Cell Death and Differentiation (1999) 6, 652–660

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Induction of apoptosis by IFNγ in human neuroblastoma cell lines through the CD95/CD95L autocrine circuit

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Received: 15.1.99; revised: 28.4.99; accepted: 12.5.99
Edited by C. Thiele

Abstract

The CD95 (APO-1/Fas) system can mediate apoptosis in immune cells as well as in tumour cells, where it may contribute to tumour immune-escape. On the other hand, its induction by anticancer drugs may lead to tumour reduction. Interferonγ (IFNγ) increases the sensitivity of tumour cell lines to anti-CD95 antibody-mediated apoptosis. We describe induction of apoptosis by IFNγ through the expression of CD95 and its ligand (CD95L) in human neuroblastoma cell lines. Neuroblastoma cells showed low constitutive expression of CD95 and CD95L. Subsequent to IFNγ-modulated increase in CD95 and CD95L mRNA as well as protein levels, apoptosis was observed. Our results demonstrated that cytokine-mediated apoptosis was mediated through the activation of the CD95/CD95L autocrine circuit since: (i) cell death occurred following CD95/CD95L expression and correlated with CD95 and CD95L expression levels, (ii) failed to occur in a clone which weakly upregulated CD95 and lacked CD95L induction after IFNγ stimulation, (iii) was at least partially inhibited by using blocking F(ab')$_2$ anti-CD95 antibody fragments and the recombinant Fas-Fc protein, that prevented the interaction between CD95 and CD95L. The intracellular molecular mechanisms elicited by IFNγ are clearly highly complex, with several signalling pathways being activated, including the CD95 system. These findings suggest that IFNγ may have a significant potential in the therapy of neuroblastoma *in vivo*.

Keywords: cell death; apoptosis; IFNγ; neuroblastoma; CD95 (APO-1, Fas)

Abbreviations: BSA, bovine serum albumine; CD95L, CD95 ligand; DD, death domain; DISC, death-inducing signalling complex; FACS, fluorescence-activated cell sorting; FADD, Fas-associated death domain protein; IFNγ, interferonγ; PE, phycoerythrin; PI, propidium iodide; TNF, tumour necrosis factor; tTG, tissue transglutaminase

Introduction

The CD95/CD95 ligand (CD95L) system is a key regulator of apoptosis. CD95 (APO-1, Fas), a 48 KDa transmembrane receptor glycoprotein, is a member of the nerve growth factor/tumour necrosis factor (TNF) receptor superfamily of surface molecules, constitutively expressed in several different cell types, including activated T and B lymphocytes, hepatocytes and epithelial cells. Ligation of CD95 by its natural ligand, CD95L, or by agonistic anti-CD95 monoclonal antibody rapidly induces apoptosis in sensitive cells.[1] The CD95L belongs to the TNF superfamily of cytokines and can exist as a membrane-bound or a soluble protein.

Binding of the CD95L causes receptor trimerization and direct interaction of the intracellular death domain (DD) of CD95, required to signal apoptosis, with a set of signal-transducing molecules. This results in the assembly of a death-inducing signalling complex (DISC) in which, several cytosolic adaptors are recruited to transduce the activated CD95 signal.[2] The DD binds a Fas-associated death domain protein (FADD) that recruits the cysteine protease pro-caspase 8 (also called FLICE, MACHα).[3] Pro-caspase 8 recruitment to the DISC and subsequent oligomerization trigger its autoprocessing and subsequent activation.[4,5] Active caspase 8 is then released into the cytosol where it can activate a cascade of downstream executioner caspases, such as caspase 3-like proteases.[6] Caspases 8-induced apoptosis is also amplified through the mitochondrial release of cytochrome c.[7] The cytochrome c/Apaf-1 signalling pathway activates a cascade involving Apaf-1, caspase 9 (Apaf-3) and downstream caspases.[8–10] The downstream executioner caspases are responsible for proteolytic cleavage of a number of cellular proteins, including poly(ADP-ribose) polymerase, the small nuclear ribonucleoprotein U1-70 kdal, DFF[11] and the ICAD/CAD complex[12,13] that are able to induce DNA fragmentation.

CD95 is also widely expressed in haematologic tumour cells including T and B cell leukaemias and lymphomas,[14,15] as well as in squamous cell carcinoma, carcinoma of the breast, renal cell carcinoma, glioblastoma, prostatic and pancreatic adenocarcinoma.[16] Expression of CD95 in these tumours however, is low when compared to lymphoblastoid cells. CD95 shows a reduced expression in several cancers such as melanomas[17] and hepatocellular carcinomas[18] which may reduce the susceptibility of these tumours to CD95L-mediated immune attack. Moreover, the anticancer drugs seem to act through an up-regulation of the CD95/CD95L circuit in several human cell lines including

neuroblastoma.[19–23] We have therefore attempted to evaluate a possible role of the CD95/CD95L mechanism in human neuroblastoma cell lines.

Interferonγ (IFNγ) is an immuno-modulating cytokine released by activated T-lymphocytes during the immune response. In addition to immuno-modulatory stimulation, IFNγ promotes cellular differentiation and partial or total reversion of the malignant phenotype in several tumour cell types. IFNγ is very effective in inhibiting cellular growth and inducing terminal differentiation of some neuroblastoma cell lines.[24] It has also been shown to induce apoptosis in a variety of cell types including neuroblastoma cell lines.[25,26] Although the molecular mechanism whereby the cytokine induces cell death is still largely unclear, IFNγ and TNFα have been shown to up-regulate CD95 expression thus increasing tumour sensitivity to anti-CD95 antibody-mediated apoptosis.[16,25,27] Recently, IFNγ has been found to positively modulate CD95L expression in human embryonal carcinoma cells.[28]

In the present study, we have characterized human malignant neuroblastoma cell lines in regard to CD95/CD95L expression, CD95-mediated cell death and its modulation by IFNγ.

Results

IFNγ-induced apoptosis in human neuroblastoma cells correlates with CD95 up-regulation

As IFNγ-mediated up-regulation of CD95 was previously demonstrated in some tumour cell lines,[16,25,27] we examined the involvement of the CD95 system in IFNγ-induced apoptosis of neuroblastoma cells. As shown in Table 1, treatment of neuroblastoma cells with 1000 IU/ml IFNγ for 48 h, increased CD95 expression levels in all cell lines tested. Analysis of apoptotic cell death upon incubation of neuroblastoma cells with 1000 IU/ml IFNγ for 72 h, showed that there were two highly sensitive cell lines (SK-N-BE(2), SH-SY5Y), with over fourfold increase in cell death, two moderately sensitive cell lines (tTG-AS8, LAN-5), with 2–3-fold increase in cell death, and three resistant cell lines (TGA, SH-SEP, IMR32). The data summarised in Figure 1 seem to indicate an overall correlation between the ability of IFNγ to up-regulate CD95 expression and apoptosis. Indeed, when CD95 is induced (SK-N-BE(2), SH-SY5Y, tTG-AS8, LAN-5), there is a good induction of death. Similarly, when CD95 is not significantly induced (SH-SEP, IMR32), there is no apoptosis. The only exception is the cell line TGA that shows a good induction of CD95, without significant induction of death.

All together the data reported suggest that the CD95 system might be the effector mechanism through which IFNγ activates cell death in human neuroblastoma cells. To better characterise the involvement of the CD95/CD95L system in IFNγ-induced cell death of neuroblastoma cells, we decided to perform all further experiments on the SK-N-BE(2) cell line, which was particularly responsive to the cytokine. In order to understand the mechanism of resistance, we also investigated the effects of IFNγ on the tTG-AS8 and TGA cell lines, which were moderately sensitive and resistant to the cytokine, respectively.

Table 1 Effects of IFNγ on CD95 expression levels and cell death of neuroblastoma cell lines

	% CD95 positive cells		% Apoptotic events	
	IFNγ		IFNγ	
Cell line	–	+	–	+
SK-N-BE(2)	2	73	5	20
SH-SY5Y	1	55	2	14
tTG-AS8	3	40	4	10
LAN-5	2	31	10	24
SH-SEP	17	30	9	14
TGA	2	28	10	12
IMR32	0	0	10	11

Data are the mean of two independent experiments, each done in duplicate. S.E. are less than 10%

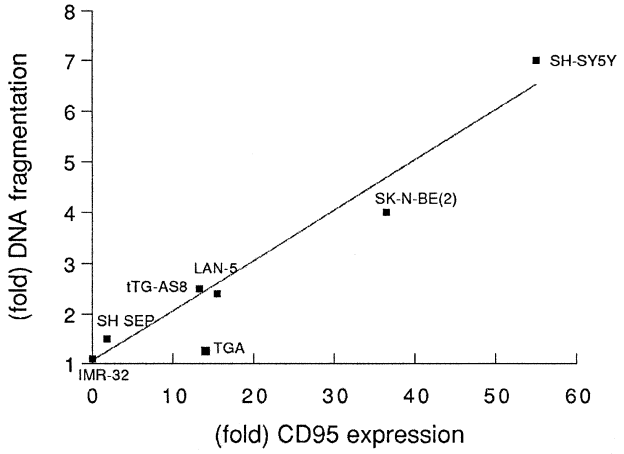

Figure 1 Correlation between IFNγ-induced CD95 up-regulation and apoptosis in human neuroblastoma cell lines. Cells were left untreated or incubated with 1000 IU/ml recombinant IFNγ for 48 and 72 h for CD95 expression and cell death analysis, respectively. CD95 expression was evaluated by FACS analysis, after staining with anti-CD95 IgG1 monoclonal antibody and subsequent incubation with goat anti-mouse IgG-PE. DNA fragmentation was assessed by FACS analysis using PI staining. Data are expressed as fold over untreated cells and given as mean of two different experiments with S.E. of less than 10%

Since CD95 expression has been shown to be induced by the combination of IFNγ and TNFα,[16,25] we investigated the constitutive and cytokine-induced expression of CD95 in the SK-N-BE(2) neuroblastoma cell line. Both mRNA analysis by RT–PCR (Figure 2A) and immunostaining (Figure 2B) revealed a low constitutive expression of CD95, which was strongly induced when cells were exposed to recombinant IFNγ (1000 IU/ml). IFNγ was found to enhance the percentage of CD95 positive cells in a time- (Figure 2B) and dose-dependent (data not shown) manner, with effects starting from 24 h and reaching a maximum at 72 h. In contrast, TNFα did not up-regulate CD95 and furthermore, the simultaneous exposure to both IFNγ and TNFα failed to increase CD95 expression significantly over the expression induced by IFNγ alone (Figure 2B).

We have performed a semi-quantitative RT–PCR to detect CD95L expression. Figure 3A shows that IFNγ

stimulated CD95L mRNA expression in SK-N-BE(2) cells (no basal expression) 48 h after treatment. The induction of CD95L mRNA expression was also confirmed by the up-regulation of CD95L protein observed 24 h after treatment with IFNγ (Figure 3B). Exposure of SK-N-BE(2) cells to IFNγ also resulted in up-regulation of the bax α-splice form, whereas the already high expression of FADD was not altered by IFNγ treatment (Figure 4).

The CD95 pathway is required for IFNγ to induce apoptosis in human neuroblastoma cells

Since both receptor and ligand were induced, we examined the susceptibility of SK-N-BE(2) cells to IFNγ-elicited cytotoxicity, testing the hypothesis that apoptosis could be mediated by the CD95/CD95L system. Apoptotic cell death was assessed by flow cytometric analysis of DNA fragmentation, after PI staining of nuclei. Cells were exposed to IFNγ

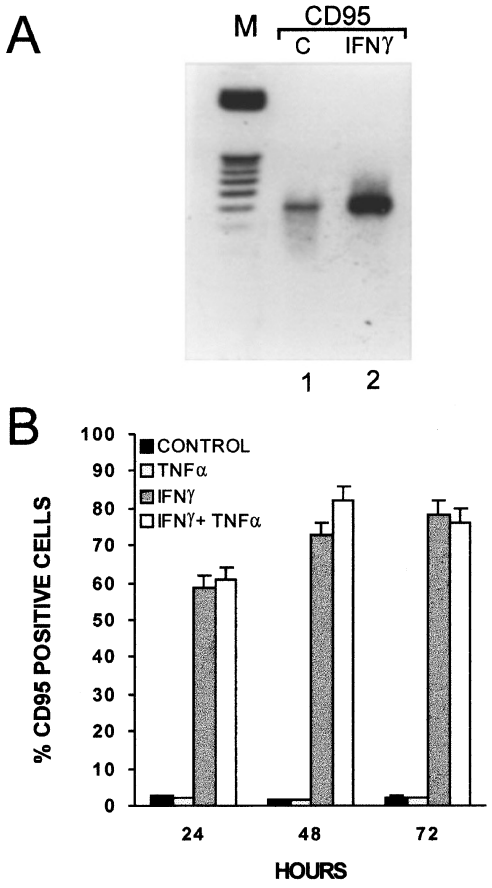

Figure 2 IFNγ-induced up-regulation of CD95 in SK-N-BE(2) cells. (**A**) Transcriptional expression of CD95 in the SK-N-BE(2) neuroblastoma cell line evaluated by RT–PCR analysis. Cells were either untreated or stimulated with 1000 IU/ml recombinant IFNγ for 48 h. RT–PCR reaction was performed as described in the Materials and Methods section. (**B**) FACS analysis of CD95 expression. SK-N-BE(2) cells were grown in medium containing IFNγ (1000 IU/ml) or TNFα (10 ng/ml) or both for 24, 48 and 72 h. Cells were stained with anti-CD95 IgG1 monoclonal antibody and subsequently, incubated with goat anti-mouse IgG-PE. Data are expressed as per cent of CD95 positive cells and given as mean of three different experiments with S.E. of less than 10%

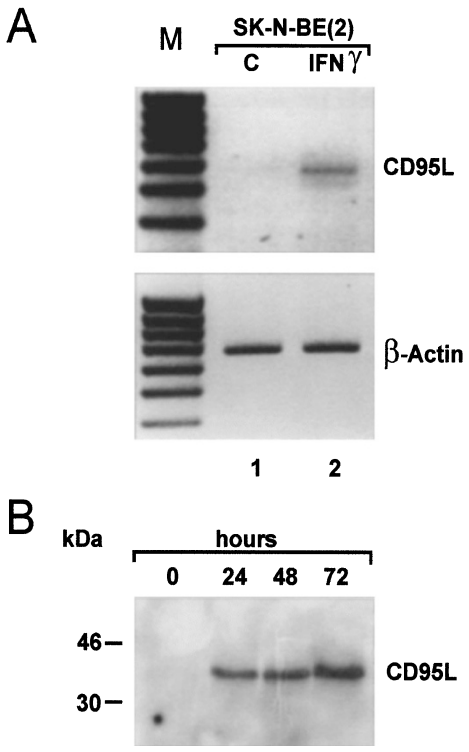

Figure 3 Effect of IFNγ on CD95L expression in SK-N-BE(2) cells. (**A**) Constitutive and IFNγ-induced CD95L mRNA expression in SK-N-BE(2) cells determined by semi-quantitative RT–PCR. Neuroblastoma cells were exposed to 1000 IU/ml IFNγ for 48 h. β-actin was used as a control for RNA loading. RT–PCR conditions are described in the Materials and Methods section. (**B**) Induction of CD95L protein by IFNγ detected by Western blot analysis. SK-N-BE(2) cells were either untreated (lane 1) or incubated with 1000 IU/ml IFNγ for 24 h (lane 2), 48 h (lane 3) and 72 h (lane 4). One hundred μg protein of cell lysates were separated by 12% SDS–polyacrylamide gels and immunodetection of CD95L protein was performed using mouse anti-CD95L monoclonal antibody and ECL

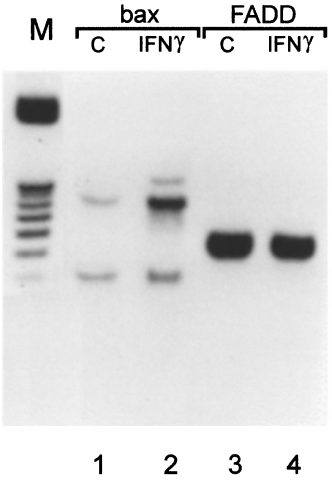

Figure 4 Effect of IFNγ on bax and FADD expression in SK-N-BE(2) cells. Transcriptional expression of bax (lanes 1 and 2) and FADD (lanes 3 and 4) was assessed by RT–PCR analysis. Cells were either untreated or exposed to 1000 IU/ml recombinant IFNγ for 48 h

(1000 IU/ml) for 24–120 h, with or without an agonistic anti-CD95 monoclonal antibody (1 μg/ml).[29] As shown in Figure 5A, IFNγ-induced DNA fragmentation was not detected until 72 h after cytokine treatment and reached its maximum after 96 h. As phosphatidylserine is exposed on the plasma membrane outer leaflet of early apoptotic cells,[30] we also detected apoptotic cells by flow cytometry using the binding of FITC-labelled Annexin V to phosphatidylserine. Significant binding of Annexin V was observed in IFNγ and IFNγ/anti-CD95-treated SK-N-BE(2) cells after 48 h of culture, when DNA fragmentation was in its early stages (data not shown).

The combined exposure to IFNγ and agonistic anti-CD95 antibody further increased the percentage of DNA fragmentation. This suggested the cytokine-mediated activation of an autocrine suicide mechanism even though the induction of CD95L was not completely sufficient to trigger all the available CD95 molecules. Indeed, the disparity between IFNγ and IFNγ plus agonistic anti-CD95 antibody declines over the time, consistently with the time course of IFNγ-activated induction of CD95L.

To further test the hypothesis that IFNγ induced apoptosis through the CD95 autocrine loop, we studied the effects of blocking $F(ab')_2$ anti-CD95 antibody fragments (antagonist), which lack agonistic function and can inhibit CD95 ligation,[31] and of the recombinant Fas-Fc protein which blocks the interaction between CD95 and CD95L by subtracting CD95L.[32,33] Both reagents disrupt the autocrine death loop, one at CD95 and the other at CD95L level (see Materials and Methods). Figure 5B shows that exposure of SK-N-BE(2) to $F(ab')_2$ anti-CD95 or Fas-Fc fusion protein for 72 h, at least partially reduced IFNγ-induced DNA fragmentation. Both CD95 and CD95L blockage systems have been shown to be quite efficient;[31–33] therefore, the observed partial inhibition of IFNγ-induced apoptosis by the blocking reagents might be explained by assuming that the cytokine-induced cell death may be only partly attributed to the up-regulation of CD95 and CD95L. Therefore, IFNγ might influence other critical biochemical events able to activate cell death.

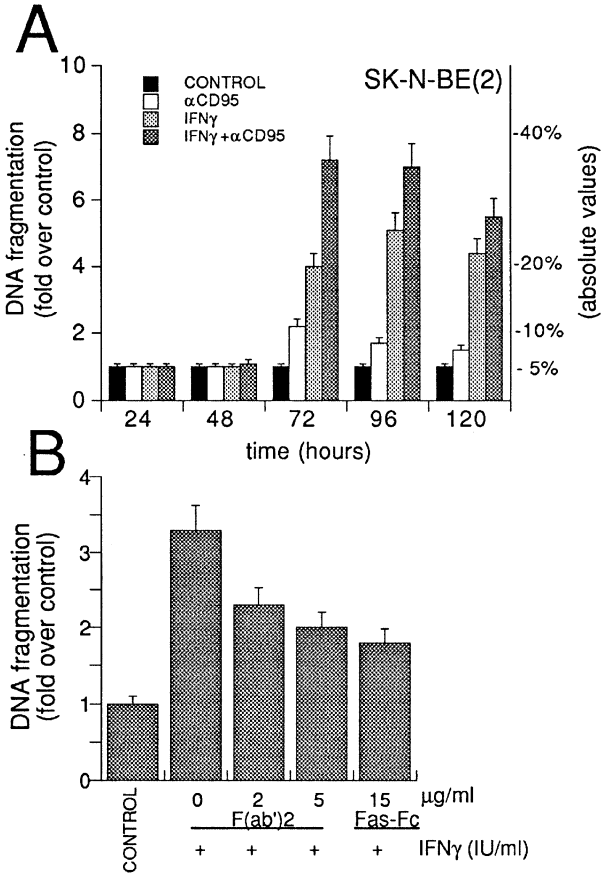

Figure 5 IFNγ-induced apoptosis in SK-N-BE(2) cells. (**A**) Time course of IFNγ-induced DNA fragmentation in the SK-N-BE(2) cell line. DNA fragmentation was measured by FACS analysis using PI staining. Cells were left untreated, exposed to IFNγ (1000 IU/ml), anti-CD95 antibody (1 μg/ml) or both for 24, 48, 72, 96 and 120 h. The percentages of DNA fragmentation were normalised over the values of untreated cells (left); the absolute values are also indicated on the right. (**B**) Inhibition of IFNγ-induced DNA fragmentation by blocking $F(ab')_2$ anti-CD95 antibody fragments and the Fas-Fc fusion protein. Cells were either untreated, exposed to 1000 IU/ml IFNγ and co-incubated with IFNγ and $F(ab')_2$ fragments (2 and 5 μg/ml) or Fas-Fc protein (15 μg/ml) for 72 h. The percentages of DNA fragmentation were normalised over the values of control cells. All the experiments were performed in duplicate and data given as mean of two different experiments with S.E. of less than 10%

IFNγ-induced apoptosis depends on CD95 and CD95L expression levels

To substantiate the involvement of a CD95/CD95L circuit in IFNγ-induced cell death, we compared the effects of IFNγ on two cell lines (see Table 1), in one of which IFNγ was unable to induce cell death. Both cell lines were sensitive to IFNγ induction of CD95, even though they showed different degrees of CD95 positivity after IFNγ treatment (Figure 6A). The TGA cell line did not show any basal expression of CD95L, and it was found to be completely insensitive to the effects of IFNγ (Figure 6B, lanes 1 and 2). IFNγ induced CD95L mRNA expression in the tTG-AS8 cell line (about twofold increase of the constitutive expression by laser densitometry) 48 h after treatment (Figure 6B, lanes 3 and 4). The induction of CD95L mRNA by IFNγ in tTG-AS8 cells was also confirmed by Western blot analysis (Figure 6C).

We studied the cytotoxic effects of IFNγ on TGA and tTG-AS8 cell lines. The TGA cell line, which weakly upregulates CD95 (27.6% positive cells after 48 h of IFNγ exposure, Figure 6A) and does not express CD95L (Figure 6B) after IFNγ stimulation, was completely resistant to IFNγ-induced apoptosis (Figure 7A). On the other hand, tTG-AS8 cells were sensitive to IFNγ-mediated apoptosis even though they presented a slight decrease in DNA fragmentation at 72 h after treatment (Figure 7B), compared with the SK-N-BE(2) cell line (Figure 5A). This may be related to a reduced IFNγ-mediated CD95 up-regulation in the tTG-AS8 cells when compared to SK-N-BE(2) cells (Figures 6A and 2B).

Tissue transglutaminase is not involved in CD95/CD95L circuit

Even though the experiments shown were performed only to address the question of the involvement of the CD95/CD95L

system, the data include some indirect evidence that tissue transglutaminase (tTG) is not involved in the CD95/CD95L circuit, at least in the experimental set under investigation. tTG is considered one of the effector elements of the apoptotic program in human neuroblastoma cells.[34–37] TGA and tTG-AS8 cell lines are tTG sense and antisense transfected clones of the SK-N-BE(2) cells, respectively.[34] As we have previously shown (34), their tTG enzymatic activity significantly differs from the parental cell line (77 ± 11, 1780 ± 190 and 27 ± 6 picomoles of [^{3}H]putrescine incorporated into N,N′-dimethylcasein/h/mg of protein, for SK-N-BE(2), TGA and tTG-AS8 cell lines, respectively). However, cell death observed after IFNγ treatment appeared to be independent of the amount of tTG expressed. Indeed, the sense transfected clone, TGA, which expresses very high levels of tTG (about 23-fold higher), was completely resistant to IFNγ-induced apoptosis (Figure 7A). Furthermore, we could not detect significant alterations of tTG enzymatic activity at 72 h after treatment of SK-N-BE(2) cells with IFNγ or IFNγ plus agonistic anti-CD95 (data not shown). The apparent lack of correlation between IFNγ-modulated CD95-mediated apoptosis and the constitutive tTG expression levels, together with the absence of modulation of enzyme activity by IFNγ, indirectly suggest that the apoptotic mechanism involving tTG activation may be different and unrelated to the CD95/CD95L pathway.

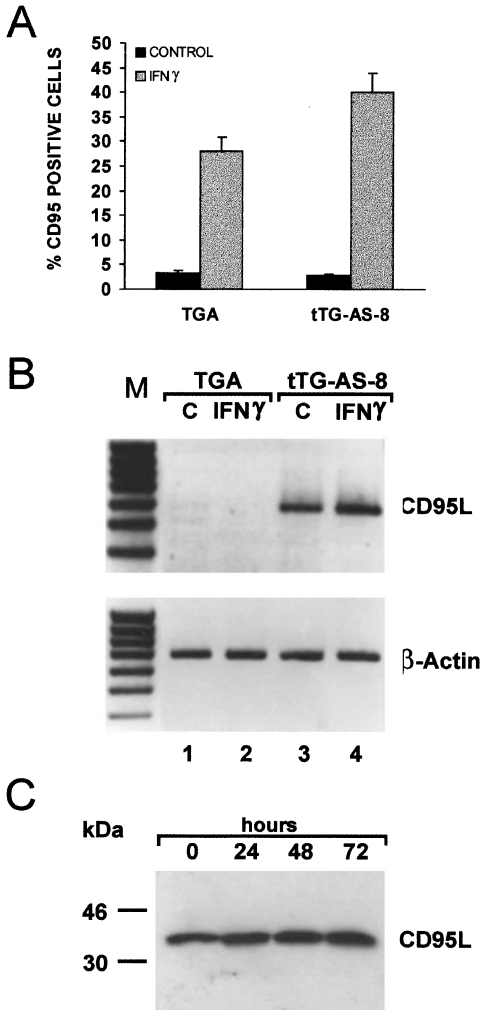

Figure 6 IFNγ-induced CD95 and CD95L expression in TGA and tTG-AS8 cell lines. (**A**) Immunostaining of TGA and tTG-AS8 cell lines with anti-CD95 IgG1 monoclonal antibody, followed by PE-coupled goat anti-mouse IgG. Cells were either left unstimulated or treated with IFNγ (1000 IU/ml) for 48 h and analyzed by FACS. Data are expressed as percent of CD95 positive cells and given as mean of three different experiments with S.E. of less than 10%. (**B**) CD95L mRNA expression was examined by RT–PCR in TGA and tTG-AS8 cells before (lanes 1–3) and after treatment with 1000 IU/ml IFNγ for 48 h (lanes 2–4). Amplification of the β-actin gene was used as control for equal conditions. RT–PCR conditions are described in the Materials and Methods section. (**C**) Analysis of CD95L protein expression by Western blot. The tTG-AS8 cells were left untreated (lane 1) and exposed to IFNγ (1000 IU/ml) for 24 h (lane 2), 48 h (lane 3) and 72 h (lane 4)

Discussion

The biological role of CD95 and CD95L in malignant disease has just begun to be established. Since the CD95/CD95L system is a key regulator of apoptosis, it may have a role as a tumour suppressor.[38] Therefore, inactivation of the CD95

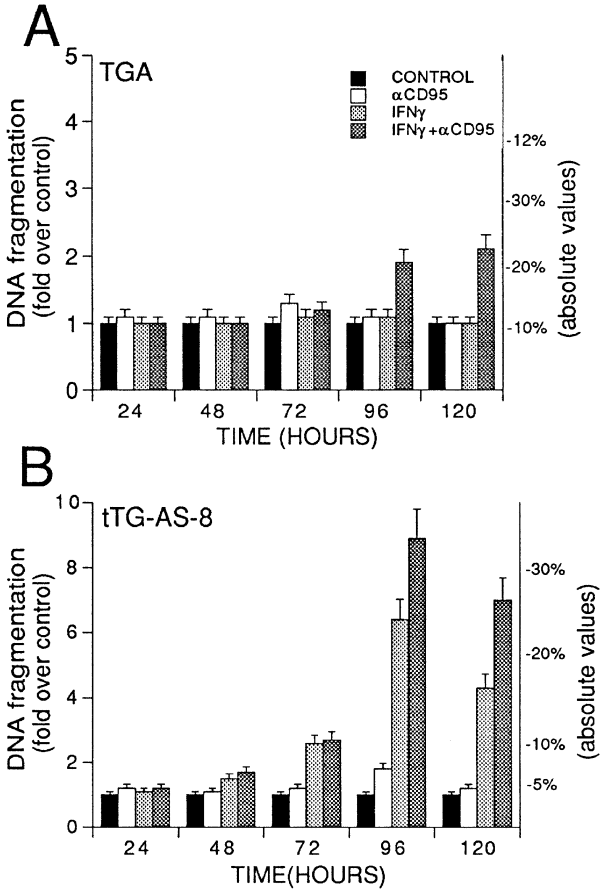

Figure 7 Cytotoxic effect of IFNγ on TGA and tTG-AS8 cell lines. DNA fragmentation was determined by FACS analysis after PI staining. TGA (**A**) and tTG-AS8 (**B**) cell lines were left untreated, exposed to IFNγ (1000 IU/ml), anti-CD95 antibody (1 μg/ml) or both for indicated times. The percentages of DNA fragmentation are expressed as fold over control (left) and absolute values (right). Data are the means of duplicate determinations carried out on two experiments with S.E. of less than 10%

signal, due to loss of CD95/CD95L expression or function, may result in reduced sensitivity of tumour cells towards cytotoxic T lymphocytes, thus contributing to tumour development or progression. In fact, complex CD95 signalling events might be modulated by several mechanisms at different levels, e.g. by NO[39] or by cross-talk with TNF-R.[40] A family of viral inhibitors (v-FLIPs) has recently been reported to interfere with apoptosis signalled by CD95 through their binding to FADD and subsequent inhibition of caspase 8 recruitment and activation.[41] The endogenous mammalian regulator of CD95 signalling has been identified and designated as FLIP (also called usurpin, Casper, I-FLICE).[42,43]

IFNγ induces apoptosis of neuroblastoma cells via the CD95/CD95L circuit

Expression of CD95L was originally thought to be restricted to activated T and NK cells. However, CD95L is also expressed in immune-privileged sites by nonlymphoid cells such as stroma cells of the eye or Sertoli cells of the testis and neurons, suggesting that CD95 may be important for maintaining a state of immune suppression.[44] In both melanomas[17] and hepatocellular carcinomas,[18] CD95 expression is partially or completely lost, while CD95L expressed by the tumour cells can actively destroy infiltrating T-lymphocytes. This mechanism enables tumour cells to protect themselves against immune effector cells and provides an immune privilege for tumours. On the other hand, level of CD95 and CD95L may be increased by cytotoxic drugs and cytokines creating a scenario of reciprocal interaction between cells of the immune system and the tumour.[45] Recent evidence indicates that IFNγ regulates a p53-independent pathway inducing apoptosis-related genes, such as TNFR 1, CD95, bak, and caspases 3, 7 and 8.[27] In the present study, we showed that all neuroblastoma cell lines tested were partially or completely negative for CD95 and CD95L expression and that both mRNA and protein levels of CD95 and CD95L were enhanced by IFNγ. Although IFNγ has been reported to modulate apoptosis in a variety of cell types including neuroblastoma cell lines,[25,26] the molecular mechanism whereby the cytokine induces cell death is still unclear. Our results show that IFNγ-induced cell death in neuroblastoma cells is regulated by CD95 and CD95L induction: (i) apoptosis induced by IFNγ strongly correlates with cell surface expression of CD95 and with CD95L levels; (ii) a clone in which CD95L is not induced did not undergo apoptosis; (iii) blocking reagents inhibiting the interaction between CD95 and CD95L were able to at least partially reduce cell death. Taken together, these observations demonstrate that IFNγ can trigger an autocrine suicide circuit by inducing the CD95/CD95L system.

Complexity of the apoptotic signalling of IFNγ

Even though the data shown unequivocally support the conclusion that IFNγ elicits cell death in neuroblastoma cells via the CD95/CD95L circuit, several pieces of information suggest that the molecular mechanisms involved are much more complex.

First, the kinetics show that IFNγ up-regulates CD95 in the SK-N-BE(2) cell line and that this effect is maximised by 48 h (Figure 2). As shown in Figure 5 however, IFNγ-treated cells remain relatively resistant to the induction of both IFNγ-induced cell death, as well as to the effects of the blocking reagents until 72 h. This result suggests that other more delayed effects of IFNγ on the SK-N-BE(2) cell line are required to induce susceptibility to CD95-mediated cell death. Taking into account the complexity of the intracellular signalling elicited by CD95 ligation, these events could either be related to the induction of adaptor and transducing molecules such as procaspase 8, or to the removal of blocking factors such as c-FLIP. Thus, IFNγ may influence the level of blocking molecules downstream of death receptor signalling similar to the effect described by c-myc,[46] or the anti-apoptotic genes of the bcl-2 family, thus increasing cell sensitivity to apoptosis. Accordingly, exposure of SK-N-BE(2) cells to IFNγ resulted in up-regulation of the bax α-splice form. Alternatively, IFNγ may induce or up-regulate both upstream and downstream caspases.

Second, as shown in Figure 5A, even at the later time points, the effect of IFNγ on cell death in the SK-N-BE(2) cell line is enhanced by incubation with anti-CD95 agonist antibodies. These results provide evidence that CD95L expression is unbalanced or relatively insufficient at recruiting all CD95 receptors available, thus inducing CD95 signalling for up to 96 h after incubation. This difference reduces at 120 h time point mainly due to a relative diminution in the effect of the anti-CD95 agonist antibody. This lessened effect might be related to a time-dependent regulation of IFNγ on the intracellular signalling molecules, as discussed above.

Third, as shown in Figure 5B, the blockade of the CD95/CD95L circuit, through two unrelated mechanisms, does not fully inhibit IFNγ-induced apoptosis. This is the strongest indication that IFNγ also exerts other critical effects, likely at the intracellular level of the cell death cascade signalling. IFNγ might therefore activate the apoptotic pathway through different molecular mechanisms, including the CD95/CD95L circuit.

On the basis of these results, IFNγ might have a therapeutic role for the treatment of human malignant neuroblastomas. In addition, IFNγ may act by exerting a powerful differentiative effect on several neuroblastoma cell lines.[24,26] Further studies are required to assess CD95/CD95L expression in neuroblastoma tumour specimens and to evaluate the susceptibility to IFNγ-mediated cytotoxicity of neuroblastoma *in vivo*; indeed, several questions still remain to be explored, such as the signalling pathway which balances IFNγ-induced apoptosis and differentiation.

Materials and Methods

Materials

Putrescine, N,N′-dimethylcasein, DL-dithiothreitol, PI, bovine serum albumine (BSA), Tween-20 and geneticin were obtained from Sigma.

[^{3}H]putrescine was from Amersham. Human recombinant IFNγ and TNFα were purchased from Pepro Tech Inc. The agonistic monoclonal anti-CD95 antibody (isotype IgG3, kappa) was prepared as previously described.[29] The blocking F(ab′)$_2$ fragments of anti-CD95 was isolated as described by Dhein *et al*.[31] The F(ab′)$_2$ anti-CD95 efficiently prevents TCR-induced apoptosis in T cells with 80–90% inhibition.[31] The recombinant Fas-Fc fusion protein[32,33] was kindly provided by Dr. Douglas R. Green, La Jolla Institute of Allergy and Immunology, San Diego, CA 92121, USA. The Fas-Fc protein, which selectively blocks the interaction between CD95 and CD95L, determines nearly complete inhibition of activation-induced apoptosis of T-cell hybridomas.[32,33]

Cell cultures

The human neuroblastoma cell line SK-N-BE(2) and its transfected clones TGA and tTG-AS8 were grown in a 1:1 mixture of minimal essential medium and Ham's F-12 medium supplemented with 10% heat-inactivated foetal calf serum, 1.2 g bicarbonate per litre, 1% non essential amino acids and 15 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid, at 37°C in a humidified atmosphere of 5% CO_2 in air. Geneticin (0.5 mg/ml) was added to the medium of transfectant clones. The SK-N-BE(2) clones, previously selected in our laboratory, have been transfected with tTG cDNA in sense and antisense orientation, respectively.[34] As they express different amounts of constitutive as well as IFNγ-stimulated CD95 and CD95L compared to the parental cell line, they represent a suitable model to study cytokine-induced apoptosis. For IFNγ and TNFα studies, cells were cultured in medium containing 1000 IU/ml IFNγ and 10 ng/ml TNFα.

RT–PCR

Total RNA was prepared using the Quiagen total RNA kit. RNA was converted to cDNA by reverse transcription and amplified for 38 cycles by PCR in a thermocycler (Stratagene) using the Gene Amplification RNA–PCR kit (Perkin-Elmer). Primers used for amplification of CD95 fragment were prepared according to the sequence of human CD95,[47] whereas primers for amplification of CD95L are described in Herr *et al*.[48] Expression of β-actin (MWG-Biotech) was used as an internal standard for RNA integrity and equal gel loading and 20 cycles of amplification were performed. PCR products were run at 60 V for 2 h on a 1.5% agarose gel stained with ethidium bromide and visualized by UV illumination.

Immunofluorescence staining

For study of CD95 expression, cells were scraped off the culture dishes, washed in PBS with 1% FCS and stained (1×10^6 cells) with 1 μg/ml anti-CD95 IgG1 monoclonal antibody[29] for 30 min at 4°C. After washing with PBS/1% FCS, cells were incubated with goat anti-mouse IgG-phycoerythrin (PE, Immunotech) for 20 min at 4°C. FII23 IgG3 antibody was used as isotype-matched nonbinding antibody to control unspecific binding. Thereafter, cells were washed once with PBS/1% FCS and analyzed on a FACScan or FACSCalibur flow cytometer (Becton-Dickinson). Fluorescence emission for PE was collected at 475 nm and 10 000 events were evaluated using the CELL Quest software.

Western blotting

Cells were incubated with 1000 IU/ml IFNγ for 24, 48 and 72 h. After treatment, cells were mechanically removed from flasks, pelleted and then washed twice with PBS. Cell lysis was achieved with lysis buffer ($1 \times$ PBS, 0.1% SDS, 1% NP40) and sonication on ice, followed by Bradford protein determination. Proteins were normalized to 100 μg/lane, separated on 12% SDS-polyacrylamide gels and blotted onto nitrocellulose sheets. Filters were washed twice with PBS containing 0.1% Tween-20 before blocking non-specific binding with PBS/5% BSA, 0.3% gelatin. The anti-CD95L monoclonal antibody was added and incubated overnight at 4°C. Nitrocellulose filters were washed five times and detection was performed by horseradish peroxidase-conjugated secondary monoclonal antibody (1:2500) for 1 h at room temperature and using the ECL method (Amersham).

Determination of apoptosis

To estimate DNA fragmentation, cells subjected to different treatments, were collected by centrifugation at $800 \times g$ for 10 min and fixed with 1:1 PBS and methanol-acetone (4:1 v/v) solution at −20°C. The hypodiploid events were evaluated by flow cytometry using a PI staining (40 μg/ml) in the presence of 13 kU/ml ribonuclease A (20 min incubation at 37°C) on a FACScan or FACSCalibur flow cytometer (Becton-Dickinson). Cells were excited at 488 nm using a 15 mW Argon laser, and the fluorescence was monitored at 275 nm, at a rate of 150–200 events/s. Ten thousand events were evaluated using the Lysis II Programme (ibid). An electronic gating Forward Scatter (FSC)/A/vs/FSC/h was used when appropriate to eliminate cell aggregates. Annexin V staining was performed as previously reported.[30] Briefly, after washing twice with PBS, 1×10^6 cells were resuspended in 100 μl of binding buffer (mM): (HEPES 10, NaCl 150, $CaCl_2$ 2.5, KCl 5, $MgCl_2$ 1, lactate 5, 0.5% BSA) and the suspension incubated with fluorescein isothiocyanate (FITC)-labeled Annexin V at a final concentration of 2.5 μg/ml for 30 min. After washing with binding buffer, cells were analyzed on the above described flow cytometer (Becton-Dickinson).

Transglutaminase activity assay

Enzymatic activity of tTG was determined by measuring the incorporation of [^{3}H]putrescine into N,N′-dimethylcasein as previously reported.[34] The reaction mixture contained 150 mM Tris-HCl buffer pH 8.3, 90 mM NaCl, 3 mM DTT, 15 mM $CaCl_2$, 12.5 mg N,N′-dimethylcasein/ml, 0.2 mM putrescine containing 1 μCi [3H]putrescine. Proteins from cellular extracts (0.1 mg) were incubated with the reaction mixture in a final volume of 150 μl at 37°C. After 20 min of incubation, the reaction was stopped by spotting 100 μl aliquots onto Whatman 3 MM filter paper. Unbound [^{3}H]putrescine was removed by washing with large volumes of 15, 10 and 5% trichloroacetic acid and absolute ethanol. Filters were then air-dried and the radioactivity was measured by liquid scintillation counting. One enzyme unit was defined as the amount of enzyme binding 1 nmol of putrescine to N,N′-dimethylcasein/h/mg protein.

Acknowledgements

The work was carried out thanks to a generous support by Ass. Neuroblastoma, Telethon (E872), AIRC, MURST 40% and Min. Sanita' to G.M.

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