

Induction of apoptosis by TNF receptor 2 in a T-cell hybridoma is FADD dependent and blocked by caspase-8 inhibitors

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Summary

Previously we reported that both human TNFR1 and TNFR2 mediate TNF-induced apoptosis in the transfected rat/mouse T cell hybridoma PC60. We show here that TNFR2-mediated apoptosis in PC60 cells can be blocked by the broad-spectrum caspase inhibitor zVAD-fmk, the caspase-8 inhibitor zIETD-fmk and by CrmA, a viral inhibitor of caspase-1 and caspase-8. This suggests an involvement of caspase-8 in TNFR2-mediated apoptosis. The upstream adaptor of caspase-8, FADD, is also involved in TNFR2-induced cell death, since transient overexpression of a dominant negative deletion mutant of FADD inhibited apoptosis induced by this receptor. TNFR2-induced apoptosis is independent of endogenous TNF or other death-inducing ligand production and

subsequent activation of TNFR1 or other death receptors. Furthermore, TNFR2 stimulation does not enhance sensitivity for a subsequent TNFR1-induced apoptotic signal, as has been reported for Jurkat cells. TRAF2 downregulation, which has been proposed as the mechanism by which TNFR2 enhances TNFR1 signaling, was observed in PC60 cells, but the TNFR1 signal was not modulated. These data confirm the capacity of TNFR2 to generate an apoptotic cell death signal independent of TNFR1.

Key words: apoptosis, cytotoxic-T-cells, TNF receptor, caspase, FADD

Introduction

TNF exerts its many different functions in the body through two different receptors. Since their discovery 15 years ago, there has been intense investigation of whether both receptors perform similar functions and act in a cooperative way or whether they perform separate biological functions (Vandenameele et al., 1995a; Wallach et al., 1997). TNF receptor 1 (TNFR1) is the main signaling receptor both for the induction of cell death and gene regulation, while the function of TNFR2 seems mostly restricted to T-cells, where it is involved in proliferation, gene activation and apoptosis (Aspalter et al., 2003; Kafrouni et al., 2003; Kim and Teh, 2001; Zheng et al., 1995). The involvement of TNFR2 in TNF-mediated cell death is controversial; does it somehow modulate the TNFR1 signal or does it mediate TNF-induced apoptosis in a direct way? Others and we have argued that TNFR2 is fully capable of direct involvement in cell death (Haridas et al., 1998; Pimentel-Muinos and Seed, 1999; Vandenameele et al., 1995b; Vandenameele et al., 1992). This view is supported by genetic in vivo studies demonstrating that cytotoxic T lymphocytes from TNFR1 knockout animals still undergo TNF-dependent apoptosis (Zheng et al., 1995). Alternatively, different mechanisms by which TNFR2 may make use of TNFR1 signaling have been proposed. One such mechanism is that TNFR2 activation induces production of endogenous TNF

that in turn activates TNFR1 (Grell et al., 1999; Vercammen et al., 1995). To our knowledge, this mechanism has not been observed in T-cells. A second mechanism by which the two TNF receptors can cooperate is ligand passing (Tartaglia and Goeddel, 1992; Tartaglia et al., 1993). It has been proposed that TNFR2 acts as a high affinity receptor with high K_{ass} and low K_{off} values, allowing TNF to pass from the highly abundant TNFR2 to a less abundant TNFR1. We previously showed that intracellular mutations of TNFR2 abolished the observed cytotoxicity in PC60 cells (Declercq et al., 1998) and that TNFR2-specific muteins of human TNF (Van Ostade et al., 1994) still induce cell death. This suggests that TNFR2 is a true signal transducing receptor system.

A recent set of publications concerning TNF-receptor cooperation in cell death models suggests that TNFR2 modulates the complex signaling machinery engaged by TNFR1. Many components of the TNFR1-induced signaling pathway have been identified, and their role in signaling has been established (reviewed by Chen and Goeddel, 2002). After ligand binding, the aggregated intracellular part of the receptor recruits a number of adaptor molecules, TRADD (TNFR1 associated protein with death domain), TRAF2 (TNFR1 associated factor 2) and RIP1 (receptor interacting protein), forming the so-called TNF-signaling complex I (Harper et al., 2003; Micheau and Tschoop, 2003; Shu et al.,

1996). This adaptor-complex dissociates from the TNFR1 receptor and TRADD recruits FADD (Fas-associated protein with death domain), caspase-8 and caspase-10, forming the TNF-signaling complex II (Micheau and Tschopp, 2003). It is important to mention that a truncated FADD molecule, FADD-DN (FADD dominant negative), which does not contain the N-terminal death effector domain (DED), will block the activation of caspase-8 in this complex (Chinnaiyan et al., 1995). Through the activation of caspase-8 and -10, TNFR1 initiates the apoptotic process and further activates downstream caspases, while the complex I molecules TRAF2 and RIP1 are responsible for signaling to gene expression and notably to anti-apoptotic mechanisms. This mechanism was described in human cells, but it is very likely that it also operates in rodent cells, with the sole difference that no mouse or rat caspase-10 has been identified (Lamkanfi et al., 2002). It has been proposed that TNFR2 influences TNFR1 signaling precisely through TRAF2. This model is primarily based on observations in Jurkat and other cell lines. These cells become sensitive to TNF after overexpression of TNFR2, but stimulating this receptor with agonistic antibodies alone does not kill the cells, or does so only with low efficiency (Chan and Lenardo, 2000; Fotin-Mleczek et al., 2002; Weiss et al., 1997). This means that in these cell lines TNFR1 activation is required for TNFR2-induced cell death. The proposed mechanism is that TNFR2 recruitment and subsequent degradation of TRAF2 interferes with the anti-apoptotic signaling of TNFR1. This model is further supported by the observation that TNFR2-specific prestimulation some hours before TNF treatment enhances the sensitivity to the cytotoxic action of TNF (Chan and Lenardo, 2000; Fotin-Mleczek et al., 2002). This model, however, does not explain the data presented by Pimentel-Muñoz and Seed who, using Jurkat cells similarly transfected with TNFR2, could not block TNF-mediated toxicity using TNFR1-neutralizing antibodies (Pimentel-Muñoz and Seed, 1999). It is unfortunate that no receptor-specific stimulation of TNFR2 was used on these cells. These authors also provide genetic evidence that RIP1 is necessary for TNFR2-mediated cell death, and they show RIP1 and TRAF2 dependent recruitment of the death-inducing adaptor FADD to the intracellular part of TNFR2 (Pimentel-Muñoz and Seed, 1999).

To study TNFR2-induced apoptosis in T-cells, we made use of PC60 cells. PC60 cells expressing both human TNF receptors (PC60 R1R2) exhibit TNF-dependent apoptosis that can be mediated by either TNFR1 or TNFR2, or by simultaneous triggering of both TNF receptors (Vandenabeele et al., 1995b). We have already shown that the TRAF2 recruitment domain of TNFR2 is required for the observed cytotoxic effect (Declercq et al., 1998). In the present study we show that TNFR2-mediated cell death is dependent on FADD and on a caspase that is inhibited by CrmA (cytokine response modifier A), zIETD-fmk and zVAD-fmk, most probably caspase-8. It was also observed that TRAF2 was downregulated by TNFR2, but not by TNFR1. However, death of PC60 cells induced by TNFR2 is not dependent on a simultaneous trigger of TNFR1. We also present data showing that TNFR2-dependent TRAF2 downregulation does not sensitize the PC60 cells to subsequent TNFR1-mediated killing. These data support the notion that TNFR2-induced apoptosis in T-cells operates independently of TNFR1.

Materials and Methods

Cells and cytokines

The hybridoma PC60.21.14.4, provided by M. Nabholz (Swiss Institute of Experimental Cancer Research, Epalinges, Switzerland), was transfected with cDNAs coding for TNFR1, TNFR2 or both, as previously reported (Vandenabeele et al., 1995b). The PC60 R1R2 were previously named 'PC60 TNF-R55/R75 cl5'. Purified *E. coli*-derived human TNF, the human TNFR1-specific mutein R32WS86T and the human TNFR2-specific mutein D143NA145R were prepared in our laboratory as described previously (Vandenabeele et al., 1995b).

DNA constructs and electroporation of PC60 cells

The expression plasmids pCAGGSCrmA (encoding Cowpox Crm) and pCDNAFADD-DN (encoding amino acids 80-205 of the wild type FADD) have been described previously (Boone et al., 2000; Vercammen et al., 1997). The pNFconluc construct contains the luciferase gene under control of the minimal chicken conalbumin promoter preceded by three NF- κ B sites (Kimura et al., 1986). pUT651, a β -gal expression plasmid, was obtained from Eurogentec (Seraing, Belgium). For stable transfections, PC60 R1R2/5 cells (10^7) were electroporated as reported previously (Declercq et al., 1995). A combination of the plasmids pCAGGSCrmA (10 μ g) and pNFconluc (10 μ g), or pNFconluc alone (10 μ g) combined with 1 μ g of the selection plasmid pSV2Neo (G418 selection) was added to the cells and the mixture was exposed to a single electropulse (1500 μ F, 300 V). Two days later, G418 (1500 μ g; Life Technologies, Paisley, UK) was added and the cells were cloned by means of limiting dilution. For transient expression, pUT651 (10 μ g) combined with pCDNAFADD-DN (20 μ g) or the control vector pCDNAI (20 μ g) were electroporated as described. Assays were performed 24 hours after electroporation.

Immunoblotting

Cell lysates were subjected to SDS-PAGE and immunoblotted using a CrmA-specific rabbit polyclonal antiserum, rabbit anti-caspase-3 antiserum (Denecker et al., 2001), a monoclonal FADD-specific antibody (Transduction Laboratories, Lexington KY), or TRAF2 (Santa Cruz Biotechnology). Detection was performed using the ECL system (Amersham Life Science, Amersham, UK).

Propidium iodide exclusion assay

Cells were seeded in 96-well microtiter plates at 5×10^4 cells/well or 24-well plates at 10^5 cells/well. They were treated, for the times indicated in the figure legends, with saturating concentrations of hTNF (100 ng/ml) or its receptor-specific muteins R32WS86T (hTNFR1, 500 ng/ml) or D143NA145R (hTNFR2, 500 ng/ml) (Loetscher et al., 1993). The number of apoptotic cells in a given culture was measured by a propidium iodide (PI) exclusion assay as described previously (Vandenabeele et al., 1995b). PI was added at a final concentration of 30 μ M and cells were analyzed on an Epics 753 fluorometer or a FACS-Calibur flow cytometer (Becton Dickinson, Sunnyvale, CA). When used, the protease inhibitors zVAD-fmk or zDEVD-fmk (Enzyme Systems Products, Dublin, CA) or zIETD-fmk, the granzyme B inhibitor zAAD-fmk or zYVAD-cmk (Calbiochem-Novabiochem International, San Diego, CA) were added 1 hour prior to TNF or the TNF muteins.

β -galactosidase cell death assay

Transiently transfected cells were stimulated overnight either with control medium, 500 ng/ml R32WS86T, 500 ng/ml D143NA145R or 100 ng/ml hTNF. Subsequently, cells were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) as described previously (Chinnaiyan et al., 1996). β -gal-stained blue cells were examined

microscopically for apoptotic features, such as membrane blebbing and nuclear changes, after which the percentage of induced apoptotic cells was determined (500 cells were counted in each assay).

Luciferase assay

PC60 transfectants were cultured in the absence or presence of hTNF and lysed after 6 hours of stimulation according to the luciferase assay protocol of Promega Biotec (Madison, WI). Luciferin (Duchefa Biochemie, Haarlem, The Netherlands) was added and luciferase activity was measured on a Topcount Luminometer (Packard Instrument Co., Meriden, CT).

Results

TNFR2 induces apoptosis in PC60 cells

As we reported previously (Vandenabeele et al., 1995b), both human TNF receptors are equally capable of inducing apoptosis when transfected in the T cell hybridoma PC60 (PC60 R1R2 cells) (Fig. 1). The percentage of propidium iodide (PI)-positive cells was taken as a measure of apoptotic death in these cultures. We previously correlated PI measurements with other parameters of apoptosis, such as annexin V staining and DNA fragmentation, in PC60 R1R2 cells and excluded other types of cell death, such as necrosis, after TNF stimulation in these cells (Denecker et al., 2000; Vandenabeele et al., 1995b). Annexin V staining occurs earlier (6-9 hours) than PI-positivity in these cells upon TNF treatment, but eventually the annexin V-positive cells will become PI positive after 16 to 20 hrs (data not shown). Hence, PI-positivity is a valid parameter for apoptotic cell death in PC60 cells. Individual triggering of either TNF receptor is achieved through stimulation with muteins of hTNF that are capable of binding only one receptor: R32WS86T triggers TNFR1 while D143NA145R is specific for TNFR2 (Loetscher et al., 1993). When PC60R1R2 cells were treated with both muteins together, this resulted in an additive amount of cell death comparable to what is observed with hTNF (Fig. 1).

Caspase-8 inhibitors block TNFR2-induced apoptosis

Apoptosis is most often dependent on caspase activity, so it is

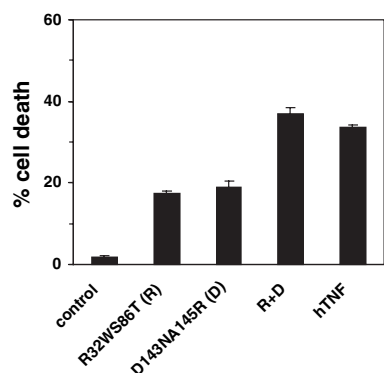


Fig. 1. Both TNF receptors induce apoptosis in PC60 R1R2 cells. PC60 R1R2 cells were treated for 20 hours with 100 ng/ml hTNF or its receptor-specific muteins (500 ng/ml). The percentage of apoptotic cells was determined using propidium iodide exclusion.

hardly surprising that the broad-spectrum caspase inhibitor zVAD-fmk prevents TNF-mediated cell death in PC60 cells (Fig. 2A). As a control, the granzyme B inhibitor zAAD-fmk had no effect in the same assay. As shown in Fig. 2B, zVAD-fmk is equally capable of inhibiting cell death induced by TNFR1 or by TNFR2. It is important to point out that the combination of zVAD-fmk and TNFR2-specific stimulation did not result in necrotic cell death, not even after extended observation of the cells (72 hours, data not shown), as has been reported for Jurkat cells (Chan et al., 2003). In an attempt to get more information about the identity of caspases involved in this process, we tested the ability of several caspase

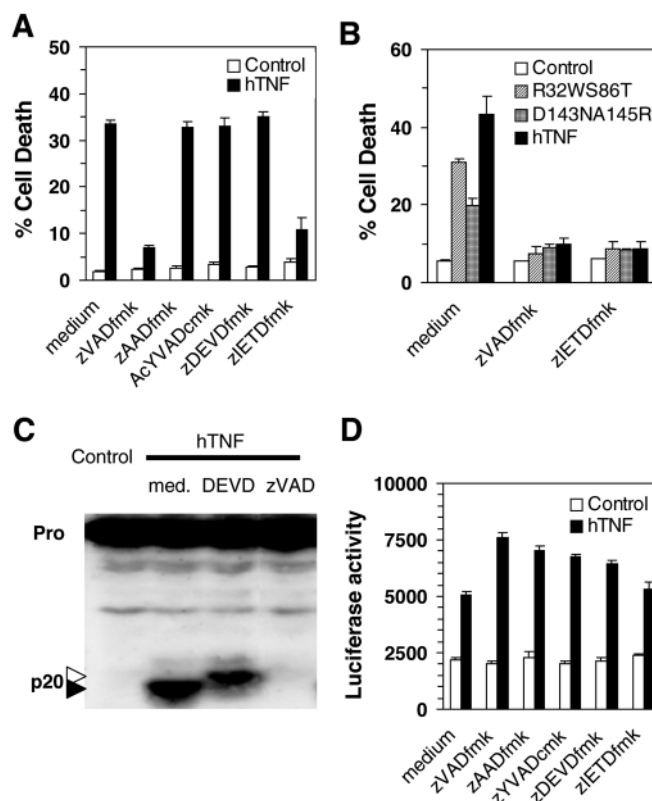


Fig. 2. Both TNFR1- and TNFR2-mediated apoptosis in PC60 R1R2 cells are blocked by the caspase inhibitor zVAD-fmk. (A) PC60 R1R2luc cells were seeded in microtiter plates and left untreated or were stimulated with saturating amounts of hTNF (100 ng/ml) in the presence (100 μ M) or absence of the synthetic peptide caspase inhibitors zVAD-fmk, zDEVD-fmk, zYVAD-cmk, zIETD-fmk or the granzyme B inhibitor zAAD-fmk. Cells were cultured for 20 hours before cell death was measured by cytofluorometric analysis of PI uptake. (B) The experimental procedure was the same as in A. Cells were incubated with hTNF or its receptor-specific muteins R32WS86T or D143NA145R (500 ng/ml) in the absence or presence of zVAD-fmk. (C) Cells were treated for 7 hours in the presence of 100 ng/ml hTNF, in the presence or absence of peptide inhibitors zDEVD-fmk or zVAD-fmk. Cell lysates were analyzed by means of western blotting with a caspase-3 antibody. Pro is the unprocessed procaspase-3, and p20 is the processed form with the black arrowhead showing the normal p20 subunit and the white arrowhead showing a shifted band indicative of a covalent bond between the p20 and zDEVD-fmk. (D) The experimental procedure was the same as in A. Cells were lysed 6 hours after stimulation and luciferase activity determined.

inhibitors to interfere with TNF-induced apoptosis in PC60 cells (Fig. 2A). The inhibitors zYVAD-fmk, zDEVD-fmk and zIETD-fmk have been shown to be specific for caspase-1-like, caspase-3-like and caspase-8-like activities, respectively. Although high concentrations of these modified peptides were used (up to 200 μ M in some experiments, data not shown), neither zYVAD-fmk nor zDEVD-fmk inhibitors were able to block the TNF-dependent cell killing, excluding a major role for caspase-1 and caspase-3 in the TNF-induced apoptotic death of PC60 cells (Fig. 2A). Since DEVD peptide inhibitors are capable of inhibiting apoptosis in many cell lines but did not affect TNF-induced apoptosis in PC60R1R2 cells, we verified whether this inhibitor is capable of entering the PC60 cells. Therefore we measured DEVDase activity in cell lysates from untreated and TNF-treated cells. The TNF-induced DEVDase activity was inhibited (90% inhibition) in zDEVD-fmk-treated cell cultures (data not shown). In addition, we could show that the p17 fragment, containing the catalytic cysteine of activated caspase-3, shows an upward shift when

cells were treated in the presence of zDEVD-fmk, indicative of a covalent bond between the enzyme fragment and the peptide-inhibitor (Fig. 2C). This shift is also seen when lysates of TNF-treated cells, containing activated caspase-3, were incubated with zDEVD-fmk *in vitro* (data not shown). Caspase-3 processing was not observed when TNF stimulation occurred in the presence of the broad-spectrum caspase inhibitor zVAD-fmk. These findings indicate that the peptide inhibitor zDEVD-fmk was capable of entering the cells and also that a zVAD-fmk-sensitive protease, most probably an initiator caspase, is activated upstream of caspase-3. In contrast to zDEVD-fmk and zYVAD-fmk, the inhibitor zIETD-fmk did substantially reduce TNF-mediated apoptosis in the PC60 R1R2, which is a first indication that caspase-8 is the predominant caspase in TNF-receptor signaling in this cell line. None of the peptide caspase inhibitors used had an effect on TNF-induced NF- κ B activation, indicating that the inhibitors did not interfere with TNF signaling in general (Fig. 2D). This was analyzed in PC60 R1R2 cells transfected with a NF- κ B-dependent luciferase expression vector. The somewhat higher luciferase values in cells treated with protease inhibitors might be due to a slower degradation of luciferase. In addition to peptide inhibitors, overexpression of protein inhibitors can be used to identify the protease involved in signaling. We stably expressed the cowpox caspase inhibitor CrmA (Los et al., 1995; Tewari and Dixit, 1995) in PC60 R1R2 cells and confirmed its expression by western blotting (Fig. 3A). CrmA inhibited induction of apoptosis by TNF and by its receptor specific muteins, which meant that signaling towards apoptosis through both receptor types was blocked (Fig. 3B). We also compared the NF- κ B activation by TNF in a control cell line and in the CrmA transfected cells, and found that CrmA did not inhibit the NF- κ B-dependent luciferase activity stimulated by TNF (data not shown). Our data point to the crucial involvement of one or more caspases in TNFR2-mediated cell death that can be inhibited by zVAD-fmk, zIETD-fmk and CrmA but not by zYVAD-cmk and zDEVD-fmk. The obvious candidate is caspase-8, whose activity is, like caspase-1, blocked by CrmA but not by the caspase-1 inhibitor zYVAD-cmk (Garcia-Calvo et al., 1998; Muzio et al., 1996).

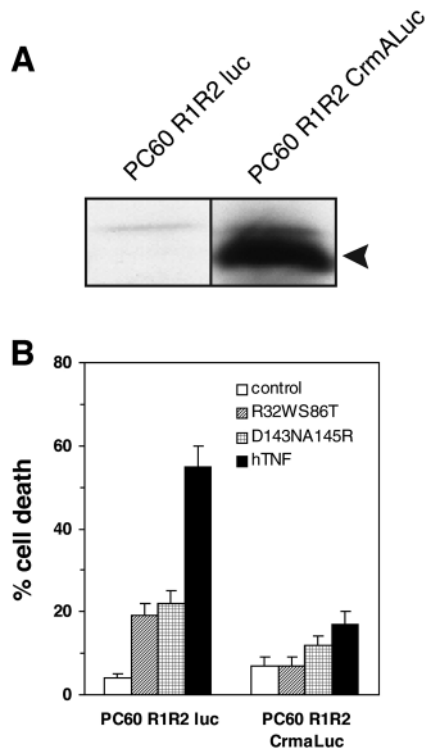


Fig. 3. TNFR1- and TNFR2-mediated apoptosis in PC60 R1R2 cells are both inhibited by the cowpox caspase inhibitor CrmA. (A) PC60 R1R2 cells were transfected with a combination of the plasmids pCAGGSCrmA and pNFconluc. After selection and subcloning, cell lines were isolated that expressed the CrmA protein and produced elevated levels of luciferase activity after TNF stimulation (named PC60 R1R2 CrmA luc). Control cells were obtained by only transfecting pNFconluc and subsequent screening for luciferase inducibility (PC60 R1R2 luc). Untreated cells were lysed and 100 μ g of protein was subjected to CrmA immunoblot. The arrowhead shows the position of the CrmA protein. (B) PC60 R1R2 luc and PC60 R1R2 CrmA luc cells were cultured in the absence or presence of hTNF (100 ng/ml) or receptor specific muteins (500 ng/ml) for 20 hours and the percentage of propidium iodide-positive cells was determined.

FADD-DN blocks TNFR2-induced apoptosis

Since we found indications of caspase-8 involvement in TNFR2-mediated apoptosis, we evaluated the potential involvement of the caspase-8 upstream adaptor FADD in this pathway. Overexpression of the dominant-negative N-terminal deletion mutant of FADD (residues 80-205, FADD-DN), lacking the death effector domain (DED), blocks cell death induced by death receptors that make use of this adaptor (Chinnaiyan et al., 1996). Transient overexpression of the FADD-DN molecule strongly inhibited TNF-induced apoptosis in PC60 R1R2 cells (Fig. 4A,B). When the contribution of each receptor was examined using the TNF-receptor specific muteins R32WS86T and D143NA145R, we found that expression of FADD-DN completely blocks not only TNFR1-induced cytotoxicity, but also that induced by TNFR2 (Fig. 4B). This finding suggests that direct TNFR2 signaling to the FADD/caspase-8 apoptosis mechanism is possible.

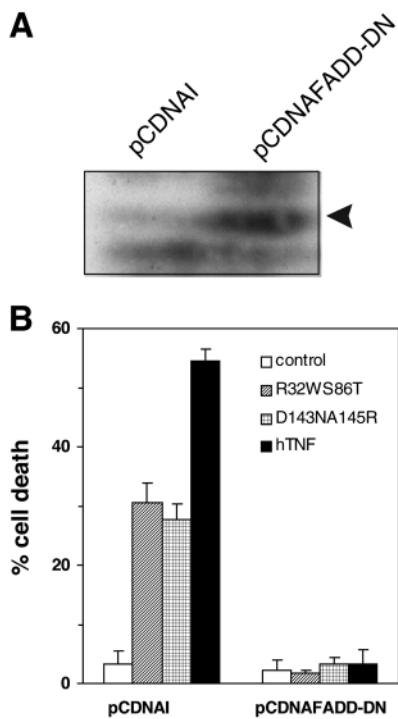


Fig. 4. Both TNFR1- and TNFR2-mediated apoptosis in PC60 R1R2 cells is FADD dependent. (A) PC60 R1R2 were electroporated with: pUT651, a β -gal expression plasmid, plus pCDNAI (control) or plus pCDNAFADD-DN, encoding dominant negative FADD (80-205). 48 hours after transfection cells were lysed and 150 μ g of protein was submitted to immunoblotting using a monoclonal FADD-specific antibody. (B) 24 hours after electroporation, cells were stimulated overnight either with 500 ng/ml R32WS86T, 500 ng/ml D143NA145R or 100 ng/ml hTNF. Subsequently, cells were stained with X-gal and β -gal-positive blue cells were examined microscopically for apoptotic features, such as membrane blebbing and nuclear changes, after which the percentage of induced apoptotic cells was determined (500 blue cells were counted in each measurement).

TNFR2-mediated cell death does not occur through secretion of endogenous TNF or other death-inducing ligands

In a previous study we reported that addition of a neutralizing, polyclonal anti-TNF antiserum did not inhibit TNFR2-induced cell death in PC60 R1R2 cells (Decoster et al., 1998; Vandenabeele et al., 1994). Since FADD and caspase-8 are generally associated with TNFR1 or TRAIL signaling (Muzio et al., 1996; Boldin et al., 1996; Sprick et al., 2000), we performed additional experiments to evaluate a potential involvement of TNFR1 or other death-inducing ligands in TNFR2-induced apoptosis in PC60 R1R2. One mechanism by which TNFR2 could make use of TNFR1 signaling is through the secretion of endogenous TNF. Therefore, we stimulated PC60 R1R2 cells with TNF-receptor-specific muteins in the presence or absence of htr-5, a TNFR1 specific antagonistic antibody. As shown in Fig. 5A, TNFR2-induced apoptosis is unaffected by htr-5, whereas TNFR1-mediated cell death can be inhibited. We used another approach to confirm the absence of a role for endogenously produced TNF or other death-inducing ligands in TNFR2-mediated apoptosis, by repeating

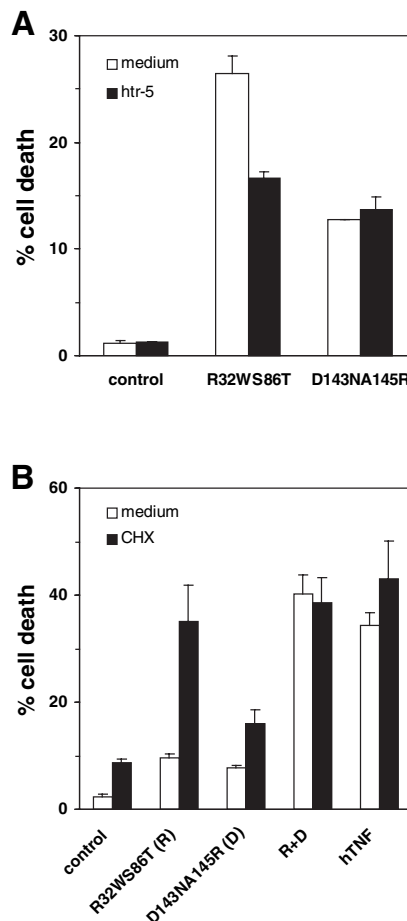


Fig. 5. TNFR2-mediated apoptosis is not dependent on endogenous production of TNF or other death-inducing ligands. PC60 R1R2 cells were treated with hTNF or its muteins as in Fig. 1 and the resulting amount of cell death determined. (A) Incubation in the absence or presence of 5 μ g/ml hTNFR1 antagonizing monoclonal antibody htr-5. (B) Incubation in the absence or presence of 10 μ g/ml cycloheximide (CHX).

the cytotoxic assay in the presence of the protein synthesis inhibitor cycloheximide. We found that apoptosis induced by TNFR1 or TNFR2 is not inhibited by cycloheximide but actually enhanced (Fig. 5B). Together, these data exclude a role for endogenous TNF in TNFR2-mediated cell death.

TNFR2 stimulation does not sensitize PC60 cells to TNFR1-mediated killing

It is clear from our experiments that killing by TNFR2 in PC60 cells occurs independently of TNFR1 ligand stimulation (Fig. 5) (Vandenabeele et al., 1994; Vandenabeele et al., 1995b). In Jurkat and other cell lines, where TNFR2 modulation of the TNFR1 pathway is suggested, TNFR2 prestimulation enhances the sensitivity of these cells for a subsequent TNFR1 trigger. To investigate whether this is a general phenomenon also occurring in PC60 cells, PC60 R1R2 cells were pretreated with the TNF mutein D143NA145R that is specific for TNFR2, and subsequently exposed to hTNF. Pretreatment with the TNFR2-specific TNF mutein does not make these cells sensitive to TNF-induced apoptosis (Fig. 6A). The percentage of cell death

observed with TNFR1-specific stimulation after TNFR2 prestimulation is higher than with TNFR1 stimulation alone, but this is just an additive effect and not a real sensitization. An enhanced sensitivity to hTNF in D143NA145R-prestimulated PC60 R1R2 cells might alternatively be masked by an upper limit in the amount of apoptosis that can be induced in these cells. To examine this possibility, TNFR2 prestimulated cells were treated with different concentrations of hTNF (Fig. 6B). TNFR2 prestimulation did not enhance sensitivity for hTNF, since the amount of hTNF needed in both prestimulated and non-prestimulated cells for half maximal killing lies in the 10–20 ng/ml range for both conditions.

In Jurkat cells, TNFR2-mediated sensitization of TNFR1-dependent cytotoxicity is attributed to downregulation of TRAF2 (Fotin-Mleczek et al., 2002). We observe that PC60

R1R2 cells show strongly decreased levels of TRAF2 expression upon TNFR2 stimulation (Fig. 6C). As this cell line does not exhibit an enhanced cytotoxic response after TNFR2 prestimulation (Fig. 6), we conclude that downregulation of TRAF2 by TNFR2 is not sufficient to enhance TNFR1-mediated cell death in PC60 R1R2 cells.

Discussion

Our results indicate that in the rat-mouse cytotoxic T-cell hybridoma PC60 transfected with human TNF receptors, TNFR2 is fully capable of inducing cell death independently of TNFR1 ligand stimulation. Simultaneous triggering of both TNF receptor types has an additive effect (Vandenabeele et al., 1995b). In most cell types, TNFR1 is the main TNF receptor that signals towards cell death, while TNFR2-mediated apoptosis *in vivo* is observed only in T-cells. When different subsets of T-cells were investigated, TNFR2 was found to regulate apoptosis only in CD8⁺ CTL (cytotoxic T lymphocytes) (Kim and Teh, 2001; Zheng et al., 1995). Much of the confusion about the role of TNFR2 in TNF-induced apoptosis could be attributed to differences in the types of cells used. In many non-lymphoid cells, TNFR2-induced signaling is attributed to the stimulation of TNFR1, ligand passing from TNFR2 to TNFR1 (Tartaglia et al., 1993), or the induced secretion of endogenous TNF (Grell et al., 1999; Vercammen et al., 1995). We have already ruled out the involvement of endogenous TNF or ligand passing in TNFR2-mediated effects on PC60 cells before (Declercq et al., 1998; Declercq et al., 1995; Vandenabeele et al., 1995b; Vandenabeele et al., 1994) and now we provide conclusive evidence that the observed TNFR2-mediated cytotoxicity is not the consequence of other endogenously produced toxic ligands such as lymphotoxin or TRAIL. Indeed, antagonistic TNFR1 antibodies could not inhibit cell death induced by TNFR2. In addition, TNFR2 killing could not be blocked by the translation inhibitor cycloheximide, which rules out the involvement of any endogenous cytotoxic factor that could be produced upon TNFR2 stimulation.

Two research groups, using TNFR2-transfected Jurkat cells, describe TNFR2 as an enhancer of TNFR1-induced cell death incapable of independently inducing apoptosis (Chan and Lenardo, 2000; Fotin-Mleczek et al., 2002). The enhancement mechanism had already been observed in non-lymphoid cells (Fotin-Mleczek et al., 2002; Weiss et al., 1997). It is proposed that TNFR2 disrupts anti-apoptotic signals generated by TNFR1 while the cell death signals remain. This mechanism could correlate with the observed TRAF2 degradation in Jurkat cells (Fotin-Mleczek et al., 2002). This degradation follows TRAF2 recruitment to TNFR2 and is dependent on ubiquitination and cIAP-1 (Li et al., 2002). TNFR2 stimulation is also reported to enhance Fas-mediated cell death (Elzey et al., 2001). Since Fas does not employ TRAF2 for signaling, the TRAF2 downregulation mechanism does not seem to be universal. In contrast to the Jurkat cell model, we observed cell death in PC60 R1R2 cells after clustering of TNFR2 alone. TRAF2 expression levels also diminished drastically upon TNFR2 triggering of PC60 cells, but this did not result in increased sensitivity to TNFR1-mediated death as was observed in Jurkat cells. This experiment suggests that in PC60 cells TNFR2-mediated cell death may occur through a mechanism distinct from that in Jurkat cells. This difference

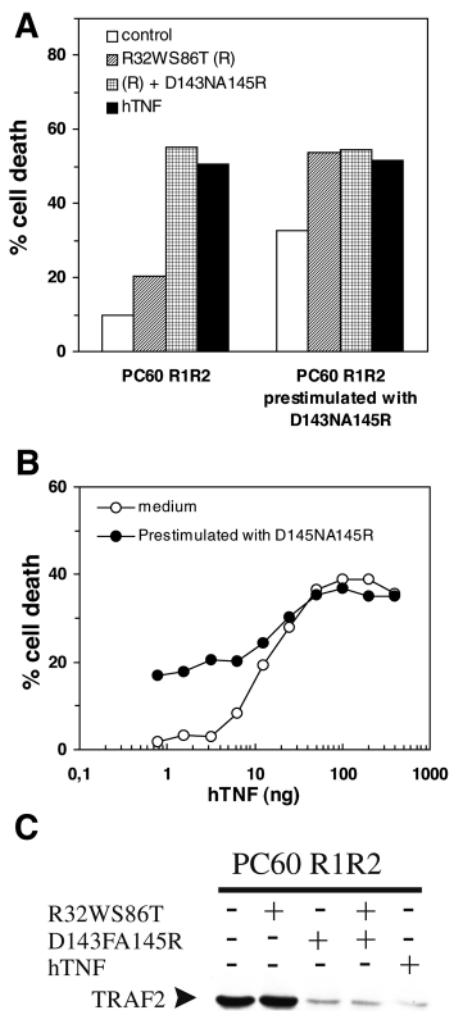


Fig. 6. TNFR2 stimulation does not sensitize PC60 R1R2 cells for a subsequent TNFR1-mediated apoptotic signal. (A) PC60 R1R2 cells, preincubated for 14 hours in medium or medium containing 500 ng/ml of the TNFR2-specific mutein D143NA145R were stimulated with 100 ng/ml hTNF, the TNFR1 specific mutein R32WS86T (500 ng/ml) or a combination of both receptor specific muteins. (B) Cells preincubated as in A were exposed to a dilution range of hTNF as indicated. (C) Western blot, revealed with anti-TRAF2, of lysates of PC60 R1R2 cells that were treated for 14 hours with hTNF (100 ng/ml) or its receptor specific muteins (500 ng/ml).

could be attributed to the CD8⁺ CTL origin of PC60 cells (Conzelmann et al., 1982) compared to Jurkat cells, which are CD4⁺ (Lifson et al., 1986) and show TNFR2 functionality reminiscent of non-lymphoid cells (Fotin-Mleczeck et al., 2002; Weiss et al., 1997). It has been shown that TNFR2 has distinct functions in CD8⁺ cells. In these cells TNFR2 can act as a costimulatory molecule for T cell receptor stimulation, (Aspalter et al., 2003; Kim and Teh, 2001) and is necessary for the efficient clearance of adenovirus-infected hepatocytes (Kafrouni et al., 2003). More importantly, genetic studies have shown that TNFR2 is required for apoptosis of CD8⁺ cells during activation-induced cell death, whereas TNFR1 is not (Alexander-Miller et al., 1998; Kim and Teh, 2001; Zheng et al., 1995). Moreover, TNFR2 is a necessary mediator of HIV-mediated apoptosis of CD8⁺ cells (Herbein et al., 1998).

Because TNFR2-mediated killing is dependent on TNFR1 stimulation in many cell systems, our PC60 R1R2 cells are valuable for the analysis of TNFR2 signaling. Our results show that TNFR2, like TNFR1, uses the intracellular signal transduction molecule FADD to activate its death-inducing pathway. In agreement with FADD involvement in TNFR2 signaling, both TNF receptors can activate the caspase cascade, which was inhibitable by CrmA and the zIETD-fmk and zVAD-fmk peptide inhibitors. A major involvement of caspase-1 or the execution caspases-3 and -7 was excluded because neither zYVAD-cmk nor zDEVD-fmk inhibited TNF-mediated apoptosis. It has been reported previously that DEVD inhibitors do not block TNF-induced apoptosis in different cell lines (Gamen et al., 1996; Tafani et al., 2000). This observation may be cell-line-specific since in several other cell lines DEVD inhibitors prevent TNF-mediated apoptosis (Higuchi et al., 1997; Wissing et al., 1997).

Most probably, the caspase that is inhibited by CrmA, zVAD-fmk and zIETD-fmk in these pathways is caspase-8 (Zhou et al., 1997). In both Fas/Apo1- and TNFR1-induced apoptosis, the N-terminal DED of caspase-8 interacts with the homologous N-terminal DED of FADD, resulting in the activation of caspase-8 (Boldin et al., 1996; Muzio et al., 1996). We think that two alternative hypotheses may be formulated to explain the mechanism of involvement of FADD and caspase-8 in TNFR2-mediated apoptosis. Because TNFR2 does not contain a death domain and direct FADD-TNFR2 interaction is not reported and seems unlikely, other adaptor molecules would be needed to recruit FADD to the receptor. Indeed, Pimentel-Muinos and Seed showed FADD recruitment via RIP1 and TRAF2 to the TNFR2 signaling complex (Pimentel-Muinos and Seed, 1999). In addition, we also demonstrated that FADD, caspase-8 and RIP1 can be recruited to the same complex (Vanden Berghe et al., 2004). Since these data are based on overexpression studies, an alternative mechanism may exist. Recent publications have shown that FADD and caspase-8 are not recruited into the primary TNFR1 signaling complex, but only associate with TRADD in a secondary complex that does not contain the receptor (Harper et al., 2003; Micheau and Tschopp, 2003). It seems possible that TNFR2 induces a similar secondary signaling complex responsible for the induction of apoptosis in PC60 cells. Despite the fact that genetic and biochemical evidence indicates that TNFR2 can signal to cell death independently of TNFR1, the signaling complexes involved in TNFR2-induced apoptosis remain elusive and need further investigation.

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