NFκB-dependent Down-regulation of Tumor Necrosis Factor Receptor-associated Proteins Contributes to Interleukin-1-mediated Enhancement of Ultraviolet B-induced Apoptosis*

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Apoptosis can be induced by different stimuli, including UV radiation and activation of cell death receptors, like tumor necrosis factor receptor 1 (TNF-R1),1 CD95 (Fas/APO-1), and tumor necrosis factor-related apoptosis-inducing ligand receptors (TRAIL-R) (1). Upon binding of the specific ligands TNFα, CD95L, or TRAIL clustering of the receptor leads to activation of the intracellular death domain resulting in apoptotic signal transduction (2, 3). In contrast to CD95L, TRAIL induces apoptosis preferentially in transformed cells, whereas normal cells remain resistant (4). This selectivity suggested TRAIL to be a promising candidate for an anticancer drug (5).

The TRAIL receptor family consists of four members, of which TRAIL-R1 and TRAIL-R2 are potent inducers of apoptosis (6, 7). Although evidence exists showing that TRAIL-R1 and TRAIL-R2 activation leads to nuclear factor κB (NFκB) activation (8, 9), the major role is induction of apoptosis, starting with recruitment of Fas-associated protein with death domain (FADD) followed by activation of initiator procaspase-8, which triggers activation of effector caspases (3, 10). Activation of procaspase-8 can be antagonized by competitive binding of FLICE-inhibitory protein (FLIP) to FADD or by heterodimerization of FLIP with procaspase-8 (11, 12). Another way to prevent apoptosis is expression of inhibitors of apoptosis proteins (c-IAP), which interfere with activation of effector caspases (13, 14).

In contrast to TRAIL, TNFα is a weaker inducer of apoptosis but plays an important role in mediation of innate and inflammatory responses (15). TNF-R1 triggers two different pathways upon TNFα binding, either a pro- or an antiapoptotic one (16). Both pathways coexist in a certain balance, and each can be promoted depending on the physiological conditions. Either recruitment of FADD leads to induction of apoptosis, or recruitment of TNF receptor-associated factor (TRAF) proteins results in activation of the transcription factor NFκB, which mediates cell survival pathways (17–19). Besides its important role in controlling immune function, cell proliferation, and differentiation, NFκB so far has mainly been described to induce expression of antiapoptotic genes (14, 20). Therefore, NFκB has been designated a tumor-promoting molecule. Accordingly,
NFκB was found to be constitutively up-regulated in a variety of tumor cells (21–23). Consequently, inhibition of the NFκB-activating pathway is a major target for alternative anticancer drugs.

Regarding the tumor selectivity of TRAIL, it has to be taken into account that tumor cells are generally surrounded by immune cells that constantly release proinflammatory cytokines like IL-1. Therefore, IL-1-mediated activation of NFκB may represent a pathway by which tumor cells can escape the cytotoxic effects of selective anticancer agents, like TRAIL.

Accordingly, earlier work from our laboratory revealed that activation of NFκB by interleukin-1 (IL-1) resulted in reduction of TRAIL-induced apoptosis in transformed keratocytes, which coincided with up-regulation of c-IAP-1 and c-IAP-2 proteins (24). The same was observed for CD95-induced apoptosis, supporting the assumption that NFκB protects from apoptosis universally. In contrast, apoptosis induced by UVB was shown to be significantly enhanced upon IL-1-mediated NFκB activation (25). UVB-induced apoptosis is a complex process involving several pathways like induction of genomic DNA damage (26), ligand-independent clustering of death receptors (27, 28), and generation of reactive oxygen species (29). All three pathways contribute independently to the complete apoptotic response to UVB. Analyzing the molecular mechanisms underlying enhancement of UVB-induced apoptosis by IL-1 we observed pronounced down-regulation of c-IAP proteins (25). Even more importantly, costimulation with IL-1 resulted in strong release of TNFα, which additively induced apoptosis (25), suggesting that, upon UVB exposure NFκB suddenly mediates effects that promote apoptosis. The present study was performed to investigate the molecular mechanisms underlying IL-1-mediated enhancement of UVB-induced apoptosis. Additionally, we wanted to determine whether NFκB has the potential to mediate proapoptotic effects, because this may have important implications for photocarcinogenesis and for the potential use of NFκB inhibitors in the prevention and therapy of skin cancer.

MATERIALS AND METHODS

Cells and Reagents—The human epithelial carcinoma cell line KB (American Tissue Culture Collection, Rockville, MD) was cultured in RPMI 1640 with 10% fetal calf serum. Stimulation of cells was carried out in colorless medium containing 2.5% fetal calf serum. For UVB irradiation a bank of six TL12 fluorescent bulbs (Philips, Eindhoven, The Netherlands) was used that emit most of their energy within the UVB range (293–302 nm) with an emission peak at 313 nm. Throughout this study a dose of 350 J/m² was used. Control cells were subjected to the identical procedure without being exposed to UVB. To induce TRAIL-mediated apoptosis, 80 ng/ml of recombinant human TRAIL was added to the cells. This recombinant protein was N-terminally labeled with [35S]-methionine (Amersham, Arlington Heights, IL). KB cells were cultured in 25 cm² flasks (Becton Dickinson and Co., Lincoln Park, NJ) at 37 °C in 5% CO₂ and 95% air.

Determination of Cell Death—16 h after stimulation cells were detached from culture dishes, and apoptosis was analyzed by a cell death detection ELISA (Roche Diagnostics GmbH, Mannheim, Federal Republic of Germany). The enrichment of mono- and oligonucleosomes released into the cytoplasm of cell lysates is detected by biotinylated antibody (BD Biosciences, San Diego, CA). The enrichment of mono- and oligonucleosomes is given as the mean ± S.D. of triplicates, representing one of three independently performed experiments. Throughout the experimental settings an enrichment of 1 corresponds to 5–7% apoptotic cells as determined by Annexin V staining followed by FACS analysis (data not shown).

Analysis of Gene Expression Rates by GeneChip® Arrays—To screen KB cells for NFκB-dependent differential gene expression, GeneChip® array analysis was performed utilizing Human Genome U95Av2 chips (Affymetrix®, Santa Clara, CA). 1.5 h after stimulation total RNA was extracted by the phenol/chloroform extraction method utilizing phase lock tubes (Eppendorf, Hamburg, Federal Republic of Germany). 25 μg total RNA was subjected to first and second strand cDNA synthesis using T7-(dT24)-primer (MWG-Biotech, Ebersberg, Federal Republic of Germany) and enzymes from Invitrogen according to the protocol provided by Affymetrix®. Biotinylated cRNA was in vitro transcribed from 10 μg of DNA utilizing the ENZO-kit from Affymetrix® and subsequently fragmented according to the manufacturer’s recommendation (see technical manual: GeneChip® Expression Analysis, by Affymetrix®). Samples were analyzed using an Affymetrix® fluidic station 200 and a GeneChip® 3000 (Normis) scanner with high density arrays. The data were scanned and generated by the software MicroArray Suite 5.0 (MAS 5.0) from Affymetrix®. The signals were log transformed and calculated according to the statistical algorithms of the MAS 5.0, which calculates an intensity value and a detection call corresponding to “absent,” “present,” or “marginal” for each transcript (Affymetrix® User Manual). Furthermore, we used the software package Expressionist (Expressionist, Basel, Switzerland). The software uses the Expressionist Refiner version 3.0.4. This allows an additional global quality control of the GeneChip® with detection and masking of defect areas on the chip and outliers, gradient correction of fluorescence signal, and regulation of variance.

The statistical analysis was performed with the Expressionist Analyst version 4.0.5. Data were first normalized on a logarithmic mean of 20 groups with four experiments per group for the IL-1 stimulation and five experiments per group for the other stimulations and the control. Data were filtered with genes with valid values (expression over background respective a detection call “present” or “on” (see Affymetrix® User Manual) in 50% or more per group. For sample comparison, we calculated each stimulation group against the control group with n-fold regulation by comparing the mean and the p value by the Student’s t test for statistical significance of the respective groups.

Validation of Gene Expression Rates by Real-time PCR—3 h after stimulation, total RNA was extracted from KB cells by the phenol/chloroform extraction method utilizing phase lock tubes (Eppendorf). 3 μg of total RNA was reverse transcribed using a SuperScript™ II kit (Invitrogen). Assays-on-demand® were used to quantify gene expression. The primers used were as follows: TRAF-1 (Hs00371508_m1), TRAF-2 (Hs00184192_m1), TRAF-6 (Hs00371508_m1) (Applied Biosystems, Foster City, CA). Expression rates were normalized to the expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (Hs99999905_m1) using the TaqMan® Universal kit in an ABI Prism 7900HT Real-time PCR System supplied with SDS 2.1 software (Applied Biosystems). Expression rates were calculated using the 2^(-ΔΔCT) method as described by Livak and Schmittgen (30).

Staining of Intracellular Proteins and FACS Analysis—6 h after stimulation cells (1 × 10⁶) were removed from culture dishes, washed with PBS and fixed with 0.8% paraformaldehyde in PBS for 5 min on ice. After washing, cells were permeated with 0.3% saponin (Sigma) in PBS and incubated with the first antibody (20 μg/ml for TRAFs and 10 μg/ml for c-IAP and FLIP) overnight at 4 °C. Monoclonal mouse-anti c-IAP and anti-TRAF antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA: anti-c-IAP (N19, sc-1867), anti-TRAF-1 (H3, sc-6253), anti-TRAF-2 (H249, sc-7187), and anti-TRAF-6 antibody (H274, sc-7221). Polyclonal rabbit-anti-FLIP (556567, BD Pharmingen) was used. After washing, the respective secondary antibody (10 μg/ml goat anti-mouse, PE labeled for c-IAP and FLIP) was used. After washing, the respective secondary antibody (10 μg/ml goat-anti-rabbit IgG, PE labeled for FLIP) was incubated in 0.5% saponin on 30 min on ice. Subsequently, cells were washed in PBS and subjected to flow cytometry analysis in PBS with 0.03% saponin. FACS analysis was performed using an EPICS® XL-MCL flow cytometer (Coulter, Miami, FL). 20,000 cells were analyzed for each sample. Data analysis was performed using the software WinMDI (Visible Cell Systems, Window-system Core Facilities, The Scripps Research Institute, La Jolla, CA).

Transient Transfection of Cells—KB cells (8 × 10⁵) were washed once with PBS and resuspended in 800 μl of ice-cold transfection buffer (140 mM NaCl; 5 mM KCl; 0.7 mM N₄HPO₄; 6 mM glucose; 0.1 mM β-ME, 20 mM HEPES, pH 7.0) containing 1.5% MgSO₄ (SO). Cells were electroporated with 25 μg of the plasmids pBS-Ie-B-DN, pCR3-cFLIP, pCR3-c-IAP, pCR3-TRAF-1, pCR3-TRAF-2, pCR3-TRAF-3, or the respective controls.
UVB-induced Apoptosis Enhanced via NFκB

FIG. 1. IL-1 causes reduction of TRAIL-induced apoptosis but enhancement of UVB-induced apoptosis. A, KB cells were left untreated or stimulated with 10 ng/ml IL-1 alone, with IL-1 plus UVB (350 J/m²), or IL-1 plus TRAIL (80 ng/ml). After 16 h, apoptosis was determined using a cell death detection ELISA. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the y-axis (mean ± S.D. of triplicate samples). B, supernatants from IL-1 plus UVB-treated cells or with IL-1 (100 pg/ml), with supernatants from IL-1 plus TRAIL-induced apoptosis in comparison to an addition of IL-1 (Fig. 1C). This indicates that other intracellular mechanisms are responsible for intensifying the proapoptotic effect of TNFα that results in enhancement of UVB-induced apoptosis.

FIG. 2. Transfection of cells with a super-suppressor of IκB (IκB-DN) reverses the effects of IL-1 on UVB- and TRAIL-induced apoptosis. KB cells were transfected with a plasmid encoding a IκB-DN variant or with the empty vector. 30 h later cells were either left untreated or stimulated with IL-1 (10 ng/ml) alone, with IL-1 plus UVB (350 J/m²), or with IL-1 plus TRAIL (80 ng/ml). 16 h later apoptosis was measured using a cell death detection ELISA. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the y-axis (mean ± S.D. of three independently performed experiments).

empty vector. Transfection efficacy of cells cotransfected with a plasmid encoding β-galactosidase (pCMV-βgal, Stratagene, La Jolla, CA) was determined 30 h later by staining cells with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal, 100 µg/ml) in 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mM MgCl₂ in PBS. Transfection efficacy ranged from 30 to 40%.

RESULTS

Stimulation of cells from the epithelial cell line KB with TRAIL or irradiation with UVB induces apoptosis. IL-1 causes activation of the transcription factor NFκB, which is known to be responsible for the expression of a number of antiapoptotic genes. Accordingly, costimulation of cells with IL-1 resulted in reduction of TRAIL-induced apoptosis, as demonstrated previously (24). In contrast, costimulation with IL-1 caused a significant enhancement of UVB-induced apoptosis (Fig. 1A). This enhancement coincided with a strong release of the proapoptotic cytokine TNFα (~100 pg/ml), whereas no TNFα was released upon stimulation with IL-1 alone or in combination with TRAIL (Fig. 1B). TNFα at 100 pg/ml does not induce apoptosis in KB cells itself. Addition of TNFα and of supernatants of IL-1 plus UVB-stimulated cells to UVB-irradiated cells only slightly enhanced UVB-induced apoptosis in comparison to an addition of IL-1 (Fig. 1C). This indicates that other intracellular mechanisms are responsible for intensifying the proapoptotic effect of TNFα that results in enhancement of UVB-induced apoptosis.

IL-1-mediated activation of NFκB essentially requires phosphorylation of two specific Ser residues (Ser-32/36) of its cytoplasmic inhibitor IκB followed by ubiquitination and proteasomal degradation. In the super-suppressor variant utilized in this experiment both Ser-32/36 had been substituted by Ala thereby preventing phosphorylation and degradation of IκB (IκB-DN). Cell death analysis revealed that in cells transiently transfected with IκB-DN the enhancing effect of IL-1 on UVB-induced apoptosis as well as its inhibiting effect of TRAIL-induced apoptosis was abolished, indicating NFκB to be critically involved in the underlying mechanisms (Fig. 2).

We have previously demonstrated that NFκB mediates differential regulation of c-IAPs at the protein level depending on the apoptotic costimulus (25). To expand on these findings and to further study transcription rates under these conditions, GeneChip® arrays were performed using cells stimulated with IL-1 alone or in combination with either TRAIL or UVB in comparison to untreated cells. Stimulation with IL-1 alone or IL-1 plus TRAIL resulted in a 16- to 18-fold up-regulation of antiapoptotic c-IAP mRNA and an up-regulation of antiapoptotic FLIP of ~4-fold, respectively, but only a 2-fold up-regulation of TNFα. In contrast, stimulation with IL-1 plus UVB caused only a 3-fold up-regulation of c-IAP and down-regulation of FLIP, whereas the TNFα gene was 5-fold up-regulated (Table I).

When screening for other genes being differentially regulated by NFκB, we found genes encoding TRAF-1 and TRAF-6 to be critically down-regulated upon IL-1 plus UVB treatment (Table I). Because TRAF proteins are involved in TNF-R1 as well as IL-1R-mediated signal transduction, differences in their expression rates may influence signal transduction path-
always triggered by the respective receptors, thereby influencing the fate of the cell.

The results concerning the different expression rates of TRAF genes obtained from GeneChip® data were verified by real-time PCR (Table I). Accordingly, TRAF-1 was shown to be ∼16-fold up-regulated upon IL-1 or IL-1 plus TRAIL stimulation, whereas it was only 7-fold up-regulated in case of IL-1 plus UVB treatment, in comparison to untreated cells. TRAF-6 expression was only minimally influenced by different proapoptotic costimuli, but down-regulated by the combination of IL-1 plus UVB. The TRAF-2 gene, which was not included in the GeneChip®, was at least 2-fold up-regulated by IL-1 or IL-1 plus TRAIL, respectively, but 2-fold down-regulated by IL-1 plus UVB (Fig. 3). Taken together, IL-1-mediated up-regulation of TRAF-1, -2, and -6 genes remains unaffected by TRAIL treatment but is antagonized by UVB radiation.

FLIP is known to be a major inhibitor of death receptor-driven apoptosis, because it prevents activation of the proximally acting procaspase-8. Efficient blockade of downstream effector caspases, like caspase-3, is procured by c-IAP protein. Intracellular FACS analysis was performed to show that differential c-IAP and FLIP regulation is also reflected at the protein level. 6 h after stimulation FLIP protein was found to be up-regulated by IL-1, whereas it took 8 h to significant increase the protein level of c-IAP by IL-1. Along this line, cotreatment with IL-1 counteracted TRAIL-mediated down-regulation of both FLIP and c-IAP. In contrast, IL-1 stimulation even enhanced the UVB-mediated down-regulation of FLIP and c-IAP (Fig. 4). Accordingly, the differential regulation of FLIP and c-IAP follows the same regulation pattern as indicated at the mRNA expression level.

To elucidate whether decreased c-IAP and FLIP protein levels contribute to the enhancement of UVB-induced apoptosis by IL-1, cells were transiently transfected with plasmids encoding c-IAP and FLIP, respectively. 16 h after IL-1 plus UVB or IL-1 plus TRAIL treatment cell death detection ELISA revealed that overexpression of either c-IAP or FLIP counteracts IL-1-mediated enhancement of UVB-induced apoptosis. In contrast, stimulation of cells with IL-1 plus UVB resulted in down-regulation of each TRAF-1, -2, and -6 in an IL-1-dependent manner. In accordance with the mRNA expression rates regulation was strongest for TRAF-1, indicating that TRAF-1 regulation plays a major role in this scenario (Fig. 6A). All the effects were very likely to be mediated by NFκB, because up-regulation of TRAF proteins by IL-1 alone or IL-1 plus TRAIL as well as their down-regulation by IL-1 plus UVB stimulation was abolished in cells transiently transfected with the IκB-DN variant described above (Fig. 6B).

Because the RNA expression rate of TRAF-1 as demonstrated in Table II did not correlate with the protein expression level upon IL-1 plus UVB stimulation (Fig. 6A), we studied whether post-translational proteolysis of TRAF-1 may be responsible for this discrepancy. In fact, inhibition of proapoptotic caspases with the pancaspase inhibitor zVAD significantly enhanced the TRAF-1 protein levels as demonstrated by intracellular FACS analysis performed 6 h after stimulation (Fig. 7).

To elucidate whether a reduction of TRAF protein level might promote proapoptotic pathways triggered by TNFα through activation of TNFα-R1 and may thereby contribute to enhancement of UVB-induced apoptosis by IL-1, cells were transiently transfected with plasmids encoding TRAF-1, TRAF-2, and TRAF-6, respectively. 16 h after IL-1 plus UVB or IL-1 plus TRAIL stimulation, cell death detection ELISA revealed that cells overexpressing either one of these TRAF proteins became more resistant against IL-1 plus UVB-induced apoptosis, whereas IL-1 plus TRAIL-induced apoptosis remained unaffected (Fig. 8). These results strongly indicate that NFκB-dependent down-regulation of TRAF-1, TRAF-2, and TRAF-6, respectively, contributes to IL-1-mediated enhancement of UVB-induced apoptosis.

**DISCUSSION**

The death ligand TRAIL as well as UVB radiation are potent inducers of apoptosis in transformed keratinocytes and epithelial cells. Previous work from our laboratory revealed that costimulation with IL-1 protects transformed keratinocytes from the cytotoxic effect of TRAIL in an NFκB-dependent manner (24). Because IL-1 can be released by a variety of tumor cells and is also released by inflammatory cells participating in the tumor-host immune response (32), cancer cells under these conditions may escape the therapeutic effect of TRAIL through NFκB activation.

The NFκB family of transcription factors (Rel c-Rel, Rel A (p65), Rel B, NFκB-1 (p105/p50), and NFκB-2 (p100/p52)) is involved mainly in stress-induced, immune, and inflammatory responses. Together, these proteins regulate the expression of...
KB cells were either left untreated or stimulated with IL-1 (10 ng/ml) alone, with IL-1 plus UVB (350 J/m²), or with IL-1 plus TRAIL (80 ng/ml). 3 h later cDNA was generated from total RNA and subjected to real-time PCR analyses utilizing assays-on-demand probes from Applied Biosystems®. Expression rates of TRAF1, TRAF2, and TRAF6, respectively, were normalized to glyceraldehyde-3-phosphate dehydrogenase expression levels. Genes with high scores and significant results are highlighted in gray.

TABLE II
Results of real-time PCR experiments, showing that IL-1-mediated and NFκB-dependent expression of TRAF proteins is critically influenced by the apoptotic costimulus.

| gene description | Co vs IL-1 | Co vs IL-1+TRAIL | Co vs IL-1+UVB |
|------------------|------------|------------------|-----------------|
| ΔCT mean* | ΔCT mean* | ΔCT mean* | ΔCT mean* | ΔCT mean* | ΔCT mean* |
| TRAF1 | 7.79 ± 0.35 | 3.04 ± 0.19 | 7.49E-05 | 3.64 ± 0.22 | 3.41E-05 |
| TRAF2 | 0.93 ± 0.14 | 0.22 ± 0.06 | 0.001 | 0.04 ± 0.24 | 0.001 |
| TRAF6 | 4.73 ± 0.25 | 4.29 ± 0.08 | 0.04 | 4.20 ± 0.30 | 0.07 |

* ΔCT values were calculated by software algorithms of AB-protocols. The table shows the mean of the ΔCT values for each gene and each group with the associated standard deviation.

§ The corresponding p value was calculated with Student’s t test for significance analysis of three independent performed experiments.

Fig. 3. IL-1-dependent expression rates of the TNFR-1 adapter proteins TRAF1, TRAF2, and TRAF6 are critically influenced by the apoptotic costimulus. Comparison of the ΔCT values of the different stimulations with the control group (see Table II) leads to n-fold regulation of the respective genes.

The observation that NFκB is capable of rescuing cells from death induced by TNFα (14, 20), TRAIL (24), CD95L (25), and ionizing radiation and chemotherapeutic drugs (36) gave rise to the speculation that NFκB protects from apoptosis universally. Thus, it was quite surprising to observe that IL-1 did not protect from, but even enhanced, apoptosis induced by UVB radiation. Enhancement of UVB, as well as reduction of TRAIL-induced apoptosis, seem to be NFκB-dependent, because prevention of NFκB activation in cells overexpressing a super suppressor of its inhibitor IκB reversed both effects (Fig. 2). Furthermore, enhancement of UVB-induced apoptosis was shown to coincide with a pronounced release of TNFα, which as an additional apoptotic stimulus triggers TNF-R1, indicating that enhancement of UVB-induced apoptosis by IL-1 correlates with TNFα release and is NFκB-dependent (25).

Surprisingly, addition of the same amount of TNFα in the range of 100 pg/ml to UVB-irradiated cells did not significantly enhance UVB-induced apoptosis neither did addition of supernatants from IL-1 plus UVB-treated cells. These two observations clearly rule out the possibility that either TNFα alone or other potential proapoptotic mediators released from the cells are sufficient to enhance UVB-induced apoptosis. In turn, these findings suggest that other intracellular mechanisms have to be involved as well, most likely mechanisms that intensify the proapoptotic effects mediated by activation of TNF-R1 through binding of autocrine released TNFα.

One of these mechanisms includes NFκB-mediated repression of c-IAP and FLIP genes. Both c-IAP and FLIP are very potent inhibitors of almost any proapoptotic pathway, because they interfere with the activation of initiator caspase-8 (FLIP) and effector caspases (c-IAP), respectively (11–13, 37). It is tempting to propose that UVB drives NFκB to exert its proapoptotic effect by a general repression of antiapoptotic genes that NFκB usually activates. A modified phosphorylation pattern of NFκB upon UV or daunorubicin treatment was recently shown to manipulate NFκB to interact with chromatin silencing histone deacetylase molecules, thereby mediating specific gene repression of antiapoptotic genes, like c-IAP and bel-2 (38). These findings strongly support our concept that UVB radiation changes the working pattern of NFκB. However, this may not be the only mechanism responsible, because NFκB upon UVB irradiation strongly accelerates the expression of the proapoptotic TNFα gene, which is not effectively induced by IL-1 alone. As displayed in Table I, TNFα was also 2-fold up-regulated upon IL-1 or IL-1 plus TRAIL stimulation but no TNFα was released (Fig. 1). This phenomenon is due to the fact that, after a short initial induction of the TNFα gene upon any stimulation including IL-1, transcription is completely repressed again after 2 h in the case of IL-1 and IL-1 plus TRAIL stimulation, respectively. This short term TNFα expression is obviously not sufficient to result in appropriate protein synthesis and autocrine secretion. In contrast, transcriptional activation stays stable for several hours in the case of IL-1 plus UVB treatment, as documented by semiquantitative PCR analysis (data not shown).

A similar way of NFκB-dependent gene repression upon UVB irradiation appears to apply for TRAF genes TRAF-1 and TRAF-6, whereas TRAF-3, TRAF-4, and TRAF-5 expression remains unaffected (data not shown). TRAF-2 is one of six TRAF protein members and functions as an activator of NFκB (39, 40), TRAF-1 and -6-like TRAF-2 are involved in NFκB activation (41). TRAF-1 was shown to heterodimerize with TRAF-2, thereby enhancing its antiapoptotic effects, i.e. NFκB activation and mediation of cell survival signals (42, 43). TRAF-6 mediates interleukin-1 receptor-induced activation of NFκB (44). Because TRAF-2 was not present on the GeneChip® real-time PCR experiments were performed that confirmed the assumption that besides TRAF-1 and TRAF-6 also the TRAF-2 gene is differentially regulated by NFκB, depending on the apoptotic costimulus. All three TRAF genes contain...
NFκB consensus sequences within their promoters and have previously been shown to become activated in an NFκB-dependent manner (45). Here we could demonstrate that TRAF proteins cannot only be up-regulated but can also be down-regulated upon costimulation with IL-1, indicating again that NFκB exerts different regulatory functions depending on the proapoptotic stimulus applied (Fig. 6, A and B). The expression rate determined for TRAF-2 and TRAF-6 was only slightly enhanced upon treatment with IL-1 alone or IL-1 plus TRAIL. Nevertheless, in comparison to this up-regulation the transcription efficacy was repressed even below the control level by IL-1 plus UVB stimulation. The differences in TRAF-1 expression were even more pronounced, revealing very strong induction by IL-1 and IL-1 plus TRAIL, respectively, but only minor by IL-1 plus UVB treatment. Taken together, the transcription efficacy of all three TRAF members was significantly impaired upon IL-1 plus UVB treatment.

Based on the previous observation of either up- or down-regulation of c-IAP by different apoptotic stimuli after 16 h (25), we determined whether early changes of c-IAP and FLIP and TRAF adapter proteins after 6 h may influence the fate of the cell upon costimulation of either UVB or TRAIL with IL-1. In accordance with the GeneChips® data, intracellular FACS analysis confirmed down-regulation of FLIP and c-IAP as well as of TRAF proteins upon costimulation with IL-1 plus UVB, whereas the proteins were up-regulated by IL-1 in the absence as well as in the presence of TRAIL. IL-1-mediated differential TRAF regulation was shown to be clearly NFκB-dependent, because inhibition of NFκB with an IκB super-suppressor almost completely reversed both IL-1 mediated up- as well as down-regulation of TRAF proteins. In the case of TRAF-1, the protein expression rate did not correlate with the pronounced enhancement of the transcription rate. This might be due to a general low mRNA protein turnover. Nevertheless, costimulation with IL-1 plus UVB resulted in a further reduction of protein amounts, although real-time PCR data still showed a 7-fold up-regulation of TRAF-1 mRNA. This phenomenon is obviously due to caspase-dependent proteolytic degradation of TRAF-1, because addition of the pancaspase inhibitor zVAD significantly enhanced the protein level of TRAF-1. Accordingly, previous studies already identified TRAF-1 as a cleavage substrate for caspases during induction of death receptor-induced apoptosis (46–48). In contrast, upon stimulation with IL-1 plus TRAIL up-regulation of antiapoptotic FLIP and c-IAP proteins seems to prevent TRAF-1 cleavage by blocking caspase activation.

The data shown imply the existence of a biological correlation between NFκB-dependent differential gene regulation and a bivalent influence on apoptosis induced by either death receptor activation or UVB radiation. Accordingly, overexpression of either of the antiapoptotic proteins c-IAP and FLIP or the adapter proteins TRAF-1, TRAF-2, and TRAF-6, respectively, counteracted the enhancing effect of IL-1 on UVB-induced apoptosis completely, whereas it had no significant impact on IL-1 plus TRAIL-induced apoptosis, in which all the antiapoptotic proteins mentioned become up-regulated in an IL-1-dependent manner.

The present study clearly demonstrates that down-regulation of antiapoptotic proteins and adapter proteins together determine the sensitivity of the cell toward UVB radiation. In fact this is a secondary effect mediated via TNF-R1 that becomes activated by autocrine release of sublethal TNFα doses. Because treatment of cells with IL-1 plus UVB additionally results in repression of antiapoptotic FLIP and c-IAP genes and NFκB-dependent down-regulation of TRAF-1, -2, and -6 proteins, the following scenario may take place within the cell. The quickly released TNFα (release starts after 1 h, data not shown) activates the TNF-R1, resulting first of all in TNF receptor-associated protein with death domain recruitment. Due to down-regulation of TRAF-1 and TRAF-2, the balance at
the TNF-R1 shifts toward binding of the proapoptotic adapter protein FADD, which transduces the proapoptotic signal, resulting in induction of cell death. Furthermore, down-regulation of TRAF-6 mitigates NFκB activation triggered by IL-1 through IL-1R. Additionally, inhibition of caspase-8 by FLIP and of effector caspases by c-IAP is strongly impaired due to NFκB-dependent down-regulation of these antiapoptotic proteins. In summary, the different molecular mechanisms de-

FIG. 6. IL-1-dependent differential regulation of TRAF-1, TRAF-2, and TRAF-6 proteins is NFκB-dependent. A, KB cells were left untreated or stimulated with UVB (350 J/m²) or TRAIL (80 ng/ml) alone and in combination with IL-1 (10 ng/ml). After 6 h intracellular FACS analysis was performed utilizing PE-labeled antibodies directed against TRAF-1, -2, and -6, respectively. Histograms show fluorescence intensity (x-axis) versus cell number (y-axis) of cells treated without IL-1 (gray shaded histogram) and with IL-1 (black line), respectively, in comparison to isotype control (dashed line). B, experiments were performed exactly as described in A using cells transiently transfected with an IκB super-suppressor. Data presented show one representative of three independently performed experiments.
TNFR1 but is not sufficient alone. Down-regulation of TRAF proteins then sets the course for initiating the apoptotic pathway. In this context down-regulation of TRAF-1 seems to play the major role. Finally down-regulation of apoptosis inhibitors FLIP and c-IAP intensify apoptosis triggered by TNF-R1. Here down-regulation of FLIP is the predominant mediator, because it inhibits receptor-driven apoptosis more upstream and its half-life seems to be shorter that the one of c-IAP, as deduced from intracellular FACS analysis.

Thus, the present findings imply that upon UVB radiation NFκB has the potential to trigger physiologically proapoptotic instead of antiapoptotic pathways within the cell. Although a mechanism underlying UV-mediated and NFκB-dependent gene repression has been proposed (38), the mechanism by which NFκB, upon UVB radiation, induces genes that it usually does not induce is still unclear. Studying the role of NFκB under different conditions on the physiological status of the cell is of primary importance, because the combination of phototherapy and activators of NFκB may be of potential practical value for the treatment of malignancies especially of the skin. The present study may contribute to a change in the dogma that NFκB exclusively mediates antiapoptotic effects. This observation should be carefully taken into account when designing new concepts for anticancer therapy, especially for those utilizing NFκB inhibitors.

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Fig. 7. Inhibition of caspase activity by zVAD enhances the protein level of TRAF-1. KB cells were left untreated or stimulated with UVB (350 J/m²) alone, in combination with IL-1 (10 ng/ml) with and without pretreatment with the pancaspase inhibitor zVAD (20 μM) for 1 h. After 6 h intracellular FACS analysis was performed utilizing a PE-labeled antibody directed against TRAF-1. Histograms show fluorescence intensity (y-axis) versus cell number (x-axis) of UVB-treated cells without IL-1 (gray shaded histogram), with IL-1 (black line), in comparison to isotype control (dashed line). Data presented show one of three independently performed experiments.

Fig. 8. Overexpression of TRAF-1, -2, and -6 antagonize the enhancing effect of IL-1 on UVB-induced apoptosis. KB cells were transiently mock transfected or transfected with plasmids expressing TRAF-1, TRAF-2, or TRAF-6 under control of a CMV promoter. 30 h later cells were either left untreated or stimulated with IL-1 (10 ng/ml), IL-1 plus UVB (350 J/m²), or IL-1 plus TRAIL (80 ng/ml). 16 h later apoptosis was determined using a cell death detection ELISA. The rate of apoptosis is reflected by the enrichment of nucleosomes in the cytoplasm shown on the y-axis (mean ± S.D. of triplicate samples). Data presented display one of three independently performed experiments.

Fig. 9. Model postulated for the pathway involving IL-1-mediated enhancement of UVB-induced apoptosis. For further explanations see text.

scribed shift the balance at the TNF-R1 toward proapoptotic signaling, resulting in enhancement of UVB-induced apoptosis (Fig. 9). In this scenario NFκB mediated TNFε release, which exclusively occurs upon IL-1 plus UVB treatment, serves as the initial trigger of apoptosis enhancement via activation of
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