DOWNREGULATION OF TUMOR NECROSIS FACTOR (TNF) SENSITIVITY VIA MODULATION OF TNF BINDING CAPACITY BY PROTEIN KINASE C ACTIVATORS

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The regulatory mechanisms of tumor necrosis factor α (TNF-α) production, as well as those controlling the differential TNF-α sensitivity of normal and malignant cells, appear complex and are still poorly understood. Like other cytokines, TNF-α confers its signal to target cells via binding to specific cell surface membrane receptors (1–3). Expression of TNF-α membrane receptors is necessary, but not sufficient to determine responsiveness of a given target cell (1, 3, 4). With increasing knowledge on the diversity of cellular responses to TNF (for review see 5 and 6), the question arises, by which mechanisms such diverse TNF responses are controlled. Control mechanisms operating at a postreceptor level (6), as well as receptor heterogeneity (7), may contribute to the differential response pattern of distinct cell types. Moreover, there are several examples that the magnitude of a particular biological response to a given cytokine is proportional to the quantity of ligand receptor interactions, indicating that the number of expressed membrane receptors is a critical parameter in determination of cellular sensitivity to the cytokine in question (6, 8, 9). While it was so far assumed that there is constitutive expression of TNF receptors in most tumor cells (3, 4), we recently have shown that, in normal peripheral lymphocytes, positive and negative regulatory mechanisms control membrane expression of TNF receptors. Thus, TNF receptors are reversibly induced in T cells and expressed in a stimulus-dependent manner (10). Moreover, TNF receptors in activated T cells can be rapidly modulated by activators of protein kinase C, resulting in loss of TNF-binding capacity (11).

We here demonstrate that protein kinase C–mediated downregulation of TNF-binding capacity is a general mechanism effective in both malignant and normal cells of various tissues. The data show that loss of TNF-binding capacity is most likely due to a drastic change in affinity rather than internalization or shedding of TNF receptor molecules. As downregulation of TNF-binding capacity was associated with a decrease in TNF sensitivity, our data suggest that protein kinase C is involved in the control of TNF responsiveness.

Materials and Methods

Materials. Phorbol myristate acetate (PMA) and oleyl acetyl glycerol (OAG)[1] were from Sigma Chemical Co., Heidelberg, Federal Republic of Germany. 1-(isoquinolinesulfonyl)-2-methyl-piperazine (H7) and N-(2-guanidinoethyl)-5-iosquinolinesulfonamide (HA 1004) were from Seikagaku Kogyo Co., Tokyo, Japan.

Cells and Culture Conditions. The human leukemic cell lines U 937 (histiocytic) and K 562 (erythroleukemia), as well as the colon adenocarcinoma line Colo 205 were obtained from American Type Culture Collection, Rockville, MD. Normal peripheral T lymphocytes were purified, stimulated with matrix-bound anti–T cell receptor antibody, and cultured in 10 ng/ml of purified recombinant IL-2 for 5–10 d before use as described (12).

Cytokines. Highly purified (>99%) recombinant human TNF-α as well as IFN-γ (Genentech, South San Francisco, CA) were kindly provided by Dr. G. Adolf, Boehringer Ingelheim, Vienna, Austria. The specific activity of TNF-α, as determined by cytotoxicity assay on murine L 929 cells, was 5×10^7 U/mg. Radioiodination of TNF-α to a specific radioactivity of ~40 μCi/μg without significant loss of bioactivity was performed as described (3). Human recombinant IL-2 with a purity of >99.5% was kindly provided by Dr. M. Wrann, Sandoz Forschungsinstitut, Vienna, Austria.

Assay Systems. Binding assay with ^{125}I-TNF-α was performed as described (3, 10). Measurements were usually performed in triplicates and included controls for unspecific binding of the ligand in the presence of a 200-fold excess of unlabelled TNF-α. Bioassay for TNF-α using murine fibrosarcoma L 929 cells was performed in flat-bottom microtiter plates (six replicates) as described (13).

Determination of Internalized TNF-α. To determine the amount of internalized ^{125}I-TNF-α, membrane-associated ligand was removed by a pH 3 wash over a three-step gradient. All steps were performed at 0–4°C. Cells were divided into two aliquots (1–2 $\times$ 10^6 cells in 0.5 ml) and one aliquot was layered on top of a discontinuous gradient consisting of the following three solutions: (a) 0.5 ml of culture medium supplemented with 20% Ficoll; (b) 3 ml of 100 mM NaCl, 50 mM glycine/HCl, pH 3.0, supplemented with 10% Ficoll; and (c) 0.5 ml of culture medium supplemented with 5% Ficoll. The second cell aliquot was used for determination of total amount of cell-associated ^{125}I-TNF-α, and was layered on top of a similar Ficoll gradient consisting of PBS (pH 7.3) supplemented with 10% Ficoll in the second layer. The cells were centrifuged for 10 min at 50 g, the solution was carefully aspirated, and cells were transferred into counting vials to determine radioactivity. Under these conditions of pH 3 wash, >90% of the membrane-receptor-bound ^{125}I-TNF-α can be dissociated from the cells.

Preparation of Cell Membranes. All manipulations were performed at 4°C (buffer A: 125 mM sucrose, 7.5 mM Tris/HCl, pH 7.4, 2 mM EDTA, 2 mM EGTA, and 1 mM PMSF; buffer B: 100 mM Tris/HCl, pH 7.4, 10 mM CaCl$_2$, 10 mM MgCl$_2$, 1 mM PMSF, and 2% FCS). Cells were harvested, washed once in buffer A, and allowed to swell for 20 min in buffer A at 10^8 cells/ml. They were subjected to homogenization in a glass/glass potter with 50 gentle strokes at 1,000 rpm. The homogenate was diluted with an equal volume of buffer A, and intact cells as well as nuclei were removed by centrifugation for 10 min at 50 g. The pellet was washed with an additional volume of buffer A, and membranes were obtained from the combined supernatants by centrifugation for 15 min at 12,000 g and washed twice with buffer B.

Results

The effect of the phorbol ester PMA on TNF-binding capacity was investigated on tumor cell lines derived from various tissues. As shown in Table I, a significant reduction of TNF binding was observed in all tumor cell lines after incubation for 20 min with PMA. Normal diploid fibroblasts, as well as normal activated T

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[1] *Abbreviations used in this paper:* CHX, cycloheximide; H7, 1-(isoquinolinesulfonyl)-2-methyl-piperazine; HA 1004, N-(2-guanidinoethyl)-5-isoquinolinesulfonamide; OAG, oleyl acetyl glycerol.

TABLE I

TNF Receptor Downregulation Mediated by PMA Is Not Tissue-specific and Is Antagonized by Protein Kinase C Inhibitors

Cell line	Origin	PMA concentration	Residual specific ^{125}I-TNF-α binding
		ng/ml	%
HL 60	Myeloid leukemia	10	46
K 562	Myeloid leukemia	100	22
U 937	Myeloid leukemia	100	17
HuT 78	T lymphoma	10	9
Colo 205	Colon carcinoma	10	3
WiDR	Colon carcinoma	10	22
PA 1	Teratocarcinoma	10	48
CCD 18	Diploid fibroblasts	10	37
T cells	Normal PBL	10	10
T cells	PK inhibitor H7	5	44
T cells	PK inhibitor HA 1004	5	16
T cells	None	5	14

Cells (6×10^6 cells/ml) were incubated for 20 min at 22°C with PMA at the indicated concentrations. Thereafter, the cells were washed twice and ^{125}I-TNF-α-binding capacity was determined in comparison with untreated cells. To determine the effect of protein kinase inhibitors H7 and HA 1004, respectively, T cells were preincubated with 50 μM of the respective agent before the addition of PMA. All cell lines were derived from American Type Culture Collection lines.

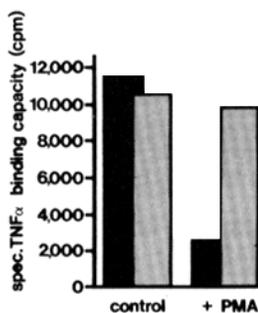FIGURE 1. PMA does not downregulate TNF-α-binding capacity of isolated plasma membranes. K 562 cell membranes were prepared as described in Materials and Methods. TNF-α-binding capacity of membrane equivalents from 4×10^6 cells (▨) was compared with that of 2×10^6 K 562 cells (■) before and after treatment with 100 ng/ml PMA for 20 min at room temperature. ^{125}I-TNF-α-binding experiments with membranes were performed in buffer B. Centrifugation steps were carried out at 12,000 g.

cells showed a similar response (Table I). PMA treatment of isolated cell membranes did not affect TNF-binding capacity, indicating that receptor modulation was not due to direct interaction of PMA with TNF-receptor molecules (Fig. 1). As shown previously (11), pretreatment of T cells with the protein kinase inhibitor H7 reduced the loss of TNF-α-binding capacity of PMA-treated T cells (Table I). In contrast, pretreatment with protein kinase inhibitor HA 1004 did not prevent downregulation (Table I). Both substances are strong inhibitors for a variety of protein kinases, however, the affinity of HA 1004 for protein kinase C is about sevenfold lower as compared with H7 (14). These data, therefore, strengthen the view that protein kinase C is the mediator of PMA-induced downregulation of TNF-α-binding capacity.

To address questions concerning the mechanisms of TNF receptor downregulation, the following experiments were performed largely with the leukemic
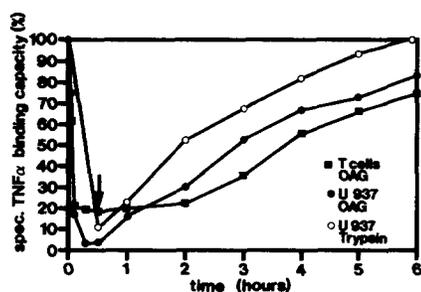
FIGURE 2. Kinetics of reappearance of TNF-α-binding capacity after treatment of cells with OAG or trypsin. Cells were incubated with 100 μM OAG at 10⁶ cells/ml at 37°C. At the times indicated, aliquots of 6 × 10⁶ cells were taken, washed once, and ¹²⁵I-TNF-α-binding capacity was determined (three replicates for each specific and unspecific binding). After 30 min of OAG treatment the cells were collected by centrifugation, washed once to continue cell culture in the absence of OAG (arrow). At the same time the culture of trypsin-treated cells was set up for comparison (0.2% wt/vol trypsin at 37°C for 15 min). Specific ¹²⁵I-TNF-α-binding is given in percent of untreated controls, which remained constant over the time of the experiment (T cells: 11,800 ± 700; U 937 cells; 8,700 ± 650 cpm).
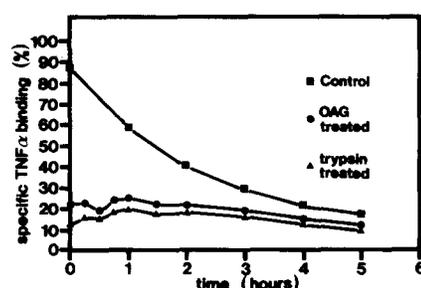

FIGURE 3. Influence of CHX treatment on TNF receptor expression. U 937 cells were preincubated for 30 min with 10 μg/ml CHX. Aliquots were either left untreated or treated with OAG and trypsin, respectively, as described in Fig. 2. Thereafter, cells were washed and again supplemented with 10 μg/ml CHX and kept at 37°C for the indicated times. At the indicated times, 6 × 10⁶ cells from each group were taken to determine TNF-α-binding capacity. Specific ¹²⁵I-TNF-α binding is given in percent of untreated cells (11,200 ± 750 cpm).

cell lines K 562 and U937 as well as with normal activated T cells, which express high numbers of TNF receptors and respond to TNF-α (3, 10, 15, 16). PMA-induced TNF receptor downregulation lasted for more than 12 h, even when the cells had been washed after PMA treatment (data not shown). In contrast, loss of TNF-binding capacity induced by the membrane permeable protein kinase C activator OAG was fully reversible. After removal of OAG, half-maximal binding capacity of both U937 cells and of activated T cells was recovered within ~3 h of incubation at 37°C (Fig. 2). Scatchard analyses of U 937 cells at different times after OAG treatment indicated an apparent loss of high-affinity TNF receptors, with no evidence for the appearance of a low-affinity set of TNF receptors, even when ¹²⁵I-TNF-α concentrations up to 1 μg/ml had been used in TNF-α binding assays (data not shown). Recovery of TNF-binding after OAG treatment occurred with similar kinetics, as upon complete removal of receptors by trypsinisation (Fig. 2). Blocking of protein synthesis with cycloheximide (CHX) revealed a half-life of TNF receptors of ~2 h in unstimulated U937 cells (Fig. 3). CHX treatment totally abolished the reexpression of TNF receptors, independent of whether receptors had been removed by trypsin treatment or whether TNF-α-binding capacity had been downregulated by OAG treatment (Fig. 3). These data indicate that (a) the internal pool of TNF receptor proteins is neglectable and that (b) recovery of TNF-α-binding capacity upon protein kinase C–mediated downregulation requires de novo protein synthesis.

We then investigated whether protein kinase C–mediated TNF receptor modulation affects internalization of ligand/receptor complexes. Fig. 4 shows the kinetics of dissociation and internalization of ¹²⁵I-TNF-α of untreated and PMA-treated U 937 cells. The data indicate that in the absence of PMA ~25%
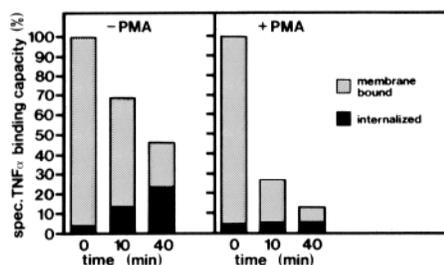
FIGURE 4. PMA treatment prevents internalization and increases dissociation of TNF-α-receptor complexes. U 937 cells were incubated at 0°C with 20 ng/ml of ^{125}I-TNF-α for 1 h to saturate cell surface TNF receptors. Then cells were washed twice and incubated at 37°C without or with 100 ng/ml PMA to follow the rate of internalization and dissociation. At the times indicated, aliquots of 6 × 10⁶ cells were taken and immediately cooled down by the addition of excess ice cold PBS supplemented with 2% FCS and 0.02% sodium azide. After collection of the cells by centrifugation, aliquots were prepared to determine the total amount of cell-bound radioactivity (centrifugation over a pH 7 gradient) as well as internalized ^{125}I-TNF-α (centrifugation over a pH 3 gradient) as described in Material and Methods. Unspecific binding was similar in all groups (<12%), and was subtracted. 100% of specific TNF-α binding represents 12,400 cpm.
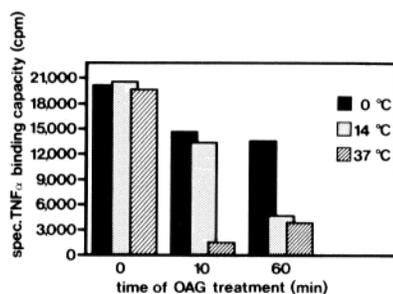

FIGURE 5. Time and temperature dependence of OAG-induced downregulation of TNF-α-binding capacity. U 937 cells were treated at the indicated temperatures with 100 μM OAG. At the times indicated, TNF-α-binding capacity was determined from aliquots of the cells, using 20 ng/ml ^{125}I-TNF-α.

of the receptor-bound TNF-α is internalized within 40 min at 37°C. Upon incubation in the presence of PMA, however, the vast majority of prebound TNF-α dissociates, while internalization is abolished (Fig. 4). Moreover, at temperatures below 17°C, where membrane fluidity is low (17), internalization of TNF-α is not detectable during a 1-h incubation period (data not shown). However, OAG treatment of U937 cells at 14°C as well as 0°C still produced a reduction of TNF-α-binding capacity, although the kinetics of receptor modulation were different at 37, 14, and 0°C, respectively (Fig. 5). Half-maximal reduction was achieved after 3 min at 37°C and after ~30 min at 14°C. Only partial decrease of TNF-α-binding capacity was noted at 0°C during a 5-h incubation (data not shown). These data show that protein kinase C−mediated downregulation of TNF-binding capacity is neither due to internalization nor due to shedding of receptor molecules.

As the magnitude of TNF-α response, e.g., cytostasis of U937 cells and HLA-A,B,C expression in K562 cells, is proportional to the number of ligand/receptor interactions (Fig. 6), we addressed the question whether downregulation of TNF-α-binding capacity would result in a respective change in TNF-α responsiveness. In fact, a clear modulation of TNF-α responsiveness was observed in distinct assay systems. In a TNF cytotoxicity assay with actinomycin D−treated L 929 cells, 10–20-fold higher concentrations of TNF-α were necessary to reach the same cytotoxic effects in the presence of 10 ng/ml of PMA (not shown) or 100 μM OAG (Fig. 7). At these concentrations, neither OAG nor PMA influenced cell viability by themselves. As in this assay system, a 2-h pulse treatment with TNF-α was sufficient to mediate a strong cytotoxic effect, it was possible to study
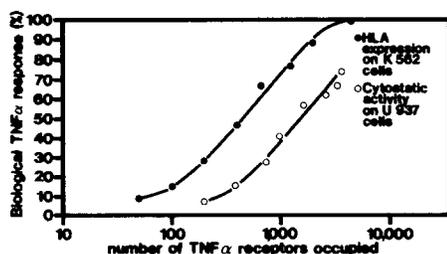
FIGURE 6. Correlation between the number of ligand-receptor interactions and the resulting biological TNF-α response. Cells were cultured for 48 h with various concentrations of TNF-α. Thereafter, HLA-A,B,C expression in K 562 cells was determined using an Epics C cytofluorograph (16), and cytostatic activity of TNF-α towards U 937 cells was determined by a standard [³H]thymidine incorporation assay as described (3). The number of occupied TNF-α receptors was calculated for each TNF-α concentration on the basis of a dissociation constant of $K_d = 1.5 \times 10^{-10}$ M (3), using the formula: percent occupied receptors $= 100 \times$ (concentration)/(concentration $+ K_d$). Biologic response is given in percent of the maximally achievable response for each cell line at saturating concentrations of TNF-α (100 ng/ml).

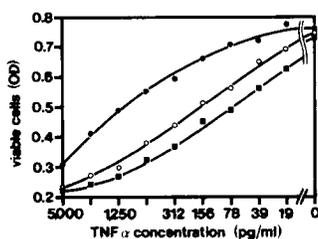
FIGURE 7. OAG-mediated reduction of TNF sensitivity is reversible. L929 cells were treated with 100 μM OAG for 30 min at room temperature. Thereafter, the cells were washed twice, and serial dilutions of TNF-α were added either in the absence (O) or presence (●) of 100 μM final concentration of OAG. In addition, control cultures were set up with untreated L cells (■). All three groups were maintained in TNF-α for 2 h at 37°C, washed once to remove unbound TNF-α, and cell culture was continued for 22 h in the presence of actinomycin D (1 μg/ml).
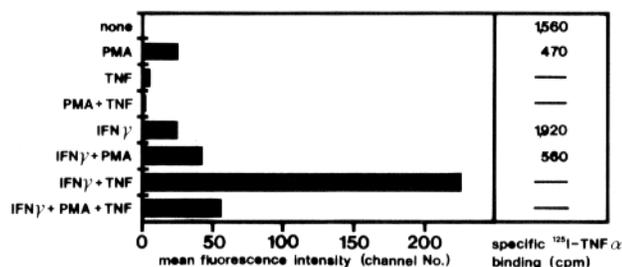

FIGURE 8. Inhibition of TNF-α-mediated enhancement of HLA-DR expression in parallel with downregulation of TNF-α-binding capacity in PMA-treated Colo 205 cells. Colo 205 cells (10^7 cells/group) were cultured with the indicated substances at the following concentrations: PMA, 10 ng/ml; TNF-α, 10 ng/ml; IFN-γ, 0.01 ng/ml. PMA was added 30 min before addition of cytokines. After 2 d of culture, in each group HLA-DR expression was determined by fluorocytometry using an FITC-labeled monoclonal antibody L 243 as described (16). In parallel, specific ¹²⁵I-TNF-α capacity was determined in all those groups, which were not treated with TNF-α in the preceding culture, using 20 ng/ml of ¹²⁵I-TNF-α.

whether TNF unresponsiveness is restricted to the period of TNF-α-binding deficiency, which can be transiently induced by pulse treatment of cells with OAG (Fig. 2). L929 cells were treated for 30 min with 100 μM OAG to obtain maximum downregulation of TNF-α-binding capacity (see Fig. 2). Thereafter, OAG was removed by two washing steps and the cells were then incubated with TNF-α for 2 h. Under these conditions, reduction of TNF-α sensitivity was only 2-fold, as compared with the 16-fold reduction when OAG was continuously present during the 2-h TNF-α treatment of L cells (Fig. 7). Thus, upon transient stimulation of protein kinase C, allowing rapid reappearance of TNF-binding capacity, a parallel reappearance of TNF-α sensitivity is observed.

PMA also strongly antagonized with TNF-mediated enhancement of antigen expression in various tumor cell lines, as well as in normal T cells. An example is shown in Fig. 8, where the capacity of TNF-α to enhance IFN-γ-induced HLA-DR expression in Colo 205 cells (15, 16) was used as a model to study the

influence of protein kinase C activation on TNF receptor expression and TNF-α responsiveness. In accordance with earlier results (15, 16), TNF-α exerted strong synergistic activity, resulting in a roughly sevenfold increase in IFN-γ-induced HLA-DR antigen expression. In the presence of PMA, the TNF-α-mediated enhancement of HLA-DR expression in Colo-205 cells was greatly diminished. In contrast, PMA by itself and in combination with IFN-γ had a slight stimulatory effect on HLA-DR expression in these cells. The reduced TNF-α sensitivity of Colo 205 cells corresponded with a reduced TNF-binding capacity, determined in parallel (Fig. 8).

Discussion

We have shown here that intracellular control mechanisms of TNF-α sensitivity exist in both normal and malignant human cells, which are linked to protein kinase C and enable the cell to rapidly downregulate TNF-binding capacity, thereby increasing resistance to TNF-α. While cells derived from various tissues show, with respect to modulation of TNF-α-binding capacity, differential sensitivity to protein kinase C activators (Table I), the basic finding of downregulation is apparently a general phenomenon. We have previously described that in activated T cells, only those activators of protein kinase C that are membrane permeable were able to modulate TNF-binding capacity (11). In extension of these results, we have here excluded the possibility that protein kinase C activators might directly interact with the ligand-binding site of TNF receptors, as PMA itself has no effect on TNF-binding capacity of isolated cell membranes (Fig. 1). Clearly some cytosolic compounds are necessary to confer PMA-induced reduction of TNF-binding capacity. The differential capacity of the selective inhibitors of protein kinases H7 and HA 1004, respectively, to interfere with PMA-induced downregulation further supports the view that protein kinase C is directly involved in this process (Table I).

TNF-receptor modulation is an extremely rapid event (11) (Fig. 2), which already suggested that neither internalization nor shedding of receptor molecules could account for this phenomenon. Indeed, the data presented in Fig. 4 show that internalization of preformed TNF-receptor complexes is not enhanced by PMA treatment. Quite in contrast, in the presence of PMA, internalization of TNF-α is strongly reduced, probably because TNF-receptor complexes more rapidly dissociate compared with the untreated control. These results indicate that loss of the binding capacity of the receptor, triggered by PMA, and internalization of the TNF-α-receptor complex are independent reactions.

At 14°C, OAG treatment produces a reduction in TNF-α binding comparable to that at 37°C (Fig. 5). At this temperature, cellular membranes are still in a frozen state, preventing both internalization and shedding. Accordingly, as receptor molecules must still be present in the cell membrane, downregulation of TNF-α-binding capacity at 14°C must be explained by a change in ligand affinity of the majority of expressed receptor molecules. As in binding assays with PMA- or OAG-treated cells using a concentration range up to 5×10^{-8} M, no specific TNF-α binding was obtained, aside from that by the few remaining high-affinity receptors, affinity of modulated TNF receptors must have changed drastically (≥ 100-fold), thereby causing functional inactivation. This process

appears irreversible, because recovery of TNF-α-binding capacity after downregulation is dependent on de novo protein synthesis (Fig. 3). Further, kinetics of reappearance of TNF-α-binding capacity after OAG treatment is comparable with that of cells, where cell-surface receptor proteins had been removed by trypsinization (Fig. 2), indicating that both processes simply reflect normal receptor turnover. The half-life of TNF receptors of ~2 h, found in CHX-treated U 937 cells (Fig. 3) is consistent with this interpretation.

As to the molecular mechanism by which protein kinase C stimulation leads to modulation of TNF-binding capacity, our data suggest a direct phosphorylation of the receptor protein(s) at its cytoplasmic site. Such a mechanism has been shown to affect the function of other membrane receptor proteins: phosphorylation of receptor proteins by protein kinase C can either lead to enhanced internalization (transferrin receptors), result in a change in ligand affinity (insulin and epidermal growth factor receptors), or change the potential tyrosine kinase activity of the receptor protein itself (insulin receptors) (for review see 18).

There is general agreement that expression of TNF receptors is a prerequisite for biological responses to TNF-α, as cells, which, a priori, lack TNF receptors (3, 19), or have completely lost binding capacity upon CHX treatment (20), are TNF resistant. Regulation of receptor expression as a potential mechanism for a quantitative regulation of TNF responses was suggested from the finding that the magnitude of a given TNF-α response is proportional to the number of receptor-ligand interactions (Fig. 6). Thus, IFN-γ-mediated upregulation of TNF-α receptors may contribute to enhancement of TNF responses (21), although this effect by itself is not sufficient to explain synergism of the two cytokines (6). However, the data presented here show direct correlation between TNF-α-binding capacity and TNF-α sensitivity. PMA, which downregulates TNF-α-binding capacity for prolonged periods of time, was able to strongly interfere with various TNF-α actions, e.g., cytotoxicity on L929 cells (data not shown) and enhancement of HLA-DR expression in Colo 205 cells (Fig. 8). Moreover, using the rapidly metabolized glycerol derivative OAG for transient stimulation of protein kinase C, we could show that reexpression of TNF-α-binding capacity is accompanied with reversion of TNF-insensitive cells to their TNF-sensitive phenotype (Fig. 7). These data, therefore, suggest that induction of TNF-α resistance by activators of protein kinase C is effective at the level of TNF receptor expression.

Summary

The regulatory action of activators for protein kinase C on the specific binding capacity for recombinant human tumor necrosis factor α (TNF-α) was studied on various human cell lines. Phorbol myristate acetate (PMA) and oleyl acetyl glycerol (OAG) both are able to rapidly downregulate TNF-binding capacity of normal and malignant cells derived from various tissues. As PMA treatment did not enhance internalization of TNF-α-receptor complexes at 37°C, and since OAG was able to downregulate TNF-binding capacity under conditions where internalization and shedding of receptor protein are prevented, we conclude that protein kinase C controls ligand affinity of the TNF-receptor protein, possibly via direct phosphorylation. Protein kinase C triggered downregulation

of TNF-α-binding capacity concomitantly resulted in reduction of TNF-α sensitivity, as revealed from decreased cytotoxic action of TNF-α on L 929 cells and from inhibition of TNF-α-mediated enhancement of HLA class II antigen expression in Colo 205 cells. Restoration of TNF-binding capacity upon abrogation of protein kinase C stimulation leads to full recovery of TNF responsiveness, further supporting the close linkage of TNF-receptor expression and TNF sensitivity. These data suggest that regulation of TNF-binding capacity by protein kinase C is one of the cellular control mechanisms of TNF responsiveness.

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