Protein kinase C-dependent phosphorylation is involved in resistance to tumour necrosis factor- α -induced cytotoxicity in a human monocytoid cell line

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To investigate the mechanism underlying resistance to tumour necrosis factor- α (TNF α)-induced cytotoxicity, we have developed a human hybrid cell line, designated A10, derived from the fusion of human U-937 monocytoid cells and human monocytes, which expressed large numbers of TNF α receptors and yet remained highly resistant to TNF α . However, in the presence of the protein kinase C (PKC) inhibitors RO-31-7549 or RO-31-8220 (donated by Roche), these cells became sensitive to TNF α -induced cytotoxicity, suggesting that PKC activity is required for protective mechanisms. On investigation of protein phosphorylation in TNF α -stimulated permeabilized A10 cells, a rapid increase in serine/threonine phosphorylation of phosphoproteins

of molecular masses 130, 90, 80, 65 and 42 kDa was found. Subsequently, we found a similar pattern of increased phosphorylation following stimulation of A10 cells with mezerein, a phorbol ester derivative which activates PKC, a serine/threonine kinase. The theory that activation of PKC was responsible for increased phosphorylation was confirmed by a dose-dependent inhibition of the TNF α -induced protein phosphorylation by the PKC inhibitors RO-31-7549 and RO-31-8220. The possible link between the TNF α -stimulated early protein phosphorylation events and the maintenance of protective mechanisms against TNF α -induced cytotoxicity is discussed.

INTRODUCTION

Tumour necrosis factor- α (TNF α) is produced primarily by activated macrophages and, besides its capacity to induce cytotoxicity in some tumour cell lines, it has a wide spectrum of other biological activities in many normal cell types (Le and Vilček, 1987; Jäättelä, 1991). However, the mechanisms that underlie the selective toxicity whereby tumour cells are killed and normal cells are spared remain enigmatic. The first crucial step in the action of TNF α is its binding to specific cell surface receptors, of which two types have been cloned (Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). Although the expression of receptors and the subsequent formation of TNF α -receptor complexes are essential for triggering TNF α -directed actions, the selective toxicity of TNF α does not appear to be due to differences in the number or type of receptors. It thus appears that selective toxicity is controlled by differences occurring in intracellular events induced after receptor occupancy and the levels at which inherent protective mechanisms operate. Inhibitors of macromolecular synthesis are known to enhance TNF α -induced cytotoxicity such that even relatively resistant cell lines become susceptible (Meager et al., 1989). This indicates that, without particular protective mechanisms that are dependent upon protein synthesis, resistance can be lost.

Although TNF α receptors do not themselves appear to have intrinsic kinase activity (Schall et al., 1990; Smith et al., 1990), several reports (Schütze et al., 1989; Kaur et al., 1989; Robaye et al., 1989; Evans et al., 1990) have described the TNF α -induced phosphorylation of distinct cytosolic proteins in different human

tumour cell lines, suggesting that TNF α can activate non-receptor protein kinases. The involvement of protein kinase A, protein kinase C (PKC) and a novel serine kinase have been described (reviewed in Vilček and Lee, 1991; Camussi et al., 1991), and PKC in particular has been reported to play a role in the modulation of resistance to TNF α -induced cytotoxicity (Aggarwal and Eessalu, 1987; Johnson and Baglioni, 1988; Scheurich et al., 1989; Belizario and Dinarello, 1991).

In order to investigate further the mechanisms which underlie resistance to TNF α -induced cytotoxicity we have used a U-937 cell $\times$ human monocyte hybrid cell line, designated A10, developed in our laboratories, as these cells were shown to express high numbers of TNF α receptors and yet were highly resistant to TNF α . We report the effects of the specific PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989), which are more selective for PKC than is staurosporine. The results suggest that PKC activity is involved in the mechanism(s) which protect cells against TNF α -induced cytotoxicity. To confirm PKC involvement, we investigated protein phosphorylation events following stimulation by TNF α . We used a permeabilized cell system (Mire-Sluis et al., 1985, 1986; Evans et al., 1990) designed to detect rapid changes in phosphorylation. In addition, we examined as a comparison the protein phosphorylation induced by mezerein, a phorbol ester derivative, which is known to activate PKC (Grove and Mastro, 1991). We show that TNF α and mezerein act similarly by rapidly inducing phosphorylation of several cytosolic proteins, and that the phosphorylation is blocked by the specific PKC inhibitors RO-31-7549 and RO-31-8220.

Abbreviations used: TNF α , tumour necrosis factor- α ; rhTNF α , recombinant human TNF α ; PKC, protein kinase C; MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; HIFBS, heat-inactivated foetal bovine serum; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; TGR, 6-thioguanine-resistant; PBMC, peripheral blood mononuclear cells; DMSO, dimethyl sulphoxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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EXPERIMENTAL

Cell lines and establishment of the A10 hybrid

The human U-937 monocytoid cell line, originally established from a patient with histiocytic lymphoma (Sundström and Nilsson, 1976), was grown at 37 °C in RPMI 1640 medium (Flow Laboratories) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (HIFBS; Imperial Laboratories) and routine antibiotics. Cells were transferred every 3–4 days at an inoculation density of approx. 1×10^5 cells/ml. To obtain a drug-selectable cell fusion partner from U-937, a culture of exponentially growing U-937 cells at 2×10^5 cells/ml was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Sigma Chemical Company) at 0.5 µg/ml of growth medium for 24 h. Following this, cells were pelleted by centrifugation and re-seeded into fresh growth medium without MNNG. The cells surviving the mutagen treatment (approx. 25% of total) were grown for 4 days at 37 °C to establish mutant phenotypes and then serially passaged in culture medium containing increasing concentrations of 6-thioguanine (0.01–10.0 µg/ml) over a period of 2 months to select hypoxanthine-guanine phosphoribosyltransferase-negative (HGPRT⁻) mutant cells. When a strongly growing population of HGPRT⁻ U-937 cells had been established, it was cloned by limiting dilution in microtitre plates (Falcon) and clones were selected that were stably defective in HGPRT activity. One such clone, designated U-937 6TGR (6-thioguanine-resistant), was grown up to mass culture and used in cell fusions with human peripheral blood mononuclear cells (PBMC) as follows. A suspension of U-937 6TGR cells (2×10^7 cells) and human PBMC (1×10^8 cells) were pelleted by centrifugation, washed twice with serum-free culture medium, repelleted and slowly resuspended in 2 ml of 50% poly(ethylene glycol) 1540 (BDH Chemicals). Following a 1 min incubation at room temperature, the fusion mixture was rapidly diluted with 20 ml of growth medium. The cells were then repelleted and resuspended in 100 ml culture medium containing 10% HIFBS before being distributed in 1 ml aliquots in 24-well sterile cluster plates. Following incubation at 37 °C for 24 h, 1 ml of growth medium containing hypoxanthine (13.61 µg) and azaserine (1 µg) was added to all wells. Thereafter, 1 ml of growth medium was removed from wells every other day and replaced with 1 ml of new growth medium containing hypoxanthine and azaserine. This was continued for 2 weeks. Growth medium was then replaced on a weekly basis until hybrid cells had grown up sufficiently for transfer. Fusion efficiency was extremely low, as reported by others (Tadayoshi et al., 1988; Sperber et al., 1990), and only five hybrid cell lines were established from six independent fusions. All five hybrids had regained HGPRT activity and were killed in the presence of 6-thioguanine (1.0 µg/ml). Morphologically they all resembled the parental U-937 6TGR cells, but two of the hybrid cell lines, designated A10 and A11, strongly expressed the monocyte/macrophage cell marker CD68 (Kelly et al., 1988; Pulford et al., 1990), detected by the CD68-specific monoclonal antibody EMB 11 (Dako Limited), and human leucocyte antigen-DR (HLA-DR). These markers were present to a lesser extent on U-937 6TGR and U-937 cells. It was therefore assumed that A10 and A11 cells were hybrids derived from the fusion of U-937 6TGR and a human monocyte or macrophage cell line.

Radiolodination of TNFα

An Eppendorf tube was coated with Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril; Sigma Chemical Co.) by addition of 40 µl of 0.1% Iodogen in trichloromethane and evaporation of

the solvent at 37 °C. Recombinant human (rh)TNFα (5 µg) (Dainippon Pharmaceutical Co.) in 30 µl of 0.25 M sodium phosphate buffer, pH 6.9, was layered over the freshly prepared film of Iodogen and incubated for 10 min at 4 °C in the presence of 1 mCi of carrier-free Na¹²⁵I (Amersham). Free iodine was removed by gel filtration on a Sephadex G-25 column (PD-10; Pharmacia) equilibrated with 2% BSA in PBS, pH 7.2.

A total of 98% of the radioactive iodine in the final product was incorporated into TNFα protein, as determined by trichloroacetic acid precipitation. The specific radioactivity of the labelled rhTNFα was approx. 40–70 µCi/µg.

Enumeration of TNFα receptors

Cells were centrifuged at 1000 *g* (4000 rev./min) for 5 min and resuspended at 1×10^6 cells/100 µl per Eppendorf tube in binding buffer (2% BSA in PBS, pH 7.2), and then incubated with various concentrations of ¹²⁵I-rhTNFα in the presence of a 100-fold excess of unlabelled rhTNFα for 1 h at 4 °C, mixing continuously.

To separate the bound ¹²⁵I-TNFα from the free fraction, cells were collected through phthalate oil (dioctyl phthalate/dibutyl phthalate, 2:3, v/v) at 1000 *g* (4000 rev./min) for 5 min in 400 µl Microfuge tubes. The tubes were then frozen in solid CO₂ so that the cell pellets could be safely cut away. Both the cell-bound and the free radioactivity were measured using a γ-radiation counter. Specific binding was determined by subtracting the non-specific binding from the total binding. Non-specific binding was not more than 10% in all experiments discussed. Calculation of the dissociation constant (*K_d*) and the number of receptors was determined by Scatchard analysis (Scatchard, 1949) of the binding data.

Induction of protein phosphorylation using permeabilized cells and characterization of phosphoproteins

Cells were washed once with serum-free RPMI medium, divided into portions of 1×10^6 cells and centrifuged in 2 ml Eppendorf tubes at 500 *g* for 5 min at room temperature. Each cell pellet was resuspended in 8 µl of reaction mixture containing 40 mM Hepes (pH 7.4), 10 mM MnCl₂, 20 mM magnesium acetate, 4 mM β-mercaptoethanol, 4 µM sodium orthovanadate, 200 nM CaCl₂ and 10 mM *p*-nitrophenyl phosphate. Then 2 µl of L-α-lysophosphatidylcholine (Lipid Products Ltd., Redhill, Surrey, U.K.), at 1 mg/ml in water, was added and the suspension gently mixed. The reaction was initiated by the addition of 5 µCi of [γ-³²P]ATP and variable amounts of rhTNFα or mezerein, with 0.1% ovalbumin used as diluent and control throughout. The PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989) were dissolved in dimethyl sulphoxide (DMSO) and added to the reaction mixture 10 min prior to the addition of rhTNFα. Labelling was usually for 3 min 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 mM sodium orthovanadate, 1 mM phenylmethanesulphonyl fluoride, 5 mM *p*-nitrophenyl phosphate, 20 mM EDTA and 50 µM ATP. The detergent-treated cells were centrifuged at 1500 *g* (4 °C) for 5 min to separate the detergent-insoluble material (nuclei and cytoskeleton) from the detergent-soluble material (cytosol plus solubilized membranes and organelles). The detergent-soluble fractions were analysed by electrophoresis in 7.5% polyacrylamide gels in the presence of SDS under reducing conditions. The resulting gels were fixed, stained with Page Blue G-90 stain (BDH) to reveal molecular mass markers (Pharmacia), destained and dried under heat and vacuum. The dried gels were

then autoradiographed using Kodak X-Omat S film at -70°C using intensifying screens.

Phosphoamino acid analysis of ^{32}P -radiolabelled proteins

After autoradiography, selected proteins appearing on the X-ray film were carefully cut out of the dried gel. The gel slices were finely chopped and placed into glass bottles. The slices were allowed to re-swell at 37°C in 10 ml of extraction fluid consisting of 0.05 M NaHCO_3 , 0.1% SDS and 5% β -mercaptoethanol for 2 h. The gel was pulverized to remove it from the paper and heated at 100°C for 1 h. The suspension was filtered and the remaining gel was re-extracted overnight. The resulting supernatants were pooled and lyophilized under vacuum. Protein was precipitated with 5 vol. of acetone/ammonia (18:1, v/v) in glass tubes and incubated at -20°C for 10 min before being centrifuged for 15 min at 600 g at 4°C . The pellets were resuspended in 1 ml of ethanol/ether (1:1, v/v) in 1 ml plastic Eppendorf tubes and centrifuged in a Microfuge. This washing procedure was repeated twice before the pellet was dissolved in 100 μl of 6 M HCl at 100°C . Hydrolysis of the protein was undertaken at 110°C for 2 h and stopped by the addition of 1 ml of water prior to lyophilization. The resulting pellet was dissolved in 20 μl of phosphoamino acid marker mixture consisting of phosphoserine, phosphothreonine and phosphotyrosine (each at 1 mg/ml) prior to being electrophoresed on 1 MM Whatman filter paper in 7% formic acid buffer, pH 1.7, for 4 h at 3 kV. Phosphoamino acid markers were identified after electrophoresis by spraying the air-dried paper with 0.2% ninhydrin solution containing 5 mM KOH. Radioactivity in the spots was identified by autoradiography. The autoradiography was analysed using an LKB Ultrosan XL laser densitometer to provide quantitative data.

Cytotoxicity assays

Cells were seeded in 96-well (Falcon microtest) plates in 50 μl of medium at 2×10^4 cells/well. Serial dilutions of $\text{TNF}\alpha$ were added in 100 μl of growth medium/well. To determine the effect of the PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989; compounds 2 and 3 respectively in Figure 1 of that paper), cells were also treated with 0.1–10 μM of these agents in 50 μl of growth medium/well, in the presence and absence of $\text{TNF}\alpha$. After 48 h, cell survival was estimated by incubation for 1 h with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (10 μl /well of a solution of 5 mg of MTT/ml of PBS, pH 7.0). The formazan product of MTT formed in metabolically active cells was eluted with 10% SDS in 0.02 M HCl (25 μl /well) for 3–4 h at 37°C . Absorbances were read at 590 nm and cell survival was calculated for each $\text{TNF}\alpha$ concentration using the formula:

$$\text{Cell survival (\%)} = \frac{A_{590} \text{ of TNF-treated cells} - A_{590} \text{ of background}}{A_{590} \text{ of untreated cells} - A_{590} \text{ of background}} \times 100$$

RESULTS

$\text{TNF}\alpha$ receptors on A10 cells

Incubation of intact viable A10 cells with increasing concentrations of ^{125}I -rh $\text{TNF}\alpha$ (up to 10 ng/ml) resulted in its binding to cell surface receptors. This binding was prevented by addition of unlabelled rh $\text{TNF}\alpha$, and results (not shown) indicated that non-specific binding was no more than 10% of the total

radioactivity bound to cells. The binding data shown in Figure 1 were treated with the Scatchard (1949) relation to obtained the total numbers of $\text{TNF}\alpha$ -binding sites or receptors and to determine the affinity constant K_d . By this analysis, A10 hybrid cells were estimated to express approx. 20000 receptors per cell with an affinity constant of 2.9×10^{-10} M.

Cytotoxicity assays

Despite having relatively high numbers of TNF receptors per cell, A10 cells were found to be highly resistant to the cytotoxic/growth-inhibitor effects of $\text{TNF}\alpha$ (Figure 2). However, in the presence of the PKC inhibitors RO-31-7549 and RO-31-8220, $\text{TNF}\alpha$ elicited a cytotoxic dose-response which was clearly demonstrable at inhibitor concentrations of 8 and 4 μM respectively (Figure 2). However, 100% cell killing was not achievable even at very high concentrations of $\text{TNF}\alpha$ with the highest levels of PKC inhibitors that could be used without significantly affecting cell proliferation. DMSO at 0.5% (v/v) in the medium, equivalent to the volume used for addition of 10 μM of PKC inhibitor, in itself had no anti-proliferative effect and was without effect on $\text{TNF}\alpha$ -mediated cytotoxicity.

$\text{TNF}\alpha$ -induced protein phosphorylation in A10 cells

To monitor the early protein phosphorylation events induced following receptor occupancy, we used a permeabilized cell system designed to detect extremely rapid changes in protein phosphorylation (Evans et al., 1990). To elicit the permeabilized state, cells were treated with L- α -lysophosphatidylcholine, an agent which has previously been shown to render cells permeable to small molecules. However, it has no deleterious effects on cellular morphology, as visualized by electron microscopy (Miller et al., 1978), or for several hours on the synthesis of DNA, RNA or protein (Reddy and Pardee, 1980). More importantly for this

Figure 1 Scatchard analysis of ^{125}I - $\text{TNF}\alpha$ saturation binding to surface $\text{TNF}\alpha$ receptors on A10 cells

Cells (1×10^6 cells/100 μl of binding buffer) were incubated for 1 h at 4°C with ^{125}I - $\text{TNF}\alpha$ in the presence or absence of a 100-fold excess of unlabelled $\text{TNF}\alpha$, mixing continuously. Bound radioactivity was separated from free radioactivity by collecting the cell pellet through phthalate oil. Specific binding was determined by subtracting the non-specific binding from total binding as described in the Experimental section. Non-specific binding was not greater than 10% in any binding study.

Figure 2 Effect of PKC inhibitors on the sensitivity of A10 cells to TNF α -mediated cytotoxicity

Serial dilutions of TNF α were added to 2×10^4 cells/well in the presence or absence of various concentrations of the PKC inhibitors as indicated. The cells were incubated for 48 h; then the surviving cells incubated with MTT and colorimetrically assayed at 590 nm as described in the Experimental section.

study, permeabilization promoted the rapid entry of [γ - 32 P]ATP into cells where phosphorylating enzymes could utilize it immediately, thus making it possible to detect phosphorylation events occurring seconds after the addition of TNF α or mezerein.

Phosphoproteins from control and TNF α -treated permeabilized A10 cells were characterized by SDS/7.5%-PAGE followed by autoradiography. It was shown that short-term TNF α treatment (3 min) resulted in a marked increase in the phosphorylation of a phosphoprotein of molecular mass 90 kDa and in the appearance of four distinct cytosolic phosphoproteins, with apparent molecular masses of 130, 80, 65 and 42 kDa (Figure 3), compared with the basal protein phosphorylation that occurred in untreated control cells. The increased phosphorylation of the 90, 80, 65 and 42 kDa proteins was regularly observed, but induced phosphorylation of the 130 kDa protein was not always readily apparent. These TNF α -induced protein

Figure 3 Dose-dependent TNF α -induced and mezerein-induced phosphorylation of A10 cell phosphoproteins

Cells were labelled with [γ - 32 P]ATP and treated with increasing amounts of TNF α for 3 min, or left untreated (control). Phosphoproteins were separated by SDS/7.5%-PAGE and analysed by autoradiography as described in the Experimental section. Lane 1, 0.2 pg of TNF α /ml; lane 2, 2 pg of TNF α /ml; lane 3, 20 pg of TNF α /ml; lane 4, 200 pg of TNF α /ml; lanes 5 and 6, ovalbumin controls; lane 7, 0.01 ng of mezerein/ml; lane 8, 0.1 ng of mezerein/ml; lane 9, 1.0 ng of mezerein/ml; lane 10, 10 ng of mezerein/ml. Positions of molecular mass markers are indicated on the left. Arrows (centre) indicate the molecular masses (kDa) of the TNF α -induced phosphoproteins.

Figure 4 Quantitative evaluation of the phosphoproteins showing induced phosphorylation following TNF α stimulation of A10 cells: temporal dependence of phosphorylation

Cells were labelled with [γ - 32 P]ATP and treated with TNF α (20 pg/ml) for various intervals. Phosphoproteins were separated by SDS/7.5%-PAGE and identified by autoradiography. Quantitative analysis of the phosphoproteins was carried out using an LKB Ultrascan XL densitometer. The increase in phosphorylation is plotted as a percentage of the maximum density of each phosphoprotein band versus time (min). Proteins: X, 42 kDa; □, 65 kDa; ■, 80 kDa; ●, 90 kDa.

phosphorylations were dose-dependent, with maximum induction occurring at approx. 20 pg/ml (Figure 3, lane 3). A concentration of TNF α of 200 pg/ml appeared to be slightly less effective in inducing/increasing phosphorylation of these five proteins (Figure 3, lane 4). The incorporation of 32 P into the proteins was time-dependent, being detected within 3 min of stimulation with TNF α , occurring maximally in most cases by 5 min, and plateauing thereafter (Figure 4). Pre-incubation of the cells with an anti-TNF α monoclonal antibody (101.4) com-

Table 1 Phosphoamino acid analysis of the phosphoproteins showing induced phosphorylation following TNF α stimulation of A10 cells

Extracts from gel slices corresponding to the phosphoproteins with the indicated molecular masses were treated as described in the Experimental section. Following autoradiography of the electrophoresis paper of separated phosphoamino acids, the autoradiograph was scanned densitometrically and the amounts of phosphoserine, phosphothreonine and phosphotyrosine in each phosphoprotein were calculated as a percentage of the total ^{32}P incorporation.

Phosphoprotein molecular mass (kDa)	Phosphoamino acid (%)		
	Phospho-serine	Phospho-threonine	Phospho-tyrosine
130	86.6	13.4	0
80	64.2	35.8	0
65	63.2	36.8	0
42	62.5	37.5	0

Table 2 Quantitative evaluation of the effect of PKC inhibitors on the phosphoproteins induced by TNF α : dose-dependent inhibition of phosphorylation events

Permeabilized A10 cells were labelled with [^{32}P]ATP and pre-incubated for 10 min at room temperature with various concentrations of the PKC inhibitors RO-31-7549 and RO-31-8220. The cells were then stimulated with 20 pg of TNF α /ml for 3 min. The phosphoproteins were separated by SDS/7.5%-PAGE and identified by autoradiography. Quantitative evaluation of the phosphoproteins was carried out using an LKB Ultrascan XL densitometer.

		Phosphorylation (% of maximum)				
Concn (μ M)	kDa ...	130	90	80	65	42
RO-31-7549						
0		100	100	100	100	100
0.1		59	62	40	40	46
1		43	52	23	22	33
10		22	25	11	10	20
RO-31-8220						
0		100	100	100	100	100
0.1		80	52	23	22	70
1		40	43	10	15	43
10		10	34	10	9	25

pletely inhibited the phosphorylation of these proteins (results not shown), thus indicating that the phosphorylations were specifically induced by TNF α and not by components of the reaction mixture.

To determine the identity of the phosphorylated amino acid residues in the TNF α -induced phosphoproteins, the corresponding bands were excised from the SDS/7.5% PAGE gel and subjected to acid hydrolysis. As shown in Table 1, TNF α -induced incorporation of ^{32}P was detectable as phosphoserine and phosphothreonine residues only, indicating that these proteins are substrates for serine and threonine kinases.

The involvement of PKC in TNF α -induced phosphorylation was also indicated by a dose-dependent inhibition of this phosphorylation by the PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989). As shown in Table 2, densitometry analysis of autoradiograms indicated that both RO-31-7549 and RO-31-8220 at 10 μM inhibited the phosphorylation of the 80 and 65 kDa proteins by up to 90%. The phosphorylation of the

130, 90 and 42 kDa proteins was also markedly reduced by these PKC inhibitors (Table 2). The solvent vehicle for PKC inhibitors, i.e. DMSO, did not appear to have any effect on the cells or protein phosphorylation.

To strengthen the evidence for the involvement of the serine/threonine kinase PKC in the TNF α signal transduction pathway, we used mezerein, a phorbol ester capable of activating PKC (Grove and Mastro, 1991). As shown in Figure 3, mezerein stimulated a pattern of increased phosphorylation similar to that produced by TNF α in A10 cells. In particular, large increases in the phosphorylation of the 90, 80, 65 and 42 kDa phosphoproteins were apparent, although phosphorylation of the 130 kDa phosphoprotein was less evident (Figure 3). Increased intensity of staining was also observable in other protein bands in the gel, e.g. in the > 130 kDa and 45–60 kDa regions. As with TNF α , the phosphorylation increases were dose-dependent, with a concentration of 0.1 ng of mezerein/ml being the most effective (Figure 3, lane 8) for the phosphorylation of the 90, 65 and 42 kDa proteins. Concentrations of mezerein higher than this were less effective for these proteins, but a further increase in phosphorylation of the 80 kDa protein was apparent at 1 ng/ml (Figure 3, lane 9).

DISCUSSION

We have shown that A10 cells, hybrid cells that display some characteristics of normal monocytes and express high numbers of TNF α receptors, are rendered sensitive to TNF α -induced cytotoxicity by incubation with the specific PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989). This suggests that PKC activity itself is involved in maintaining protective mechanisms against the potential cytotoxic activity of TNF α , and that the TNF α -induced PKC-mediated phosphorylation events may be of functional significance in augmenting such protective mechanisms. However, 100% cell killing could not be achieved (Figure 2), even in the presence of PKC inhibitors, suggesting that protective mechanisms other than those regulated by PKC activity still remained active in A10 cells. Alternatively, the PKC inhibitors may not be able to eliminate all PKC activity, which could be due to the presence of more than one member of the PKC isoenzyme family (Parker et al., 1989), and the residual PKC activity could be sufficient to maintain partial resistance to TNF α . Nevertheless, our results are in agreement with those of Hamamoto et al. (1990), who showed that the PKC inhibitor staurosporine augmented TNF α -mediated cytotoxicity in U-937 cells, and are also consistent with previous reports in which activation of PKC was demonstrated to lead to desensitization to TNF α -induced cytotoxicity (Aggarwal and Eessalu, 1987; Johnson and Baglioni, 1988; Scheurich et al., 1989; Belizario and Dinarello, 1991).

Since PKC was implicated in the maintenance and augmentation of protective mechanisms against TNF α -induced cytotoxicity, we investigated protein phosphorylation in A10 cells following stimulation by TNF α . Using a permeabilized cell system (Mire-Sluis et al., 1985, 1986; Evans et al., 1990), which allowed rapid changes in phosphorylation to be detected, we showed that physiological amounts of TNF α (2.0–200 pg/ml) stimulated the rapid phosphorylation of at least five proteins of molecular mass 130, 90, 80, 65 and 42 kDa, in permeabilized A10 cells (Figure 3). We could, however, detect little or no further increases in phosphorylation of the five proteins monitored after 5 min from the point of TNF α stimulation (Figure 4). This suggests either that the phosphorylating enzyme(s) is rapidly deactivated following its initial TNF α -induced activation, or that the activity of phosphatases is also increased. We found that

higher concentrations of TNF α (≥ 200 pg/ml) were less effective than lower concentrations (e.g. 20 pg/ml) in stimulating phosphorylation of proteins, suggesting that, above the optimal TNF α concentration, the competing processes of enzyme deactivation and dephosphorylation become increasingly predominant. The concentrations of TNF α required to induce protein phosphorylation in A10 cells were found to be lower than the concentrations needed to induce cytotoxicity. This can be explained if it is assumed that cytotoxicity is only manifested at TNF α concentrations that are apparently ineffective in inducing PKC-dependent phosphorylation or that induce PKC down-regulation.

In our A10 cells the kinase activated by TNF α was probably PKC, since all of the inducibly phosphorylated phosphoproteins were similar and probably identical to those induced by mezerein, a known PKC activator (Grove and Mastro, 1991). We have only found evidence for serine and threonine phosphorylation (Table 1) in our TNF α -stimulated cells, again indicating involvement of a serine/threonine kinase such as PKC. In addition, the TNF α -inducible phosphorylation of these phosphoproteins was inhibited by the specific PKC inhibitors, RO-31-7549 and RO-31-8220 (Table 2). The lower PKC inhibitor concentrations required to inhibit phosphorylation compared with those required to increase TNF α -induced cytotoxicity are probably attributable to the permeabilization of A10 cell membranes and the consequent increased access of inhibitors to the intracellular compartment.

The identity of the proteins inducibly phosphorylated in TNF α -stimulated A10 cells is not known, but there are some similarities to the molecular masses of proteins previously shown to be phosphorylated in hormone- or cytokine-stimulated cells. For example, our 80 kDa phosphoprotein is similar in mass to an 82 kDa myristoylated alanine-rich PKC substrate (designated MARCKS) whose phosphorylation is induced by TNF α and a number of other stimuli, including phorbol esters, in a variety of cell types (Thelen et al., 1990; Easom et al., 1990). Similarly, the 65 kDa phosphoprotein may be identical to a phosphoprotein of the same molecular mass which is inducibly phosphorylated in cells stimulated with interleukin-1, a cytokine which has overlapping biological activity with TNF α (Matsushima et al., 1987, 1988; Le and Vilček, 1987). The identities of the 130, 90 and 42 kDa phosphoproteins remain elusive, but it is unlikely that the latter is the widely described 42 kDa serine/threonine protein kinase, an enzyme termed 'MAP kinase', which has been found to be phosphorylated on tyrosine residues even following stimulation by phorbol ester (Rossomando et al., 1989).

We did not find that 26–28 kDa proteins (Schütze et al., 1989; Robaye et al., 1989; Kaur et al., 1989) were phosphorylated in our cell system. However, it would appear that TNF α -induced phosphorylation of specific cytosolic phosphoproteins can be cell-type-dependent, and is probably largely a reflection of the diversity of signal transduction pathways and their operating levels within individual cell types (Schütze et al., 1989). Divergent phosphorylation patterns could also be due to either the employment of different experimental conditions or the use of more specific and/or more sensitive methods of analysis, e.g. immuno-precipitation (Kaur et al., 1989) or two-dimensional gel electrophoresis (Guy et al., 1991).

In conclusion, we have shown in a monocyte-like hybrid cell line, A10, that the specific PKC inhibitors RO-31-7549 and RO-31-8220 (Davis et al., 1989) sensitize cells to TNF α -induced cytotoxicity, suggesting that PKC activity is involved in

cellular protective mechanisms. We investigated early protein phosphorylation in TNF α -stimulated A10 cells and found that this was probably due to TNF α -induced augmented PKC activity. Several proteins showed increased phosphorylation; their identities, and thus their function and potential role in TNF α signal transduction pathways culminating in enhancement of protective mechanisms, require further investigation.

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