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# Early Events in the Antiproliferative Action of Tumor Necrosis Factor Are Similar to the Early Events in Epidermal Growth Factor Growth Stimulation

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The process of TNF-induced cytotoxicity is complex but appears to be mediated through a TNF-specific cell surface receptor. Recent evidence suggests that TNF action on tumor cells may be antagonized by epidermal growth factor (EGF) and other EGF-receptor modulatory peptides implicating a role for EGF-R in the process of TNF-induced cytotoxicity. In the present report, we investigated the biochemical actions of TNF on several biochemical events known to occur in the process of EGF signal transduction in intact cells. The actions of TNF were compared directly to those of EGF in both TNF-sensitive and -resistant tumor cell lines.

In TNF-sensitive ME-180 cervical carcinoma cells, TNF (20 ng/ml) stimulated the tyrosine protein kinase activity of the EGF-receptor (EGF-R) fivefold when measured by receptor autophosphorylation in an immune complex kinase assay. TNF activation of EGF-R kinase activity in ME-180 was measurable 10 min after TNF incubation and enzymatic activity remained elevated 20 min after TNF addition. Activation of the receptor by TNF correlated with increased $^{32}P$ incorporation into EGF-R protein when receptor was immunoprecipitated from $^{32}P$-equilibrated cells following a 20 min incubation with TNF. Acid hydrolysis of EGF-R protein isolated from TNF-treated ME-180 cells demonstrates an increase in the phosphotyrosine content of EGF-R when compared to receptor isolated from untreated cells. The results suggest that TNF increased EGF-R tyrosine protein kinase activity and the state of EGF-receptor tyrosine phosphorylation in a manner similar to that reported for EGF. However, TNF does not appear to be structurally related to EGF since TNF was unable to directly activate EGF-R when incubated with extensively washed immunoprecipitates of EGF-R.

In TNF-resistant T24 bladder carcinoma cells, TNF failed to alter EGF-R tyrosine protein kinase activity although both EGF and phorbol ester were shown to modulate the enzymatic activity of the receptor in these cells. These results indicate that the ability of TNF to modulate EGF-R kinase in target cells may correlate with its cytotoxic actions on TNF-sensitive tumor cells.

Other biochemical activities associated with the induction or regulation of cellular growth were examined in TNF- or EGF-treated tumor cells. EGF stimu-

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lated a rapid 8–16-fold increase in the expression of the proto-oncogene c-*myc* when analyzed by dot-blot analysis of total cellular RNA or Northern blot hybridization of polyadenylated RNA. TNF treatment failed to alter c-*myc* expression in ME-180 cells when analyzed by either technique. The two structurally distinct peptides, TNF and EGF, induced similar patterns of ornithine decarboxylase activity (the rate-limiting enzyme in polyamine biosynthesis) in ME-180 cells but differed in their relative magnitude of maximal induction. Similar results were obtained in TNF- or EGF-treated T24 cells, suggesting the effects of TNF on polyamine biosynthesis are not related to its cytotoxic mechanism of action. These results indicate that TNF shares some of the early biochemical actions of EGF in tumor cells and some of these effects may be related to the mechanism of TNF-induced cytotoxicity.

**Key words: epidermal growth factor receptor, tyrosine kinase activity, phosphorylation, c-myc, control of cell growth**

Tumor necrosis factor (TNF) has been shown to induce cytotoxicity or cytostatis in several tumor cell lines while other tumor cells appear resistant to the growth-inhibitory effects of this peptide. The heterogeneous effects of TNF on the growth of tumor cells are not explained by differences in the ability of TNF to bind or associate with TNF receptors on the surface of target cells [1,2]. Equivalent TNF binding and processing of the TNF binding "complex" have been measured in both TNF-sensitive and -resistant tumor cells [3,4]. Chemical cross-linking of TNF to its cell surface binding proteins suggests that resistance may be due to lack of a component or activity normally associated with TNF receptor in sensitive cells [2]. This component may be structurally associated with the cellular TNF receptor so that TNF signal transduction or post-receptor-mediated events are altered. Therefore, to understand the process of TNF-mediated cytotoxicity and the biochemical elements which control sensitivity and resistance to this peptide, experiments which assess the early biochemical events in TNF signal transduction in tumor cells displaying sensitivity and resistance to TNF are important.

The process of TNF signal transduction is poorly understood. Early observations on TNF action in normal cells suggested that TNF influences cell growth in a manner which may share a common mechanism or metabolic pathway with epidermal growth factor (EGF) [5]. EGF is known to transduce its growth-stimulatory signal through a cell surface receptor (EGF-R) which upon binding EGF leads to an increase in the intrinsic tyrosine-specific protein kinase activity of the receptor [6] and an increase in the phosphotyrosine content in EGF-treated cells. In addition, EGF has been shown to increase the expression of the proto-oncogene c-*myc* [7,8] and to lead to the induction of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) [9–11]. Enhanced c-*myc* expression and ODC activity have been shown to occur following stimulation of cell growth by a variety of agents [12–14].

TNF has been shown to increase the number of EGF receptors on the surface of normal fibroblasts [15]. This biological effect of TNF may correlate with the mitogenic properties of TNF on these cells. Additionally TNF has recently been shown to stimulate phosphorylation of EGF-R which correlates with decreased EGF-R affinity in gingival fibroblasts [16].

In a study of tumor cell sensitivity to TNF, Sugarman et al [17] demonstrated that pretreatment of TNF-sensitive tumor cells with exogeneous growth factors can block the ability of TNF to induce cytotoxicity, suggesting a role for growth factor receptors in TNF action.

In the present report, we compared the effects of EGF and TNF on several key biochemical events associated with the cellular actions of EGF. These events include activation of EGF receptor tyrosine protein kinase activity, altered phosphorylation state of the EGF receptor, and induction of *c-myc* mRNA expression and ODC activity. These biochemical processes were examined in TNF-treated tumor cells previously shown to express equivalent TNF binding properties but differing in their sensitivity to the antiproliferative effects of TNF.

## MATERIALS AND METHODS

### Cell Lines

Human cervical carcinoma (ME-180) and bladder carcinoma cells (T24) were obtained from American Type Culture Collection (Rockville, MD). A431 epidermoid carcinoma cells were provided by Dr. Peter Steck (Department of Neuro-oncology, M.D. Anderson Cancer Center). All cells were grown and maintained in minimal essential media (MEM) supplemented with 10% fetal calf serum (GIBCO, Grand Island, NY) and 50 $\mu$g/ml gentamycin.

### Assays

**Effect of TNF on the growth of human tumor cells.** The effect on the growth of ME-180 and T24 cells incubated with various doses of TNF was analyzed by using Bio-Rad protein dye reagent (Bio-Rad Laboratories, Richmond, CA) as previously described [18]. Briefly, cells were dispensed into 96-well plates (4–6 $\times 10^3$ cells/well) and allowed to attach (overnight at 37°C) prior to the addition of appropriate dilutions of TNF to the culture media (200 $\mu$l final volume/well). After incubation for 72 h, medium was aspirated and the cell monolayers were rinsed three times with $Ca^{+2}$- and $Mg^{+2}$-free phosphate-buffered saline (PBS). Following the final rinse, wells were tapped dry, 10% ethanol was added to each well (50 $\mu$l), and the cells were subjected to three cycles of freeze-thawing. Two hundred microliters of Bio-Rad reagent protein dye was added to each well and following a 20 min incubation, the absorbance was read at 600 nm (Bio-Tek Instruments Winooski, VT) and compared to control wells (medium alone). Values represent the average of six determinations with no more than 3% variance in any point. Under the conditions of this assay the relative percentage of protein remaining in each well is directly proportional to the number of remaining cells and is used as an indicator of cell growth. The percentage of control growth is calculated by using the formula:

$$100 \times \frac{\text{O.D.}_{600}\text{ treated cells} - \text{blank}}{\text{O.D.}_{600}\text{control cells} - \text{blank}}$$

**EGF-receptor tyrosine protein kinase assay.** Tyrosine kinase activity of the EGF receptor was measured by immune complex kinase assay as previously described [19–21] and is based upon utilization of an antibody in an assay similar to that reported by Downward et al. [22]. Cells grown to confluence in 25 cm$^2$ flasks ($5 \times 10^6$) were treated with TNF (20 ng/ml), EGF (20 ng/ml), or phorbol ester (500 ng/ml) for various times as indicated. Monolayers were rinsed three times with ice-cold PBS, collected by scraping into tubes, and pelleted by centrifugation (500*g*—3 min) at 4°C. Supernatants were drained and tapped dry on absorbent paper. Cell pellets were lysed by

sonication in 1 ml RIPA "B" buffer (1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 $\mu$g/ml aprotinin, 5 mM PMSF, 10 $\mu$g/ml leupeptin, 20 mM sodium phosphate, pH 7.4). The lysates were incubated on ice 5 to 10 min and then clarified by centrifugation (10,000*g* for 15 min); 0.5 $\mu$g of anti-EGF receptor monoclonal antibody (R1 MAb from Amersham Corporation, Arlington Heights, IL) was added to each lysate, incubated 1 h on ice, and then treated with 50 $\mu$l pansorbin (10% w/v solution) (Calbiochem, San Diego, CA). After 15 min, immune complexes were washed three times with 0.1% Triton X-100 in PBS, pH 7.4. EGF-receptor kinase assay was initiated by the resuspension of the pellet in 50 $\mu$l of 20 mM Hepes (pH7.4) containing 10 $\mu$Ci [$^{32}$P]-ATP, 6 mM $MnCl_2$, 100 $\mu$M $Na_3VO_4$, and 0.1% Triton X-100. The reaction was incubated at 30°C for 10 min and terminated by the addition of 1 ml of ice-cold RIPA "A" buffer (same as RIPA "B" with the addition of 0.1% SDS, 0.5% deoxycholate, 1 mM sodium vanadate, and 5 mM sodium pyrophosphate). Immune complexes were washed two additional times with RIPA "A" buffer; pellets were heated in Laemmli sample buffer; and the proteins were subjected to SDS–polyacrylamide (8%) gel electrophoresis [23]. Prestained molecular weight markers were applied to companion lanes. Following completion of electrophoresis, gels were fixed in 10% acetic acid/40% methanol, heated to 60°C in 1 M KOH for 60 min and then neutralized in 5% methanol/7% acetic acid. Gels were dried and exposed on Kodak XAR-5 film.

**Ornithine decarboxylase activity assay.** ODC activity was measured as previously described [24]. Briefly, cells were grown to confluence in 100 × 20 mm dishes, shifted to serum-free medium for 12 h, and stimulated with hormone (20 ng/ml) for various intervals. Monolayers were rinsed three times in ice-cold PBS, collected by scraping, and pelleted by centrifugation. The cell pellet was sonicated in 0.6 ml of ODC assay buffer (50 mM sodium potassium phosphate, pH 7.2, 0.1 mM EDTA, 1.0 mM dithiothreitol, 40 $\mu$M pyridoxal phosphate) and following centrifugation (12,000*g* for 15 min), the supernatant was assayed for ODC activity. The activity is defined in units of ODC activity (picomoles $CO_2$ evolved/h/mg protein). Protein was determined by Bio-Rad protein assay by using bovine serum albumin as a standard.

## Modulation of c-*myc* expression

Modulation of c-*myc* proto-oncogene expression in ME-180 cells was analyzed by dot-blot hybridization of total RNA as previously described [25] and Northern blot of poly A selected RNA as reported by Thomas [26]. Briefly, RNA was extracted from EGF- or TNF-treated quiescent ME-180 cells ($10^8$ cells per time point) by using a modified quanidium isothiocyanate/cesium chloride method [27]. Northern blot analysis was performed by selection of polyadenylated RNA by chromatography on oligo(dT)-cellulose columns [28]. The mRNA (5 $\mu$g/lane) was size fractionated by electrophoresis in a 1.1% agarose gel containing 2.2M formaldehyde and transferred to nylon filters with 20 × SSC (1 ×SSC = 0.15M NaCl, 0.015M Na-citrate). For dot-blot analysis total cellular RNA was serially diluted in a solution containing "special" SSC (12× NaCl and 8× Na citrate) and 12.8% formaldehyde. The mixtures were then heated for 15 min at 60°C and spotted onto nylon filters which were subsequently baked in a vacuum oven for 1 3/4 h at 80°C. Both dot-blot and Northern blot filters were prehybridized overnight and hybridized for 48 to 72 h at 42°C with DNA probes that had been radiolabeled with $^{32}$P by the oligo-primer extension method [29] to a specific activity of 1–3 × $10^9$ cpm/$\mu$g of DNA. The filters were subsequently washed at 60°C for 60 min by using a solution of

0.1× SSC and 0.1% SDS, air dried, and autoradiographed using x-ray film. A chick beta-actin probe was used to verify the content of nucleic acid in each lane. Molecular cloning of the probes used in this study has been described as follows: c-*myc*, a 1.3 kb ClaI/EcoR1 genomic clone from the 3′ end of the gene [30] and chick beta-actin, a 1.8 kb PstI cDNA [31].

### Immunoprecipitation of EGF-R from $^{32}$P-equilibrated cells

Confluent ME-180 cells (in 60 mm dishes) were equilibrated in phosphate-free MEM containing 0.2% fetal bovine serum and 1 mCi/ml $^{32}P_i$ (carrier-free) for 24 h prior to the addition of 20 ng/ml TNF (or BSA as control). After a 20 min incubation, cell monolayers were rinsed twice in ice-cold PBS, scraped by rubber policeman, and pelleted by centrifugation (600*g*—5 min) at 4°C. Cell pellets were retained and sonicated (10 s—70% power, Kontes Micro-Ultrasonic Cell disrupter) in 1 ml of cell lysis buffer (CLB) consisting of 10 mM Tris-HCl pH 7.5, 1% BSA, 10 μg/ml aprotinin and leupeptin, 50 mM NaF, 0.5% deoxycholate, 1 mM $Na_3VO_4$, and 1 mM phenylmethyl sulfonyl fluoride. Following a 5 min incubation on ice, lysates were centrifuged at 15,000*g* for 20 min and the supernatant fraction was retained, made 0.5 M in NaCl concentration, and incubated with 2 μg of anti-EGF-R monoclonal antibody (R1) for 2 h on ice. Immune complexes were precipitated with the addition of 100 μl pansorbin and incubated an additional 30 min prior to pelleting the complexes by centrifugation (10,000*g*—2 min). Immune complexes were washed with CLB containing 0.5M NaCl followed by a wash in CLB containing 0.5M NaCl and 0.1% SDS. Precipitated EGF-R protein was released from the immunocomplex by heating the pellet in 20 mM DTT containing 1% SDS. Extracts were centrifuged, supernatants were retained, and phosphoproteins were resolved by SDS-PAGE (8%). The gel was dried and exposed to x-ray film for autoradiography.

### Phosphoamino acid analysis of EGF-R

ME-180 cells equilibrated in $^{32}$P (as described above) were treated with 20 ng/ml TNF (or BSA as control) for 30 min. Receptor protein was immunoprecipitated, washed, and resolved by SDS-PAGE. The unfixed gel was dried under vacuum and EGF-receptor protein was identified by autoradiography. Bands representing EGF-R were excised and rehydrated in 0.1M ammonium bicarbonate. Gel slices were incubated with 100 μg/ml trypsin (in 0.1M $NH_4CO_2$ buffer) in a total volume of 5 ml at 37°C for 24 h. An additional 500 μg of trypsin was added to each sample and incubated an additional 24 h prior to recovery of phosphopeptides. Supernatants containing phosphopeptides were lyophilized and acid hydrolyzed in 1 ml of 5.8M HCl in sealed ampules at 110°C for 2 h. After removal of the acid by drying in a vacuum oven, hydrozylates were resuspended in 250 μl of 15 mM potassium phosphate buffer (pH 3.8) containing 1 mg/ml of phosphoamino acid standards. Phosphoamino acids were resolved by HPLC on a SynChroPak AX 300 anion exchange column (25 × 0.4 cm) equilibrated in $KH_2PO_4$ buffer as previously described [32].

Samples were injected and resolved at a flow rate of 2 ml/min and fractions of 1 ml were collected and analyzed for $^{32}$P by Cerenkov counting. Counts are plotted vs. fraction number and compared to the elution of phosphoamino acid standards chromatographed individually (or in combination) under similar elution conditions.

## Materials

Recombinant human TNF was kindly supplied by Dr. H. Michael Shephard of Genentech Corporation (South San Francisco, CA). TNF had a specific activity of 5 × $10^7$ units/mg and migrates as a single 17,000 molecular weight species on SDS-polyacrylamide gels. Murine EGF was purchased from Collaborative Research Inc. (Bedford, MA). Phorbol ester (PMA) was obtained from CCR, Inc. (Chanhassen, MN). Monoclonal antibody against EGF receptor (R1), [$^{14}$C-1] ornithine (specific activity = 60 mCi/mmol), and carrier-free $^{32}$P were all purchased from Amersham Corporation (Arlington Heights, IL). [γ-$^{32}$P]-ATP (specific activity >3,000 Ci/mmol) was purchased from New England Nuclear (Wilmington, DE). Pansorbin was obtained from CalBiochem Corp. (La Jolla, CA).

## RESULTS

The effect of increasing doses of TNF on the growth of two human tumor cell lines is shown in Figure 1. Cervical carcinoma ME-180 cells demonstrate sensitivity to the antiproliferative effects of TNF in agreement with other investigations [1,2] and this correlates with a decrease in thymidine incorporation as previously described [18]. TNF inhibited ME-180 cell growth by 50% (IC 50) at a concentration of 100 units/ml (2 ng/ml). The growth of T24 bladder carcinoma cells was not influenced by TNF even at doses greater than 10,000 units/ml (>200 ng/ml). The apparent resistance to TNF antiproliferative effects is unlikely to be due to lack of TNF receptors since several studies have demonstrated the presence of TNF receptors on tumor cells independent of their sensitivity or resistance to TNF cytotoxic actions. The cell lines used in this study have previously been shown to bind TNF equivalently at their cell surface, expressing both similar affinity and equal number of binding sites for TNF [1]. Thus the biochemi-

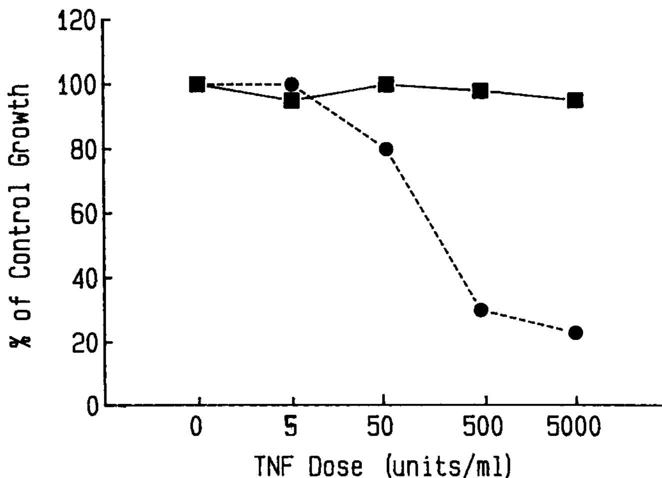


Fig. 1 Effect of TNF on the growth of human tumor cells. Increasing doses of TNF were incubated with ME-180 (●) or T24 (■) cells for 72 h. The effect on the growth of these cells was analyzed as described in Materials and Methods. Values represent the difference between TNF-treated and control cell density and are based upon the average of six determinations with no more than 3% variance in any one point.

cal effects of TNF were examined in these two cell lines to determine whether TNF influences a cascade of events in sensitive cells distinct from those induced in resistant populations.

Because EGF-R synthesis [15] and phosphorylation [16] are known to be effected by TNF we compared the actions of TNF with those of EGF on several biochemical elements associated with the process of EGF signal transduction.

One of the earliest biochemical responses to EGF following specific binding to its cell surface receptor is the activation of the EGF receptor (EGF-R) tyrosine kinase activity [33]. To investigate whether TNF affects EGF-R kinase activity, EGF-R tyrosine kinase activity was measured by specific immunoprecipitation of EGF-R and autophosphorylation of receptor protein in the presence of [$\gamma$-$^{32}$P] ATP (immune complex kinase assay). EGF-R kinase activity was measured after 0–20 min of TNF treatment in ME-180 cells (Fig. 2) and T24 cells (Fig. 3). TNF (1,000 units/ml) stimulated EGF-R tyrosine kinase activity in a time-dependent fashion in ME-180 cells. TNF induced a rapid activation of EGF-R kinase activity when compared to kinase activity in untreated ME-180 cells. Densitometric quantitation of the effect of TNF on EGF-R kinase activity demonstrates a 4–5-fold increase in kinase activity when measured 10–20 min after TNF incubation. Similar results were obtained in three separate experiments which demonstrate 2–6-fold activation of kinase activity following TNF incubation. These results demonstrate that TNF induced an early stimulatory effect on

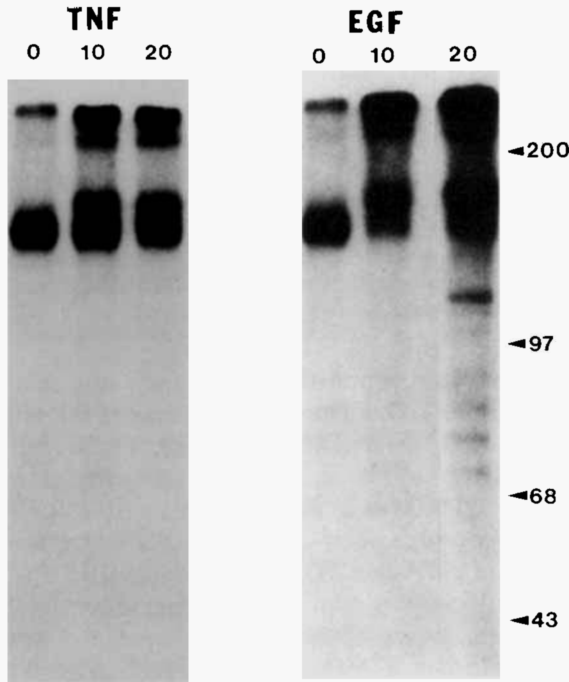


Fig. 2. Effect of TNF or EGF treatment on EGF-R tyrosine kinase activity in ME-180 cells. Serum-free quiescent ME-180 cells were treated with 20 ng/ml TNF or 20 ng/ml EGF for 0–20 min at 37°C as noted. Following incubation, cells were rinsed and collected and EGF-receptor kinase activity was measured as described in Materials and Methods. Gels was exposed to x-ray film for autoradiography. TNF treatment of ME-180 cells stimulated EGF-receptor kinase activity in a time-dependent, EGF-like fashion.

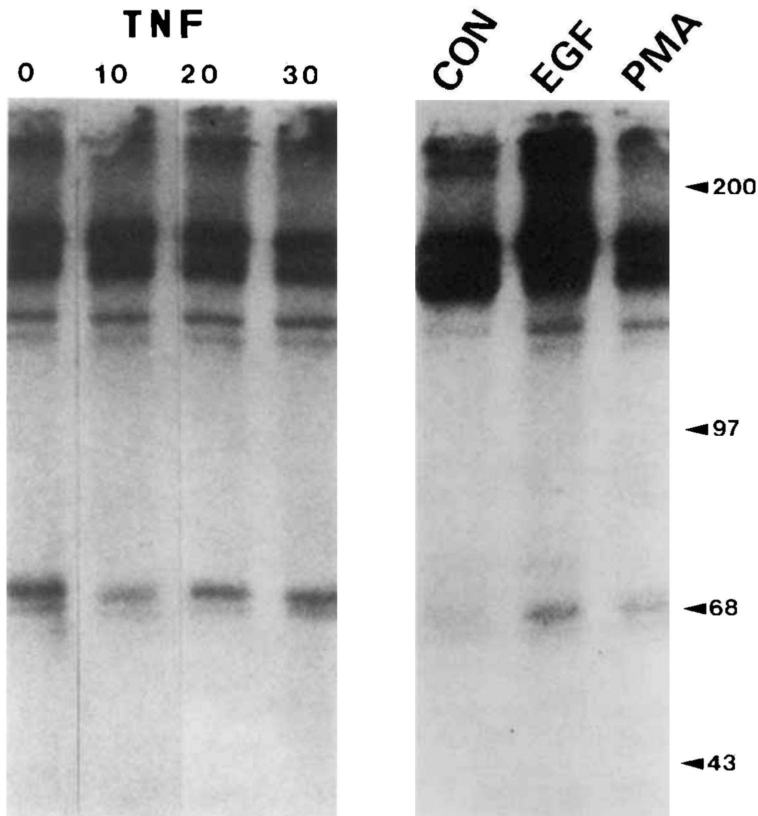


Fig. 3. Effect of treatment on EGF-receptor kinase activity in T24 cells. T24 cells equilibrated in serum-free medium were treated with 20 ng/ml TNF for 0–30 min at 37°C as noted (**left**). Additionally, T24 cells were treated with 20 μl dilutent as control (CON), EGF (20 ng/ml), or PMA (500 ng/ml) for 20 min at 37°C. After incubation, EGF-receptor kinase activity was measured as described above. TNF had no significant affect on T24 cell EGF-receptor kinase activity, whereas EGF and PMA treatment resulted in stimulation or reduction in kinase activity, respectively.

tumor cell EGF-receptor tyrosine protein kinase activity in a manner similar to that previously reported for EGF. To examine the significance of this effect and its relationship to the mechanism of TNF-induced cytotoxicity, TNF-resistant T24 cells were treated with TNF and EGF-R kinase activity was assayed as described above. As demonstrated in Figure 3, TNF was unable to modulate EGF-R kinase activity in T24 cells which previously have been shown to express TNF receptors and to induce an anti-viral state in response to TNF [1,34]. Increased incubation time (>90 min) or dose of TNF (>100 ng/ml) also failed to modify EGF-R kinase activity in T24 cells.

These results suggest that modulation of EGF-R tyrosine kinase activity by TNF is an early and important mediator in the process of TNF-induced cytotoxicity. It also appears that tumor cell sensitivity may be related to the ability of TNF to modulate EGF-R kinase activity in TNF-receptor-positive cells. Other biological agents have been shown to modulate EGF-R tyrosine kinase activity in a variety of cell types. Incubation with EGF stimulates endogeneous tyrosine kinase activity of the EGF receptor through direct binding of growth factor to EGF-R expressed at the membrane surface [35,36].

Additionally, phorbol ester treatment has been demonstrated to reduce EGF-receptor tyrosine kinase activity through phosphorylation of a regulatory threonine residue on EGF receptor [37,38]. To determine whether the inability of TNF to alter EGF-R tyrosine kinase activity in T24 cells was due to a general resistance to other modulatory agents, EGF-R tyrosine kinase activity was measured following incubation with EGF or phorbol ester. As demonstrated in Figure 3, treatment of T24 cells with EGF resulted in increased EGF-R tyrosine kinase activity while phorbol ester treatment reduced tyrosine kinase activity. These results indicate that T24 EGF-R tyrosine kinase activity can be modulated by both exogeneous activating or inactivating agents and the inability of TNF to modulate tyrosine kinase (as demonstrated with TNF treatment of ME-180) could be related to the resistant of this tumor cell line to the antiproliferative actions of TNF.

Phosphorylation of EGF-R has been shown to represent a major mechanism of tyrosine kinase activation or modulation [35–38]. To determine whether TNF modulation of receptor kinase activity is accompanied by alterations in the state of EGF-R phosphorylation, ME-180 cells were equilibrated in $^{32}P$ and treated with TNF for 20 min. EGF-R protein was isolated by immunoprecipitation and resolved by SDS-PAGE. As shown in Figure 4, TNF increased $^{32}P$ incorporation into EGF-R protein 2–3-fold when compared to control cells. Similar results were obtained in three separate experiments. To determine the amino acid residue which is modified following exposure to

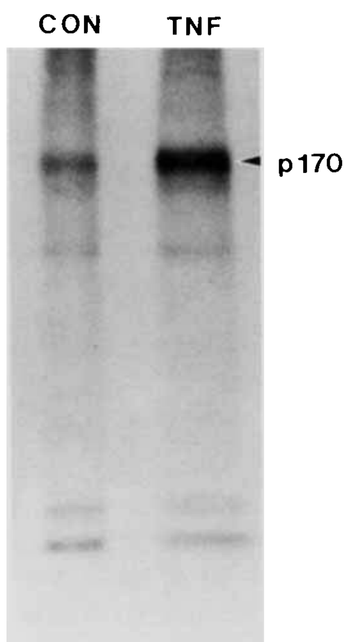


Fig. 4. $^{32}P$ Incorporation into EGF-receptor protein during TNF treatment. ME-180 cells grown to confluence and equilibrated in $^{32}P$ were treated with 20 ng/ml TNF for 20 min. The cell monolayers were rinsed in PBS, scraped, and collected by centrifugation. Cell pellets were lysed and the EGF receptor was immunoprecipitated with R1 antibody and pansorbin as described in Materials and Methods. Immunoprecipitates were washed extensively and proteins were electrophoresed on 8% polyacrylamide gels. Gels were dried and EGF-R was visualized by autoradiography. A photograph of the autoradiograph is shown with the position of 170 kDa EGF-R illustrated. TNF stimulated $^{32}P$ incorporation into receptor protein 2–3-fold over that of control.

TNF, EGF-R protein bands were excised from the polyacrylamide gel by proteolysis and acid hydrolyzed as described in Materials and Methods; $^{32}$P-labelled phosphoamino acids were resolved by HPLC separation on an anion exchange column. The peak elution position of phosphoamino acid standards is shown in Figure 5. Phosphoamino acid analysis performed under these conditions demonstrates the presence of phosphotyrosine residues in EGF-receptor isolated from control or TNF-treated ME-180 cells. TNF treatment appears to increase the phosphotyrosine content of the receptor without effecting phosphoserine or threonine accumulation. To determine whether the endogeneous phosphotyrosine content on EGF-R was distinct to ME-180 cells, parallel studies of A431 epidermoid carcinoma cells were performed. Treatment of $^{32}$P-equilibrated A431 cells with EGF stimulated phosphorylation of EGF-R protein threefold when compared to untreated cells as shown in Figure 6. Phosphoamino acid analysis of EGF-R, performed as described for ME-180 cells, demonstrates a high endogenous content of phosphotyrosine in unstimulated A431 cell EGF-R in comparison to both phosphoserine and phosphothreonine (Fig. 7). EGF stimulated increased phosphorylation on tyrosine residues with smaller but significant alterations in serine and threonine phosphorylation as reported by other investigators utilizing alternate methods of phosphoamino acid analysis [38,41]. Based upon these studies it appears that the high endogenous content of phosphotyrosine on EGF-R is related to the analytical procedure of phosphoamino acid analysis and is not unique to ME-180 cells. It appears however that TNF and EGF induce similar effects on EGF-R phosphorylation and activation of the intrinsic tyrosine kinase activity of the EGF receptor.

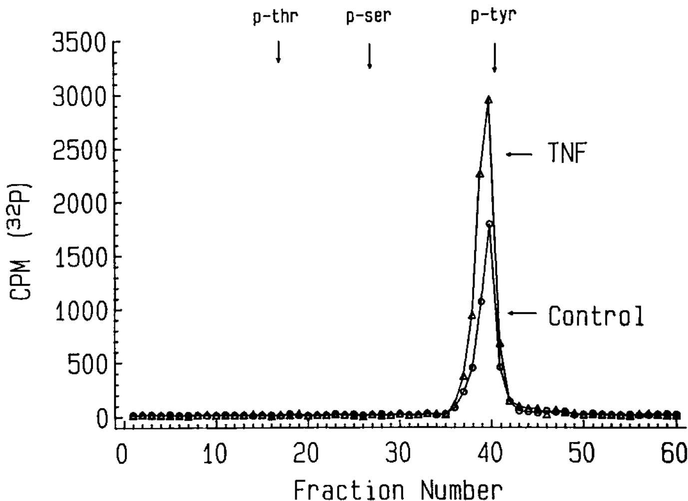


Fig. 5. Phosphoamino acid content in EGF-R following TNF incubation. $^{32}$P-labeled EGF-R protein from control or TNF-treated ME-180 cells was isolated from gel slices containing EGF-R protein by trypsinization as described in Materials and Methods. Phosphopeptides were acid hydrolyzed at 110°C for 2 h, dried over NaOH in vacuo, and resuspended in 15 mM potassium phosphate buffer pH 3.8 containing 1 mg/ml of phosphoamino acid standards. Samples were applied to an HPLC anion exchange column equilibrated in $K_2HPO_4$ buffer and eluted at 2 ml/min, and 1 ml fractions were collected. Samples were counted for Cerenkov radiation and plotted vs. fraction number. The peak elution of phosphoamino acid standards is shown above the figure. TNF stimulates incorporation of $^{32}$P into tyrosine residues on EGF-R based upon this analysis.

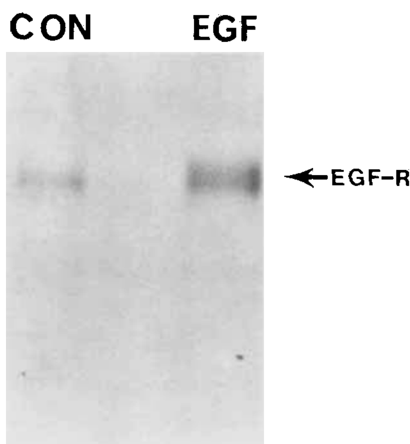


Fig. 6. Effect of EGF on the Phosphorylation of A431 cell EGF receptor. Confluent A431 cells (in 60 mm culture dishes) were equilibrated in $^{32}P$ as described in Figure 4. EGF (20 ng/ml) was added for the final 20 min of incubation and EGF receptor was immunoprecipitated and resolved by SDS-PAGE. The gel was exposed to x-ray film for autoradiography (60 min at −70°C). EGF stimulated phosphorylation of EGF-receptor protein whose migration is indicated by the arrow (170 kDa).

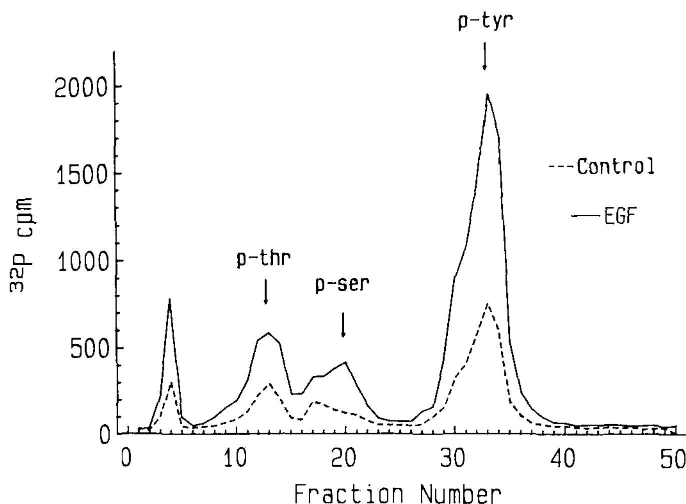


Fig. 7. Phosphoamino acid analysis of EGF-R from A431 cells. $^{32}P$-labeled EGF-R from control or EGF-treated A431 cells was eluted from polyacrylamide gels (Fig. 6) and analyzed for phosphoamino acid content as described for ME-180 cells (Fig. 5). Fractions of 1.2 ml were collected from the anion exchange column and analyzed for $^{32}P$ by Cerenkov radiation and plotted vs. fraction number. EGF stimulated the formation of phosphothreonine, phosphoserine, and phosphotyrosine on EGF-R based upon this analysis.

Due to the similarities between EGF-R kinase activation by TNF and EGF, we further investigated the effects of TNF on several EGF-regulated biochemical events. To determine whether TNF shares the ability to directly stimulate EGF-R kinase, by interaction at the ligand binding domain of this receptor, EGF-R was immunoprecipitated from ME-180 cells and washed extensively as described in the legend to Figure 8.

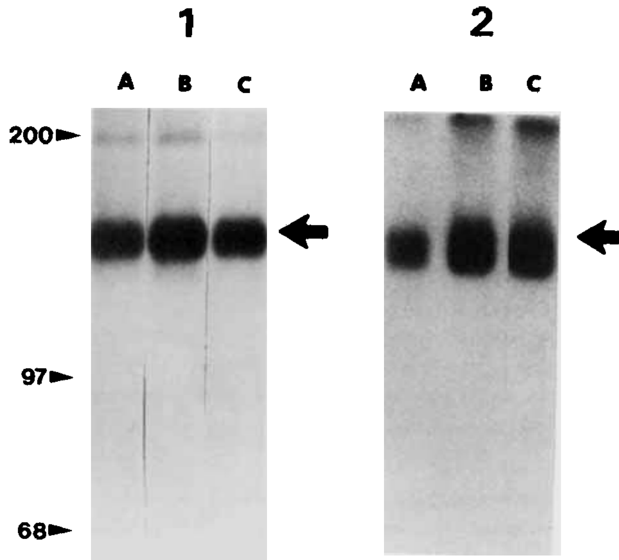


Fig. 8. Effect on TNF or EGF addition to immunoprecipitated EGF receptor from quiescent ME-180 cells. EGF receptor from quiescent ME-180 cells was immunoprecipitated with R1 antibody followed by pansorbin as described in Materials and Methods. Following centrifugation, the immune complexes were washed **1**) five times in 0.1% Triton X-100 in PBS, pH 7.4, or **2**) one time in 0.01% Triton X-100 in PBS, pH 7.4. The immunoprecipitated pellets were resuspended in 120 $\mu$l of 20 mM Hepes containing 6 mM $MnCl_2$, separated into three equal aliquots, and treated with 10 $\mu$l of **A**) diluent, **B**) EGF (200 ng), or **C**) TNF (200 ng) and incubated at 30°C for 10 min. The immune-complex kinase assay was initiated with the addition of 10 $\mu$Ci [$^{32}$P]-ATP and incubated 10 min at 30°C. The reaction was terminated, washed, and analyzed as previously described. An autoradiograph of the dried gel is shown. The arrow indicates position of the EGF receptor.

The immune complexes of EGF-R were aliquoted into three equal fractions and treated with either bovine serum albumin (diluent) or TNF (200 ng) or EGF (200 ng) for 10 min and assayed for tyrosine kinase activity. As illustrated in Figure 8A, TNF, under these conditions (lane 1C), was unable to alter the tyrosine kinase activity of the EGF-R when monitored by receptor autophosphorylation whereas EGF stimulated kinase activity threefold over that of control sample under these conditions. Modulation of EGF-R kinase does not appear to result from TNF binding EGF-R directly and its presence does not alter EGF-receptor tyrosine kinase activity. The effect of TNF on EGF-R may require whole cells or additional cellular components to enhance the enzymatic activity of the EGF receptor. Evidence which supports this hypothesis was obtained from EGF-R immunoprecipitates from ME-180 cells washed once in low concentrations of detergent. These less vigorously washed immunoprecipitates of EGF-R were incubated with TNF or EGF as described above and the resultant tyrosine kinase activity of the receptor was analyzed and compared to control. As shown in Figure 8B, both TNF and EGF enhanced EGF-R tyrosine kinase activity under these conditions. This result suggests that a component non-covalently associated with EGF-R or co-immunoprecipitated with EGF-R antibody may be capable of altering EGF-R enzymatic activity in the presence of TNF. The biochemical nature of this component (or components) is unknown.

EGF, as well as other growth factors, has been shown to enhance the expression of several growth-regulatory genes or proteins [14]. To determine whether TNF shares the

ability to modulate growth-regulated gene or protein expression due to its activation of EGF-R tyrosine kinase we measured the effect of TNF on the expression of the proto-oncogene c-*myc* and on the induction of the polyamine-biosynthetic enzyme ornithine decarboxylase (ODC). Total cellular RNA was isolated from ME-180 cells after 0, 1, 4, and 8 h of TNF or EGF treatment. Equal amounts of RNA were serially diluted and blotted onto a nylon membrane. The blot was hybridized with a c-*myc* or $\beta$-actin probe to demonstrate evenness of nucleic acid loading. As shown in Figure 9, TNF failed to modulate the expression of c-*myc* mRNA, whereas EGF enhanced c-*myc* expression 8–16-fold when measured 1 h after treatment which remained elevated 4–8 h after EGF treatment.

Northern blot analysis of poly A–selected RNA from EGF- or TNF-treated ME-180 cells (Fig. 10) demonstrates similar results. EGF treatment resulted in 4–8-fold induction of c-*myc* mRNA 1 h after EGF addition. Treatment of ME-180 cells with TNF at a dose which enhances activation of EGF-R kinase activity and phosphorylation failed to alter c-*myc* expression. In addition, c-*myc* mRNA isolated from control or TNF- or EGF-treated ME-180 cells had identically sized c-*myc* transcript based upon migration in agarose gels. These results suggest that the early effects of TNF and EGF on ME-180 cell EGF-R can be differentiated based upon their effect on c-*myc* expres-

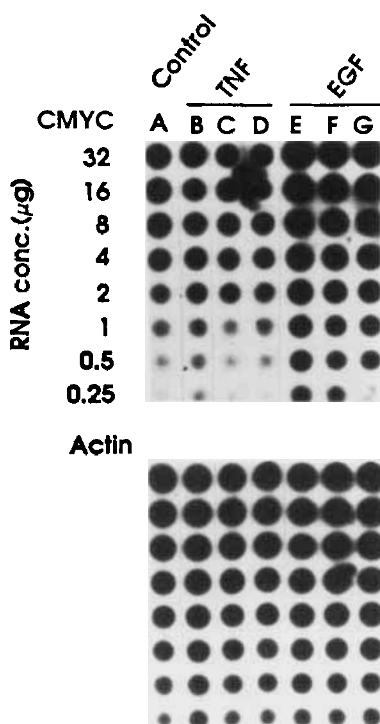


Fig. 9. Dot-blot analysis of c-*myc* expression in TNF- or EGF-treated ME-180 cells. Total cellular RNA was extracted from ME-180 cells following treatment with 20 ng/ml of EGF or TNF for 0 (A), 1 (**B,E**), 4 (**C,F**), or 8 (**D,G**) h of treatment. RNA was serially diluted, blotted, and hybridized to the c-*myc* and $\beta$-actin probes as described in Materials and Methods. An autoradiograph of the blot is shown. (NOTE—lane B contains twofold-greater RNA content than other lanes as judged by $\beta$-actin hybridization.)

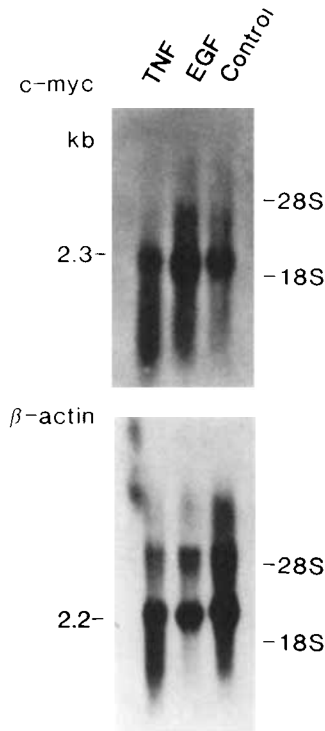


Fig. 10. Northern blot analysis of c-*myc* transcript from TNF- or EGF-treated ME-180 cells. Quiescent ME-180 cells ($10^8$) were treated with 20 ng/ml TNF or EGF and harvested 1 h after incubation. Cells were pelletted and RNA was extracted as described in Materials and Methods. Polyadenylated RNA was selected by oligo-(dT) cellulose chromatography and was size fractionated by electrophoresis in 1.1% agarose. RNA was transferred to nylon membranes and hybridized with nucleic acids probes for c-*myc* of β-actin as described. An autoradiograph of the resultant blot is shown. Based upon relative RNA content by β-actin hybridization, TNF treatment did not alter c-*myc* expression in ME-180 cells.

sion. Activation of EGF-R kinase activity by TNF without a concomminant increased c-*myc* expression may contribute to the cytotoxic process induced by TNF.

The influence of both EGF and TNF on ODC activity is shown in Table I. Both factors induced maximal ODC induction 3 to 4 h after treatment but differed markedly in the magnitude of ODC induction. TNF stimulated a 5–6-fold increase in ODC activity while EGF stimulated ODC activity 18-fold compared to that of control ME-180 cells. Based upon the time course studies of ODC induction and stimulatory actions of TNF on EGF-R kinase activity the results suggest a relationship between the mechanism of EGF- and TNF-stimulated ODC induction. To determine whether ODC induction correlates with the cytotoxic actions of TNF, ODC activity was measured in TNF-resistant T24 cells following exposure to either TNF or EGF. As demonstrated in Table I, TNF stimulated ODC activity in T24 cells, suggesting that alterations in ODC enzymatic activity are not directly involved in the cellular response of tumor cells to TNF-induced toxicity. Similar patterns of ODC activity were obtained following EGF treatment of T24 cells with maximal induction occurring 12 h after addition of either factor. However, as shown in Figure 3, TNF failed to effect EGF-R kinase activity in any

TABLE I. Effect of TNF or EGF on ODC Activity in ME-180 and T24 Cells*

| | ODC activity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | ME-180 cells | | | | T24 cells | | | |
| | TNF-treated | | EGF-treated | | TNF-treated | | EGF-treated | |
| Time (h) | Units/mg | Fold increase | Units/mg | Fold increase | Units/mg | Fold increase | Units/mg | Fold increase |
| 0 | 34 ± 8 | 1.0 | 81 ± 13 | 1.0 | 9.6 ± 4.0 | 1.0 | 9.8 ± 4.5 | 1.0 |
| 3 | 184 ± 5 | 5.5 | 1,488 ± 149 | 18.5 | 26.3 ± 4.1 | 2.7 | 43 ± 5.0 | 4.4 |
| 6 | 138 ± 9 | 4.1 | 269 ± 46 | 3.3 | 49.4 ± 14.5 | 5.1 | 63.8 ± 6.6 | 6.5 |
| 9 | 139 ± 1 | 4.1 | 389 ± 7 | 4.8 | N.T. | — | N.T. | — |
| 12 | 75 ± 3 | 2.2 | 123 ± 9 | 1.5 | 109.3 ± 5 | 11.4 | 83.0 ± 22.9 | 8.5 |

*ME-180 or T24 cells were grown to confluence in 100 × 20 mm dishes and cultured in serum-free medium overnight. Triplicate dishes were treated with 20 ng/ml of EGF or TNF and were assayed for ODC activity (as described) after 0, 3, 6, 9, or 12 h of treatment. Each time point represents the average of duplicate assays on triplicate samples ± SEM. ODC activity is given in units of activity defined as pmoles $CO_2$ evolved/h.

of our experimental trials, suggesting at least two mechanisms are involved in the induction of ODC following TNF treatment. These mechanisms may be distinct in TNF-sensitive vs. -resistant tumor cells. Increased ODC activity following TNF treatment of ME-180 cells appears to correlate with the ability of TNF to modify EGF-R tyrosine kinase activity. However, TNF-stimulated ODC induction in T24 cells appears to be independent of modified EGF-R tyrosine kinase activity. The mechanism of ODC induction in TNF-treated T24 cells has not been elucidated.

## DISCUSSION

In the present report, we examine the early biochemical events of TNF action on TNF-sensitive and -resistant tumor cell lines previously shown to express equivalent TNF binding activity. We demonstrate that TNF activates EGF-R tyrosine kinase activity in TNF-sensitive ME-180 cells but not in TNF-resistant T24 cells. Activation of ME-180 cell EGF-R kinase represents the earliest reported (10 min) enzymatic activity modified in TNF-sensitive tumor cells following incubation with this cytokine. These data suggest that one factor which may be involved in tumor sensitivity to TNF may be the ability of TNF to modulate EGF-receptor tyrosine kinase activity in tumor cells. Our results further suggest that EGF-R may play a role in the genesis or mediation of TNF-induced cytotoxicity in at least some tumor cell lines. We are currently examining the relationship between expression of EGF-R in squamous cell carcinoma cell lines and their corresponding sensitivity to TNF.

We have demonstrated that increased tyrosine kinase activity of the EGF-R correlates with increased phosphorylation of tyrosine residues on receptor protein in TNF-treated ME-180 cells. Several tyrosine residues on the EGF-receptor protein are known to be sites of autophosphorylation [22]. Additionally, protein kinase C has been shown to phosphorylate a threonine residue on the EGF receptor at a site in close proximity to its transmembrane domain [37]. Phosphorylation at these sites correlates with both increased (autophosphorylation at tyrosine residues) and decreased (threonine phosphorylation) tyrosine kinase activity of the EGF-R [33]. The information presented in this report demonstrates that TNF action on EGF-R in TNF-sensitive tumor cells is

similar to that reported for EGF, stimulating both tyrosine kinase activity and phosphorylation of tyrosine residues on the receptor. Whether TNF induces phosphorylation at a known site or a unique site is under investigation.

Recently, TNF and the biologically related molecule interleukin-1 have been shown to enhance phosphorylation of EGF-R in gingival fibroblasts [16]. Phosphorylation of receptor induced by these cytokines correlates with a transient decrease in EGF-R affinity without alterations in receptor number. Interestingly, basal phosphorylation of fibroblast EGF-R protein was predominantly on serine residues. Cytokine treatment of these cells with TNF/IL-1 was demonstrated to increase both threonine and serine phosphorylation with a reported decrease in phosphotyrosine content. In this report, we demonstrate that the basal phosphorylation of EGF-R in ME-180 tumor cells was predominantly at tyrosine residues and treatment with TNF stimulated an increase in phosphotyrosine content on EGF-R. While the HPLC phosphoamino acid procedure employed in this study does appear to result in greater phosphotyrosine recovery in relation to phosphoserine and phosphothreonine, we have demonstrated that this technique does resolve both phosphoserine and phosphothreonine, and metabolic alterations in their content can be measured by this procedure (Fig. 7). However, we were unable to detect any alterations in phosphoserine or phosphothreonine content in ME-180 cell EGF-R in response to TNF. While the disparate effects of TNF on tumor cell vs. fibroblast cell growth may be related to several parameters, it is possible that TNF-induced alterations in cell growth may be related to its ability to alter the basal phosphorylation state of specific cellular proteins. The dependence of tumor cell growth on tyrosine kinase activity and the ability of cells to adjust to changes in receptor activity or function induced by TNF may result in divergent growth effects on normal vs. tumor cells. Our studies suggest that modulation of EGF-R kinase activity (and phosphorylation of tyrosine residues) is related to the ability of TNF to induce cytotoxicity. However, the mechanism of TNF-induced activation of EGF-R is not understood.

The data provided in this report demonstrate that TNF does not alter EGF-R kinase activity directly through interaction with the EGF receptor at its extracellular binding domain, as has been shown to occur with other EGF-R-modulatory peptides. This observation is consistent with current TNF binding analyses which demonstrate that $^{125}$I-TNF binding could not be displaced by EGF, thus providing evidence for a discrete binding site for TNF [17]. Therefore, it appears likely that TNF occupancy with its receptor initiates a series of biochemical events which alter EGF-R kinase activity and activate the process of induced cytotoxicity. Understanding the process of TNF-induced EGF-R activation will be essential to elucidating the mechanism of TNF cytotoxicity.

Several reports have demonstrated that modulation of EGF-R kinase can be achieved with non-proteinaeous membrane components (e.g., gangliosides [39,40], sphingosine [41,42]). TNF, acting at the surface of tumor cells, may alter the structure, chemical composition, functional distribution, or association of these membraneous components with membrane-associated proteins to alter their function. Treatment of various cell types with TNF or incubation with artificial phospholipid bilayers has shown TNF to be very active in altering composition, mobilization, or degradation of lipid structures [43–47]. Thus the mechanism of TNF action may depend upon the presence of these components and the dependence of tumor cells on the expression of membrane-associated tyrosine kinase activity for their continual growth. Interestingly, we have

found that TNF is able to modulate EGF-R kinase activity in preparations of unwashed and immunoprecipitated EGF-R, suggesting that an additional cellular component associated with EGF-R or co-immunoprecipitated with receptor protein is capable of modifying EGF-R kinase activity in the presence of TNF. We are currently examining the biochemical nature of the components associated with EGF-R in TNF-sensitive tumor cell lines.

A major distinction between the activation of EGF-R kinase activity by EGF and TNF is their individual effects on c-*myc* expression. EGF increased c-*myc* mRNA 8–16-fold 1 h after treatment, whereas TNF was inactive in modifying c-*myc* expression. Therefore, although both peptides appear to activate a major growth factor–tyrosine kinase signal transduction process at the cell surface (EGF-R), their actions diverge in subsequent biochemical actions involving nuclear participation (c-*myc* expression, DNA synthesis). EGF stimulation of cell growth is thought to be a consequence of increased phosphotyrosine content in treated cells due to enhanced tyrosine kinase activity of EGF-R or through its translocation to the cell nucleus [48–50]. Whether TNF alters target cell growth by interfering with EGF-R translocation or metabolism is unknown at this time.

Interestingly, TNF failed to inhibit c-*myc* expression in ME-180 cells even after 8 h of TNF incubation. Other investigators have demonstrated a rapid reduction in c-*myc* expression following TNF treatment of HeLa cells [51]. The data obtained in this study suggest inhibition of c-*myc* expression is not a primary effect of TNF in ME-180 cells.

TNF did induce ODC activity in ME-180 cells in a manner similar to that of EGF. Several mechanisms have been shown to be responsible for the rapid and marked induction of ODC activity including transcriptional [52], translational [53,54], and post-translational events [55]. Due to the effects of TNF on EGF-R activity in ME-180 cells, it appears attractive to propose that activation of EGF-R and increased tyrosine kinase activity following TNF treatment is partially responsible for the effects of TNF on ODC activity in these cells. However, TNF also altered ODC activity in TNF-resistant tumor cells which we have demonstrated express EGF-R kinase activity which is not influenced by TNF incubation. These results suggest that induction of ODC by TNF may be due to a mechanism unrelated to activation of EGF-R kinase activity. It also suggests that ODC induction by TNF is not directly related to its cytotoxic mechanism. We are investigating the overall mechanisms involved in TNF-stimulated ODC induction to determine the role of EGF-R in this process.

In summary, TNF shares some of the early biochemical effects of EGF on several biological processes. These activities include activation of EGF-R tyrosine kinase activity, incorporation of phosphate into EGF receptor protein, and the induction of ODC. Distinct differences also exist between these two factors including the mechanism of activation of EGF-R and the expression of the proto-oncogene c-*myc*. Evidence suggests that some or all of these activities may be involved in the induction of TNF-mediated cytotoxicity. Therefore, although both factors appear to activate the same signal transduction pathway, they do so through independent and unique mechanisms which result in distinct patterns of proto-oncogne expression and growth-related enzyme induction. The factors responsible for the generation of altered cellular responsiveness to EGF and TNF may play a major role in the dissociation of the growth-promoting effects of EGF and the growth-inhibitory effects of TNF.

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