Tumor Necrosis Factor (TNF)-Induced Protein Phosphorylation in a Human Rhabdomyosarcoma Cell Line Is Mediated by 60-kD TNF Receptors (TR60)

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In the present study, we used a cloned derivative, KYM-1D4, of the human rhabdomyosarcoma cell line, KYM-1, known to express high numbers of the two tumor necrosis factor (TNF) receptors, TR60 and TR80, and to be highly sensitive to TNFα-mediated cytotoxicity/antiproliferation, to investigate the role of TR60 and TR80 in protein phosphorylation. Using permeabilized KYM-1D4 cells, it was found that TNFα strongly induced phosphorylation of proteins of molecular weight 80, 65, 58, 42, and 30 kD. Addition of a monoclonal antibody (MoAb) against TR60 was shown to induce cytotoxicity/antiproliferation in KYM-1D4 cells and the same pattern of protein phosphorylation as TNFα, whereas addition of an MoAb against TR80 was both noncytotoxic and ineffective in inducing protein phosphorylation. In contrast, in a highly TNFα-resistant KYM-1–derived cell line, 37B8R, no protein phosphorylation was induced with either TNFα or the agonistic anti-TR60 MoAb. However, when 37B8R was allowed to revert to partial TNF sensitivity by culture in the absence of TNFα, the resultant cell line, 37B8S, was found to regain inducibility of protein phosphorylation by TNFα. These results indicate that expression of functional TR60 in KYM-1–related cell lines is principally involved in TNFα-mediated cytotoxicity/antiproliferation and is necessary for the induction of protein phosphorylation. Nevertheless, the latter, although apparently strongly associated with cytotoxicity, was probably involved in protective mechanisms because protein kinase C inhibitors that inhibited TNFα and anti-TR60–induced phosphorylation increased the cytotoxic/antiproliferative response to these mediators.

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a valuable tool to investigate TNFα-induced effects because the normal human biochemical pathways linked to the TNF receptors should still be intact. This approach allows, for the first time, an investigation of TNF-induced phosphorylation in a human cell line containing normal numbers of TR60. Furthermore, the effects of receptor-specific antibodies and inhibitors of phosphorylating enzymes on phosphorylation and cytotoxicity can also be investigated and interesting comparisons can be made between the KYM-I-D4 cells and the TR80-negative cells. To study TNFα-induced phosphorylation we used a permeabilized cell system\(^3\)\(^4\) designed to enable detection of rapid changes in phosphorylation. We have also used specific anti-TR60 and anti-TR80 monoclonal antibodies (MoAbs)\(^5\)\(^6\) together with specific protein kinase inhibitors\(^5\)\(^6\) to further define the role of each type of TNF receptor, both in phosphorylation and cytotoxicity. We report results that indicate that rapid phosphorylation of several proteins occurs after TNFα occupancy of TR60 and that these receptors appear to be principally responsible for TNFα-induced cytotoxicity.

**MATERIALS AND METHODS**

**Cell lines.** The human rhabdomyosarcoma cell line KYM-i was generously supplied by Dr M. Sekiguchi (Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan). The KYM-ID4 subline that is more sensitive to TNFα-mediated cytotoxicity than the parental cell line was established after limiting dilution cloning of KYM-I cells and the testing of randomly selected clones.\(^3\)\(^5\) The KYM-1-related TNFα-resistant cell line 37B8R was obtained through selection of KYM-I cells with increasing dosages of TNFα.\(^1\) All cell lines were cultured in RPMI 1640 medium containing 7% heat-inactivated fetal calf serum (HIFCS) and antibiotics and subcultured 1:20 every 7 days.

**Cytotoxicity/antiproliferation assay.** This assay was performed as previously described for KYM-I cells.\(^1\) Briefly, serial dilutions of a stock TNFα solution (recombinant human [rh] TNFα was a kind gift from The Dainippon Pharmaceutical Co Ltd, Osaka, Japan)
calibrated against the International Standards for human TNFα, 87/650, 40,000 International Units (IU), were made in RPMI 1640 medium supplemented with 2% HIFCS in the wells of 96-well microtiter plates (Nunc, Copenhagen, Denmark). Kym-1D4 cells or 37B8R cells were spun down by centrifugation and resuspended in RPMI 1640 medium plus 2% HIFCS at 2 × 10^5 cells/mL. These were then added to the microtiter plates at 2 × 10^5 cells/well and the assays were incubated at 37°C for 3 days. Cell survival was estimated by direct addition of MTT (10 μL/well of 5 mg MTT/mL phosphate-buffered saline [PBS]) to all wells and elution of the formazan product of MTT formed in metabolically active cells after 1 hour at 37°C with 10% sodium dodecyl sulfate (SDS) in 0.02 N HCl (25 μL/well). Absorbances were read after a further 4 hours of incubation at 37°C at 590 nm.

Purified MoAbs to human TNF receptors, htr-9 against the TR60 and utr-1 against TR80, were generously donated by Dr. M. Brockhaus (Hoffmann-La Roche, Basel, Switzerland). The MoAbs were diluted in RPMI medium plus 2% HIFCS and assays with either Kym-1D4 or 37B8 were performed in the same manner as with TNFα.

Induction of protein phosphorylation using permeabilized cells and characterization of phosphoproteins. Cells were washed once with serum-free RPMI medium, aliquoted in 1 × 10^6 cell lots, and centrifuged in 2-mL eppendorf tubes at 500g for 5 minutes at room temperature. Each cell pellet was resuspended in 8 μL of reaction mixture containing 40 mmol/L HEPES (pH 7.4), 10 mmol/L manganese chloride, 20 mmol/L magnesium acetate, 4 mmol/L β-mercaptoethanol, 4 μmol/L sodium orthovanadate, 200 mmol/L calcium chloride, and 10 mmol/L p-nitrophenyl phosphate. Then, 2 μL of L-α-lysophosphatidylcholine (Lipid Products Ltd, Redhill, Surrey, UK), 1 mg/mL in water, was added and the suspension was gently mixed.33 The reaction was initiated by the addition of 5 μCi (γ-32P) ATP and variable amounts of rhTNFα with 0.1% ovalbumin used as diluent and control throughout. The PKC inhibitor, RO-31-8220, compound 3 depicted in Fig 1 of Davis et al41 (donated by Roche), was dissolved in dimethyl sulfoxide (DMSO), diluted in 0.1% ovalbumin, and added to the reaction mixture 5 minutes before the addition of rhTNFα. Lavendustin A, genistein42 and calphostin C43 (LC Laboratories, Nottingham, UK) were dissolved in 0.1% ovalbumin and added in a similar manner to RO-31-8220. Labeling was normally for 5 minutes at room temperature, unless indicated otherwise. The reaction was stopped by the addition of 20 μL of an ice-cold solution containing 2% Nonidet P40, 20 mmol/L sodium orthovanadate, 1 mmol/L phenylmethylsulphonyl fluoride, 5 mmol/L p-nitrophenyl phosphate, 20 mmol/L EDTA, and 50 μmol/L ATP. The detergent-treated cells were centrifuged at 1,500g (4°C) for 5 minutes to separate the detergent-insoluble material (nuclei and cytoskeleton) from the detergent-soluble material (cytosol plus solubilized membrane and organelles). The detergent-soluble fractions were analyzed by electrophoresis in 7.5% polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS) under reducing con-
RESULTS

In accordance with previous results, KYM-1D4 cells were shown to be very sensitive to the cytotoxic and antiproliferative action of rhTNFα (Fig 1, top). Fifty percent of cells survived at an rhTNFα concentration of approximately 50 pg/mL (equivalent to 2 IU/mL). At 1 ng/mL and greater, cell killing was essentially 100%. KYM-1D4 cells are known to express relatively high numbers of TNF receptors, with 35,000 TR80 and 2,500 TR60. However, little is known about the early events after TNF receptor occupancy in these cells. We chose to investigate protein phosphorylation because this often precedes other markers of cellular activation, eg, protein synthesis, and is frequently associated with signalling pathways that culminate in specific cellular responses, eg, mitogenesis and differentiation. To monitor the early protein phosphorylation events induced after receptor occupancy, we used a permeabilized cell system designed to detect extremely rapid changes in protein phosphorylation.

Fig 3. rhTNFα and htr-9 failed to induce phosphorylation in resistant KYM-1 37B8R cells. rhTNFα-induced phosphorylation in KYM-1D4 cells is inhibited by PKC inhibitors. (A) Permeabilized KYM-1 37B8, rhTNFα-sensitive cells, and KYM-1 37BS, rhTNFα-sensitive clone of 37B8R, were incubated with rhTNFα or htr-9 at the levels indicated and [γ-32P] ATP for 5 minutes. NP40-soluble fractions were analyzed as described in Materials and Methods. (B) Permeabilized KYM-1D4 cells were incubated with various protein kinase inhibitors for 5 minutes, followed by 100 ng/mL rhTNFα and [γ-32P] ATP for 5 minutes. NP40-soluble fractions were analyzed as described in Materials and Methods. TNF, 10 ng/mL rhTNFα; G, 50 μg/mL genistein plus 10 ng/mL rhTNFα; RO, 1 μmol/L RO31-8220 plus 10 ng/mL rhTNFα; CH, 10 μmol/L chelerythrine plus 10 ng/mL rhTNFα.
Fig 4. Anti-TRGO (htr-9) induction of cytotoxicity/antiproliferation and protein phosphorylation in KYM-ID4 cells in a dose-responsive manner. (Top) KYM-ID4 cells were incubated with various levels of htr-9 and survival of cells was measured by MTT reduction, as described in Materials and Methods. (Bottom) Permeabilized KYM-ID4 cells were incubated with the levels of htr-9 indicated and $[\gamma^{32}P]ATP$ for 5 minutes. NP40-soluble fractions were analyzed and autoradiograms were densitometrically scanned as described in Materials and Methods.

Types of TNF receptors was mainly responsible for triggering phosphorylation, KYM-ID4 cells were first treated with the anti-TR60 MoAb, htr-9, which is known to act as a TNF agonist. In cytotoxicity/antiproliferation assays, it was shown that htr-9 was cytotoxic and antiproliferative to KYM-ID4 cells (Fig 4, top), and led to their cytosis in a manner similar to that induced by rhTNF-α, except that proportionately higher concentrations of htr-9 were required. The htr-9 MoAb, which acts only via TR60, was shown to induce protein phosphorylation in KYM-ID4 cells at antibody concentrations leading to marked cytosis, ie, 100 ng/mL (Figs 2 and 4), whereas at nontoxic concentrations, ie, 10 ng/mL, evidence of phosphorylation as shown by SDS-PAGE analysis was practically identical to that found for KYM-ID4 cells stimulated by rhTNF-α, ie, proteins of MW 80, 65, 58, 43, and 30 kD showed increased band density (Figs 2 and 4). In contrast, the utr-1 MoAb that interacts with TR80, but that has no demonstrable cytotoxic/antiproliferative action even up to 1,000 ng/mL, completely failed to induce any increased protein phosphorylation in KYM-ID4 cells (Fig 2). Furthermore, the combination of utr-1 with rhTNF-α failed to block the ability of rhTNF-α to induce protein phosphorylation (Fig 2). A time course of phosphorylation illustrates that rhTNF-α-induced phosphorylation is rapid, occurring maximally at 4 to 5 minutes and gradually decreasing over 1 hour (Fig 5).

To further highlight the involvement of TR60 in triggering protein phosphorylation, the response of a TNF-α-resistant KYM-1-related cell line, 37B8R, known to express 2,500 TR60 and undetectable TR80, was investigated. This cell line, 37B8R, was resistant to TNF-α-mediated cytotoxicity/antiproliferation at concentrations of 10 ng rhTNF-α/mL or even higher (Fig 6, top) and no sign of increased protein phosphorylation was evident from SDS-PAGE analysis of proteins extracted from rhTNF-α-treated 37B8R permeabilized cells (Figs 3A and 6, bottom). 37B8R is also resistant to the cytotoxic/antiproliferative effects of htr-9, and htr-9 failed to induce any changes in phosphorylation patterns.
DISCUSSION

For the human cell line KYM-1D4, which is highly sensitive to TNFα-mediated cytotoxicity/antiproliferation, we were able to show that rhTNFα rapidly induces protein phosphorylation in proteins of MW 80, 65, 58, 43, and 30 kD. KYM-1D4 cells are known to express both TR60 and TR80, with the latter predominating. However, an MoAb (htr-9) to TR60 had cytotoxic and antiproliferative activity against KYM-1D4 cells and was also able to induce rapid protein phosphorylation. The MW of the proteins phosphorylated after htr-9 treatment were the same as those in rhTNFα-stimulated cells, suggesting that only TR60 was functioning as a signal transducer leading to activation of protein phosphorylation pathways. In comparison, binding of an anti-TR80 MoAb, utr-1, to KYM-1D4 did not lead to a cytolytic response, nor was protein phosphorylation induced. However, although utr-1 can behave as a TNFα antagonist in certain circumstances, it has not been shown to act as a TNFα agonist. This may be because it fails to cross-link TR80 receptors; cross-linking of TR60 and TR80 appears to be crucial for receptor activation. For instance, when TR80-bound antibodies are cross-linked using a second antibody, a TNFα-like cytolytic response is induced in KYM-1D4 cells and in the parental cell line, KYM-1. Presently, we have no evidence that cross-linking of TR80 induces protein phosphorylation. However, rhTNFα, which might be reasonably assumed to cross-link TR80 present in KYM-1D4 cells, failed, apparently, to induce protein phosphorylation different to that induced by htr-9, the anti-TR60 MoAb. Thus, our results suggest that TR60 mediate TNF-induced cytotoxicity/antiproliferation that is strongly associated with early protein phosphorylation events, whereas TR80, which have also been shown to mediate TNF-induced cytotoxicity, appear not to activate protein phosphorylation or not to an extent that we can detect it. It is possible that the two types of receptor mediate two distinct cytotoxic responses, eg, the apoptic and necrotic forms, one of which requires or is associated with protein phosphorylation and the other that does not. Evidence for two mechanisms involving two distinct signalling pathways by which TNFα kills cells has also been reported.

The role of TR60 in mediating TNFα-induced cytotoxicity/antiproliferation and its link to early protein phosphorylation is reinforced by our finding that, in a TNFα-resistant KYM-1-related cell line, 37B8R, TR60-induced protein phosphorylation was not detected, even though these cells expressed similar numbers of TR60 to KYM-1D4. This finding suggested that resistance occurred in this case because TR60 were unable to act as signal transducers and thus activate kinases involved in protein phosphorylation. However, when sensitivity of TNFα returned after prolonged culture in the absence of TNFα to yield the cell line 37B8S, it was found that inducibility of protein phosphorylation by...
TNFα was also restored without overt changes to TR60 expression. The pattern of phosphorylation was similar to that found in KYM-1D4, but some additional phosphoprotein bands were observed in autoradiographed polyacrylamide gels. This ability of receptors to disconnect from signal transduction pathways adds another mechanism to the control of receptor-based activities and is not unique to TNF receptors, having been shown for the receptors of interleukin-3 and granulocyte-macrophage colony-stimulating factor.45

The identity and role of any of the proteins inducibly phosphorylated in TNFα-treated KYM-1D4 and 37B8S cells remain enigmatic. It is also not clear whether TNFα-induced phosphoproteins are responsible for triggering events culminating in cell death. The cytotoxic mechanism of TNFα requires oxygen, suggesting that oxygen-dependent metabolic processes or free radicals facilitate killing,46 probably through mitochondrial damage,47 and may be independent of protein phosphorylation. Indeed, we have recently shown that, in a TNFα-resistant U-937–like cell line, A10, that protein phosphorylation is mediated by PKC58 and is associated with protective mechanisms against TNFα-induced cytotoxicity, rather than the reverse. The proteins inducibly phosphorylated in KYM-1D4 show some overlap in MW (80, 65, 58, 42, and 30 kD) to those inducibly phosphorylated in A10 (130, 90, 80, 65, and 42 kD) and phosphorylation in KYM-1D4 was inhibited by the PKC inhibitors RO-31-8220,35 chelerythrine,36 and calphostin C,40 suggesting PKC involvement. The PKC inhibitor RO-31-822035 was also shown to increase both TNFα- and htr-9–mediated cytotox-
Fig 7. Effects of protein kinase inhibition on rhTNFα- and htr-9–induced cytotoxicity/antiproliferation and rhTNFα-induced phosphorylation on KYM-1D4 cells. KYM-1D4 cells incubated with various levels of rhTNFα (left) or htr-9 (right) in the presence or absence of 0.6 μmol/L RO-318220 and survival of cells measured by MTT reduction as described in Materials and Methods. Permeabilized KYM-1D4 cells were preincubated with various kinase inhibitors for 5 minutes before the addition of 10 ng/mL rhTNFα and [γ-32P] ATP for 5 minutes. NP40-soluble fractions were analyzed and autoradiograms were densitometrically scanned as described in Materials and Methods. Concentration of kinase inhibitors were as follows: 1 μmol/L RO-318220, 10 μmol/L chelerythrine, 0.5 μmol/L calphostin C, 50 μg/mL genistein, and 0.1 μmol/L lavendustin A.

In summary, our results clearly indicate that functional TR60 are required for the activation of signalling pathways leading to protein phosphorylation and cytotoxicity/antiproliferation, and are in agreement with a recent report whose findings indicate that TR60 is coupled to signal transduction cascades, eg, stimulation of PKC, sphinomyelinase, and phospholipase A2. Initially, we found that protein phosphorylation and cytotoxicity/antiproliferation were strongly associated, suggesting that increased phosphorylation of certain proteins could trigger metabolic processes that lead to cytology. However, the recent demonstration that TR80 may also mediate TNFα-induced cytotoxicity in KYM-1- and KYM-1-derived cells and the lack of evidence for protein phosphorylation being mediated by this receptor, as reported herein, lead to the alternative conclusion that protein phosphorylation is coincidental to process(es) culminating in cytotoxicity. Furthermore, we found increases of TNFα- and htr-9–cytotoxicity/antiproliferation in KYM-1D4 cells in the presence of the PKC inhibitor RO-31-8220 that not only support this conclusion, but also suggest that protein phosphorylation could be involved in mechanisms protecting against cytotoxicity.

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