TNF Receptors TR60 and TR80 Can Mediate Apoptosis Via Induction of Distinct Signal Pathways[1]

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TNF membrane receptors are usually co-expressed in many tissues but their relative contribution to cellular TNF responses is for most situations unknown. In a TNF cytotoxicity model of KYM-1, a human rhabdomyosarcoma cell line, we recently demonstrated that each of the two TNFRs is on its own capable of inducing cell death. Here we show that both receptors are able to induce apoptosis, as revealed from a similar onset of DNA fragmentation and typical morphologic criteria. To obtain additional information about the signaling pathways involved in TR60- and TR80-induced programmed cell death, we have used a series of selective inhibitors of intracellular signaling molecules. The overall pattern emerging from these experiments provides strong evidence for distinct signal pathway usage of TR60 and TR80, indicating protein kinase(s)-mediated control of TR60 signaling and a tight linkage of TR80 to arachidonate metabolism. The subsequent establishment of KYM-1-derived cell lines that display TNFR selective resistance further supports a segregation of TR60 and TR80 signaling pathways for induction of apoptotic cell death. Moreover, these results demonstrate an independent control of the distinct signaling cascades used by TR60 and TR80. This allows a highly flexible regulation of a cellular TNF response in those cases in which both receptors contribute to overall TNF responsiveness. *The Journal of Immunology,* 1994, 153: 1963.

TNF is a pleiotrophic cytokine that is primarily produced by activated macrophages. Originally named for its ability to induce tumor necrosis in certain model systems, TNF is now known to play a major role in many processes of inflammation affecting both hematopoietic and nonhemopoietic tissues. Beside a physiologic role of TNF in ontogeny and control of certain infections, TNF is also recognized as an important pathogenic factor in several chronic diseases (for reviews see Refs. 1, 2). This dual role of TNF in vivo is on the basis of the ability of TNF to modulate the expression of a variety of genes, including several other proinflammatory cytokines such as IL-1, IL-6, granulocyte-macrophage CSF, cytokine receptors, adhesion molecules, and enzymes of various metabolic and catabolic pathways (reviewed in Ref. 3). Furthermore, TNF has been demonstrated to possess direct cytotoxic activity in vitro for certain tumor cells and for some normal cells (4–6).

TNF and lymphotoxin initiate their broad range of cellular responses by interaction with cell surface membrane receptors. Two distinct but related receptor molecules have recently been molecularly cloned (7–10), here referred to as TR60 (type I) and TR80 (type II). Both receptors have significant homologies in their extracellular domains with repeat cysteine-rich sequences, defining them as members of a recently established growing receptor family (11). In contrast, the cytoplasmic domains of TR60 and TR80 are unrelated and give no indication by which mechanisms they are coupled to and activate intracellular signaling pathways.

In addition to differences in primary structure, expression of the two TNFRs seems to be differentially regulated and shows tissue-specific prevalence (12–14). Thus, although co-expression of both receptors is found in many cells, there is typically a quantitative dominance of one of the two receptors. For example, lymphoid cells predominantly express TR80 molecules, whereas epithelial cells typically express TR60 (15).

Concerning the cytotoxic activity of TNF, recent findings have drawn new attention to this particular action as

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an important physiologic function. First, a role of TNF as a possible stimulating and effector molecule of NK/lymphokine-activated killer cells is apparent from several studies (16, 17). Second, TNF seems to function as a transducer of cellular cytotoxicity in its membrane-integrated form, e.g., on macrophages (18). Third, and most intriguing, recently, TNF has been proposed to act as an inducer of apoptotic cell death during thymocyte maturation (6, 19). Clearly, TNF has the potential to exert cytotoxic effects for different cell types in differential ways, because it can induce both necrotic and apoptotic forms of cell death (20, 21).

The cellular events in TNF-mediated cytotoxicity after ligand binding to membrane receptors are still poorly understood. Involvement of different signal transduction pathways has been suggested. These include activation of phospholipase (PL)[3] A_2 with release of arachidonate metabolites (22, 23), stimulation of PLs D (24) and C (25, 26) with activation of protein kinase C (PKC) by diacylglycerols, activation of sphingomyelinase activity with subsequent release of ceramides (27), and, finally, hydroxyl radical production by mitochondrial enzymes (28). On the other hand, most tumor cell lines are sensitive to the cytotoxic action of TNF only in the presence of synergizing biologic or chemical reagents. This seems to be true independent of the pattern of TNFR expression. Therefore, it is likely that intracellular regulatory circuits control the sensitivity toward the cytotoxic action of TNF. For example, IL-1 and TNF, itself, have been demonstrated to induce TNF resistance in a protein synthesis-dependent manner (29). Key molecules in induction of TNF resistance could represent manganese-dependent superoxide dismutase (30), heat shock proteins (31), and PKs (32, 33).

Until recently the role of the two TNFRs for induction of cytotoxic effects of TNF was unclear. As of now, the vast majority of cellular responses have been attributed to signaling via TR60, whereas only a few examples exist to demonstrate TR80-initiated cellular answers. In particular, TNF-mediated cytotoxicity has been linked to TR60 in a number of different human cell lines (12, 34). The principal capability of TR60 to trigger cytotoxicity on its own has been clearly demonstrated in heterologous human/mouse bioassays in which a selective cross-species activation of the murine TR60, but not TR80, was achieved (35). Furthermore, studies with receptor subtype-specific agonistic antisera have indicated that Abs specific for murine TR60 induce cytotoxicity, whereas otherwise agonistic TR80-specific sera failed to do so (36).

A recent report has attributed this capability of TR60 to a cytoplasmic region with homology to the apoptosis inducing Ag Fas/APO-1, another member of the TNFR family (37). Although these data suggest that TR60 plays a major role for induction of a cytotoxic response, other studies indicate that TR80 may also be involved. Thus, in U937 cells, the blocking of each of the receptor subsets resulted in a significant reduction in TNF-induced cytotoxicity (38). Furthermore, it was shown that overexpression of TR80 in HeLa cells strongly enhanced TNF sensitivity (39). An even more complicated situation might be found in thymocytes in which the pattern of cytotoxic responses to TNF could be controlled by both receptors in a very subtle way (6).

Using the human rhabdomyosarcoma cell line KYM-1, which co-expresses high numbers of both receptors, we recently demonstrated that each receptor is able to induce cytotoxicity on its own (40). Moreover, limited receptor triggering that used the respective specific Abs indicated additive action (40). Here we show that, on cross-linking, each receptor induces apoptosis, as revealed from the induction of DNA fragmentation and typical morphologic changes. To obtain additional information about the signal transduction pathways involved, we used a number of selective inhibitors to interfere with either TR60- or TR80-induced apoptosis. Furthermore, we established KYM-1-derived cell clones showing receptor-type selective resistance. Both experimental approaches indicate clearly that the two TNFRs initially activate different signal pathways that eventually merge and lead to the identical cellular response, i.e., apoptosis.

Materials and Methods

Cell lines and reagents

The human rhabdomyosarcoma cell line KYM-1 (41) was generously supplied by Dr. M. Sekiguchi (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Cells were cultured at 37°C and 5% CO_2 in Clicks RPMI 1640 medium (Biochrom, Berlin, Germany) containing 10% heat-inactivated FCS and antibiotics. The generation and specificity of the mAb H398, directed against TR60 (12), the rabbit anti-human TR80 serum M80 (40), and the agonistic mAb htr-1 (15) has been described. To generate resistant KYM-1 subclones, cells were grown under culture conditions in the presence of increasing concentrations of htr-1 for KYM-60res or M80 serum for KYM-80res cells. Human rTNF-α (sp. act. 2×10^7 U/mg) was kindly provided by Knoll AG, Ludwigshafen, Germany). The following reagents were purchased from Sigma Chemical Co. (Deisenhofen, Germany): amytal (amobarbital); antimycin; bromophenacyl bromide (BPB); genistein; lithium chloride, 2-nitro-4-carboxyphenyl N,N-diphenylcarbamate (NCDC); neomycin; nordihydroguaiaretic acid (NDGA); pargyline; quinacrine; and thenoyltrifluoroacetone (TTFA). H7, H8, H89, K-252a, K-252b, staurosporine, and calphostin C were from Calbiochem, Bad Soden, Germany. D609 was from Kamiya Biomedical Company, Thousand Oaks, CA. For use in cytotoxicity assays, stock solutions of the inhibitors were prepared in medium, ethanol, or DMSO as appropriate, such that the final concentration never exceeded 0.5%, which was controlled not to affect cell viability or TNF cytotoxicity.

Electron microscopic histology

Cell cultures were fixed in 2.5% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 M PBS at pH 7.1 for 1 h at room temperature. Postfixation in 1% osmium tetroxide (Merck, Darmstadt, Germany) in 0.1 M PBS was followed by dehydration in ethanol (8 min in 40, 50, 60, 70, and 80% ethanol, 2×15 min in 96 and 100% ethanol), and propylene oxide (2×15 min). After impregnation overnight with 2:1 and 1:1 mixtures of propylene oxide and the epoxy resin, respectively, cells were embedded in

[3] Abbreviations used in this paper: PL, phospholipase; PC, phosphatidylcholine; PK, protein kinase; ROI, radical oxygen intermediate; BPB, bromophenacyl bromide; NCDC, 2-nitro-4-carboxyphenol *N,N*-diphenylcarbamate; NDGA, nordihydroguairetic acid; TTFA, thenoyltrifluoroacetone; PKC, protein kinase C; PKA, protein kinase A.

gelatin capsules and polymerized for 12 h at 40°C and at least 48 h at 70°C. Specimens were thin sectioned with a diamond knife on a Reichert OM U3 ultramicrotome (Reichert-Jung, Nußloch, Germany), stained with uranyl acetate and lead citrate, and examined with a Zeiss EM 10A electron microscope at 60 kV.

Detection of DNA fragmentation

Cells were incubated under culture conditions with the indicated reagents for different times, then harvested, and DNA was isolated by standard procedures. DNA was analyzed by electrophoresis on a 1.2% agarose gel and visualized by ethidium bromide staining.

Cytotoxicity assay

This assay was conducted essentially as described previously (5). Briefly, cells were seeded into 96-well microtiter plates at 1×10^4 cells/well and allowed to grow overnight before the addition of the different substances to a final volume of 200 μl. For inhibitor studies, the respective concentrations of the different substances not exhibiting more than 10% cytotoxic effect, per se, over the assay period were initially determined. The substances were added 30 to 60 min before TNF or the receptor-specific agonists. Experiments that used calphostin C as a specific PKC inhibitor were performed under illumination (42). After 18 h of culture MTT, dimethylthiazol bromide (10 μl/well of a 5 mg/ml solution in PBS) was added to all wells and metabolically active cells were allowed to produce the formazan product for 2 h at 37°C. Subsequently, 90 μl of a 15% SDS solution in 0.02 N HCl was added. ODs were read after an additional 4-h incubation at room temperature at 560 nm.

Results

Both TNFRs can independently mediate cytolysis in the human rhabdomyosarcoma cell line KYM-1 (40). To approach the molecular mechanisms involved, we first investigated, by using electron microscopic analyses, the ultrastructural changes preceding TNF-induced cell death in KYM-1 cells. After stimulation with either TNF or the respective receptor-specific agonistic Abs, the typical onset of apoptotic cell death was revealed in all cases (Fig. 1). Accordingly, treatment with either TNF, the TR60-specific mAb H398 plus a secondary cross-linking reagent (goat anti-mouse IgG), or the polyclonal TR80-specific rabbit serum M80 (40) initiated strong vacuolization, condensation of chromatin at the nuclear membrane, and cellular fragmentation. All of these morphologic criteria have been associated with programmed cell death (43). Additional support for the induction of apoptosis, rather than necrosis, comes from studies of DNA fragmentation caused by induction of an endonuclease(s) that preferentially digests internucleosomal DNA (44). As shown in Figure 2, incubation of KYM-1 cells with TR80-specific agonistic Abs resulted in a typical DNA degradation pattern with approximately 180-bp steps similar to the DNA ladder observed on TNF-treatment or TR60-specific stimulation. Thus, independent and selective triggering of TNFRs in each case resulted in typical apoptotic cell death.

Because TR80, like TR60, is capable of conferring apoptotic signals to the cell nucleus, we next asked whether both receptors use identical intracellular signaling pathways. To determine this, we used several inhibitors of well-defined signal transduction pathways that have been implicated in TNF responses and asked whether they would interfere with TNF-induced cytolysis of KYM-1 cells. Initially, we tested a broad variety of kinase inhibitors for their ability to interfere with TNF-mediated cytotoxicity. Neither the potent tyrosine kinase inhibitor genistein nor the selective PKC inhibitor calphostin C showed significant effects on TNF-induced cytotoxicity. In contrast, the isoquinoline sulfonamide H8 clearly antagonized with the cytotoxic effects of TNF (Fig. 3). Only partial inhibition, however, was noted at the concentrations used. Higher concentrations of this PK inhibitor could not be used because of toxic side effects of the compound itself. Interestingly, the related compounds H89 and H7 did not significantly interfere with TNF cytotoxicity. In contrast, the highly potent serine/threonine and tyrosine PK inhibitor staurosporine, similar to the compounds K252a and K252b, strongly enhanced TNF-induced cytotoxicity (Fig. 3).

Radical oxygen intermediates (ROIs) have been shown to be involved in TNF-induced cytotoxicity in different cellular systems (28, 45, 46). Therefore, we investigated several inhibitors of the mitochondrial electron transport system for interference with TNF-mediated cytotoxicity in KYM-1 cells. Clearly, amytal, a complex I inhibitor, exerted protective effects, whereas TTFA, an inhibitor of complex II formation, was ineffective (Fig. 3). Antimycin A, a complex III inhibitor, which leads to the accumulation of ROIs in the mitochondria (46), enhanced TNF cytotoxicity. The latter data are in accordance with the proposed role of oxygen radical generation as a causal mechanism of TNF cytotoxicity (28) in KYM-1 cells.

Regarding the other compounds tested, the lipoxygenase inhibitor, NDGA, the PL inhibitor, neomycin, and the inhibitor of monoamine oxidase, pargyline, all significantly inhibited TNF-mediated cytotoxicity in KYM-1 cells. However, in all cases only partial protection was observed at the highest possible (nontoxic) concentrations used (Fig. 3). These results are in agreement with reports demonstrating the involvement of PLA_2 and lipoxygenase metabolites in cell killing by TNF (23, 24). However, a number of additional PLA_2 inhibitors used, such as BPB, NCDC, or quinacrine, and the PC-PLC-specific inhibitor D609 failed to protect KYM-1 cells from TNF-mediated cytotoxicity, although they have been shown to interfere with TNF signaling in other cellular systems (22, 27).

Some compounds that interfere with TNF action have been shown to be effective via modulation of TNFR expression (47, 48). To verify that the substances used here are indeed effective at a postreceptor, i.e., signal transduction level, binding competition studies with radiolabeled TNF and the respective receptor-specific Abs were performed. These experiments revealed that none of the TNF response-inhibiting substances, i.e., H8, neomycin, NDGA, pargyline, and amytal, significantly changed the expression of the two TNFRs or interfered with ligand or Ab binding to membrane expressed receptors (data not shown).

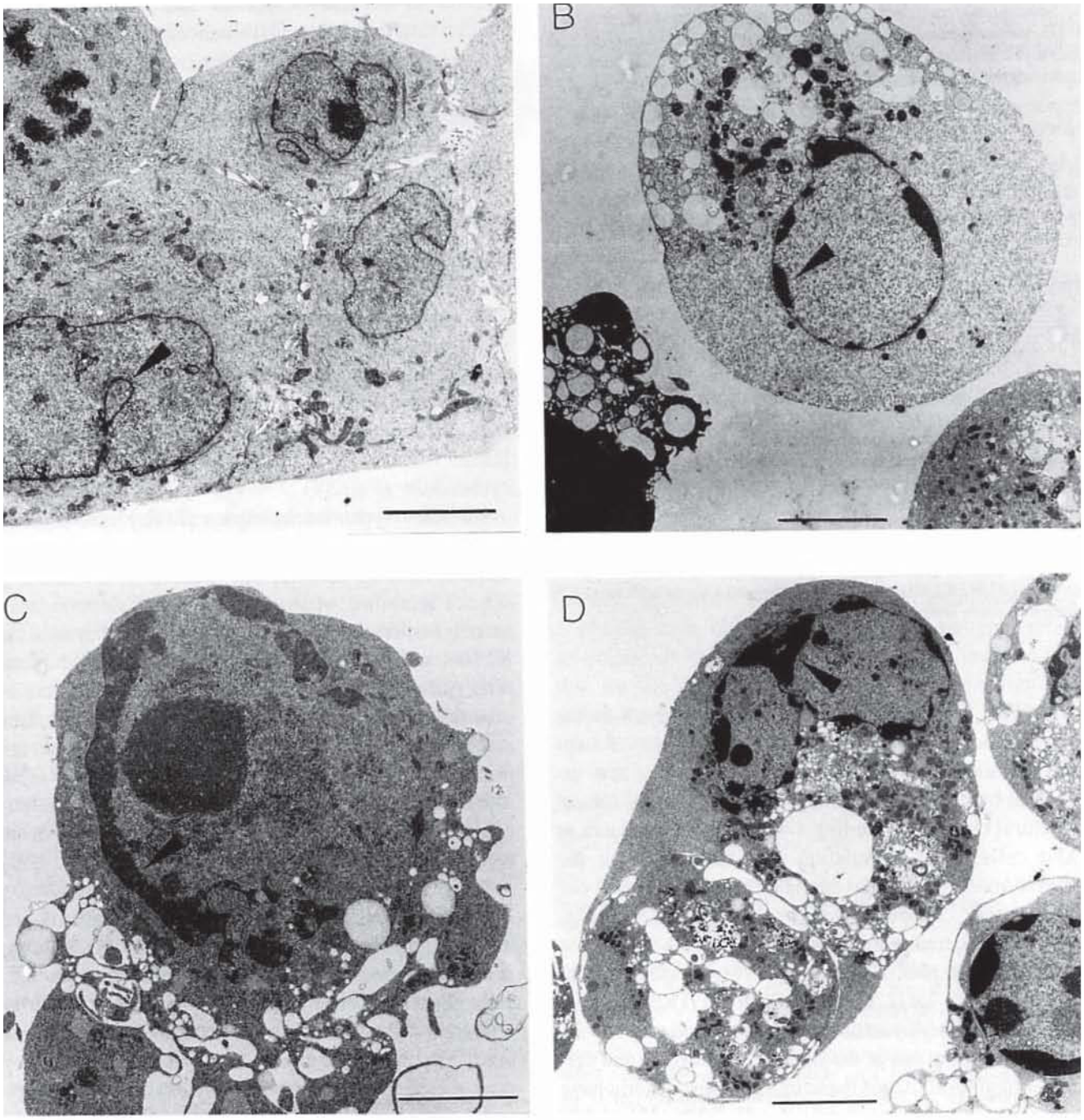

FIGURE 1. Apoptosis of KYM-1 cells induced via both TNFRs. Transmission electron micrographs were taken after 8 h of treatment of KYM-1 cells with medium (*A*), 20 ng/ml TNF (*B*), 1:200 dilution of TR80-specific rabbit serum M80 (*C*), or 3 μg/ml TR60-specific mAb H398 plus 10 μg/ml goat anti-mouse IgG (*D*). Arrowheads indicate nuclear membrane without (*A*) or with condensed chromatin (*B* through *D*). Bars = 5 μm.

Currently, all of the known TNF signal transduction pathways have been linked to TR60 rather than TR80 (27, 49–51). Therefore, it was of great interest to determine whether the above inhibitors could be used to distinguish between the signals used by the two distinct TNFRs in KYM-1 cells. Receptor-specific agonistic Abs were used to selectively induce cytolysis in KYM-1 cells. In a first set of experiments, inhibitors were used at a constant subtoxic concentration and the respective TNFR stimuli were titrated. The data obtained (Fig. 4) indeed reveal a differential sensitivity to the inhibitors and suggest a segregation in the signal pathways used by TR60 and TR80 for induction of apoptosis in KYM-1 cells. Thus, TR60-triggered cytotoxicity could be antagonized by pargyline and H8 (Fig. 4, *A* and *B*), but not by NDGA (Fig. 4*C*). In contrast, the inhibitor NDGA specifically interfered with TR80-mediated cytolysis induced by a TR80-specific serum (Fig. 4*G*), but no inhibition could be obtained with either H8 or pargyline (Fig. 4, *E* and *F*). To ensure that the observed selectivity of H8, pargyline, and NDGA was not concentration dependent, we titrated the inhibitors in a second set of experiments. Representative results are

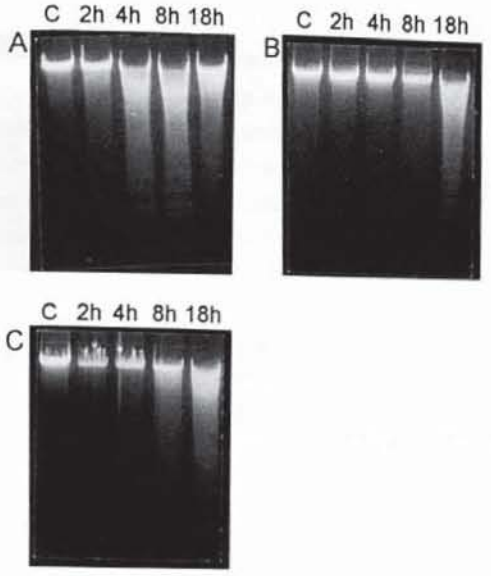

FIGURE 2. Kinetics of DNA fragmentation on selective TNFR activation. KYM-1 cells were treated as described in Figure 1 with TNF (*A*), M80 serum (*B*), or H398 plus goat anti-mouse IgG (*C*) for the indicated times. Control lanes (c) represent 18-h stimulation with medium (*A*), control rabbit serum (*B*), or goat anti-mouse IgG alone (*C*). DNA was extracted and subjected to electrophoresis on a 1.2% agarose gel in the presence of ethidium bromide.

shown in Figure 5, which clearly demonstrates that the selectivity of the inhibitors was independent of the concentration used. Again, only partial protection could be obtained because of the intrinsic toxicity of these compounds.

Because in KYM-1 cells the selective activation of each of the TNFRs finally leads to the development of apoptosis, it seems likely that both signal pathways merge at a point before the relevant intracellular effector molecules of apoptosis are activated. Indeed, we found that down-regulation of ROI formation by amytal significantly ameliorated both TR60- and TR80-induced cytolysis (Figs. 4, *D* and *H* and 5*D*). Taking into consideration that amytal possesses a considerable intrinsic cytotoxicity at the concentrations used, TR60-triggered effects could be blocked approximately 30% (Fig. 4*D*; 0.8 μg/ml H398 mAb) and TR80-mediated effects were inhibited to a lesser, but significant, extent (20%; Fig. 4*H*; serum dilution 1:800). On the basis of these results we conclude that, in KYM-1 cells, the signals induced by both receptors might be connected to the mitochondrial ROI system.

We have recently shown that induction of TNF resistance in KYM-1 cells affects both TNFRs (52). Provided the signaling cascades triggered by the two TNFRs segregate, it would be possible to establish selectively resistant cell lines derived from KYM-1 by prolonged specific stimulation of only one of the two TNFRs. Indeed, after 2 to 4 wk of culture in the presence of the TR60-specific agonistic mAb, htr-1, the surviving cells had become highly resistant toward a subsequent challenge with the same stimulus (Fig. 6*A*). Remarkably, the sensitivity of these cell lines, designated KYM-60res, toward M80-induced apoptosis was only slightly reduced (Fig. 6*B*). The reciprocal results were obtained after a selection with M80 serum. In this case, a strong desensitization of TR80 signaling (Fig. 6*B*) hardly affected sensitivity toward TR60-induced cytotoxicity (Fig. 6*A*).

Next, we wanted to know the effects of TR60 or TR80 desensitization on the expression of TNFRs by ligand competition studies with iodinated TNF and receptor specific Abs. As published previously (52), neither stimulus had any effects on TR60 cell surface expression, whereas TNF or M80 treatment led to a reduction in TR80 receptor expression. When TR80-resistant cell lines had been established after approximately 14 days of continuous stimulation, these cells expressed 3000 to 6000 TR80 molecules/cell. On additional culture in the presence of TR80 agonists, this number further declined to levels below detection (<100/cell) within 2 to 3 mo (52; and data not shown). However, induction of resistance in the KYM-80res cells shown in Figure 6 cannot be attributed to homologous receptor down-regulation, because these cells still expressed 4000 TR80 molecules/cell (data not shown). This suggests a postreceptor level of resistance induction preceeding complete loss of membrane receptors. In contrast, KYM-60res cells, having gained strong resistance toward TR60-specific agonists (Fig. 6*A*), showed no change in either TR60 or TR80 membrane expression. These data indicate that the sensitivity changes in this subline are strictly confined to a postreceptor level, i.e., affect signal transduction.

Although the development of selective receptor resistance was reproducible in a number of independent experiments, it was not possible to generate cell lines with stable phenotypes. First, the KYM-60res line could be reverted readily to higher sensitivity within 1 wk after abrogation of the receptor-specific stimulus. Second, with a prolonged period (>2 mo) of constant selective pressure, both cell lines, KYM-60res and KYM-80res, converted to a cross-resistant phenotype in which both TNFR signal pathways were affected (data not shown).

Discussion

The role of the two defined TNF membrane receptors is currently discussed in a controversial manner. In the majority of experimental systems, the ability of TNF to exert cytotoxic effects has been clearly attributed to TR60 (12, 34, 36, 37). This investigation, however, unambiguously demonstrates that TR80 is per se able to trigger apoptosis, i.e., programmed cell death. Typical symptoms of apoptosis, like DNA fragmentation and chromatin condensation at the nuclear membrane, were noted on independent triggering of each of the two TNFRs (Figs. 1 and 2). A

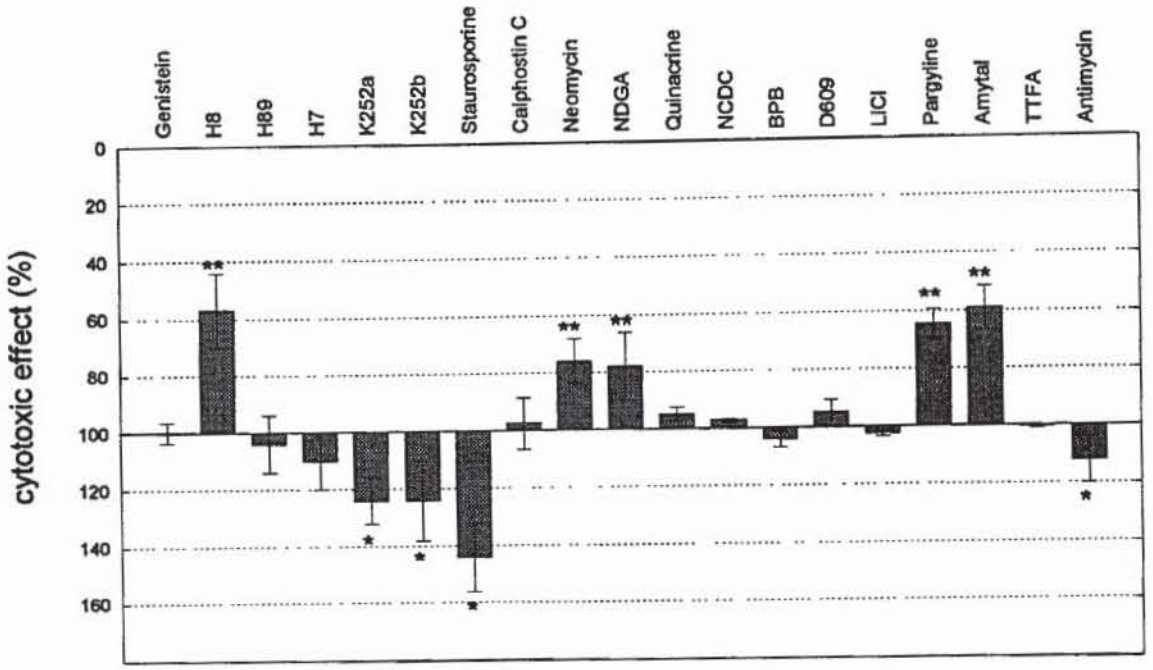

FIGURE 3. Influence of various inhibitors of intracellular signal pathways on TNF-mediated cytotoxicity. KYM-1 cells were cultured for 18 h in microtiter plates with or without 250 pg/ml TNF (approximately ED_{50}) in the absence or presence of genistein (3.2 μM), H8 (15 μM), H89 (0.5 μM), H7 (6.3 μM), K-252a (250 nM), K-252b (2.5 μM), staurosporine (25 nM), calphostin C (50 nM), neomycin (1 mM), NDGA (10 μM), quinacrine (2.5 μM), NCDC (50 μM), BPB (2.5 μM), D609 (120 μM), LiCl (2.5 mM), pargyline (1.25 mM), amytal (400 μM), TTFA (45 μM), or antimycin (5 μM). The maximum concentration for each drug that did not, on its own, exhibit more than 10% cytotoxic effect over the assay period was determined and chosen for the experiments. Cell viability was determined by formazan production and evaluated in an ELISA reader at 560 nm. Shown is the influence of the drugs on TNF-induced cytotoxicity setting the TNF effect without the drug as 100% and the OD in the presence of the inhibitor alone as 0%. Experiments were performed in triplicate and the mean SD of at least three independent experiments is presented (significance was evaluated by using Student's *t*-test: $^*p < 0.05$; $^{**}p < 0.01$).

minor difference observed was the slower kinetics of apoptotic development after stimulation of TR80 when compared with those of selective TR60 stimulation or of combined receptor triggering. Significant DNA fragmentation could be detected after 4 h of stimulation with TNF, whereas 8 h and 12 to 18 h of TR60 and TR80 stimulation, respectively, were necessary to obtain comparable effects (Fig. 2). These differential kinetics in apoptotic development, confirmed by visual microscopic controls, could either reflect differences in signal transduction pathways or be a result of the different receptor cross-linking efficiencies by the Abs vs TNF. In addition, because antagonistic Abs specific for each of the two receptors do inhibit TNF cytotoxicity in KYM-1 cells (40), cooperative effects of TR60 and TR80 on TNF triggering are conceivable.

Because KYM-1 cells express extraordinarily high numbers of TR80 compared with normal tissues the ability of this receptor to induce programmed cell death could be attributed to this fact and, consequently, could represent a specific property of only this particular cell line. However, investigations performed with the myeloid granulocyte-macrophage CSF-dependent cell line, GMSO (53), revealed that overexpression of TR80 is not a prerequisite to function as an inducer of apoptosis. GMSO cells express TR80 at levels comparable to primary cells (~2000/cell) and also develop apoptosis on selective stimulation of TR80 (S. Öz, manuscript in preparation). Therefore, it seems conceivable that TR80 receptors could be involved in TNF-mediated apoptosis in biologically relevant situations, e.g., in negative selection of thymocytes. The recent work of Hernández-Caselles and Stutman (6) is in accordance with this view. These authors suggested that TR80 is involved in both growth stimulation and induction of apoptosis by TNF, dependent on the culture conditions used.

The availability of a cell line that shows an identical cellular response on selective stimulation of each of the two TNFRs prompted us to study identity or differences in the intracellular signals involved. In a first step, we tested a broad range of inhibitors of potential intracellular signaling molecules for their effects on TNF-induced apoptosis in KYM-1 cells. From the emerging pattern of modulation of TNF responsiveness, the following conclusions can be drawn. A number of PKC inhibitors were found to act in an additive/synergistic way with TNF, suggesting that they either enhance TNF-mediated cytotoxicity directly or block concomitantly induced protection mechanisms. These enhancing compounds include H7, staurosporine, and K252A and B, substances that have been often used as inhibitors of PKC, but certainly have a broader target spectrum (32, 54). On the other hand, calphostin C, currently regarded as a highly potent and specific inhibitor

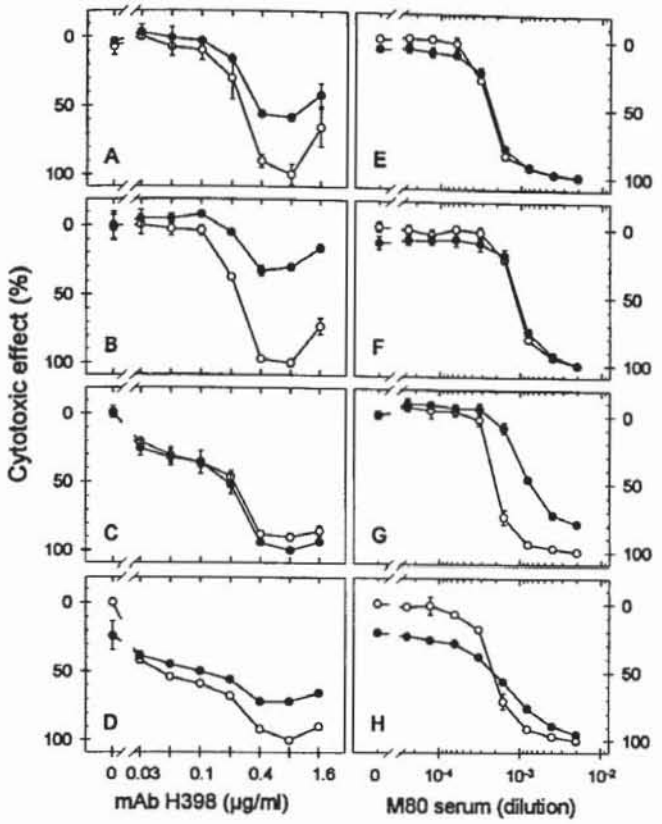

FIGURE 4. Inhibition of receptor-specific induction of cytotoxicity. KYM-1 cells were incubated with increasing concentrations of either TR60-specific mAb H398 plus 3 µg/ml goat anti-mouse IgG (*A* through *D*) or TR80-specific rabbit serum (*E* through *H*) in the absence (○) or presence (●) of 1.25 mM pargyline (*A* and *E*), 15 µM H8 (*B* and *F*), 10 µM NDGA (*C* and *G*), or 0.5 mM amytal (*D* and *H*). Viability was determined as described in Figure 3. The maximum cytotoxic effect of the respective agonist under the given experimental conditions was 100% and the medium control, 0%, i.e., the intrinsic cytotoxic effect of the respective inhibitor was not subtracted. A representative experiment is shown.

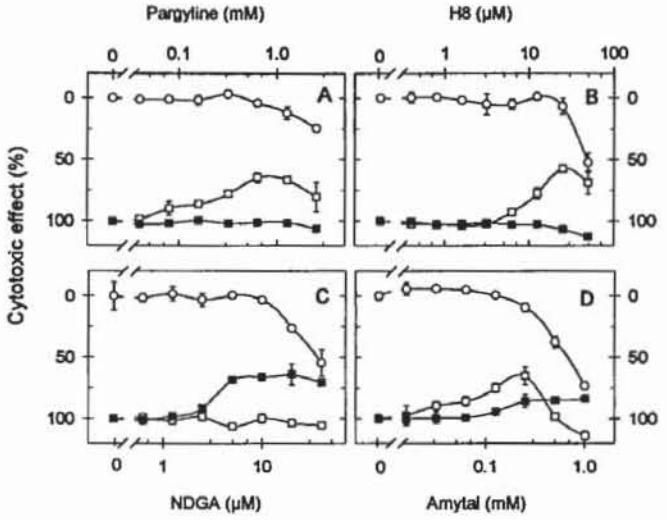

FIGURE 5. Dose-response curves of inhibitors. KYM-1 cells were treated with serial dilution of inhibitors alone (○) or with 0.75 µg/ml H398 plus 3 µg/ml goat anti-mouse IgG (□) or M80 serum (1:1500; ■) in the presence of serial diluted inhibitors. Viability was determined as described in Figure 3. The maximum cytotoxic effect of the respective agonist under the given experimental conditions was 100% and the medium control, 0%. Representative experiments are shown.

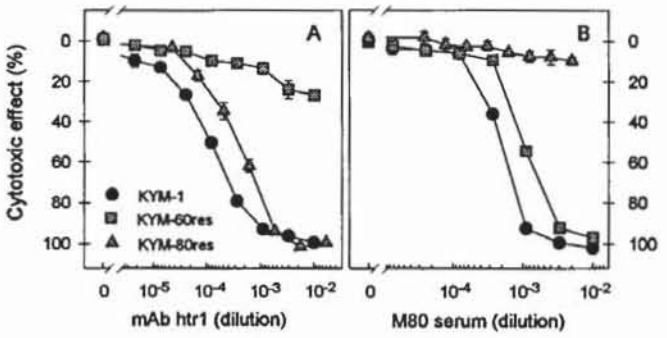

FIGURE 6. Development of receptor-specific resistance in KYM-1-related cell lines. KYM-1 cells had been cultured in the presence of increasing concentrations of TR60- (KYM-60res cells) or TR80- (KYM-80res cells) specific agonists. Typical sensitivity patterns after 3 wk of culture are displayed in comparison with untreated KYM-1. Cells were washed extensively then stimulated with the TR60-specific mAb htr-1 (*A*) or M80 serum (*B*) for 18 h, and viability was determined as described in Figure 3. The maximum cytotoxic effect of the respective agonist under the given experimental conditions was 100%. A representative experiment is shown.

of several PKC isoenzymes, did not affect TNF-triggered cytolysis (Fig. 3). These results question the role of calphostin C-sensitive PKC isoenzymes in signaling of, and in protection from, TNF cytotoxicity in KYM-1 cells (26, 32), although the role of serine/threonine-specific PKs in TNF signal transduction seems to be evident (2).

Quite remarkable protective effects were found with another kinase inhibitor, the isoquinoline sulfonamide derivative H8. Although often used as a PKA-specific reagent, this inhibitor is now also known to affect various other kinases, depending on the concentration used (54). Moreover, specific involvement of PKA in TNF signaling seems unlikely because the related inhibitor H89 was totally inefficient. This derivative has a very similar structure to H8 but has a higher affinity for cAMP-dependent kinases (IC_{50} of 0.048 vs 0.48 µM) and is, thus, expected to block PKA more effectively (55; and information of the supplier). Taken together, the data obtained with the PK inhibitors indicate a role of PKs in TNF signaling and TNF-induced protective mechanisms but suggest that neither conventional PKC subtypes nor PKA are critically involved in either process in KYM-1 cells.

Furthermore, the use of various inhibitors of phospholipid metabolism suggested involvement of the PLA_2/lipoxygenase pathway in TNF-mediated cytotoxicity, which is in full agreement with data obtained by other investigators (22–24). The experimental results obtained in this study, however, did not reveal exact components involved

in this process because the PLA_2 inhibitors BPB and quinacrine, in contrast with neomycin and NDGA, were ineffective. Regardless of the precise location of inhibitory action, the identification of inhibitors that interfere with TNF-induced cytotoxicity in KYM-1 enabled us to investigate whether TNFR signaling pathways segregate. Indeed, some of the inhibitory compounds revealed a clear segregation in this regard. The H8-sensitive PKs that are involved in TNF-mediated cytotoxicity can be clearly linked to TR60-induced pathways (Fig. 4). On the other hand, NDGA, often used as an inhibitor of lipoxygenase pathway, but also known as an inhibitor of mono-oxygenase and as a radical scavenger (56), selectively inhibited TR80-mediated apoptosis (Fig. 4). Accordingly, TNF-mediated apoptosis in KYM-1 cells can be initiated by both TNFRs using at least initially distinct signal pathways. These two pathways apparently function in an additive manner, as demonstrated previously (40), and most likely merge at some point upstream of mitochondrial ROI production before the activation of the apoptotic effector molecules. This is suggested from data obtained with the ROI production inhibitor amytal, which reduced cytotoxicity induced by both TR60 and TR80 (Fig. 4).

KYM-1 cells have been shown to readily develop TNF resistance on prolonged treatment with TNF (52). The differential sensitivity of TR60- and TR80-mediated apoptosis to various inhibitors of the signal transduction mechanisms raised the possibility to selectively induce resistance at a given receptor subtype. Cell lines developing such a receptor-specific resistance were, in fact, reproducibly obtained and, thus, further support the view of a distinct signal transduction pathway usage of the two TNFRs. Together, these data suggest that for both TNFRs response limiting steps in the respective signaling pathways must exist which 1) are distinct from each other and 2) can be regulated independently. On the other hand, a limited cross-desensitization was reproducibly found in all 60res and 80res cell lines (Fig. 6), indicating that some overlap in TR60 and TR80 action must exist. This could be effective at the stage of generation of apoptotic effector molecules and/or directly upstream at a late step in the intracellular signal pathway that is shared by both receptors.

In the case of KYM-80res cells, an additional mechanism might be involved in the development of resistance because these cells reduced TR80 membrane receptor expression (52; data not shown). The importance of this gradual membrane receptor loss for generation of resistance is unclear at present. For example, we noted that TNF resistance preceeded complete loss of receptors, and fully resistant lines were obtained that still expressed significant numbers of TR80 membrane receptors. Although the mechanism of receptor down-regulation has not been addressed in this study, Ab-induced receptor shedding is possible, because TR80-specific mRNA levels remained unchanged (data not shown). In any case, TR80 down-regulation is induced by TR80 activation, itself, and does not represent cross-modulation triggered by TR60.

Currently, the majority of TNF responses has been attributed to TR60, whereas the overall contribution and in particular the separate functional role of TR80 in TNF signaling was unknown. Nevertheless, in a number of different cellular systems, cooperative effects of TR60 and TR80 have been described. These include cytotoxic effects of TNF on U937 cells (38), stimulatory activity for T cells and thymocytes (57), and enhancement of surface Ag expression in colon carcinoma cells (14). We could now show that TR80 is involved in TNF-mediated signaling and present evidence for the existence of receptor selective signal pathways. These pathways may lead to the same cellular response yet are subjected to largely independent cellular control mechanisms. This finding suggests that it might be possible to develop strategies that circumvent the usually rapid process of TNF desensitization. Accordingly, an alternative short-term treatment with receptor-specific agonists could prevent a general TNF unresponsiveness and, therefore, could be superior to a treatment with the natural ligand that affects both receptors simultaneously.

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