Interleukin-1 Protects Transformed Keratinocytes from Tumor Necrosis Factor-related Apoptosis-inducing Ligand- and CD95-induced Apoptosis but Not from Ultraviolet Radiation-induced Apoptosis*

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Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a new member of the tumor necrosis factor (TNF) family, induces apoptosis primarily of transformed cells. Interleukin-1 was previously found to protect the keratinocyte cell line KB from TRAIL-induced apoptosis, thus we studied whether interleukin-1 also protects from other apoptotic stimuli (ultraviolet radiation (UV), CD95-ligand). Interleukin-1 rescued KB cells from TRAIL- and CD95-induced apoptosis, which was critically dependent on nuclear factor-κB, because cells transfected with a super-repressor form of the nuclear factor-κB inhibitor IκB were less protected. In contrast, UV-mediated apoptosis was not only not prevented by interleukin-1 but even enhanced. This opposite effect of interleukin-1 was also observed for the expression of the inhibitor of apoptosis proteins (IAPs). Whereas TRAIL- and CD95-mediated suppression of IAP expression was partially reversed by interleukin-1, UV-mediated down-regulation of IAPs was not reversed but even further enhanced. Increased apoptosis induced by interleukin-1 plus UV was accompanied by excessive TNFα release, implying that enhanced cytotoxicity is due to the additive effect of these two apoptotic stimuli. Accordingly, enhanced apoptosis was reduced by blocking the TRAIL receptor-1. The opposite effects of interleukin-1 indicate that different mechanisms are involved in UV-induced apoptosis compared with CD95- and TRAIL-mediated apoptosis. Furthermore, the data suggest that whether a signal acts in an antiapoptotic way or not does not only depend on the signal itself but also on the stimulus causing apoptosis.

During apoptosis, a complex death program becomes initiated that ultimately leads to the fragmentation of the cell. The death program can be either initiated by the cell itself when the time has come to die (programmed cell death) or by certain external stimuli activating death receptors on the cell surface (for review, see Ref. 1). Thus, apoptosis does not only play an important role in the development and maintenance of tissue homeostasis but also represents an effective mechanism by which harmful cells can be eliminated. Induction of apoptosis allows the organism to get rid of infected cells and also of tumor cells. According to resistance to apoptosis was identified as an important event in tumors. Hyper. Apoptosis of tumor cells can be initiated by triggering cell death receptors, leading to activation of the intracellular apoptotic machinery (2). Chemotherapeutic drugs used in cancer treatment may exert their therapeutic effects by activating these pathways (3–5). On the other hand, it is known that defects in the apoptotic pathways or activation of antiapoptotic machineries can confer resistance to chemotherapy (for review, see Refs. 6 and 7). In addition, tumor cells can escape apoptotic elimination by down-regulation of apoptosis-related molecules on the cell surface (8, 9). Consequently, control of the balance between pro- and antiapoptotic processes within the cell has been recognized as an important target for therapeutic intervention. Thus, elucidation of the molecular mechanisms regulating these processes is of primary interest.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), one called APO-2 ligand, is a recently identified molecule belonging to the tumor necrosis factor (TNF) family that was characterized by its ability to induce apoptosis (10, 11). Among the TNF family members, TRAIL displays highest homology to CD95-ligand (CD95L), which induces apoptosis by triggering the surface receptor CD95 (Fas/APO-1) (12). In contrast to CD95L, TRAIL was found to induce apoptosis in numerous transformed cell lines but to kill normal cells less effectively (10, 11). Because of the unique ability to induce apoptosis preferentially in cancer but not in normal cells, TRAIL may be highly efficient in specifically eradicating tumor cells in vivo (13). Thus, TRAIL has prospects of becoming an effective anticancer drug of the future. Recently, we observed that preincubation with the pro-inflammatory cytokine interleukin-1 (IL-1) renders transformed keratinocytes resistant to the apoptotic effect of TRAIL (14). The protective effect of IL-1 against TRAIL-induced apoptosis seems to be mediated via activation of the transcription factor nuclear factor-κB (NFκB). The observation that transformed cells become resistant to TRAIL upon exposure to IL-1 was the first demonstration of a

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§ The abbreviations used are: TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; CD95-Ah, agonistic anti-CD95-antibody; CD95L, CD95 ligand; IAP, inhibitor of apoptosis protein; IL-1, interleukin-1; NFκB, nuclear factor-κB; PARP, poly(ADP-ribose) polymerase; TNF, tumor necrosis factor; PBS, phosphate-buffered saline.

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Fig. 1. IL-1 protects from TRAIL- and CD95-induced apoptosis but not from UV-induced apoptosis. KB cells were exposed to TRAIL (32 ng/ml), CD95-Ab (1 μg/ml), or UV light (300 J/m²) in the absence or presence of recombinant human IL-1β (10 ng/ml). Control cells (Co) were left untreated. Apoptosis was examined 16 h later by determining nucleosomal DNA fragmentation using an apoptosis determination kit. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown by the values on the y axis. Data presented show the representative results of one of three independently performed experiments.

pathway that allows tumor cells to escape the killing effect of TRAIL. Because IL-1 is secreted by a variety of tumor cells (15) and is also released by inflammatory cells participating in the host immune response (16), tumors under these conditions could become resistant to TRAIL in vivo.

Therefore, we were interested in expanding these observations by studying whether the antia apoptotic effect of IL-1 is restricted to TRAIL or whether other apoptotic stimuli are inhibited as well. Here, we show that IL-1 protects transformed keratinocytes from TRAIL- and CD95-mediated apoptosis. In contrast, apoptosis induced by ultraviolet (UV) irradiation was not only not prevented by IL-1 but was even further enhanced. This opposite effect of IL-1 was also observed when studying the expression of the antia apoptotic proteins c-IAP1 and c-IAP2. Thus, these data suggest that antia apoptotic activity of a stimulus does not only depend on its nature but also on the stimulus causing apoptosis.

EXPERIMENTAL PROCEDURES

Cells—The epitheloid carcinoma cell line KB (American Type Culture Collection, Manassas, VA) and the spontaneously transformed human keratinocyte cell line HaCat (kindly provided by N. Fusenig, Deutsches Krebsforschungszentrum, Heidelberg, Germany) (17) were cultured in RPMI containing 10% fetal calf serum and 1% glutamine at 37 °C with 5% CO₂ in a humidified atmosphere. Irradiation of cells with UV light was performed using a bank of 4 FS20 bulbs (Westinghouse Electric Corp., Pittsburgh, PA) which emit most of their energy within the UVB range (290–320 nm) as described (18). Subconfluent cells were exposed through PBS to a dose of 300 J/m², unless otherwise stated.

Reagents—Recombinant human TRAIL protein was provided from Immunex Corp., Seattle, WA. This is a leucine zipper form of TRAIL that requires no further cross-linking for induction of maximal apoptotic activity (19). Recombinant CD95L and an agonistic antibody against CD95 (CD95-Ab) were obtained from Alexis and Immunotech, respectively. Antibodies directed against caspase-3 and poly(ADP-ribose) polymerase (PARP) were obtained from Dianova, Hamburg and Roche Molecular Biochemicals, Mannheim, Germany, respectively. Antibodies directed against c-IAP1, c-IAP2, and the fluorescein isothiocyanate-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Recombinant human IL-1β was obtained from Roche Molecular Biochemicals. TNFα was measured by use of an ultrasensitive TNFα enzyme-linked immunosorbent assay (Diaclone, Besancon, France). Plasmids allowing overexpression of a mutated IxB variant were kindly provided by K. Schulze-Osthoff, Münster, Germany (20).

Detection of Cell Death—For the detection of DNA fragmentation, a cell death detection enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) was used. The enrichment of mono- and oligonucleosomes released into the cytoplasm is calculated using the formula: absorbance of sample cells/absorbance of control cells. Enrichment factor was used as a parameter of apoptosis and shown on the y axis as mean ± S.D. of triplicates.

Quantitation of apoptosis by annexin V binding was performed using a commercially available kit (Bender Corp., Vienna, Austria). Briefly, cells were washed and resuspended in annexin V binding buffer. Fluorescein isothiocyanate-conjugated annexin V was added, and the samples were analyzed by flow cytometry (Epics XL, Coulter, Miami, FL).

Western Blot Analysis—Cells were harvested and lysed in RIPA buffer (10 mM Tris, pH 8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 1 mM sodium orthovanadate) for 15 min on ice. After centrifugation, supernatants were collected, and the protein content measured using a Bio-Rad Protein Assay kit (Bio-Rad). Protein samples were subjected to SDS-polyacrylamide gel electrophoresis, blotted onto nitrocellulose membranes, and incubated with antibodies of interest. To monitor equal loading, membranes were reprobed with an antibody directed against α-tubulin (Pharmingen, San Diego, CA). Signals were detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

Transfection—Cells (1.5 × 10⁵) were washed once with PBS and resuspended in 600 μl of PBS, 1.25% Me₂SO. Cells were electroporated with 20 μg of each plasmid DNA according to the method described by Melkonyan et al. (21). Transfection efficiency of cells cotransfected with a plasmid encoding β-galactosidase (pcDNA6/V5-His/αCoE, Invitrogen, San Diego, CA) was determined 24 h later by staining with X-gal (100 μg/ml) in 5 mM potassium ferriyanide, 5 mM potassium ferrocyanide, and 1 mM MgCl₂ in PBS.

Staining of Intracellular Proteins—Aliquots of cells (2 × 10⁵) were harvested 16 h after stimulation, washed once with PBS, and fixed with 0.8% parafomaldehyde for 5 min on ice. After washing, cells were treated with 0.3% saponine for 5 min on ice. Following centrifugation, cells were resuspended with the antibodies directed against c-IAP1 or c-IAP2 in 0.3% saponine at 4 °C overnight. Purified goat IgG was used as an isotype control. None of the stimuli used (TRAIL, CD95-Ab, UV radiation, IL-1) changed the isotype controls. Cells were washed with PBS and incubated with the respective fluorescein isothiocyanate-conjugated secondary antibody in 0.3% saponine for 30 min. Cells were washed, resuspended in 0.03% saponine/PBS, and subsequently analyzed in a flow cytometer.

RESULTS

IL-1 Protects Transformed Keratinocytes from TRAIL- and CD95-induced Apoptosis but Not from UV-induced Apoptosis—Because we had recently observed that IL-1 protects cells from TRAIL-induced apoptosis (14) we were interested in studying whether this effect is specific for TRAIL or whether IL-1 also protects cells from other apoptotic stimuli. Therefore, KB cells were exposed to TRAIL or to an agonistic CD95-Ab, which induces apoptosis via activation of CD95 (12). Both TRAIL and CD95-Ab induced apoptotic cell death of KB cells, as determined by a cell death detection enzyme-linked immunosorbent assay (Fig. 1). When KB cells were preincubated with IL-1 for 15 min, cells were almost completely protected from the apo-
Opposite Effects of IL-1 on Apoptosis

Caspase-3 is a member of the family of interleukin-1β-activated caspases (26). Thus, caspase-3 cleavage can serve as an additional read-out system to evaluate apoptosis. Therefore, KB cells were exposed to TRAIL, CD95-Ab, or to UV light either in the absence or presence of IL-1. Cell lysates were prepared 16 h later for Western blot analysis using an antibody against caspase-3. Because this antibody is directed against the caspase-3 proform, it cannot recognize the processed 17-kDa form, and a loss of the immunoreactive band in samples in which caspase-3 is activated can be observed. Significant reduction of the caspase-3 proform was observed in protein extracts of KB cells that were treated either with TRAIL, CD95-Ab, or UV light (Fig. 3). Preincubation of cells with IL-1 almost completely prevented TRAIL- and CD95-Ab-induced caspase-3 activation. In contrast, IL-1 enhanced UV-induced caspase-3 activation, which is shown by the complete disappearance of the immunoreactive band.

Caspase-3 cleaves the death substrate PARP (27). Accordingly, PARP was cleaved from its intact 116-kDa form into the inactive 85-kDa fragment in samples of TRAIL-, CD95-Ab- and UV-exposed KB cells (Fig. 3). IL-1 pretreatment significantly reduced TRAIL- and CD95-Ab-induced PARP cleavage, whereas UV-mediated cleavage of PARP was again enhanced in the presence of IL-1. Taken together, these data clearly demonstrate that IL-1 rescues cells from CD95- and TRAIL-induced apoptosis, whereas UV-induced apoptosis is even enhanced by IL-1.

The Protective Effect of IL-1 Is Critically Dependent on NFκB—We recently proposed that IL-1 might rescue cells from TRAIL-induced apoptosis via activation of NFκB, because IL-1 activated NFκB in KB cells. Furthermore, inhibition of NFκB activation by the proteasome inhibitor MG132 antagonized the protective effect of IL-1 (14). Activation of NFκB is associated with degradation of the inhibitory protein IκB by the proteasome pathway. Upon triggering of the IL-1 receptor, IκB becomes phosphorylated at the serine residues 32 and 36, which acts as a signal for ubiquination and subsequent degradation of IκB by the 26S proteasome (28, 29). Thus, IκB degradation and consequently NFκB activation can be blocked by proteasome inhibitors such as MG132 or lactacystin (29). However, the approach using proteasome inhibitors provides only indirect evidence that the protective effect of IL-1 is due to activation of NFκB, because one cannot exclude that the inhibitors like MG132 may affect other pathways as well. Thus, we addressed whether IL-1 protects KB cells from TRAIL- and CD95-mediated apoptosis via activation of NFκB by overexpressing a super-repressor form of IκB. In this mutant form, two point mutations (Ser-32 → Ala, Ser-36 → Ala) prevent phosphorylation and subsequent proteasomal degradation of IκB (20). As a
consequence, NFκB release, nuclear translocation, and functional DNA binding is prevented. Whereas KB cells transfected with the empty cytomegalovirus vector were still rescued by IL-1, the protective effect of IL-1 on TRAIL- and CD95-induced apoptosis was significantly reduced in cells transfected with the IκB super-repressor (Fig. 4). These results support the concept that IL-1-mediated protection from TRAIL- and CD95-induced apoptosis is dependent on NFκB activation.

Differential Regulation of Antiapoptotic Proteins by IL-1—Because IL-1 protected KB cells from CD95- and TRAIL-induced apoptosis but enhanced UV-mediated cell death, we were interested in elucidating the mechanism underlying this heterogeneous effect. It has recently been reported that NFκB may exert its antiapoptotic effect via induction of several proteins including the inhibitor of apoptosis proteins (IAP) c-IAP1 and c-IAP2 (30). Therefore we investigated how IL-1 affects c-IAP1 and c-IAP2 expression in KB cells exposed to the different apoptotic stimuli. Intracellular protein expression evaluated by fluorescence-activated cell sorter analysis revealed that KB cells express c-IAP1 and c-IAP2 constitutively (Fig. 5). Exposure of KB cells to either TRAIL, CD95-Ab, or UV light caused a down-regulation of both c-IAP proteins. IL-1 alone only marginally enhanced constitutive c-IAP1 and c-IAP2 expression (data not shown), but reversed CD95- and TRAIL-induced down-regulation of c-IAP1 and c-IAP2 partially (Fig. 5). The opposite effect was exerted by IL-1 when cells were exposed to UV light. In this case, IL-1 did not reverse reduced c-IAP1 and c-IAP2 protein levels, but even slightly further enhanced the down-regulation. Taken together, IL-1 appears to affect c-IAP levels differentially depending on the stimulus that induces apoptosis. The further reduction of c-IAP expression in UV-exposed cells by IL-1 might be an explanation why IL-1 enhances UV-induced apoptosis.

Enhancement of UV-induced Apoptosis by IL-1 Is Due to Endogenous Release of TNFα—TNFα can generate two types of signals, one that induces apoptosis (31) and one that activates NFκB (32). The overall result in a specific cell type under specific conditions appears to be dependent on the balance of the two signals (32). When KB cells were treated with TNFα in addition to TRAIL, an enhancement of apoptosis was observed (data not shown), indicating that under these conditions the death signal caused by TNFα overrules activation of NFκB. Consequently, both stimuli induce apoptosis in an additive way. Because TNFα can be released by KB cells (18) we looked into whether the enhancing effect of IL-1 on UV-induced apo-
Opposite Effects of IL-1 on Apoptosis

Recently, it was reported that CD95 is critically involved in UV-mediated apoptosis (24, 39). UV radiation can directly activate the death receptor by inducing functional aggregation of CD95 (24, 39). Inhibition of CD95 clustering following UV exposure reduces but does not completely block apoptosis (24), implying that besides the CD95 pathway other mechanisms must be involved as well. This assumption is supported by the present findings: if UV-induced apoptosis was to be exclusively mediated by CD95, both CD95- and UV-induced apoptosis would be inhibitable by the same interventions. In this case, IL-1 should have protected KB and HaCaT cells not only from CD95- but also from UV-induced apoptosis.

According to recent reports (30, 33, 41, 42), NFkB appears to exert its antiapoptotic effects via the induction of antiapoptotic proteins, including c-IAP1, c-IAP2, X-linked IAP, and IEX-1L. Therefore, we were interested in the effect IL-1 may exert on the expression of IAPs in cells exposed either to TRAIL, CD95-Ab, or UV light. Intracellular protein measurements revealed that all three apoptotic stimuli reduced levels of c-IAP1 and c-IAP2, although at different levels. c-IAP1 and c-IAP2 appear to exert their antiapoptotic activity by specifically binding to the terminal effector domains of caspase-3 and -7 (43). In contrast to the mode of action of these proteins, little is known about how c-IAPs are regulated, except that they are under control of NFkB and that c-IAP2 can exert a positive feedback control on NFkB via an IxB targeting mechanism (44). This study for the first time demonstrates negative regulation of c-IAP expression by apoptotic stimuli. Because this was assessed only by determining the protein expression, we do not as yet know whether this inhibition is transcriptionally regulated. Although the mechanisms by which c-IAPs are down-regulated remain to be determined, this phenomenon might be of rele-

**TABLE I**

| Treatment | KB     | HaCaT    |
|-----------|--------|----------|
| 0 ng/ml   | 0.49   | 0.5      |
| IL-1      | 0.80   | 1.00     |
| TRAIL     | 0.41   | 0.46     |
| IL-1 + TRAIL | 0.88 | 0.97     |
| CD95-Ab   | 0.48   | 0.50     |
| IL-1 + CD95-Ab | 0.90 | 0.95     |
| UV        | 1.00   | 10.00    |
| IL-1 + UV | 55.00  | 70.00    |

*KB and HaCaT cells were left untreated (−) or exposed to TRAIL (32 ng/ml), CD95-Ab (1 µg/ml), or UV radiation (300 J/m²) in the absence or presence of IL-1β (10 ng/ml).

**FIG. 6.** Enhancement of UV-induced apoptosis by IL-1 is inhibited by blocking the TNF receptor type 1. KB and HaCaT cells, respectively, were exposed to UV radiation (300 J/m²) in the absence or presence of IL-1β (10 ng/ml). An antibody blocking the TNF receptor type 1 was added (500 ng/ml). Apoptosis was examined 16 h after treatment by determining nucleosomal DNA fragmentation using an apoptosis determination kit. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown by the values on the y axis. Data presented show the representative results of one of three independently performed experiments.
opposing effects of antiapoptotic cytokines and the TNF pathway on keratinocytes and keratinocyte-like cells. Our findings suggest that the mechanism by which IL-1 affects c-IAP expression in such cells can differ depending on the cell type, with c-IAP1 being upregulated by IL-1 in keratinocytes and downregulated in keratinocyte-like cells. Overall, these results highlight the complex and context-dependent nature of the TNF pathway in keratinocytes and its implications for understanding the regulation of apoptosis in this cell type.

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