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TNFα-Induced IEC-6 Cell Apoptosis Requires Activation of ICE Caspases whereas Complete Inhibition of the Caspase Cascade Leads to Necrotic Cell Death

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Tumor necrosis factor (TNF)α is considered to play a key pathogenetic role in inflammatory bowel diseases. In this study we analyzed the mechanisms by which TNFα induces intestinal epithelial cell apoptosis. TNFα alone, and more potently in combination with IFNγ, induced a high degree of IEC-6 cell apoptosis. This effect was more than 100-fold stronger if both of the TNF-R were stimulated, compared to stimulation of the p55-TNF-R alone, indicating an important apoptosis enhancing effect of the p75-TNF-R. TNFα-induced apoptosis required activation of ICE caspases and was completely abolished by its inhibitor, zVAD-fmk. Specific inhibition of caspase-3 with zDEVD-fmk did not alter the effect of TNFα. Western blot analyses confirmed that caspase-3 was not activated in response to TNFα. In the presence of complete inhibition of the caspase cascade with zVAD-fmk ($\geq$50 μM), TNFα induced cell necrosis rather than apoptosis. Our data reveal that TNFα can trigger enterocyte cell death via apoptosis or necrosis, depending upon the activation or blockade of specific caspases.

Tumor necrosis factor (TNF)α, a pleiotropic proinflammatory cytokine, is considered to play a crucial role in the pathogenesis of various inflammatory states, including idiopathic chronic inflammatory bowel disease (IBD) (1, 2). TNFα binds to two distinct membrane receptors, the p55 and p75 TNF-R, both of which are expressed by intestinal epithelial cells (3). In addition to their involvement in immune regulation and inflammation (4), the TNF-R have been implicated in the induction of apoptosis, activation of nuclear factor kappa-B and regulation of cell proliferation via mitogen-activated protein kinases or N-terminal c-Jun kinase (5–7).

Studies have shown that the p55 TNF-R is primarily responsible for TNF-induced apoptosis of target cells (8, 9). Analysis of the molecular mechanisms revealed that the cytoplasmatic death-domain (DD) of the p55 TNF-R is needed to trigger the apoptotic response upon stimulation (10). The interaction of the DD with intracellular adapter molecules, such as TRADD, FADD or others, allows the formation of a death-inducing signaling complex (DISC), resulting in rapid activation of pro-caspase-8 and subsequently, the caspase-cascade, leading to apoptotic cell death (10, 11). In contrast to the p55, the p75 TNF-R lacks an intracellular domain that induces apoptosis. To date, the contribution of the p75 TNF-R in mediating apoptosis is controversial and its signaling mechanisms remain unclear.

The cascade of intracellular aspartate-specific cysteinyl proteases is critical to apoptosis. These intracellular caspases are present as inactive zymogens that become activated after cleavage into two subunits in response to apoptotic stimuli. Recently, a model was proposed (12), dividing caspases into initiator proteases such as caspases-8 or -9, while others, such as caspases-3, -6 or -7, act as potent effectors of apoptosis. This latter group of caspases is responsible for the degradation of various enzymes and structural proteins involved in apoptotic cell death (12).

The aim of this study was to analyze the effect of TNFα on intestinal epithelial cell apoptosis and to identify the signaling mechanisms involved. The model employed was the IEC-6 cell line, composed of non-transformed immature crypt jejunal intestinal epithelial cells of rat origin (13). TNFα-induced apoptosis was more than 100-fold stronger if both TNF-R were stimulated, compared to stimulation of the p55 TNF-R alone, indicating an important enhancing effect of the p75 TNF-R. The apoptotic signaling required activation of ICE-caspases, but was independent of caspase-3. Complete inhibition of the caspase-cascade with zVAD-fmk switched the apoptotic response of TNFα into a necrotic form of cell death.

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MATERIAL AND METHODS

Cell culture and reagents. IEC-6 cells (passages 17–20, ATCC, Rockville, MD) were cultured at 37°C in a humidified atmosphere of 5% CO_2 in Dulbeco's Modified Eagle Medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) (Gibco, Grand Island, NY), 1 mM sodium pyruvate, 1% penicillin/streptomycin, as we previously described (14). The cell monolayers were stimulated with recombinant human (h) TNFα, which selectively stimulates the p55-TNF-R of rat cells without binding to the p75 TNF-R (15), or recombinant rat (r) TNFα, which stimulates both the p55 and p75 TNF-R (both from Genzyme, St-Louis, MO, at 0.01 to 1000 ng/ml). To control for the specificity of each cytokine source, neutralizing rabbit anti-human TNFα (Peprotech, Rocky Hill, NJ) or rabbit anti-rat TNFα (Genzyme) antibodies were employed (50 neutralizing U/ml). Further reagents used in this study were: recombinant rat IFNγ (0.1–100 U/ml, R + D Systems), caspase-3 specific inhibitor zDEVD-fmk, and the ICE-like caspase specific inhibitor zVAD-fmk (both from Kamyia, Thousand Oaks, CA), anti-caspase-3 antibodies (polyclonal, Pharmingen, Mississauga, Ont. and rat-specific from Santa Cruz, Santa Cruz, CA) which both recognize the native (32 kDa) and the proteolytically active forms (17 kDa). Horseradish peroxidase-labelled antibodies directed against mouse or rabbit immunoglobulins were obtained from Promega (Madison, WI), while anti-goat-IgG was from Biosource (Camarillo, CA). Propidium iodide (PI) and HOECHST 33342-DNA stains were from Sigma and Boehringer-Mannheim (Germany), respectively.

Apoptosis assays. IEC-6 cells were cultured in 24-well-plates (Falcon) at a density of $1–6 \times 10^5$ cells/ml. Cells were allowed to adhere overnight, after which the medium was changed. The cells were then treated for up to 48 h with 0.1 to 1000 ng/ml hTNFα or rTNFα alone, or combined with IFNγ (1–100 U/ml). After harvesting (floating cells included), the cells were stained with PI (5 μg/ml, Sigma, St.-Louis, MO) or the Hoechst 33342 DNA-dye (5 μg/ml) on ice and immediately analyzed by flow cytometry (PI staining), as well as by fluorescence microscopy (PI + Hoechst staining). In parallel, the TUNEL-assay (Boehringer-Mannheim) was used to confirm apoptotic cell death, as previously described (14). Furthermore, the expression of phosphatidylserine on the outer plasma membrane, characteristic of early apoptotic cells, was monitored using the Apo-Alert Annexin V kit (Clontech, Palo Alto, CA), according to the manufacturer's instructions. Apoptosis was then quantified by flow cytometry (FACScan, Becton Dickinson, Missisauga, ON). Dose-response studies showed that 50 ng/ml concentrations of rTNFα or IFNγ + hTNFα induced a submaximal rate of apoptosis Therefore, this concentration was used to perform inhibitor studies, unless otherwise indicated. To investigate the signaling pathways involved in TNFα-induced apoptosis, specific anti-caspase-3 (zDEVD-fmk) and anti-ICE-like caspases (zVAD-fmk) peptide-inhibitors, which interact with the active site of the cleaved caspases, were used (0.01–500 μM) under the same experimental conditions as above. To confirm the biological effect of these caspase-inhibitors, FAS-induced apoptotic Jurkat cells served as positive control cells (data not shown).

Immunoblotting. The activation of caspase-3 was determined by western blotting. TNFα stimulated IEC-6 cell lysates were prepared using an ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 1% Triton) with a mixture of protease inhibitors (Boehringer), as previously described (7). After determination of protein concentrations, equivalent samples were resolved on 14% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated overnight with either of two polyclonal anti-caspase-3-antibodies (1:1000, Pharmingen or 1:50, Santa Cruz) diluted in Tris-buffered saline/Tween-20-1% milk powder, followed by incubation with the corresponding alkaline phosphatase-conjugated antibody (anti-rabbit-IgG 1:2500, or anti-goat-IgG 1:5000). The bands were read by enhanced chemiluminescence (ECL-kit, Amersham). Both antibodies detected active caspase-3 as a 17 kDa band in IEC-6 cell lysates, when the membranes were overexposed, indicating minimal caspase-3 degradation under unstimulated conditions. Butyrate-induced apoptotic Caco-2 cells served as a positive control for caspase-3 detection (14).

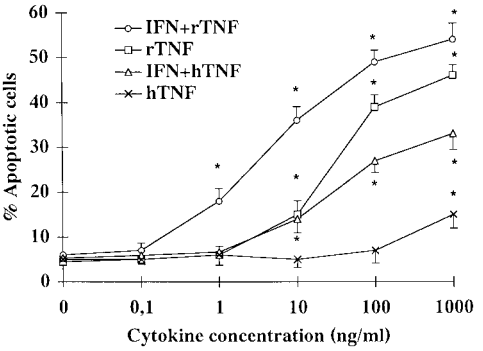

FIG. 1. TNFα-induced apoptosis in IEC-6 cells. Selective stimulation of the p55 TNF-R with hTNFα alone induced apoptosis only at very high concentrations. However, in response to rTNFα, which stimulates both the p55 and the p75 TNF-R, a high degree of apoptosis was observed, in a dose-dependent manner. In addition, co-stimulation with IFNγ rendered the cells more susceptible to TNFα-induced apoptosis with either h or rTNFα. Apoptosis was mesured by flow cytometry using Annexin-V staining, as described under Materials and Methods. The mean of six independent experiments ($\pm$ standard deviations) is shown. * $p < 0.01$.

Proliferation assays. IEC-6 cell growth was examined by monitoring changes in DNA synthesis, as measured by the incorporation of ^{3}H-thymidine (1 μCi/ml) into cellular DNA for 2 h prior to the termination of cultures, as we previously described (15).

Experimental design and statistical analysis. All apoptosis and proliferation experiments were performed in triplicate and were repeated at least ten times. Western blot experiments were repeated at least four times to ascertain reproducibility. Representative experiments or mean values $\pm$ standard deviation are shown, as indicated. Significance was established at 95%, and determined by the Mann-Whitney U-test.

RESULTS

Effect of TNFα on IEC-6 cell apoptosis. Recombinant rTNFα potently induced IEC-6 cell apoptosis, in a dose- and time-dependent manner, as analyzed by both the TUNEL-assay and Annexin-V staining (Fig. 1). To determine whether TNFα-induced IEC-6 cell apoptosis was mediated solely via the p55 TNF-R, as described for other cells, this receptor was selectively stimulated with hTNFα. As shown in Fig. 1, only a weak apoptotic response was observed despite high hTNFα concentrations (1000 ng/ml). At least 100-fold higher TNFα doses were required to induce IEC-6 cell apoptosis when the p55 TNF-R was stimulated alone, compared to the effect of rTNFα, which stimulates both TNF-R. Co-stimulation with IFNγ, alone without effect (15), ren-

dered IEC-6 cells more susceptible to TNFα-induced apoptosis (Fig. 1). IFNγ enhanced the apoptotic response to both hTNFα and rTNFα. However, no change in the constitutive expression of either the p55 or p75 TNF-R was observed after stimulation with IFNγ (data not shown). The apoptotic response of IEC-6 cells to isolated stimulation of the p55 TNF-R in the presence of IFNγ was still weaker than with co-stimulation of the p75 TNF-R by rTNFα. IEC-6 cell apoptosis in response to rTNFα was confirmed by morphological analysis (Fig. 2), as shown by nuclear condensation and fragmentation, with the formation of apoptotic bodies. These morphological findings were similar to that induced by IFNγ + hTNFα, via the p55 TNF-R alone (Fig. 2D).

TNFα-induced apoptosis requires activation of ICE caspases. In order to unravel the signaling mechanisms of TNFα-induced apoptosis, IEC-6 cells were stimulated with hTNFα or rTNFα alone or in combination with IFNγ in the presence of irreversible caspase-inhibitors. As shown in Fig. 3a, the ICE-like caspase inhibitor zVAD-fmk (0.01–10 μM) potently blocked the apoptotic effect of rTNFα alone, in combination with IFNγ, as well as the combination of IFNγ + hTNFα. In contrast, the caspase-3 specific peptide-inhibitor zDEVD-fmk failed to block rTNFα and IFNγ-facilitated TNFα mediated apoptosis. Only at zDEVD-fmk concentrations above 200 μM was an anti-apoptotic effect observed. In this dose range however, the peptide's effect is less specific and interaction with caspases other than the CPP-32 group cannot be excluded. In addition, western blot analysis revealed no proteolytic activation of caspase-3 in response to TNFα (Fig. 3b), typically seen as a 17 kDa band or indirectly, as diminished expression of the inactive caspase form (32 kDa).

Effect of complete caspase cascade inhibition on TNFα-induced IEC-6 cell death. Unlike the anti-apoptotic effect of zVAD-fmk at low concentrations ($\leq$10 μM), a dramatically increased number of dead cells in response to rTNFα (maximally 63%) was observed with zVAD-fmk doses of 50 μM or higher (Fig. 4). This cell death sensitizing effect was also observed after isolated stimulation of the p55 TNF-R with hTNFα, even at low cytokine concentrations which alone, failed to induce apoptosis. Morphological analysis of these cells showed no signs of apoptosis, but rather, necrotic cell death with swelling of the cell membrane and nucleus (Fig. 2E). In addition, no formation of apoptotic bodies was detected in these cells. No cytotoxic effect however, was seen with zVAD-fmk alone, at all doses tested. Control experiments with the DMSO solvent alone, or in combination with hTNFα or rTNFα, did not induce IEC-6 cell necrosis either. These results indicate that the observed effect was due to complete inhibition of the caspase-cascade at high zVAD-fmk concentrations. No shift towards necrotic cell death was observed in response to TNFα in the presence of high dose zDEVD-fmk, in contrast to zVAD-FMK.

TNFα stimulation of the p55 TNF-R is necessary and sufficient to induce IEC-6 cell growth. In contrast to the apoptotic response, which was more potent after simultaneous activation of both TNF-R, stimulation of the p55 TNF-R alone was sufficient to enhance IEC-6 cell proliferation. TNFα resulted in increased DNA synthesis, as evidenced by enhanced ^{3}H-thymidine incorporation (Fig. 5). The growth promoting effect of hTNFα and rTNFα was detectable at doses as low as 0.1 ng/ml, and was maximal at 10 ng/ml (291 $\pm$ 18% and 311 $\pm$ 30.6%, respectively, $p < 0.001$). This promitogenic effect was not significantly different when results were compared for hTNFα and rTNFα.

DISCUSSION

TNFα mRNA expression and production is increased in actively inflamed IBD tissue, and is believed to play a key role in the pathogenesis of these disorders (16). Recent reports on the successful use of anti-TNFα antibodies in the treatment of Crohn's disease further underscores the importance of this particular cytokine in the pathogenesis of this disorder (17). To date however, our understanding of the underlying molecular mechanisms as to how TNFα contributes to intestinal inflammation and to the tissue damage is incomplete. In this study, we demonstrate that TNFα is a potent inducer of crypt intestinal epithelial cell death.

The parallel use of recombinant hTNFα, which selectively stimulates the p55 TNF-R, and rTNFα, which stimulates both TNF-R (15), allowed us to analyze the distinct roles and signaling mechanisms of these receptors in mediating IEC-6 cell apoptosis. Costimulation of both TNF-R was significantly more potent in inducing IEC-6 cell apoptosis, compared to the p55 TNF-R alone. In order to explain these findings, ligand passing mechanisms or cross-talk between the two TNF-R have been proposed, rather than independent signaling via the p75 TNF-R (18). Using Hela transfectants expressing wild-type p75 TNF-R or deletion mutants, Weiss *et al.* (19) suggested that distinct intracellular signaling mechanisms are required for the synergistic activity between the two TNF-R. It was proposed that the interaction of TNF receptor-associated factor (TRAF)-2 and the p75 TNF-R is relevant to the enhancement of p55 TNF-R mediated cytotoxicity by the p75 TNF-R. Our data largely support this concept of receptor crosstalk. To induce IEC-6 cell apoptosis, over 100-fold higher TNFα doses were required when the p55 TNF-R alone was stimulated, compared to activation of both

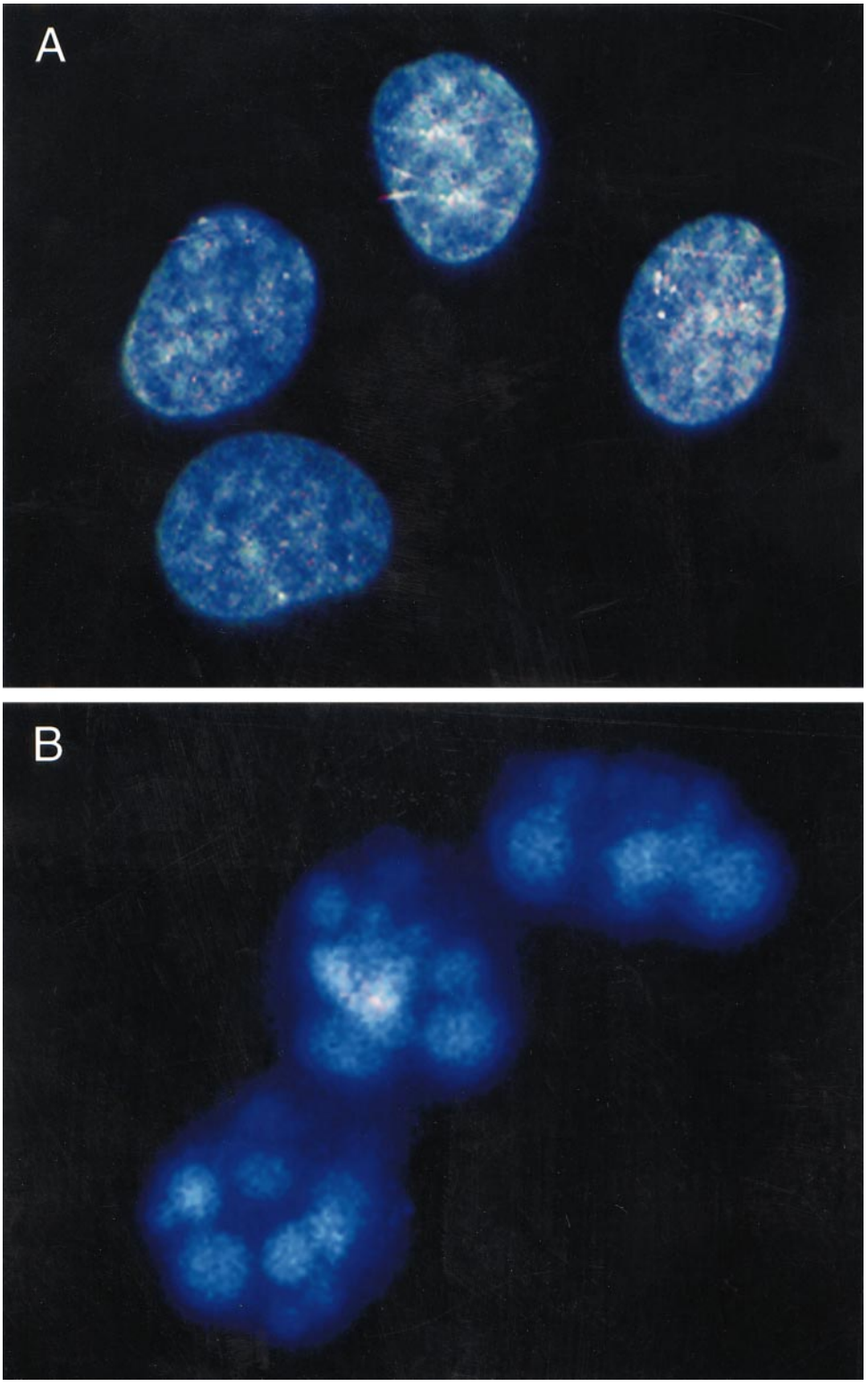

FIG. 2. Morphological analysis of TNFα-induced IEC-6 cell apoptosis. Staining with the HOECHST 33342 DNA dye was utilized in order to obtain a detailed morphological analysis confirming apoptosis. Control cells (A) displayed ovaloid nuclei with normal heterochromatin structure. In contrast, rTNFα-stimulated IEC-6 cells showed typical nuclear fragmentation with the formation of apoptotic bodies (B), or marked DNA-condensation (C), both characteristic of advanced stages of apoptosis. Similarly, IFNγ-facilitated p55 TNF-R triggered apoptotic cells were observed to be fragmented into typical apoptotic bodies (D). However, in the presence of the caspase inhibitor zVAD-fmk (100 μM), rTNFα-stimulated cells died by necrosis (E), characterized by swelling of the nuclei and loss of the normal chromatin structure.

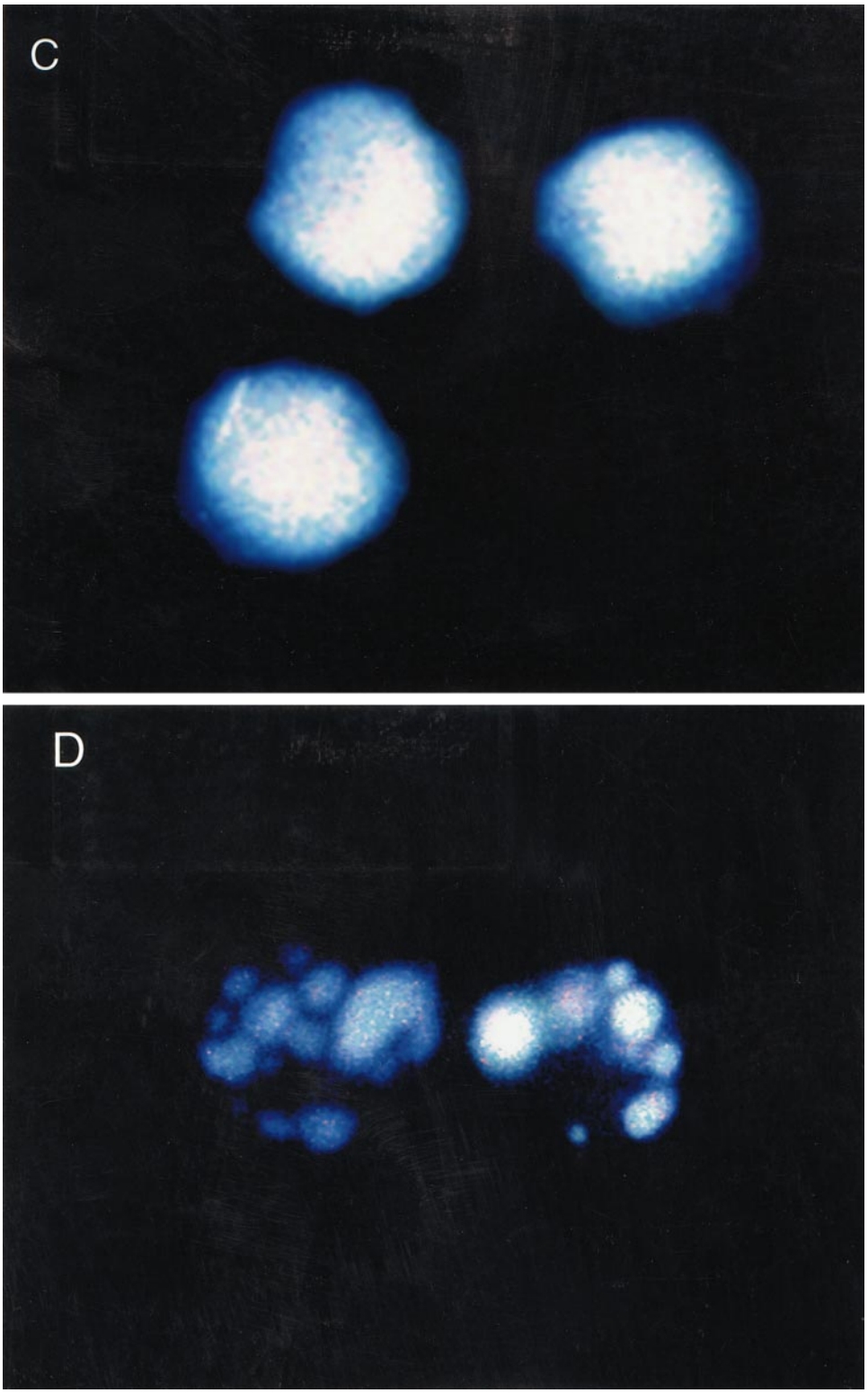

FIG. 2—*Continued*

TNF-R, confirming the enhancing effect of the p75 TNF-R. On the other hand, TNFα-induced proliferation, which was solely mediated via the p55 TNF-R, was similar in response to either cytokine, indicating comparable biological activities of the two recombinant factors.

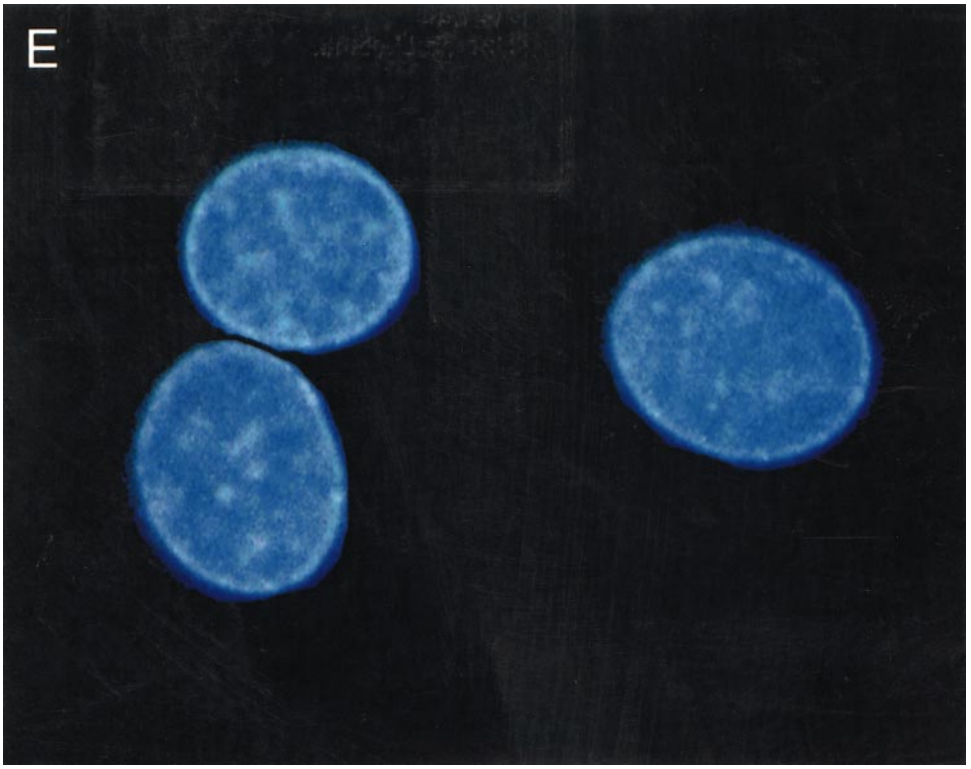

FIG. 2—*Continued*

In an attempt to gain insight into the signaling mechanisms underlying TNFα induced apoptosis in our enterocyte cell model, we selectively blocked different caspases, known to be essential to apoptosis (12). The specific caspase-3 inhibitor zDVED-fmk failed to block TNFα-induced apoptosis. A moderate anti-apoptotic effect was only observed when high concentrations of this inhibitor were used. However, non-specific inhibition of caspases other than the CPP-32 group cannot be excluded at these doses. Furthermore, western blot analyses confirmed that caspase-3 was not activated in response to TNFα, even after prolonged stimulation (48 h). Even in the presence of IFNγ, which markedly enhanced TNFα-induced apoptosis, no significant activation of caspase-3 was observed. Ossina *et al.* (20) recently showed that the sensitizing effect of IFNγ to TNFα-induced apoptosis in HT-29 colon cancer cells was mediated via increased gene expression of several caspases, such as caspase-3. We did not detect increased caspase-3 expression after stimulation of IEC-6 cells with IFNγ, as analyzed by western blots (data not shown). The expression of ICE-caspases was particularly enhanced by IFNγ in HT-29 cells (20). This is in accordance with our findings in IEC-6 cells, in that TNFα-induced apoptosis required activation of ICE-caspases and IFNγ potently enhanced the pro-apoptotic effect of TNFα. Treatment with the ICE-inhibitor zVAD-fmk completely abolished the apoptotic response to rTNFα and to a high degree, the IFNγ-enhanced apoptosis with co-stimulation by hTNFα or rTNFα. This is in keeping with the observation that TNFα-induced apoptosis required the activation of ICE-caspases to cleave of the membrane-associated cytoskeletal protein α-fodrin (21). Recently, Garcia-Calvo *et al.* (22) showed that zVAD-fmk is a broad-range inhibitor, binding to all caspases, although with varying affinity. At low doses, it selectively inhibits caspases-1, 4 and 11. At higher concentrations, it also inhibits caspases of the other groups and probably, yet unidentified caspases (23, 24).

We did not observe any differences in apoptotic signaling in response to isolated stimulation of the p55 or both TNF-R, supporting the hypothesis that the two receptors activate similar signal transduction pathways. Surprisingly, complete inhibition of ICE-caspases with zVAD-fmk at concentrations of 50 μM or higher markedly increased the number of dying IEC-6 cells in response to isolated p55 TNF-R stimulation or to the simultaneous activation of both TNF-R. Morphological analysis confirmed that the initially apoptotic cell death was switched to a necrotic form, likely due to inhibition of effectors essential to the apoptotic pathway. These findings expand upon the recent observations of FAS and TNF-induced dual signaling resulting in apoptosis or necrosis in the L929 cell model (25, 26). Vercammen *et al.* (25) proposed two different death

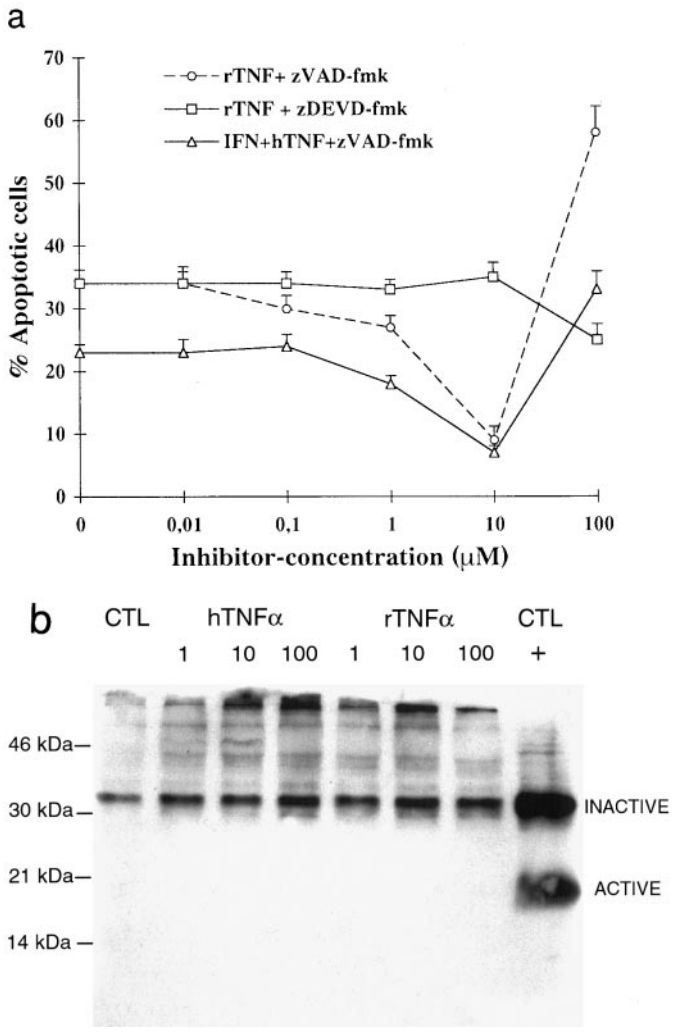

FIG. 3. IEC-6 cell p75 TNF-R-mediated apoptosis requires the activation of ICE caspases. (a) Specific inhibition of caspase-3 with zDEVD-fmk failed to abolish rTNFα (50 ng/ml)-induced IEC-6 cell apoptosis, whereas the ICE caspase inhibitor zVAD-fmk inhibited the apoptotic effect. However, at zVAD-fmk doses of 50 μM or higher, a marked increase in the number of dead cells in response to rTNFα was noted. Similarly, apoptosis in response to isolated stimulation of the p55 TNF-R (hTNFα, 50 ng/ml) in the presence of IFNγ (100 U/ml) was significantly suppressed by zVAD-fmk. Apoptosis was quantified by flow cytometry using the Annexin-V-assay. The mean data of six independent experiments performed in duplicate are shown ($\pm$ standard deviation). (b) Western blot analysis of hTNFα or rTNFα stimulated IEC-6 cell showed no proteolytic activation of caspase-3, confirming the observation that TNFα-induced apoptosis was independent of caspase-3. The positive control consisted of butyrate-induced apoptotic Caco-2 cells.

pathways originating from FAS: one rapidly inducing apoptosis, and if it is blocked, a second resulting in necrotic cell death involving mitochondrial generation of oxygen radicals. Similar conclusions might apply to the TNF-R pathways in the IEC-6 cell model. It is important to note that TNFα doses which failed to induce apoptosis potently triggered necrosis in the

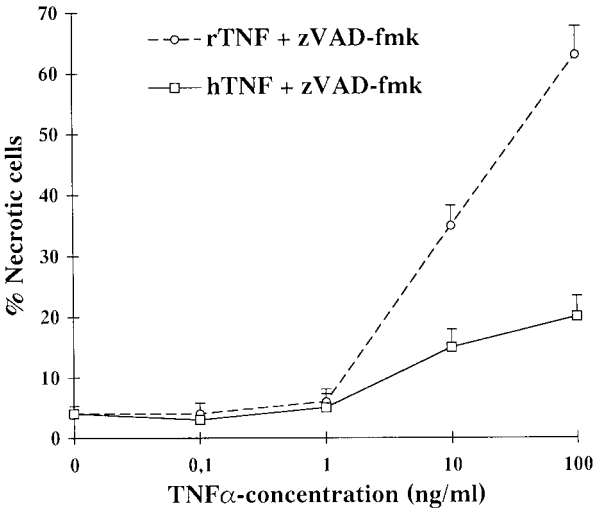

FIG. 4. TNFα-induced IEC-6 cell necrosis after complete inhibition of the caspase cascade. In the presence of high doses zVAD-fmk (100 μM), isolated stimulation of the p55 TNF-R, and more potently, stimulation of both TNF-R, induced IEC-6 cell necrosis in a dose-dependent manner.

presence of complete caspase-inhibition. This might be of particular relevance in view of potential clinical trials with specific caspase-inhibitors for GI-diseases.

In summary, TNFα is a potent inducer of enterocyte death. Examination of the underlying signaling mechanisms revealed that TNFα can activate two different cell death pathways. Apoptosis required activation of ICE-caspases, whereas the necrotic pathway occurred

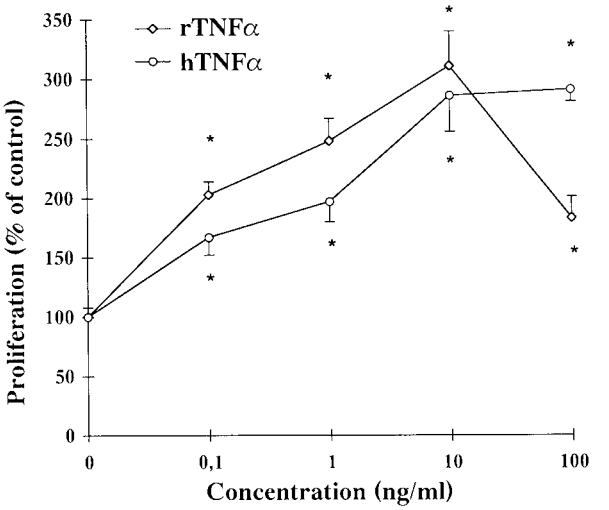

FIG. 5. TNFα-induced IEC-6 cell proliferation. Both r TNFα and hTNFα stimulated IEC-6 cell proliferation in a dose-dependent way, as determined by [^{3}H]thymidine incorporation. However, at rTNFα concentrations of 100 ng/ml, this trophic effect was due less to the simultaneous cytotoxic effect. Mean data of five independent experiments are shown ($\pm$ standard deviation). * $p < 0.05$ versus control.

only after complete inhibition of the caspase-cascade. These findings suggest that caspases may play a protective role against necrotic cell death in the intestinal epithelium.

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