The heat shock protein 90 (Hsp-90) inhibitor, geldanamycin, and the proteasome inhibitor, MG-132, both inhibited tumor necrosis factor receptor 1 (TNF-R1)- but not TRAIL-induced apoptosis in Kym-1 cells, suggesting that TNF-R1-induced cell death is dependent on NF-κB activation in this model. Triggers of TNF-R1 by agonistic antibodies led to cell-type specific induction of endogenous TNF and apoptosis, the latter of which was abrogated by neutralizing TNF specific antibodies. TNF-R1-stimulated cells expressed TNF mainly in its membrane-bound form. Geldanamycin failed to inhibit apoptosis induction by a combination of agonistic TNF-R1- and TNF-R2-specific antibodies, indicating that both TNF receptors co-operate in TNF-R1-triggered apoptosis in Kym-1 cells. Thus, TNF-R1 stimulation can elicit a strong and rapid apoptotic response via induction of membrane TNF and subsequent cooperation of TNF-R1 and TNF-R2. Moreover, we give evidence that this mechanism circumvents the need of the prolonged presence of exogenous soluble TNF for TNF-R1-mediated apoptosis induction.

Tumor necrosis factor (TNF) is a pleiotropic cytokine fulfilling a broad variety of immunoregulatory functions. TNF occurs in two forms: a type II membrane protein (memTNF) and a soluble form (sTNF) derived therefrom by proteolytic processing (1). Two distinct receptors for TNF, TNF-R1 and TNF-R2, transduce TNF signals (1). Although memTNF can potently bind TNF, selective stimulation of this receptor, sTNF acts predominantly via TNF-R1 (1). The TNF receptors are the prototypic name given to representatives of the TNF receptor superfamily (2). Based on the presence of a death domain, the TNF receptor family can be subdivided into death receptors and receptors without a death domain (2). Although TNF-R1 is a member of the death receptor group, TNF-R2 belongs to the non-death domain-containing receptors (2). Stimulation of death receptors typically leads to the recruitment of cytoplasmic death domain-containing adaptor proteins that couple death receptors to the apoptotic machinery of the cell (2). In contrast, non-death domain-containing receptors interact with members of the TNF receptor-associated factor (TRAF) family of adaptor proteins that mediate activation of NF-κB, JNK, and p38 (2, 3). In contrast to other death receptors, e.g. Fas, which are predominantly recognized as death inducers, TNF-R1 has a variety of non-apoptotic immunoregulatory functions. At first glance, the less apparent activity of TNF-R1 can be explained by the fact that TNF-R1 is a potent activator of the anti-apoptotic NF-κB pathway. Indeed, if TNF-R1-induced NF-κB activation is compromised, e.g. in knock-out mice deficient in components of the NF-κB pathway, TNF-R1 triggering leads to apoptosis induction in normally TNF-insensitive cells (1, 3). Although TNF-R2 has no death domain, selective stimulation of this receptor, e.g. by use of agonistic antibodies, is sufficient to induce apoptosis in some cells (4–7). TNF-R2-induced apoptosis is not caused by a direct activation of the apoptotic caspase cascade but rather is mediated indirectly by production of endogenous TNF, which subsequently signals cell death via TNF-R1 (8, 9). In addition, there is evidence that TNF-R2 can enhance TNF-R1-induced cell death independently of endogenous TNF by depletion of cytoplasmic TRAF2-containing anti-apoptotic protein complexes (10–14).

In this study we show that induction of endogenous memTNF and subsequent triggering of TNF-R2 act as a powerful amplification mechanism in TNF-R1-induced apoptosis. Accordingly, by that mechanism, a short TNF pulse can be sufficient to trigger cell death, whereas prolonged TNF treatment is necessary for apoptosis induction in cells in which TNF-R1 triggering does not up-regulate endogenous TNF.

**Experimental Procedures**

**Cell Culture and Reagents**—The human rhabdomyosarcoma cell line, Kym-1, was a generous gift from M. Sekiguchi (University of Tokyo, Japan), and the human cervical carcinoma cell line, HeLa, was obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in Click’s RPMI medium (Biochrom, Berlin, Germany) supplemented with 10% fetal calf serum (Kym-1) or in RPMI medium (Biochrom) supplemented with 5% fetal calf serum (HeLa). TRAIL-M2 complex was generated by mixing the respective concentration of Flag-tagged TRAIL with the anti-Flag monoclonal antibody M2 (Sigma-Aldrich) to a final concentration of 1 μg/ml of antibody. After 15 min of incubation at room temperature, the formed TRAIL-M2 complex was transferred to the cells. The anti-Flag monoclonal antibody M2 was purchased from Sigma-Aldrich. Flag-tagged TRAIL and Flag-tagged TWEAK were generously supplied by P. Schneider and J. Tschopp (University of Lausanne, Switzerland). The TNF-R1-specific mAb Htr1 (15) and the polyclonal TNF-R2-specific rabbit IgG (16) have been described elsewhere. The neutralizing TNF-specific mAb 101-4 was a kind gift from A. Meager (National Institute for Biological Standards and Technologies), and the polyclonal TNF-R2-specific rabbit IgG (16) have been described elsewhere.
endogenous TNF is involved in TNF-R1-dependent apoptosis

**Fig. 1.** Inhibitors of TNF-induced NF-κB activation interfere with rapid TNF-R1-initiated apoptosis. A–J, Kym-1 cells (2 × 10^6) were seeded in 100 μl of medium in 96-well plates 1 day before adding the indicated concentration of the following apoptosis inducers: sTNF (A, B, G, H), αTNF-R1 mAb Htr1 (C), anti-TNF-R2-specific rabbit IgG (E), TWEAK (F), and cross-linked Flag-tagged TRAIL (D, I, J). Where indicated, the cells were incubated overnight before adding the apoptotic reagents in the absence (open circles) or presence (filled circles) of geldanamycin (0.5 μM). In experiments analyzing the effect of MG-132 (filled circles, G–J), this compound (10 μM) was added 15 min before giving the apoptotic challenge. At 8 (A, C, D, G, I) or 24 h (B, E, F, H, J) after adding the apoptotic reagent, cell viability was determined by crystal violet staining.

and Control, South Mimms, UK). Geldanamycin was from Sigma-Aldrich.

**Cytotoxicity Assays**—Cells (2 × 10^6) were grown overnight in 100 μl of culture medium in 96-well plates. The next day cells were treated with the indicated concentrations of TNF, TNF-R1/2-specific antibody, TRAIL-M2 complex, or TWEAK. Where indicated cells were pretreated overnight with geldanamycin. Cell death assays with HeLa cells (20 × 10^6) well) were performed in the presence of 0.5 μg/ml cycloheximide. Cell viability was determined using crystal violet staining as described elsewhere (14).

**RNase Protection Assay**—Kym-1 cells (2 × 10^6) were seeded in 100-mm Petri dishes and cultivated overnight. The next day cells were treated as indicated. Total RNA was isolated with the RNA INSTA-PURE kit (Eurogentech, Seraing, Belgium) according to the manufacturer’s recommendations. The presence of transcripts of the indicated cytokine genes, including TNF, and the internal controls L32 and glyceraldehyde-3-phosphate dehydrogenase were analyzed using the Multi-Probe template set (hCK-3, Pharmingen, Hamburg, Germany). Probe synthesis, hybridization, and RNase treatment were performed with the RiboQuant Multi-Probe RNase Protection Assay System (Pharmingen) according to the manufacturer’s recommendations. After RNase treatment the protected transcripts were resolved by electrophoresis on a denaturing polyacrylamide gel and analyzed on a Phosphor-Imager with Image Quant software (Amersham Biosciences). To correct the signals of protected transcripts, the background intensities were determined for each individual transcript in the close neighborhood of the respective band and subtracted from the value of the protected transcript. Finally, TNF signal intensities were normalized using the intensities of the corresponding endogenous controls.

**Western Blotting**—Cell lysates were prepared in radioimmunoprecipitation buffer supplemented with 0.1 volume of a protease inhibitor mixture stock solution (Roche Molecular Biochemicals). After removal of cell debris by centrifugation at 10,000 × g for 10 min, protein concentrations were determined by the Bradford assay. Lysates (100 μg) were resolved by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting, and nonspecific binding sites were blocked by incubation in phosphate-buffered saline containing 0.05% Tween 20 and 3% (w/v) dry milk. Procaspe-8 (p55/p53) and the p18 and p41/43 fragments derived therefrom by proteolytic processing were visualized with a caspase-8-specific mAb (kindly provided by Klaus Schulze-Osthoff) and horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma) using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates. For detection of I-κBα, a 1:2000 dilution of a polyclonal rabbit IgG preparation (Pharmingen) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Sigma) were used.

**ELISA**—For ELISA of endogenously produced TNF, cells were treated as indicated, harvested, and lysed in 300 μl of 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 complemented with a protease inhibitor mixture for 30 min on ice. After removal of debris (40,000 × g for 10 min) lysates were adjusted with respect to protein concentration. Calibrated lysates, the corresponding cell culture supernatants, and supernatants concentrated 15-fold by polyethylene glycol dialysis were analyzed with a human TNF-specific ELISA (PharMingen) according to the manufacturer’s protocol. For ELISA of TNF-R1-induced IL-6 production, HeLa cells were treated as indicated, and the corresponding cell culture supernatants were analyzed by a human
IL-6-specific ELISA (PharMingen) according to the manufacturer’s protocol.

Transient Transfection and Reporter Gene Assays—HeLa were seeded with a density of 1.5 × 10^5 cells/well in 96-well plate, and the next day cells were transfected with 250 ng/ml of a mixture of the following plasmids: empty vector (70%), a luciferase reporter plasmid driven by three consensus NF-κB sites (20%), and a β-galactosidase expression vector driven by the SV40 promoter (10%) using the Superfect reagent (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. After 1 day of recovery, cells were stimulated as indicated and lysed by the addition of 50 μl of luciferase lysis solution (GalactoLysis Kit, Tropix) and one freeze-thaw cycle. 25 μl of each extract was mixed with 50 μl of luciferase substrate (Luciferase Assay System, Promega), and the luminescence was determined in the single photon mode using an Anthos microplate luminometer (Lucy 2). In addition, 25 μl of each cell extract was incubated for 1 h with 25 μl of Galacto Plus substrate (Galacto Plus, 1:100 in Galacto reaction buffer diluent). Finally, lysates were mixed with 100 μl of Accelerator II solution to determine relative β-galactosidase activity (GalactoLysis Kit) again with the Anthos microplate luminometer (Lucy 2). To obtain relative luciferase units, luciferase activities were normalized with respect to activities of the cotransfected β-galactosidase.

RESULTS

Geldanamycin and MG-132 Completely Block TNF-R1-dependent Gene Induction and Inhibit TNF-R1-but Not TRAIL-induced Cell Death in Kym-1 Cells—We have recently shown that apoptosis induction by the non-death domain-containing receptor TNF-R2 critically depends on the induction of endogenous TNF and subsequent triggering of the death receptor TNF-R1 in Kym-1 cells (8). As TNF can also be induced via TNF-R1, we asked whether induction of endogenous TNF also contributes to the cytotoxic effect of TNF-R1 itself. We therefore examined whether interference with the gene-inducing capacity of TNF-R1 affects apoptosis induction by exclusive stimulation of this receptor, i.e. by soluble TNF or TNF-R1-specific agonistic antibodies. To block TNF-R1-mediated gene induction, cells were pretreated with geldanamycin (GA), which interferes with heat shock protein 90 (Hsp-90) function (17). Impairment of Hsp-90 function prevents TNF-R1-induced NF-κB activation in at least two ways: first, by degradation of the Hsp-90-associated kinase RIP (18), a molecule having an essential role in TNF-R1-induced NF-κB activation (19), and second, by inhibition of the recruitment of the IKK complex to TNF-R1 (20). Soluble TNF or agonistic TNF-R1-specific antibodies induce a full apoptotic response in Kym-1 cells within 8 h. In contrast, apoptosis induction by stimulation of the non-death domain-containing receptor TNF-R2 needs a prolonged incubation overnight to become detectable (data not shown and Ref. 21). The delayed onset of TNF-R2-induced apoptosis is in good agreement with the absolute dependence of this process from newly synthesized endogenous TNF. Surprisingly, rapid TNF-R1-dependent apoptosis, triggered by sTNF or the agonistic TNF-R1-specific mAb Htr1, was almost completely prevented by GA when measured after 8 h (Fig. 1, A and C) but was only slightly affected when determined after 24 h (Fig. 1B). In contrast, apoptosis induction by TRAIL, which induces apoptosis by a similar FADD- and caspase-8-dependent pathway like TNF (22), was not inhibited but rather was enhanced by geldanamycin in 8 h assays (Fig. 1D). Slow cell death induction by agonistic TNF-R2-specific antibodies as well as by TWEAK, a member of the TNF ligand family that also induces cell death in Kym-1 cells by endogenous TNF (21), was also blocked by geldanamycin (Fig. 1, E and F). Thus, RIP and/or the IKK complex also could play a role in these apoptotic processes. Remarkably, the proteasome inhibitor MG-132, which is able to block NF-κB activation by inhibition of IκB degradation, conferred significant protection against sTNF-induced apoptosis in 8 h assays (Fig. 1G) but sensitized cells for the apoptotic action of sTNF in 24 h assays (Fig. 1H). In contrast, TRAIL-induced apoptosis was sensitized by MG-132 in short (8 h) and long (24 h) time assays (Fig. 1, I and J). In comparison with GA, the inhibitory effect of MG-132 on TNF-induced apoptosis after 8 h was less efficient. Moreover, in 24 h assays MG-132 showed even a strong pro-apoptotic capacity. This might reflect that MG-132, in contrast to GA, not only inhibits the sTNF-induced production of NF-κB-dependent genes (TNF and also the anti-apoptotic factors TRAF1 (23, 24), cIAP1 (24), cIAP2 (24, 25), cFLIP (26, 27), and Bfl-1/A1 (28)) but also interferes with the constitutive expression of anti-apoptotic factors. In accordance with this idea, we found that MG-132, but not GA, sensitized several cell lines for death receptor-induced apoptosis in the absence of cycloheximide (data not shown), which is necessary in many cell lines to allow efficient apoptosis-induction. It has been shown recently that death receptors can induce necrosis instead of apoptosis by a RIP-dependent but caspase-independent pathway (29). To check whether in Kym-1 cells necrosis induction is responsible for the observed cytotoxic effects of TNF, agonistic TNF receptor-specific antibodies, and TRAIL, we analyzed the cytotoxic effects of these reagents in the presence of the caspase inhibitor z-VAD-fmk, which is able to completely block apoptosis triggered by death receptors. In all cases, z-VAD-fmk treatment conferred complete protection (data not shown), therefore ruling...
out the idea that necrosis induction plays an important role. Accordingly, we found that GA and MG-132 inhibited TNF and Htr1- but not TRAIL-induced processing of caspase-8 (Fig. 2). Together, these data suggest that TNF-R1-dependent induction of protein synthesis significantly contributes to TNF-R1-dependent apoptosis.

**Geldanamycin and MG-132 Inhibit TNF-R1-mediated Up-regulation of TNF**—Next, we analyzed the impact of GA and MG-132 on TNF receptor-mediated gene induction by RNase protection assay analyses. Soluble TNF, the TNF-R1-specific agonistic mAb Htr1, and to a lesser extent agonistic TNF-R2-specific antibodies and TWEAK indeed up-regulated TNF-mRNA (Fig. 3A) as well as the well known NF-κB target genes TRAF1 and cIAP2 (data not shown). In accordance with the role of endogenously produced TNF in apoptosis induction, GA blocked TNF induction by the above mentioned reagents.

**Endogenous TNF Is Involved in TNF-R1-dependent Apoptosis**

A, Kym-1 cells (20 × 10^3) were cultivated in 100 μl of medium in 96-well plates overnight. The next day cells were challenged for 8 h with αTNF-R1 mAb Htr1 (250 ng/ml) in the absence (open circles) or presence of 10 μg/ml of the neutralizing TNF-specific mAb 101-4 (filled circles). Viability was again determined by crystal violet staining. B, a TNF pulse is sufficient for apoptosis induction in Kym-1 cells. Kym-1 cells (20 × 10^3) were cultivated in 100 μl of medium in 96-well plates overnight. The next day, cells were treated with the indicated concentration of αTNF. After 30 min TNF was removed by three washes with medium. Subsequently, fresh medium was added with (open circles) or without (filled circles) new TNF. From the experimental group replenished with medium only, a part of the supernatant was taken after 30 min and transferred to an independent group of Kym-1 cells (filled squares) to control the efficiency of the wash procedure. After overnight incubation, cell viability was determined by crystal violet staining. C, Kym-1 cells were stimulated with a short pulse of sTNF (10 ng/ml) followed by a past pulse incubation of 8 h with the indicated combinations of TNF (10 ng/ml) and z-VAD-fmk (20 μM). Cell culture supernatants were then transferred to HT29 cells and co-treated with 10 ng/ml IFNγ. After 36 h the viability of HT29 cells was determined by crystal violet staining. The supernatant of Kym-1 cells stimulated with a TNF pulse followed by treatment with z-VAD-fmk was also analyzed upon 5-fold concentration to increase the chance to detect low concentrations of TNF. In experimental groups in which the TNF pulse was followed by z-VAD-fmk treatment, the latter was also given during the TNF pulse. Please note that TNF kills IFNγ-treated HT29 cells by necrosis, which is not inhibited by z-VAD-fmk, with an ED50 between 30 and 100 pg/ml (data not shown). D, Kym-1 cells were stimulated for 8 h with the agonistic TNF-R1-specific mAb Htr1 (250 ng/ml) in the presence of 20 μM z-VAD-fmk (open bars). TNF concentrations were determined in supernatants (SN), in cell lysates, and in the detergent-soluble cell fraction (2 × 10^6 cell equivalents/ml) by a TNF-specific ELISA. Concentrated supernatants and cell lysates were adjusted to represent similar cell equivalents. E, Kym-1 cells were incubated for 24 h with the agonistic TNF-R1-specific mAb Htr1 (250 ng/ml) in the presence of 20 μM z-VAD-fmk (solid line) or with z-VAD-fmk alone (gray plot, dotted line). Expression of memTNF was detected by immunofluorescent staining and fluorescence-activated cell sorter analysis using the TNF-specific mAb T1. Before incubation with the TNF-specific antibody, cells were washed for 1.5 min at pH 3 to disrupt complexes of memTNF and TNF receptors. F, Kym-1 cells (20 × 10^3) were cultivated overnight in 96-well plates in the absence or presence of GA (0.5 μM). The next day cells were challenged for 8 h with the indicated combinations of αTNF-R1 mAb Htr1 (250 ng/ml) and TNF-R2-specific rabbit IgG. Finally, cell viability was again determined by crystal violet staining.
Endogenously Produced TNF Has a Pivotal Role in Rapid TNF-R1-initiated Apoptosis in Kym-1 Cells—To demonstrate directly the importance of endogenous TNF in TNF-R1-triggered apoptosis, we analyzed the impact of neutralizing TNF-specific antibodies. In a former study, we showed that a neutralizing TNF-specific antibody blocks TNF-R2- but not TNF-R1-triggered apoptosis and failed to find evidence for a measurable contribution of endogenous TNF in TNF-R1-induced apoptosis (8). However, in this study, we have searched for an involvement of endogenous TNF in overnight assays (18–24 h) and hence, under conditions in which GA and MG-122 conferred only limited (GA) or no (MG-122) protection (compare Fig. 1, A and B with G and H). Thus, we redesigned our experimental conditions and looked for an inhibitory effect of neutralizing TNF-specific antibodies in TNF-R1-induced apoptosis triggered by Htr1 in short (8 h) time assays. At this early time point TNF-R1-induced cell death was completely blocked by neutralizing TNF-specific antibodies, demonstrating that endogenously produced TNF has a pivotal role in TNF-R1-dependent apoptosis in Kym-1 cells (Fig. 4A).

Geldanamycin blocked the apoptotic effect of TNF concentrations high enough to allow complete occupancy of TNF-R1. It is therefore very unlikely that the endogenously produced TNF exerts its potent enhancement of apoptosis simply by delivering an additional trigger for TNF-R1. Endogenously produced TNF therefore more likely fulfills a function that cannot be readily mediated by soluble TNF. If one takes into consideration that endogenously produced TNF is primarily expressed in its membrane-bound form, such a pro-apoptotic mechanism could reside in the triggering of TNF-R2 and thereby in TRAF2-dependent depletion of anti-apoptotic proteins, as discussed previously (10–14). To estimate the amount of TNF-R1-induced TNF in Kym-1 cells, we challenged TNF-R1 with sTNF for 30 min. This short TNF pulse was sufficient to induce complete cell death, albeit with a somewhat reduced efficiency compared with permanent TNF treatment (Fig. 4B). After removal of this initial TNF-R1 trigger by a pH3 wash or three washes with culture medium, the cells were cultivated for an additional 8 h in the presence of z-VAD-fmk to inhibit apoptosis. The cytotoxic effect of the supernatants was then tested on HT29 cells. HT29 cells were used because in these cells sTNF in combination with IFNγ induces necrosis instead of apoptosis (Ref. 30; own data not shown). As necrosis is not inhibited or even enhanced by caspase-inhibitors (31, 32), HT29 cells are useful in detecting sTNF in the supernatants of
Endogenous TNF Is Involved in TNF-R1-dependent Apoptosis

Fig. 6. Model of TNF receptor co-operation in rapid TNF-R1-initiated apoptosis of Kym-1 cells. “Primary” events (solid lines): selective stimulation of TNF-R1 by soluble TNF or agonistic antibodies (1) results in the NF-κB-dependent (2) induction of TNF (3b) and anti-apoptotic factors (3a). The TNF-R1-induced, and possibly already existing, anti-apoptotic factors (e.g. IAPs) interact with TRAF2 (3a-1) and block TNF-R1-induced apoptosis (3a-2). “Secondary” events (dotted lines): the endogenously produced TNF acts primarily in its membrane-bound form (3b-1) and can therefore stimulate TNF-R1 and more importantly TNF-R2 (3b-2). As a consequence of the stimulation of the TRAF2-interacting receptor TNF-R2 by endogenous membrane TNF (3b-3), the anti-apoptotic TRAF2-containing complex is deviated from TNF-R1, and the aforementioned block on TNF-R1-dependent apoptosis is released (3b-4). Because of the pivotal role of TNF-R2 stimulation by endogenously produced membrane TNF, reagents interfering with its action (A, GA and Mg-123 by inhibiting its production; B, anti TNF by blocking its action) strongly inhibit rapid TNF-R1-induced apoptosis. Please note that with extended incubation times TNF-R1 stimulation alone, without the support of TNF-R2, can also induce apoptosis.

cells treated with caspase-inhibitors. Using this assay we found no evidence for bioactive sTNF in the supernatants of z-VAD-fmk-protected, TNF-R1-stimulated Kym-1 cells even at 15 fold concentration (Fig. 4C). To detect TNF-R1-induced TNF directly, we analyzed supernatants and cell lysates of Htr1-stimulated cells. In accordance with the bioassays described above, only very limited amounts of TNF were found in concentrated supernatants of Htr1-treated cells. In contrast, in detergent-soluble fractions derived from Htr1-treated Kym-1 cells, significant amounts of TNF were detectable (Fig. 4D). Dependent on the experiment, the detected amount of TNF corresponded to 500–1500 molecules/Kym-1 cell. In accordance with the idea that the cell lysate-associated TNF represents the membrane-bound form of TNF, fluorescence-activated cell sorter analysis revealed consistently weak membrane TNF expression after treatment with a mixture of TNF and z-VAD-fmk (Fig. 4E).

According to our model, the pro-apoptotic action of TNF is not absolutely essential to it. According to our model, the pro-apoptotic action of TNF-R2 relies on the capability of this receptor to recruit the aforementioned anti-apoptotic TRAF-IAP complex. As a consequence of the competition of TNF-R1 and TNF-R2 for the TRAF2-dependent recruitment of the anti-apoptotic TRAF-IAP complex, the availability of the latter for TNF-R1 is reduced (33). Therefore, the blockage on procaspase-8 processing in the TNF-R1 signaling complex is relieved and the apoptotic pathway can be triggered. TRAF2-dependent competition for IAP proteins can be effective only when these proteins are of limited availability. Indeed, Western blot analysis of endogenous and green fluorescent protein-tagged recombinant IAP proteins suggest that in Kym-1 cells, even after TNF-R1-induced up-regulation, only a few thousand of these molecules are expressed per cell (data example, HeLa-TNF-R2 cells, which are not capable of producing TNF after TNF-R1 triggering (data not shown) but express high numbers of TNF-R2 (14), required prolonged exposure to sTNF (3–8 h) for significant induction of cell death (Fig. 5A) and activation of caspase-8 (Fig. 5B). In accordance with the lack of sTNF-induced TNF production in HeLa-TNF-R2 cells, GA failed to inhibit TNF-R1-dependent cell death in these cells (Fig. 5C). Importantly, a short pulse with sTNF was sufficient to induce IκB degradation (Fig. 5D) and DNA binding of NF-κB (Fig. 5E). Consequently, a TNF pulse activated a NF-κB reporter gene and induced the NF-κB target, IL-6, almost as efficiently as under conditions in which sTNF was present during the whole assay (Fig. 5A). These data indicate that in HeLa cells the rapid activation of gene-inducing pathways (NF-κB) is normal. Thus, the possibility arises that the formation of apoptosis-competent TNF-R1 signaling complexes is a secondary, rather ineffective event occurring only with time after the predominant formation of non-apoptotic gene-inductive receptor signaling complexes. However, the formation of pro-apoptotic TNF-R1 signaling complexes can be facilitated by memTNF/TNF-R2-dependent depletion of TRAF2 and TRAF2-interacting anti-apoptotic factors (Fig. 6).

DISCUSSION

Based on the aforementioned experimental data and data from the literature, we propose the following model of rapid TNF-R1-induced apoptosis (Fig. 6). Exclusive stimulation of TNF-R1 by soluble TNF or TNF-R1-specific agonistic antibodies leads to activation of the NF-κB pathway. Among the NF-κB-induced target genes in Kym-1 cells are the anti-apoptotic factors cIAP2 and TRAF1 (data not shown) but also the TNF gene itself (Fig. 3). Newly produced (e.g. cIAP2 and eventually TRAF1) and already existing (e.g. cIAP1) anti-apoptotic factors form a complex with TRAF2 (24, 33). By virtue of the capability of TRAF2 (and eventually TRAF1) to interact with TNF-R1-bound TRADD, this complex translocates to the TNF-R1-signaling complex, thus preventing the proteolytic activation of procaspase-8 (24). However, in TNF-R2-expressing cells that respond, with the up-regulation of TNF, to TNF-R1 triggering, the protective effect of the TRAF-IAP complex becomes secondarily compromised. As the endogenously produced TNF occurs primarily in its membrane-bound form (Fig. 4), it has the capability to stimulate TNF-R2 in an autocrine/paracrine manner. The importance of TNF-R2 stimulation by endogenously produced membrane TNF is reflected by the fact that reagents interfering with the action of membrane-bound TNF strongly inhibit rapid TNF-R1-induced apoptosis (Figs. 1 and 4A). However, there is no absolute requirement for endogenous membrane-bound TNF to induce cell death, because Kym-1 cells undergo apoptosis even in the presence of GA after stimulation with soluble TNF for 24 h (Fig. 1B). Therefore, endogenous TNF enhances and accelerates TNF-R1-induced cell death but is not absolutely essential to it.
not shown). It has been shown recently that cIAP1 can act as an E3 ligase upon recruitment into the TNF-R2 signaling complex, triggering the proteasomal degradation of TRAF2 (34). Thus, this process might intensify the competition of the TNF receptors for the anti-apoptotic TRAF-IAP complex.

In cells not responding with TNF production toward TNF-R1 stimulation, apoptosis-induction, but not NF-κB activation, can be dependent on the prolonged presence of exogenous TNF (Fig. 5). Thus, it is tempting to speculate that endogenously produced TNF can also exert a pro-apoptotic effect in the absence of TNF-R2 by facilitating cell-autonomous prolonged TNF-R1 triggering. In conclusion, our study reveals a novel mechanism of TNF-R2 by facilitating cell-autonomous prolonged TNF-R1 induction.

Acknowledgments—We thank P. Schneider and J. Tschopp (University of Lausanne, Switzerland) for kindly providing Flag-tagged TRAIL and Flag-tagged TWEAK.

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