Brief Definitive Report

Nuclear Factor (NF)-κB-regulated X-chromosome-linked iap Gene Expression Protects Endothelial Cells from Tumor Necrosis Factor α-induced Apoptosis

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Summary

By differential screening of tumor necrosis factor α (TNF-α) 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 hiap were found to be strongly upregulated upon treatment of ECs with the inflammatory cytokines TNF-α, interleukin 1β, and LPS, reagents that lead to activation of the nuclear transcription factor κB (NF-κB). Indeed, overexpression of iκBα, an inhibitor of NF-κB, suppresses the induced expression of iap genes and sensitizes ECs to TNF-α-induced apoptosis. Ectopic expression of one of the human iap genes, human X-chromosome-linked iap (xiap), using recombinant adenovirus overrules the iκBα effect and protects ECs from TNF-α-induced apoptosis. We conclude that xiap represents one of the NF-κB-regulated genes that counteracts the apoptotic signals caused by TNF-α and thereby prevents ECs from undergoing apoptosis during inflammation.

Key words: activation • inhibitor of apoptosis gene family • endothelial cells • adenovirus • nuclear factor κB

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-α, 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-κB (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-κB or of the upstream parts of its signaling pathway during TNF-α activation results in apoptosis in a variety of cell types originally resistant to TNF-α-induced apoptosis (3, 4). Furthermore, fibroblasts and macrophages from NF-κB subunit p65-deficient mice are more sensitive to TNF-α-induced apoptosis (5). Therefore, it has been proposed that activation of NF-κB 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-α-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-κB (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-α-stimulated primary ECs, which are resistant to TNF-α-induced apoptosis. How-
ever, adenovirus-mediated overexpression of IκBα (18, 19), an inhibitor of NF-κB, renders primary ECs sensitive to TNF-α-induced apoptosis and at the same time inhibits iap gene upregulation. Thus, iap gene expression appears to be dependent on NF-κB activation. Importantly, we show that ectopic expression of iap is sufficient to overcome the IκBα effect in IκBα-overexpressing ECs and protects these cells from TNF-α-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 M 199 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 10^6 cells/ml.

Northern Analysis

Total RNA was isolated using Trizol reagent (GIBCO BRL, Gaithersburg, MD). 10 μg total RNA was separated on a 1.3% formaldehyde agarose gel. Samples were run in 0.2 M MOPS (3-[N-morpholino]propanesulfonic acid), pH 7.0, 5 mM sodium acetate, 1 mM EDTA. The gel was blotted overnight using 10× SSC onto a GeneScreen Plus nylon membrane (Dupont-NEN, Boston, MA), dried, and fixed by UV-light (UV-cross-linker 120,000 μJ; Stratagene Inc., La Jolla, CA). Membranes were hybridized with 32PdATP-labeled (Terminal Transferase, Boehringer Mannheim, Mannheim, Germany) oligonucleotides specific to hiap1 (5′-agagattcagctgcatctccaccaacagctgctc-3′), hiap2 (5′-aggacttctccacaaagaaatccctaggtgctagctacactccc-3′), hiap (5′-ggagaaggctggctgtgggagacacagcctggagggc-3′), and hiap2 (5′-agcagctggctgctgggagacacagcctggagggc-3′) using Quikhyb-solution (Stratagene, Inc.) at 65°C. Membranes were washed twice for 15 min at room temperature in 1× SSPE/3× SSC/20 mM sodium phosphate buffer, pH 7.2, and twice for 30 min at 65°C in 1× SSPE/0.1× SSC. Signals were analyzed on a Phosphorimager SF (Molecular Dynamics, Sunnyvale, CA).

A denovirus Construction and Infection

Adenovirus IκBα 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.

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-α-inducible gene, piap was found also to respond to the inflammatory stimuli LPS and to a lesser degree to IL-1β. Subsequently, we have tested whether members of the human iap gene family (xiap [hIAP, M I H A], hiap1 [iap2, M I H C], and hiap2 [iap1, M I H B]; 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-α in HSMECs and HUVECs. Treatment of HSMECs or HUVECs with TNF-α for up to 24 h did not lead to apoptosis, whereas the well-established TNF-α-sensitive monocytic cell line U 937 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 speculate that induced iap gene expression may prevent ECs from undergoing programmed cell death in response to TNF-α.

TNF-α 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-κB that has been shown to be the central mediator of gene regulation in the inflammatory
response of activated ECs leading to leukocyte adhesion and thrombosis (24, 25). Therefore, we tested whether NF-κB was involved in upregulation of iap genes in response to inflammatory stimuli. Having shown previously that expression of IκBα from a recombinant adenovirus vector abolishes NF-κB-dependent upregulation of inflammatory genes such as IL-1β, IL-6, IL-8, and vascular cell adhesion molecule 1 in LPS-stimulated ECs (26), we used this adenovirus-IκBα construct to investigate whether NF-κB inhibition also impairs iap gene expression. HUVECs and HSMECs were infected with either a control adenovirus or the recombinant adenovirus IκBα (27). After 2 d, cells were stimulated with TNF-α 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 IκBα-infected ECs was suppressed, indicating that the upregulation of iap genes is controlled by activation of NF-κB.

We then raised the question whether blocking the activation of NF-κB would actually sensitize ECs to TNF-α-induced apoptosis. Indeed, ECs infected with the recombinant adenovirus IκBα construct started to die ∼6 h after TNF-α 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 DNA from IκBα-expressing and TNF-α-treated cells, but not from control virus-infected or nontreated cells, showed the DNA fragmentation pattern characteristic for apoptosis. Thus, inhibition of NF-κB activation renders ECs TNF-α sensitive, indicating that induction of apoptosis in ECs can occur independent of NF-κB.

These data suggested that TNF-α-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-α-induced apoptosis, we coinjected HUVECs with recombinant adenovirus constructs expressing myc-tagged xiap and IκBα, respectively. Infection with recombinant adenovirus IκBα alone and stimulation with TNF-α–induced apoptosis in HUVECs (Fig. 4 B, c and d). Coexpression of xiap and IκBα (Fig. 4 B, f) reduced the percentage of apoptotic cells to background levels obtained in TNF-α–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-α in IκBα-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-α–induced apoptosis when compared to primary ECs, we analyzed whether this cell line also differs with respect to TNF-α–inducible upregulation of iap genes. U937 and HUVECs were not infected, were infected with a control adenovirus, or were infected with the recombinant adenovirus IκBα construct. Cells were either left untreated or treated with TNF-α (500 U/ml) for 4 h. The membranes were probed with oligonucleotides specific to hiap1, hiap2, and xiap. Expression of IκBα was controlled by reprobing the membranes with an IκBα cDNA probe. Equal loading was confirmed by hybridization with a GAPDH cDNA probe. AdV, adenovirus.
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 IkBα renders ECs sensitive to TNF-α-induced apoptosis; and (d) ectopic expression of xiap in IkBα-overexpressing ECs overrules the IkBα/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...
with the TNFR 2, leading to NF-κB activation (9), and hiap 2 is also part of the TNFR 1 signaling complex (15). On the other hand, hiap 1 and hiap 2 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-α-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-1β. Pretreatment of a human fibrosarcoma line (HT 1080V) with the nonapoptotic, NF-κB-inducing IL-1 protects these cells from apoptosis induced by the later addition of TNF-α even in the presence of a protein synthesis inhibitor (3). In cells expressing a super-repressor form of the NF-κB inhibitor IκBα, IL-1β does not have this protective effect, suggesting that IL-1β also induces the expression of NF-κB-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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