

## **Nuclear Factor (NF)- $\kappa$ B-regulated X-chromosome-linked *iap* Gene Expression Protects Endothelial Cells from Tumor Necrosis Factor $\alpha$ -induced Apoptosis**

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### **Summary**

By differential screening of tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) and lipopolysaccharide (LPS)-activated endothelial cells (ECs), we have identified a cDNA clone that turned out to be a member of the inhibitor of apoptosis (*iap*) gene family. *iap* genes function to protect cells from undergoing apoptotic death in response to a variety of stimuli. These *iap* genes, *hiap1*, *hiap2*, and *xiap* were found to be strongly upregulated upon treatment of ECs with the inflammatory cytokines TNF- $\alpha$ , interleukin 1 $\beta$ , and LPS, reagents that lead to activation of the nuclear transcription factor  $\kappa$ B (NF- $\kappa$ B). Indeed, overexpression of I $\kappa$ B $\alpha$ , an inhibitor of NF- $\kappa$ B, suppresses the induced expression of *iap* genes and sensitizes ECs to TNF- $\alpha$ -induced apoptosis. Ectopic expression of one member of the human *iap* genes, human X-chromosome-linked *iap* (*xiap*), using recombinant adenovirus overrules the I $\kappa$ B $\alpha$  effect and protects ECs from TNF- $\alpha$ -induced apoptosis. We conclude that *xiap* represents one of the NF- $\kappa$ B-regulated genes that counteracts the apoptotic signals caused by TNF- $\alpha$  and thereby prevents ECs from undergoing apoptosis during inflammation.

Key words: activation • inhibitor of apoptosis gene family • endothelial cells • adenovirus • nuclear factor  $\kappa$ B

Endothelial cells (ECs) are located at the strategic interface between blood stream and tissue and regulate local exchange of cells and nutrients. They are critically involved in local and systemic inflammatory responses at the sites of transmigration of immune cells such as neutrophils, monocytes, and lymphocytes. The concentration of inflammatory cytokines at the site of transmigration is expected to be high, and in fact inflammatory cytokine-mediated activation of ECs is responsible for the attraction, adhesion, and extravasation of white blood cells to the inflamed tissue.

Stimulation of cells with TNF- $\alpha$ , a potent inflammatory cytokine, generates two types of signals: one that initiates programmed cell death (1), and one that leads to activation of the transcription factor NF- $\kappa$ B (2), and subsequently to the inflammatory response. The overall result in a specific cell type is dependent on the balance of the two signals. Direct inhibition of NF- $\kappa$ B or of the upstream parts of its signaling pathway during TNF- $\alpha$  activation results in apoptosis in a variety of cell types originally resistant to TNF- $\alpha$ -induced apoptosis (3, 4). Furthermore, fibroblasts and macrophages from NF- $\kappa$ B subunit p65-deficient mice are more sensitive to TNF- $\alpha$ -induced apoptosis (5). Therefore, it has been proposed that activation of NF- $\kappa$ B induces

the expression of genes that counteract apoptotic signals and prevent cell death.

Members of the inhibitor of apoptosis (*iap*) gene family have been demonstrated to suppress apoptosis induced by a variety of stimuli in different cell types (6–13, and for review see reference 14). The *iap* genes have also been shown to play a role in TNF- $\alpha$ -induced programmed cell death. Different *iap* gene family members appear to interfere with the cell death-triggering cascade at different levels. *hiap1* and *hiap2* can bind to the TNFR-associated factor 2 (TRAF2), a molecule that is associated with the cytoplasmic part of the TNFR complex and is essential for the activation of NF- $\kappa$ B (9, 15). Both have also been shown to be direct inhibitors of cell death proteases caspase 3 and caspase 7 (16). Another *iap* gene family member, the X-chromosome-linked *iap* (*xiap*), protects embryonic kidney 293T cells from bax-triggered apoptosis by inhibiting the same proteases, but in contrast it has not been found to be associated with members of the TRAF family (16, 17).

The studies presented here demonstrate that three human *iap* gene family members (*xiap*, *hiap1*, and *hiap2*) are strongly upregulated in TNF- $\alpha$ -stimulated primary ECs, which are resistant to TNF- $\alpha$ -induced apoptosis. How-

ever, adenovirus-mediated overexpression of I $\kappa$ B $\alpha$  (18, 19), an inhibitor of NF- $\kappa$ B, renders primary ECs sensitive to TNF- $\alpha$ -induced apoptosis and at the same time inhibits *iap* gene upregulation. Thus, *iap* gene expression appears to be dependent on NF- $\kappa$ B activation. Importantly, we show that ectopic expression of *xiap* is sufficient to overcome the I $\kappa$ B $\alpha$  effect in I $\kappa$ B $\alpha$ -overexpressing ECs and protects these cells from TNF- $\alpha$ -induced apoptosis.

## Materials and Methods

### Cell Culture

Cell culture flasks were coated with 1% gelatine for 30 min at 37°C. Human umbilical vein endothelial cells (HUVECs) and human skin microvascular endothelial cells (HSMECs) were grown in medium M199 supplemented with 20% bovine calf serum (HyClone, Logan, UT), endothelial cell growth factor supplement (Technoclone, Vienna, Austria), penicillin, streptomycin, fungizone, and heparin (3 U/ml). Confluent cells were split in a 1:3 ratio and used up to the sixth passage.

U937 cells were cultivated in RPMI-1640 medium supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. Cells were split 1:10 when grown to a density of 10<sup>6</sup> cells/ml.

### Northern Analysis

Total RNA was isolated using Trizol reagent (GIBCO BRL, Gaithersburg, MD). 10  $\mu$ g total RNA was separated on a 1.3% formaldehyde agarose gel. Samples were run in 0.02 M MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0, 5 mM sodium acetate, 1 mM EDTA. The gel was blotted overnight using 10 $\times$  SSC onto a GeneScreen Plus nylon membrane (Dupont-NEN, Boston, MA), dried, and fixed by UV-light (UV-cross-linker 120.000  $\mu$ J; Stratagene Inc., La Jolla, CA). Membranes were hybridized with  $\alpha$ -[<sup>32</sup>P]dATP-labeled (Terminal Transferase, Boehringer Mannheim, Mannheim, Germany) oligonucleotides specific for *hiap1* (5'-agaat-gtttcagtgccattcaatcaaccacaagatgtaagtgtgactcatgaagcttct-3'), *hiap2* (5'-aagattccaccacaaaagaaatcaatgatagactcttatgtagaattactacacttc-3'), *xiap* (5'-gaagggtgggtgggtgggaacaacacagctccctaggaagagcacaggatagtcacggggg-3'), and *naip* (5'-actgcatctaggccagaagagcagacagctctggcagcaattgtgacaaactgggaga-3') using Quickhyb-solution (Stratagene, Inc.) at 65°C. Membranes were washed twice for 15 min at room temperature in 1% SDS/3 $\times$  SSC/20 mM sodium phosphate buffer, pH 7.2, and twice for 30 min at 65°C in 1% SDS/1 $\times$  SSC. Signals were analyzed on a PhosphorImager SF (Molecular Dynamics, Sunnyvale, CA).

### Adenovirus Construction and Infection

Adenovirus I $\kappa$ B $\alpha$  has been described previously (26), and construction of *xiap* adenovirus was done by firstly introducing a fragment encoding the myc peptide sequence MEQKLISEEDL into the adenovirus transfer vector pACCMVpLpASR+ (20). Subsequently, a 1,600-bp BamHI/XbaI cDNA fragment containing the entire coding region of human *xiap* was ligated and the construct was cotransfected together with pJM17, a plasmid containing the adenoviral genome with a deletion in the E1 region into 293 cells (21). Plaques appearing after 10 d of culture were subcloned on 293 cells and were tested for *xiap* expression on immunoblots using anti-myc mAb 9E10 (22). Purification of a large batch of the recombinant adenovirus was done by two consecutive cesium chloride centrifugations as previously described (23).

Postconfluent HSMECs and HUVECs were washed once with complete PBS and incubated at a multiplicity of infection of

100 with the respective adenovirus constructs in PBS. After 30 min at 37°C, the adenovirus was washed off and fresh medium was added. Cells were maintained for an additional 2 d before being assayed.

### Analysis of DNA Fragmentation

**Electrophoresis of Genomic DNA.** Cells were incubated for 3 h at 55°C (100 mM NaCl, 10 mM Tris HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, 0.47 mg/ml Proteinase K), and then incubated with 100  $\mu$ g/ml RNaseA for 1 h at 37°C. After phenol-chloroform extraction and isopropanol precipitation, the DNA was dissolved in 50  $\mu$ l Tris/EDTA and resolved on 1.3% agarose gel.

**Quantification of Fragmented DNA.** For quantification of apoptosis fragmented DNA was determined by sandwich ELISA with antihistone coated microtiter plates and peroxidase-conjugated anti-DNA antibodies using the Cell Death Detection ELISA system from Boehringer Mannheim, according to the manufacturer's protocol.

### Flow Cytometry

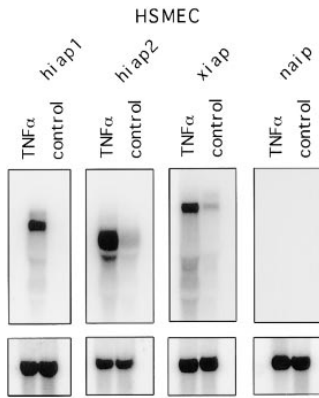
48 h after infection cells were treated with TNF- $\alpha$  (500 U/ml) for 6 h or left untreated. Cells were harvested, fixed in 70% ethanol, and the proportion of cells undergoing apoptosis was determined by flow cytometric analysis (FACSort<sup>®</sup>, Becton Dickinson, San Jose, CA) after staining with propidium iodide. Cells with a DNA content <2 N appear in the sub-G1 region (M1).

## Results and Discussion

Using a modified differential screening technique to identify and clone genes regulated by inflammatory mediators in porcine aortic ECs (PAECs) (23a) we have obtained a porcine homologue (*piap*) of the human *iap* gene family. Initially identified as a TNF- $\alpha$ -inducible gene, *piap* was found also to respond to the inflammatory stimuli LPS and to a lesser degree to IL-1 $\beta$ . Subsequently, we have tested whether members of the human *iap* gene family (*xiap* [*hILP*, *MIHA*], *hiap1* [*ciap2*, *MIHC*], and *hiap2* [*ciap1*, *MIHB*]; references 6–12) show similar responses to inflammatory cytokines. Using oligonucleotides specific for the different *iap* genes, we performed Northern blot analysis of HSMECs (Fig. 1) and HUVECs (data not shown). We demonstrate that, apart from the neuronal inhibitor of apoptosis (*naip*) that is not expressed in ECs, the *xiap*, *hiap1*, and *hiap2* genes were strongly upregulated in response to TNF- $\alpha$  in HSMECs and HUVECs.

Treatment of HSMECs or HUVECs with TNF- $\alpha$  for up to 24 h did not lead to apoptosis, whereas the well-established TNF- $\alpha$ -sensitive monocytic cell line U937 became apoptotic under these experimental conditions. *iap* gene expression has been shown to inhibit apoptosis induced by a variety of apoptotic stimuli (12). Thus, we speculated that induced *iap* gene expression may prevent ECs from undergoing programmed cell death in response to TNF- $\alpha$ .

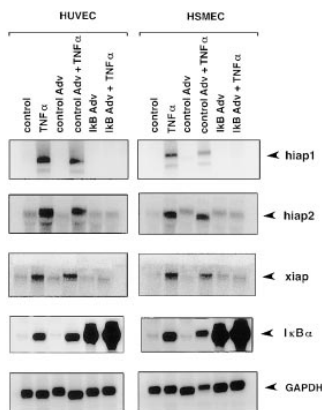
TNF- $\alpha$  is a proinflammatory cytokine whose pleiotropic biological effects are signaled through two distinct cell surface receptors, TNFR 1 and TNFR 2 (2). It is known to be a potent activator of NF- $\kappa$ B that has been shown to be the central mediator of gene regulation in the inflammatory



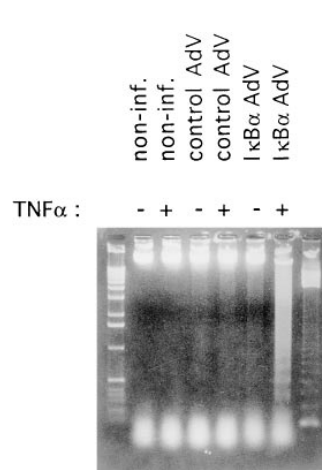
**Figure 1.** Northern blot analysis of *iap* gene expression in HSMECs. 10  $\mu$ g of total RNA from either nontreated or TNF- $\alpha$ -treated (500 U/ml) HSMECs was loaded in each lane and hybridized to oligonucleotides specific to *hiap1*, *hiap2*, *xiap*, and *naip*. The predicted transcript size corresponds to the published one (7) for *hiap1* (6.5 kb), *hiap2* (4.5 kb), and *xiap* (9 kb). To confirm the equal loading of RNA, membranes were stripped and reprobed with GAPDH.

response of activated ECs leading to leukocyte adhesion and thrombosis (24, 25). Therefore, we tested whether NF- $\kappa$ B was involved in upregulation of *iap* genes in response to inflammatory stimuli. Having shown previously that expression of I $\kappa$ B $\alpha$  from a recombinant adenovirus vector abolishes NF- $\kappa$ B-dependent upregulation of inflammatory genes such as IL-1 $\beta$ , IL-6, IL-8, and vascular cell adhesion molecule 1 in LPS-stimulated ECs (26), we used this adenovirus-I $\kappa$ B $\alpha$  construct to investigate whether NF- $\kappa$ B inhibition also impairs *iap* gene expression. HUVECs and HSMECs were infected with either a control adenovirus or the recombinant adenovirus I $\kappa$ B $\alpha$  (27). After 2 d, cells were stimulated with TNF- $\alpha$  for 4 h and probed for *xiap*, *hiap1*, and *hiap2* expression. As shown in Fig. 2, the expression of all three *iap* genes tested in adenovirus I $\kappa$ B $\alpha$ -infected ECs was suppressed, indicating that the upregulation of *iap* genes is controlled by activation of NF- $\kappa$ B.

We then raised the question whether blocking the activation of NF- $\kappa$ B would actually sensitize ECs to TNF- $\alpha$ -induced apoptosis. Indeed, ECs infected with the recombinant adenovirus I $\kappa$ B $\alpha$  construct started to die  $\sim$ 6 h after TNF- $\alpha$  stimulation. To demonstrate that the apoptotic program is involved in cell death, genomic DNA was isolated from dying cells. As shown in Fig. 3, genomic



**Figure 2.** Northern blot analysis of *iap* gene expression in adenovirus I $\kappa$ B $\alpha$ -infected ECs. HUVECs and HSMECs were not infected, were infected with a control adenovirus, or were infected with the recombinant adenovirus I $\kappa$ B $\alpha$  construct. Cells were either left untreated or treated with TNF- $\alpha$  (500 U/ml) for 4 h. The membranes were probed with oligonucleotides specific to *hiap1*, *hiap2*, and *xiap*. Expression of I $\kappa$ B $\alpha$  was controlled by reprobing the membranes with an I $\kappa$ B $\alpha$ -cDNA. Equal loading was confirmed by hybridization with a GAPDH cDNA probe. Adv, adenovirus.

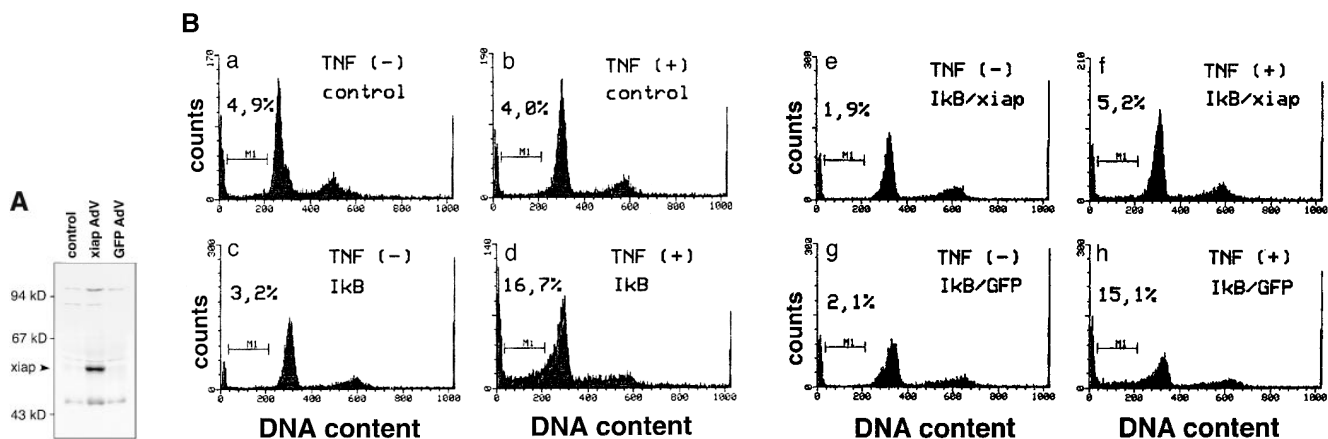


**Figure 3.** DNA fragmentation in adenovirus I $\kappa$ B $\alpha$ -infected and TNF- $\alpha$ -stimulated HUVECs. HUVECs were not infected, were infected, with a control adenovirus, or were infected with the recombinant adenovirus-I $\kappa$ B $\alpha$  construct. Noninfected cells and infected cells were left untreated or treated with TNF- $\alpha$  (500 U/ml) for 6 h. Appearance of fragmented genomic DNA was analysed by 1.3% agarose gel electrophoresis. Left lane: 1-kb ladder molecular weight standard; right lane: 123-bp ladder molecular weight standard. Non-inf.: noninfected cells; Adv: adenovirus.

DNA from I $\kappa$ B $\alpha$ -expressing and TNF- $\alpha$ -treated cells, but not from control virus-infected or nontreated cells, showed the DNA fragmentation pattern characteristic for apoptosis. Thus, inhibition of NF- $\kappa$ B activation renders ECs TNF- $\alpha$  sensitive, indicating that induction of apoptosis in ECs can occur independent of NF- $\kappa$ B.

These data suggested that TNF- $\alpha$ -induced expression of *iap* genes could be required to protect ECs from undergoing apoptosis. To directly demonstrate the ability of *iap* genes to prevent ECs from TNF- $\alpha$ -induced apoptosis, we coinfecting HUVECs with recombinant adenovirus constructs expressing myc-tagged *xiap* and I $\kappa$ B $\alpha$ , respectively. Infection with recombinant adenovirus I $\kappa$ B $\alpha$  alone and stimulation with TNF- $\alpha$ -induced apoptosis in HUVECs (Fig. 4 B, c and d). Coexpression of *xiap* and I $\kappa$ B $\alpha$  (Fig. 4 B, f) reduced the percentage of apoptotic cells to background levels obtained in TNF- $\alpha$ -treated or nontreated HUVECs (Fig. 4 B, a and b). A recombinant adenovirus expressing green fluorescent protein (27) was used as a control to show that adenovirus infection itself had no influence on apoptosis induced by TNF- $\alpha$  in I $\kappa$ B $\alpha$ -overexpressing cells (Fig. 4 B, g and h). Expression of myc-tagged *xiap* in infected HUVECs was demonstrated by Western blots stained with anti-myc mAb (Fig. 4 A).

Since the monocytic cell line U937 is sensitive to TNF- $\alpha$ -induced apoptosis when compared to primary ECs, we analyzed whether this cell line also differs with respect to TNF- $\alpha$ -inducible upregulation of *iap* genes. U937 and HUVECs were treated with TNF- $\alpha$  for 4, 6, and 9 h. At the same time points we monitored and quantified apoptosis by analysis of fragmented genomic DNA using an ELISA assay for histone-associated DNA fragments. Fig. 5 C shows that *xiap* gene was barely expressed in nontreated U937 cells and expression could not be induced by TNF- $\alpha$ . Consistently, U937 cells became significantly apoptotic after 4 h (Fig. 5 D). In contrast, as shown in Fig. 5, A and B, *xiap* was upregulated in HUVECs and no increase in fragmented DNA could be assayed in response to TNF- $\alpha$ . Identical results were obtained for *hiap1* and *hiap2* in U937 cells and HSMECs (data not shown).



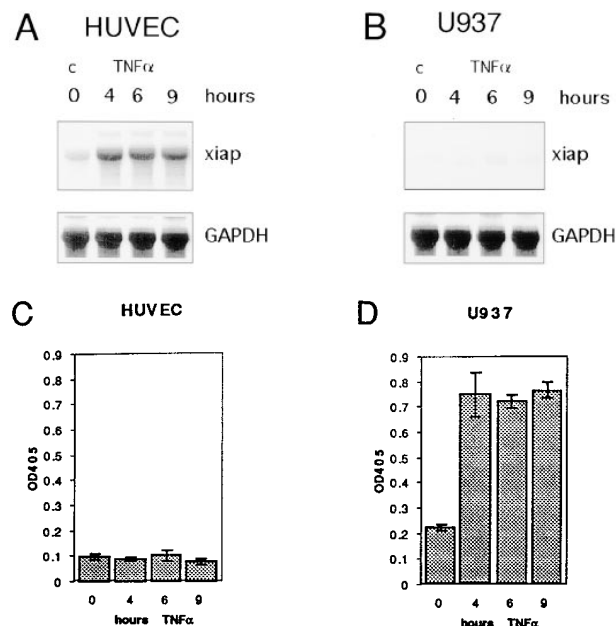
**Figure 4.** Inhibition of apoptosis by ectopic *xiap* expression. (A) Lysates of noninfected or infected HUVECs were separated by SDS-PAGE, blotted onto nylon membranes, and stained for myc-tagged XIAP protein. *AdV*, adenovirus; *GFP*, green fluorescent protein. (B) HUVECs were infected with IκBα alone (c and d), together with *xiap* (e and f), or together with GFP (g and h) recombinant adenovirus. 48 h after infection cells were treated with TNF-α (500 U/ml) for 6 h or left untreated and analyzed by FACS<sup>®</sup> after propidium iodide staining. Cells with a DNA content <2 N appear in the sub-G1 region (M1). The percentage of cells found in the M1 region is indicated. The data show one out of three representative experiments.

Our findings provide several lines of evidence that the *iap* gene products are regulated by NF-κB and that *xiap* appears to be sufficient to protect primary ECs from undergoing apoptosis in response to TNF-α: (a) *iap* genes are expressed in response to TNF-α, IL-1β, and LPS, respectively; (b) inhibition of NF-κB activation suppresses inducible *iap* gene expression; (c) inhibition of NF-κB activation by

overexpressing its inhibitor IκBα renders ECs sensitive to TNF-α-induced apoptosis; and (d) ectopic expression of *xiap* in IκBα-overexpressing ECs overrules the IκBα/TNF-α effect.

These data show that ECs and presumably other cells have developed cellular mechanisms that protect them from apoptosis and keep them able to function properly in an inflammatory situation. Fast activation of NF-κB in response to proinflammatory signals, like TNF-α, would be an appropriate mechanism to ensure the prompt expression of antiapoptotic gene(s). This hypothesis is supported by the demonstration that NF-κB p65 is necessary to protect fibroblasts from TNF-α-induced apoptosis (5).

Whether under physiological circumstances the expression of *xiap* is sufficient or whether simultaneous expression of all three *iap* genes (or other genes such as A20 [28], manganese superoxide dismutase [29], plasminogen activator-inhibitor type 2 [30], A1 [31], or other as yet undefined genes) is required to protect ECs from TNF-α-induced apoptosis remains open. Chu et al. (32) have shown recently that *hiap1* expression is dependent on activation of NF-κB in Jurkat cells and *hiap1* protein is able to protect these cells from apoptosis. However, in contrast to primary ECs, *hiap2* showed a steady state level of expression in Jurkat cells and was not controlled by NF-κB. The data indicate that expression of the *iap* gene family members and their involvement in protection from apoptosis varies in certain cell types and follows a rather complex scheme. *iap* gene expression appears to be specific for the cell type and the given stimulus. This view is supported by our finding that *iap* gene expression seems to be not involved in the TNF-α response of the monocytic cell line U937. These cells become partially apoptotic upon TNF-α treatment but do not express *iap* genes, suggesting that other protective mechanisms are operative. Recent reports demonstrated that *hiap1/2* can interfere at different levels with the apoptotic program. *hiap1* and *hiap2* associate via TRAF 2



**Figure 5.** Lack of TNF-α-inducible *xiap* gene expression correlates with apoptosis in U937 cells. Northern blot analysis of *xiap* gene expression in HUVECs (A) and U937 cells (C). HUVECs and U937 cells were treated for 4, 6, and 9 h with TNF-α. To confirm equal loading of RNA, membranes were stripped and reprobed with GAPDH. TNF-α-induced genomic DNA fragments from HUVECs (B) and U937 cells (D) were determined by colorimetric enzyme immunoassay. Columns represent the mean of three independent experiments. SD is indicated by error bars. TNF-α (500 U/ml); c, nontreated cells.

with the TNFR 2, leading to NF- $\kappa$ B activation (9), and *hiap2* is also part of the TNFR 1 signaling complex (15). On the other hand, *hiap1* and *hiap2* as well as *xiap* directly inhibit caspase 3 and caspase 7 activity, two members of the caspase family of cell death proteases, in embryonic kidney 293T cells (16, 17). However, inhibition by *xiap* is two to three orders of magnitude more potent, suggesting *xiap* as the physiological inhibitor of caspase 3 and 7 (16). These data and our finding that *xiap* expression is sufficient to prevent TNF- $\alpha$ -induced apoptosis in ECs support the concept that *xiap* plays a central role in inhibition of programmed cell death. It remains to be established whether *xiap* operates via an identical mechanism in ECs as in 293T cells and which other cell-type specific and stimulus-dependent mechanisms exist.

Unexpectedly, *iap* gene expression is also induced by LPS and IL-1 $\beta$ . Pretreatment of a human fibrosarcoma line (HT1080V) with the nonapoptotic, NF- $\kappa$ B-inducing IL-1 protects these cells from apoptosis induced by the later addition of TNF- $\alpha$  even in the presence of a protein synthesis inhibitor (3). In cells expressing a super-repressor form of the NF- $\kappa$ B inhibitor I $\kappa$ B $\alpha$ , IL-1 $\beta$  does not have this protective effect, suggesting that IL-1 $\beta$  also induces the expression of NF- $\kappa$ B-regulated antiapoptotic genes. A mechanism to overrule apoptotic signals during inflammation would enable ECs to respond properly by upregulation of inflammatory mediators such as tissue factor and adhesion molecules and at the same time to survive inflammation in order to maintain homeostasis of the inflamed tissue and initiate the healing process.

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