at 100 mM in ethanol, was used at 100 μM. PI (Becton Dickinson, Sunnyvale, CA, USA), dissolved at 3 mM in PBS, was used at 30 μM. Dihydrorhodamine 123 (DHR123; Molecular Probes, Eugene, OR, USA), dissolved at 1 mM in DMSO, was used at 0.1 M. The caspase peptide inhibitors zVAD-fmk and zDEVD-fmk were supplied by Bachem (Bubendorf, Switzerland). The caspase fluorogenic substrate acetyl-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-aminomethylcoumarin (Ac-DEVD-amc) was obtained from Peptide Institute (Osaka, Japan). Rabbit anti-murine TNF antiserum was prepared in our laboratory. Antibodies to cytochrome *c* and human Bcl-2 were from PharMingen (San Diego, CA, USA), and antibodies to human/mouse Bid from R&D Systems (Minneapolis, MN, USA). Anti-murine caspase-9 antibodies were from New England Biolabs (Beverly, MA, USA). Rabbit polyclonal antibodies against recombinant murine caspase-3 and caspase-7 were prepared at the Centre d'Economie Rurale (Laboratoire d'Hormonologie Animale, Marloie, Belgium).

Cell survival assay

L929 and Jurkat cells were seeded at 5×10^3 and at 2×10^4 cells per well, respectively, in 96-well plates. The next day cells were left untreated or were treated with IFNs; 24 h later dsRNA or anti-Fas were added. Cell survival was assessed 18 h later using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.[71] The percentage of cell death was calculated using the equation 100% x $(1-(A_{595/655}$-treated cells $-A_{595/655}$ medium)/$(A_{595/655}$-untreated cells $-A_{595/655}$ medium).

Induction of cell death in Jurkat cells for measuring DEVDase activity

Jurkat cells seeded at 5×10^5 cells/ml were treated or not with IFN. After 24 h, cells were treated or not with anti-Fas or dsRNA. The percentage of dead cells was monitored by trypan blue exclusion after addition of anti-Fas or dsRNA. Cells were collected by centrifugation and assayed for DEVDase activity.

Induction of cell death in L929 cells for FACS analysis, DEVDase activity measurement and Western blotting

For flow fluorocytometric analysis, L929sAFas or L929sAFas.Bcl2 cells were seeded at 10^5 cells/ml/well and kept in suspension in uncoated 24-well tissue culture plates (Sarstedt, Newton, NC, USA) in serum-containing DMEM. Following 24-h culture, cells were treated with or without IFN-β (200 IU/ml). TNF (10 000 IU/ml), with or without 10 mM LiCl or anti-Fas (100 ng/ml) or dsRNA (100 μg/ml) or medium as control, were added to the cells after 24 h. In each experiment three replicate plates were prepared and used for FACS analysis, determination of DEVDase activity and Western blotting.

FACS analysis

Cells were harvested and kept on ice before analysis with a FACScalibur flow cytometer (Becton Dickinson). Changes in cellular morphology were determined by plotting side-scatter *vs* forward-scatter. Loss of cell membrane integrity as a measure of cell death was determined by PI fluorescence.[30] DNA degradation was measured by PI staining after one freeze/thaw cycle to permeabilize cells, and was determined as the percentage of cells containing hypoploid DNA.[30] Production of reactive oxygen species (ROS) was measured at 525 nm as mean rhodamine 123 (R123) fluorescence, resulting from DHR123 oxidation by intact cells. Cells were incubated with DHR123 for 30 min before analysis. Relative R123 was defined as the ratio of fluorescence emitted at a given time point and that of initial control.

DEVDase activity

The fluorogenic substrate assay for caspase activity was carried out as described previously.[30] Cells were transferred to Eppendorf tubes, washed in cold phosphate buffer and lysed in 100 μl caspase lysis buffer, Cell debris was removed by centrifugation and caspase activity was determined by incubating 25 μl of the soluble fraction with 50 μM Ac-DEVD-amc in 150 μl cell-free system buffer, containing 220 mM mannitol, 68 mM sucrose, 2 mM $MgCl_2$, 2 mM NaCl, 2.5 mM PO_4H_2K, 0.5 mM EGTA, 0.5 mM sodium pyruvate, 0.5 mM L-glutamine, 10 mM HEPES-NaOH pH 7.4, and 10 mM dithiothreitol. The release of fluorescent 7-amino-4-methylcoumarin was measured for 60 min at 2-min intervals by fluorometry (excitation at 360 nm and emission at 480 nm) (Cytofluor; PerSeptive Biosystems, Cambridge, MA, USA); the maximal rate of increase in fluorescence was calculated (ΔF/min).

Western blot analysis

Cell lysate supernatants used for measuring DEVDase activity were also analyzed by Western blotting for detection of caspase and Bid proteolysis. Cells form each well analyzed for cytochrome *c* release were washed in cold phosphate buffer and permeabilized with 100 μl 0.02% digitonin dissolved in cell-free system buffer and left on ice for 1 min. This treatment allows selective lysis of the plasma membrane without affecting the organelle membranes. The pellet (organelle fraction) was dissolved separately in Laemmli buffer. Samples were analyzed by 15% SDS–PAGE and Western blotting using antibodies to cytochrome *c*, Bid, caspase-9, caspase-3 and caspase-7, and developed with Enhanced Luminol reagent (Du Pont, Wilmington, DE, USA).

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