NF-κB Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation

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Tumor necrosis factor α (TNF-α) binding to the TNF receptor (TNFR) potentially initiates apoptosis and activates the transcription factor nuclear factor kappa B (NF-κB), which suppresses apoptosis by an unknown mechanism. The activation of NF-κB was found to block the activation of caspase-8. TRAF1 (TNFR-associated factor 1), TRAF2, and the inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2 were identified as gene targets of NF-κB transcriptional activity. In cells in which NF-κB was inactive, all of these proteins were required to fully suppress TNF-induced apoptosis, whereas c-IAP1 and c-IAP2 were sufficient to suppress etoposide-induced apoptosis. Thus, NF-κB activates a group of gene products that function cooperatively at the earliest checkpoint to suppress TNF-α–mediated apoptosis and that function more distally to suppress genotoxic agent–mediated apoptosis.

TNF engagement of TNFR1 leads to the recruitment of TRADD (TNFR1-associated death domain protein) and RIP (receptor-interacting protein) to the receptor complex (*1*). TRADD interacts with FADD (Fas-associated death domain protein; also called MORT1, FADD-like ICE; also called MACH, Mort1-associated CED-3 homolog) with the death domain protein FADD on TNFR1 initiates the caspase cascade (*3*). Caspase-8 is activat-

apex of the caspase pathway and limits death domain protein signaling and caspase activation. Little is known about the regulatory events that render cells resistant to programmed cell death. The transcription factor NF-κB (*2*) inhibits the apoptotic response induced by TNF and other stimuli (*5, 6*). We now identify a mechanism to explain the ability of NF-κB to suppress apoptosis in response to TNF signaling or other apoptotic agents, thereby defining a mechanism to explain how cells can resist an apoptotic response.

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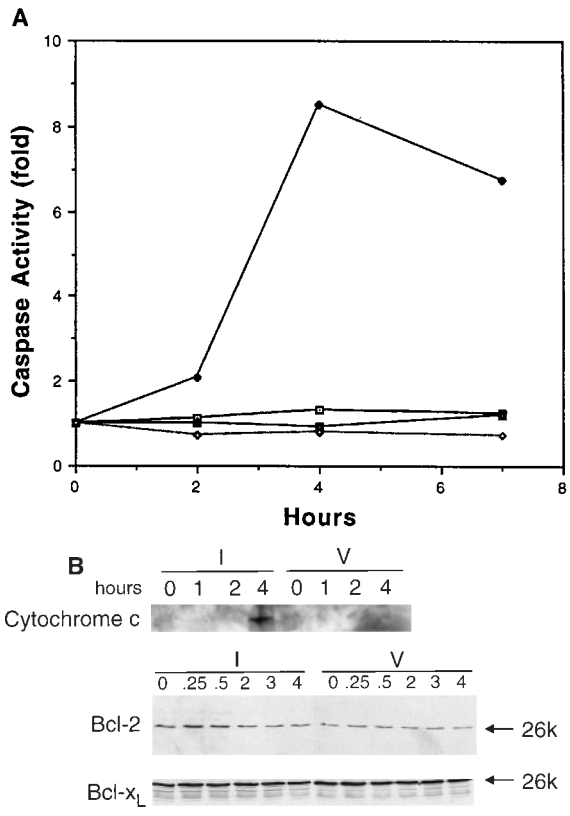

Fig. 1. The activation of caspases is inhibited in HT1080I cells but not in HT1080V cells after TNF treatment. (**A**) NF-κB blocks caspase activation. HT1080V and HT1080I cells were treated with TNF-α at 100 ng/ml for the indicated time periods. Cells were lysed in hypotonic buffer [20 mM tris-Hcl (pH 7.5), 1 mM EDTA, 100 μM phenylmethylsulfonyl fluoride, aprotonin (2 μg/ml), pepstatin (2 μg/ml), and leupeptin (2 μg/ml)], and the supernatants were incubated with DEVD-pNA (100 μM) and YVAD-pNA (100 μM) substrate. The reaction was measured with a plate reader at 405-nm wavelength. ◆, DEVD-specific caspase activity in HT1080I cells; □, DEVD-specific caspase activity in HT1080V cells; ◇, YVAD-specific activity in HT1080I cells; ■, YVAD-specific caspase activity in HT1080V cells. The results represent the average value from two independent experiments. (**B**) NF-κB activation blocks cytochrome c release from mitochondria. HT1080I and HT1080V cells were treated with TNF-α (100 ng/ml) for the indicated times. Cytosolic proteins were extracted and separated by 10% SDS–polyacrylamide gel electrophoresis. The blot was probed with monoclonal antibody to cytochrome c (1:1000). For detecting Bcl-2 and Bcl-x_L, whole-cell extracts were prepared and immunoblotted with antibodies to Bcl-2 or Bcl-x_L. Molecular mass is given at right in kilodaltons. (**C**) The processing of caspase-8 in response to TNF-α is inhibited by NF-κB activation. Cells were treated with TNF-α (100 ng/ml) for the indicated time periods. Whole-cell extracts (60 μg) were probed with antibody to caspase-8 (1:1000). For the internal control, the same extracts were also probed with antibody to tubulin (α-tubulin) (1:2000). (**D**) Caspase-8 protease activity is inhibited by NF-κB activation. Cells were treated with TNF-α (10 ng/ml) for 8 hours, and cytosolic extracts were prepared according to manufacturer's protocol (Chemicon). Three hundred micrograms of protein was incubated with IETD-pNA substrate. The reaction was measured with a plate reader at 405-nm wavelength. Solid bar, untreated control; hatched bar, treated with TNF-α. The results represent the average value from two independent experiments.

The HT1080 fibrosarcoma cell line (HT1080I) expresses a modified, super-repressor form of the NF-κB inhibitor IκBα that cannot be phosphorylated by the recently identified IκB kinases (*7*). This cell line, but not a control cell line, inhibits NF-κB nuclear function (*5*). Inhibition of NF-κB activation renders the HT1080I cells sensitive to TNF killing (*5*). The caspase family of proteins controls apoptosis induced by multiple stimuli such as Fas ligand and TNF (*8*). We investigated whether NF-κB inhibits TNF-mediated apoptosis through modulation of the caspase cascade. A known inhibitor of caspase-3, -7, and -8, DEVD (Z-Asp-Glu-Val-Asp-fluoromethylketone), and a broad spectrum caspase inhibitor, VAD (Z-Val-Ala-Asp-fluoromethylketone), blocked TNF-induced histone-associated DNA fragmentation in the HT1080I line, as determined by enzyme-linked immunosorbent assay (ELISA) (*9*). Similar results were obtained with embryonic fibroblasts from RelA/p65-null ($p65^{-/-}$) mice (*9*). As expected, TNF did not induce apoptosis in the parental HT1080V line or in wild-type embryonic fibroblasts (*9*). An in vitro substrate assay showed that DEVD-specific caspase activity was induced in the HT1080I line after TNF treatment, but not in the HT1080V line control (Fig. 1A). Thus, DEVD-specific caspases participate in TNF-mediated apoptosis when NF-κB is inhibited.

Caspase-3 (CPP32) is a critical downstream protease in the caspase cascade (*10*), responsible for cleavage of important substrates such as poly(adenosine diphosphate–ribose) polymerase (PARP) (*11*) and DNA fragmentation factor–45 [DFF-45; also called inhibitor of caspase-activated deoxyribonuclease, ICAD] (*12*). The addition of TNF to HT1080I cells resulted in the cleavage of the caspase-3 precursor within 5 hours, whereas caspase-3 proenzyme remained unchanged in the control HT1080V cells (*9*). The caspase substrates PARP and DFF-45 were almost totally cleaved after 8 hours of TNF treatment in the HT1080I line, but not in the HT1080V control cells (*9*). Cytochrome c is released from mitochondria during apoptosis and is a coactivator of caspase-3 (*13*). The antiapoptotic functions of Bcl-2 and Bcl-x_L involve the inhibition of cytochrome c release from mitochondria (*14*). Cytochrome c was released from mitochondria into the cytoplasm after a 4-hour TNF stimulation of HT1080I, but not HT1080V cells (Fig. 1B). Cytochrome c oxidase II was not detected in these extracts (*9*); thus, release was not due to nonspecific effects. NF-κB may, therefore, regulate Bcl-2 and Bcl-x_L proteins. However, the amount of Bcl-2 and Bcl-x_L remained unchanged after TNF treatment in both HT1080V and HT1080I cells (Fig. 1B). Thus, NF-κB controls an antiapoptotic mechanism upstream of the proteolytic activation of caspase-3 and upstream of mitochondria.

We examined whether NF-κB blocked apoptotic signaling directly at the activation of caspase-8, the apical caspase. In contrast with vector control cells, HT1080I processed caspase-8 within 4 hours of TNF addition (Fig. 1C), preceding the activation of caspase-3. The caspase-8 antibody we used did not detect the active subunit of caspase-8, but a specific inhibitor of caspase-8, IETD, inhibited TNF-mediated apoptosis and indicated that caspase-8 was activated by TNF in HT1080I cells (*9*). Both TRADD and FADD levels remained constant in HT1080I and HT1080V cells over the time course of the experiment (*9*). Additionally, an in vitro assay showed that caspase-8 protease activity was substantially induced by TNF-α only in the HT1080I cells (Fig. 1D). Thus, NF-κB activation suppressed the initiation of caspase activation by blocking the activation of caspase-8.

The inhibitor-of-apoptosis (c-IAP) proteins suppress caspase-1–induced apoptosis (*15*) and associate with TNFR1 (*16*). TRAF1 and TRAF2 associate with TNFR1 and control signal transduction pathways (*1*). The mRNAs for TRAF1 and c-IAP2 were essentially unexpressed in HT1080I cells but were induced by TNF in HT1080V cells (Fig. 2), indicating that

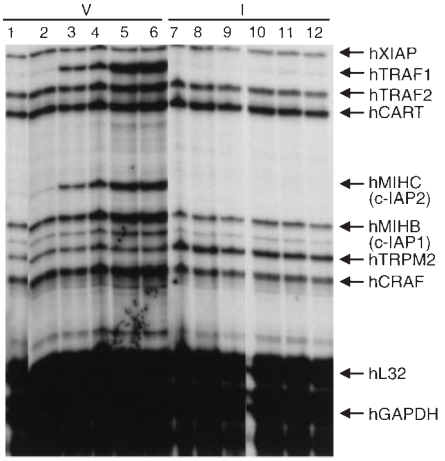

Fig. 2. TRAFs and c-IAPs are induced by TNF through activation of NF-κB. HT1080V and HT1080I cells were treated with TNF (20 ng/ml) with or without 25 μM VAD (to prevent the onset of apoptosis in HT1080I cells). Total RNA was extracted with the RNeasy kit (QIAGEN). The ribonuclease (RNase) protection assay was performed according to the supplier's instructions (PharMingen, San Diego, California). Briefly, human apoptosis template set hAPO-5 was labeled with [α-^{32}P]uridine triphosphate. RNA (10 μg), and 8×10^5 cpm of labeled probes were used for hybridization. After RNase treatments, the protected probes were resolved on a 5% urea-polyacrylamide-bis-acrylamide gel. Lanes 1 and 7, 2 and 8, 3 and 9, and 5 and 11 represent RNAs from cells treated with TNF-α for 0, 0.5, 1, and 4 hours, respectively. Lanes 4 and 10 and 6 and 12 were treated with the caspase inhibitor VAD (20 μM) for 1 and 4 hours, respectively. Lanes 1 to 6 represent RNA from HT1080V cells, and lanes 7 to 12 represent RNA from HT1080I cells. The nomenclature is from the supplier's kit. hTRPM2 stands for human testosterone–repressed prostate message–2, hCRAF for TRAF3, and hCART for TRAF4.

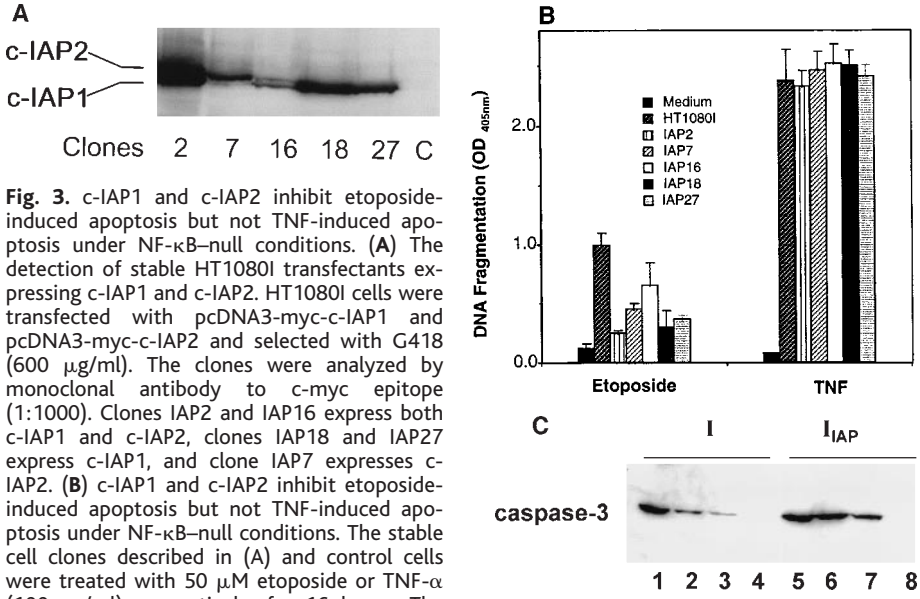

Fig. 3. c-IAP1 and c-IAP2 inhibit etoposide-induced apoptosis but not TNF-induced apoptosis under NF-κB–null conditions. (**A**) The detection of stable HT1080I transfectants expressing c-IAP1 and c-IAP2. HT1080I cells were transfected with pcDNA3-myc-c-IAP1 and pcDNA3-myc-c-IAP2 and selected with G418 (600 μg/ml). The clones were analyzed by monoclonal antibody to c-myc epitope (1:1000). Clones IAP2 and IAP16 express both c-IAP1 and c-IAP2, clones IAP18 and IAP27 express c-IAP1, and clone IAP7 expresses c-IAP2. (**B**) c-IAP1 and c-IAP2 inhibit etoposide-induced apoptosis but not TNF-induced apoptosis under NF-κB–null conditions. The stable cell clones described in (A) and control cells were treated with 50 μM etoposide or TNF-α (100 ng/ml), respectively, for 16 hours. The supernatants were collected and measured by cell death ELISA as described in Fig. 1A to measure apoptosis. $OD_{405\ nm}$, absorbance at 405 nm. (**C**) TNF and etoposide differentially induce the processing of caspase-3 in HT1080I (I) cells expressing c-IAP1 and c-IAP2 (I_{IAP}). Clone IAP2 described in (A) was used. Lanes 1 and 5, 2 and 6, and 3 and 7 represent cells treated with etoposide for 0, 4, and 8 hours, respectively. Lanes 4 and 8 represent cells treated with TNF-α for 8 hours. The processing of caspase-3 was detected by protein immunoblot with monoclonal antibody to caspase-3 (1:1000).

NF-κB activity controls the expression of these genes. Additionally, TRAF2 and c-IAP1 mRNAs were induced in HT1080V cells but had a reduced, basal expression in HT1080I cells (Fig. 2), indicating that NF-κB activation was also required for full induction of these genes. Virtually identical results were obtained in other cell types when NF-κB was inhibited (*9*). Thus, our data indicate that NF-κB controls the expression of TRAF1 and TRAF2 and c-IAP1 and c-IAP2.

c-IAP1 and c-IAP2 specifically inhibit the active forms of caspase-3 and -7 and also block the etoposide-induced processing of caspase-3 (*17*). c-IAP2 is activated by NF-κB but cannot inhibit TNF-mediated apoptosis in cells in which NF-κB is inhibited (*18*). v-Rel, the viral homolog of the NF-κB family member c-Rel, induces expression of an avian form of IAP1 (*19*). To compare the inhibitory potential of c-IAP1 and c-IAP2 relative to TNF- and etoposide-induced apoptosis, we established stable HT1080I cell lines that express c-IAP1, c-IAP2, or both (Fig. 3A). Some chemotherapeutic compounds activate NF-κB, and inhibition of NF-κB renders cells sensitive to these drugs (*5*). Etoposide activated NF-κB in HT1080V cells but not in HT1080I cells; activation of NF-κB inhibited the processing of caspase-3 and delayed cell death in response to etoposide (*9*). Expression of c-IAP1, c-IAP2, or both blocked the etoposide-induced apoptosis in HT1080I cells during the 16-hour time course (Fig. 3B). However, these stable transfectants were still susceptible to TNF-induced apoptosis (Fig. 3B). The etoposide-induced processing of caspase-3 was partially inhibited in the HT1080I cells expressing c-IAP1 and c-IAP2 as compared with the parental HT1080I cells (Fig. 3C). The TNF-induced processing of caspase-3 was not suppressed in the clone expressing c-IAP1 and c-IAP2 (Fig. 3C).

Transient expression of TRAF1, TRAF2, or both also had no suppressive effect on TNF-α–induced apoptosis in HT1080I cells (*9*). Therefore, HT1080I cells stably expressing different combinations of the TRAF and c-IAP proteins (Fig. 4A) were tested for their ability to suppress TNF-α–induced cell death. These cells expressed equivalent levels of TNFR1 (*9*). Expression of TRAF1 and c-IAP2 was sufficient to partially inhibit cell death (Fig. 4B) and to suppress caspase-8 activation (Fig. 4C). However, the expression of TRAF1 with c-IAP1 and c-IAP2 or the expression of TRAF1 and TRAF2 with c-IAP1 and c-IAP2 substantially enhanced the antiapoptotic response (Fig. 4B) and the suppression of the caspase-8 activity (Fig. 4C).

Transient expression assays with the LacZ expression vector and with expression vectors for TRAF1 and TRAF2 and c-IAP1 and c-IAP2 were performed in embryonic fibroblasts from $p65^{-/-}$ animals. The expression of the inducible TRAF and c-IAP proteins from the appropriate expression vectors was confirmed by protein immunoblot (*9*). The individual expression of TRAF1, TRAF2, c-IAP1, or c-IAP2 had little or no protective function on TNF-induced apoptosis in p65-null embryonic fibroblasts (Fig. 4D). Coexpression of c-IAP1, c-IAP2, TRAF1, and TRAF2 protected the cells from TNF-α–induced killing. However, coexpression of c-IAP1 and c-IAP2 with another TRAF (TRAF3) could not suppress cell death induced by TNF-α (Fig. 4D). As a positive control, the expression of the FADD dominant negative construct inhibited TNF-induced apoptosis. Thus, our data indicate that the NF-κB–controlled expression of TRAF1 and TRAF2 along with c-IAP1 and c-IAP2 provides maximum protection against TNF-induced apoptosis.

We show here that NF-κB activation serves as a primary mechanism to protect cells against an apoptotic stimulus such as TNF. In the case of TNF, the activation of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 functions to suppress apoptosis at the level of caspase-8. Consistent with our data, it has been shown that cells from TRAF2-null mice are more susceptible to killing induced by TNF plus cycloheximide (*20*) and that

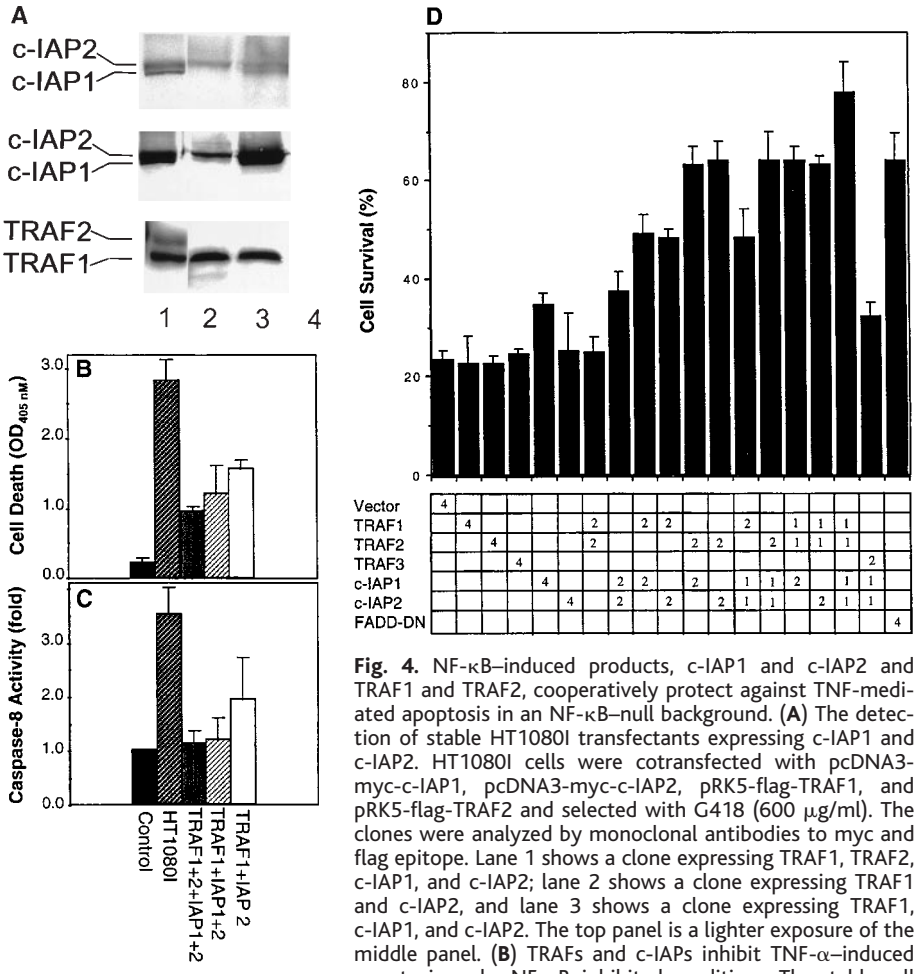

Fig. 4. NF-κB–induced products, c-IAP1 and c-IAP2 and TRAF1 and TRAF2, cooperatively protect against TNF-mediated apoptosis in an NF-κB–null background. (**A**) The detection of stable HT1080I transfectants expressing c-IAP1 and c-IAP2. HT1080I cells were cotransfected with pcDNA3-myc-c-IAP1, pcDNA3-myc-c-IAP2, pRK5-flag-TRAF1, and pRK5-flag-TRAF2 and selected with G418 (600 μg/ml). The clones were analyzed by monoclonal antibodies to myc and flag epitope. Lane 1 shows a clone expressing TRAF1, TRAF2, c-IAP1, and c-IAP2; lane 2 shows a clone expressing TRAF1 and c-IAP2, and lane 3 shows a clone expressing TRAF1, c-IAP1, and c-IAP2. The top panel is a lighter exposure of the middle panel. (**B**) TRAFs and c-IAPs inhibit TNF-α–induced apoptosis under NF-κB–inhibited conditions. The stable cell clones described in (A) and control cells were treated with TNF-α (10 ng/ml) for 8 hours. The supernatants were collected and measured by cell death ELISA as described in Fig. 3B to measure apoptosis. The results represent the mean value from three independent experiments. (**C**) TRAFs and c-IAPs inhibit TNF-α–induced caspase-8 activity. Cell extracts from (B) were incubated with IETD-pNA substrate. The reaction was measured as described in Fig. 1D. (**D**) Coexpression of c-IAP1, c-IAP2, TRAF1, and TRAF2 restores cell resistance to TNF-induced apoptosis in $p65^{-/-}$ embryonic fibroblasts. $p65^{-/-}$ cells were either cotransfected (with Superfect) with pcDNA3-lacZ (0.5 μg) and one or a combination of four expression constructs encoding c-IAP1, c-IAP2, TRAF1, and TRAF2 or with pcDNA3-lacZ and empty control vector as labeled on the figure. The amount of transfected plasmid was performed as indicated. The total amount of transfected plasmid DNA was kept at 4.5 μg. All experiments were performed in six wells of a 12-well plate. After 36 hours, cells were left untreated or were treated with TNF-α for an additional 16 hours. The attached cells were fixed with 0.5% glutaraldehyde for 15 min at room temperature and stained with X-Gal for 6 hours or overnight. The number of blue cells in four randomly chosen fields was counted. The results represent the average of three independent experiments. FADD-DN, FADD-dominant negative construct.

TRAF1 may inhibit antigen-induced apoptosis in a transgenic animal model (*21*). Also, NF-κB can regulate c-IAP2, but this protein is unable, on its own, to inhibit TNF-mediated apoptosis under NF-κB–null conditions but rather provides survival function through activating NF-κB (*18*). IAPs can inhibit cytochrome c–induced caspase activity and the proteolytic processing of caspase-3 (*17*). Consistent with this, c-IAP1 and c-IAP2 expression alone blocked etoposide-induced processing of caspase-3 and apoptosis in HT1080I cells. However, although cytochrome c release and caspase-3 activation occurred in TNF-mediated apoptosis in NF-κB–inhibited cells, the overexpression of c-IAP1 and c-IAP2 was insufficient to inhibit TNF-induced processing of caspase-3 and to render cells resistant to apoptosis. Thus, TNF and etoposide may have different caspase requirements to efficiently kill cells, which supports our conclusion that the recruitment of c-IAP1 and c-IAP2 to the receptor complex in response to TNF, presumably through interactions with TRAF1 or TRAF2, is required to inhibit the apical caspase, caspase-8. However, it is also possible that TNF-induced killing is somehow stronger than that induced by etoposide and requires inhibition at the apex of the cell death pathway. These observations underscore the importance of the activation of TRAF1 and TRAF2 as well as c-IAP proteins in suppressing TNF-induced cell death. The fact that most cells survive a TNF-α challenge supports the hypothesis that a rapid defense mechanism induced by the activation of NF-κB is required to block death signaling at the initiating and not at the executing stage of apoptosis because inhibiting the latter response may only delay cell death (*8, 14*).

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Biological Action of Leptin as an Angiogenic Factor

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Leptin is a hormone that regulates food intake, and its receptor (OB-Rb) is expressed primarily in the hypothalamus. Here, it is shown that OB-Rb is also expressed in human vasculature and in primary cultures of human endothelial cells. In vitro and in vivo assays revealed that leptin has angiogenic activity. In vivo, leptin induced neovascularization in corneas from normal rats but not in corneas from *fa/fa* Zucker rats, which lack functional leptin receptors. These observations indicate that the vascular endothelium is a target for leptin and suggest a physiological mechanism whereby leptin-induced angiogenesis may facilitate increased energy expenditure.

Leptin, a circulating hormone secreted by adipocytes, influences body weight homeostasis through effects on food intake and energy expenditure (*1*). It also modulates other physiological actions, including lipid metabolism, hematopoiesis, pancreatic β cell function, ovarian function, and thermogenesis (*2*). Despite this multiplicity of biological effects in extraneural tissues, the leptin receptor is expressed predominantly in the hypothalamus (*3*). Alternative splicing of a single transcript encoded by the *db* gene produces several variants of the leptin receptor, including a transmembrane full-length, long form (OB-Rb) expressed at high levels in discrete hypothalamic regions (*4*). The OB-Rb form has a cytoplasmic domain that transduces the leptin signal through the Jak-STAT pathway (*5*, *6*).

The discovery of leptin and its receptor strongly supports the hypothesis that adipose tissue mass is regulated by a hormone that is

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NF-κB Antiapoptosis: Induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to Suppress Caspase-8 Activation

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