The Involvement of Protein Tyrosine Kinase Activity in a Tumor Necrosis Factor Resistance Mechanism (43920)

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Abstract. **Certain cytokines activate pathways involving protein phosphorylation. Serine and threonine phosphorylation are most common, whereas tyrosine phosphorylation is a rare post-translational event, accounting for a very small percentage of phosphorylated amino acids. Nonetheless, protein tyrosine kinase activity is associated with several cell surface receptors and is involved in intracellular signaling. Here, we show that tumor necrosis factor (TNF) treatment of cells resistant to TNF-mediated lysis resulted in an increase in protein tyrosine kinase activity. Moreover certain TNF-resistant cell lines became sensitive to TNF-mediated cytolysis when treated with the inhibitors of protein tyrosine kinases, genistein, and herbimycin A. In contrast, genistein had no effect on the lysis a TNF-sensitive cell line. The increase in TNF-mediated lysis affected by genistein occurred only when it was present during TNF treatment, and the effect was maximal when the inhibitor was added 30 min after the TNF. These findings suggest that, in TNF-resistant cells, TNF activates a protein tyrosine kinase that contributes to the cell's resistance to lysis and this resistance mechanism does not function in the TNF-sensitive cell line.** [P.S.E.B.M. 1995, Vol 210]

Tumor necrosis factor-α (TNF) is a pleotropic cytokine derived from activated macrophages and other cells. One of its putative roles is to induce necrosis of solid tumors as described by Carswell (1). Accordingly, TNF has been viewed as a potential anticancer agent. Other effects of TNF include: stimulating the immune system during bacterial and viral infections, activation of polymorphonuclear cell function by induction of granulocyte-monocyte colony-stimulating factor, the induction of myeloid cell differentiation, fibroblast growth stimulation, increase expression of MHC, shock associated with endotoxin, and cachexia as a result of infection or neoplastic disease.

Not all cells possessing TNF receptors respond to TNF in a detectable way. Cytotoxic effects of TNF are limited to certain cells. It has been documented that some cells resistant to TNF cytolysis express receptors but are incapable of internalizing TNF (2, 3). Other cells possess a high molecular weight component associated with the human TNF receptor which affects cytotoxicity (4). Furthermore, *in vitro* studies have shown that some TNF-resistant cells become sensitive to the cytotoxicity of TNF when inhibitors of DNA transcription or protein synthesis are admixed, either simultaneously with, or subsequent to the addition of TNF (5–7); This suggests TNF resistance is dependent on *de novo* protein synthesis. Several RNAs and proteins are induced in TNF-resistant cells after TNF treatment (e.g., Ref. 5). Whether these proteins are involved in TNF resistance is unknown.

Intracellular signaling subsequent to TNF stimulation has been extensively investigated using numerous cell types. Albeit sometimes conflicting and inconclusive, such experiments seem to indicate protein phosphorylation occurs as a result of TNF-receptor binding (8–11). The kinases and substrates involved in TNF-mediated signal transduction are poorly understood. There are reports of TNF-mediated activation

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of serine kinases, microtubule-associated protein 2 kinases, cap-binding protein kinases, protein kinase C, and tyrosine kinases (12–14).

Here, we report that the TNF treatment of cell lines normally resistant to TNF-mediated lysis manifest an increase in protein tyrosine kinase activity. Furthermore, these resistant cells exhibit a marked sensitivity to TNF lysis in the presence of the tyrosine kinase inhibitor genistein (15, 16). Thus, certain cells resist TNF-mediated lysis via a mechanism that appears to be dependent upon the phosphorylation of tyrosine residues on proteins.

Materials and Methods

Cell Lines and Reagents. The cloned mouse fibroblast cell lines B/C-N, 10ME, L88, L929, and 3T3A31 were used. B/C-N, L88, and 3T3A31 are resistant to lysis by TNF; 10ME and L929 are TNF sensitive. All cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum.

Recombinant human TNF was kindly provided by Dr. L. Lin of Cetus Corp. Genistein (ICN, Costa Mesa, CA), staurosporine (Sigma Chemical Co., St. Louis, MO), and herbimycin A (Gibco-BRL, Gaithersburg, MD) were dissolved in DMSO, and subsequently diluted at least 300-fold for the assays (see tables and figures). Sodium orthovanadate (Fisher Scientific) was dissolved in DMEM and subsequently diluted as indicated in the figures.

Protein Tyrosine Kinase Assay. As previously described (17), the determination of protein kinase activity was via enzyme-linked immunosorbent assay (ELISA). Cells grown near confluency were treated with 667 units/ml of TNF for various time periods (see figures). After treatment, the cells were washed twice with cold phosphate-buffered saline (PBS) and lysed in solubilization buffer by freeze-thaw. Various concentrations of cell lysates from untreated and TNF-treated cells (see figures) were added, in triplicates, into microtiter wells coated with poly(GluNa:Tyr) (Sigma) and incubated 30 min at 37°C in reaction buffer containing ATP (50 n*M;* Sigma). Then the wells were washed four times with buffer. Detection of phosphorylated tyrosine was by the use of a mouse monoclonal anti-phosphotyrosine antibody, PY.72.10.5 (1:100) (a generous gift from Dr. Bartholomew M. Sefton), and Goat anti-mouse Ig-horse raddish peroxidase (1:2000) (Amersham, Arlington Heights, IL). The presence of bound immunoglobulin was determined with 5-aminosalicilyate (Sigma) and read in an ELISA plate reader at 450 nm.

Absorbance of control wells, from which ATP was omitted, was subtracted from the sample absorbances. Representative experiments are shown.

^{51}Cr Release Assay for TNF-Mediated Cytotoxicity. TNF cytolysis was assayed as follows: various concentrations of TNF were prepared in an enriched RPMI medium (18) and admixed with 1×10^4 ^{51}Cr-labeled target cells in microtiter plate wells. The TNF concentrations are indicted in the tables or figures. Genistein was added as indicated in the tables and figures. The wells with no genistein contained DMSO, the genistein solvent. The wells contained a final volume of 0.15 ml. The amount of target lysis was determined after 18 hr by ascertaining the percentage of ^{51}Cr released. Percentage specific release = 100 × (sample cpm − spontaneous cpm)/(total cpm − spontaneous cpm). Each data point represents the average of triplicate samples with a range of less than 10%. The spontaneous release in all assays was less than 35%. Representative experiments are shown.

Results

Treatment of TNF Resistant Cells with TNF Resulted in an Increase in Protein Tyrosine Kinase Activity. It is possible that tyrosine phosphorylation is involved in the TNF signaling pathway. To investigate this, cells that are resistant or sensitive to TNF were treated with TNF for various length of time. The cell lysates were then analyzed for the induction of protein tyrosine kinase activity. As shown in Figure 1, TNF treatment of the TNF resistant cell line B/CN

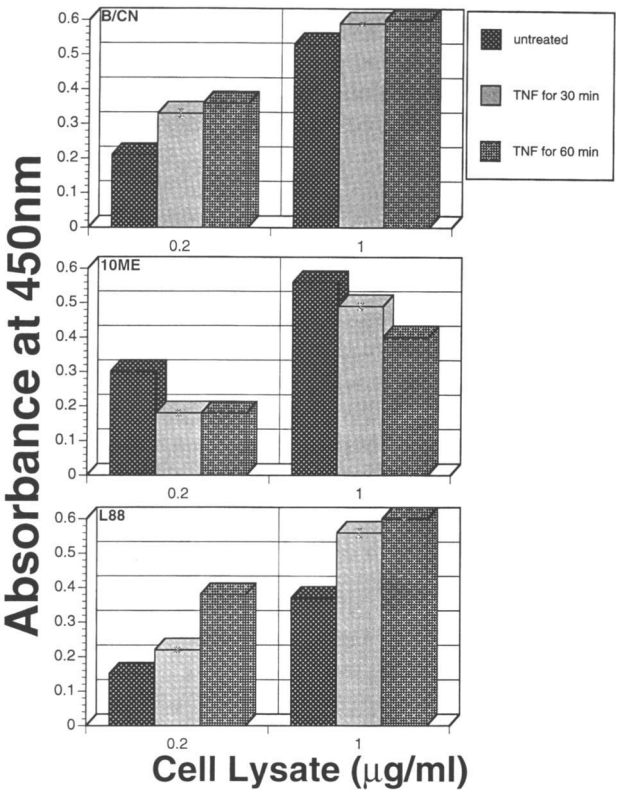

Figure 1. Treatment of TNF-resistant cell lines, B/CN and L88, with TNF results in an increase in protein tyrosine kinase activity; but not in the TNF-sensitive cell line, 10ME.

resulted in an increase of protein tyrosine kinase activity. Furthermore, an increase in protein tyrosine kinase activity was observed with another TNF resistant cell line, L88. In contrast, with the TNF sensitive cell line 10ME, there was a decrease in protein tyrosine kinase activity following TNF treatment. Furthermore, the tyrosine kinase activity was inhibited by protein tyrosine kinase inhibitors genistein and herbimycin A (data not shown). Hence, induction of protein tyrosine kinase activity was consistantly observed in TNF resistant cells after TNF treatment; whereas, TNF treatment of the TNF sensitive cells always resulted in a decrease in protein tyrosine kinase activity.

TNF-Resistant Cells Became Sensitive to TNF Lysis with the Addition of Genistein or Herbimycin A, Inhibitors of Protein Tyrosine Kinases. If the protein tyrosine kinase activity detected in the TNF resistant cells (Fig. 1) is important to maintaining TNF resistance, then inhibitors of protein tyrosine kinase should increase lysis of the resistant cells. Genistein, at low concentrations, has been demonstrated to be a specific inhibitor of the tyrosine kinase activity of EGF receptors. Moreover, genistein is capable of inhibiting tyrosine kinases other than the EGF receptor. Based on the fact that many growth factors rely on protein phosphorylation for signal transduction and that there appears to be an increase in protein tyrosine kinase activity upon TNF stimulation, we investigated the effects of genistein on TNF-mediated lysis of the TNF-resistant cell lines B/C-N, L88, and 3T3A31, and the TNF-sensitive cell line 10ME. Despite their TNF resistance, B/C-N, L88, and 3T3A31 express TNF receptors (2, 7). As shown in Figure 2, minimal cytotoxicity was observed when either B/C-N, L88, or 3T3A31 were treated with low levels of TNF. TNF treatment of 10ME resulted in significantly higher cytotoxicity. For example, at 0.1 units TNF/well, there was about 20% lysis of 10ME; comparable killing of B/CN, L88, or 3T3A31 required approximately 100-fold more TNF; at higher doses of TNF there was similarly higher lytic activity on 10ME than the other cells. However, with the addition of genistein to the cytotoxicity assay, the resistant cell lines became sensitive to TNF, whereas the lysis of the sensitive cell line was unaffected (Fig. 2). Although the percentage increase in TNF-mediated lysis of TNF-resistant cells with genistein was small, the TNF-sensitivity after genistein treatment was increased approximately 10-fold in that 10 units of TNF, in the absence of genistein, were required to achieve the same level of cytotoxicity as 1 unit of TNF in the presence of genistein (Fig. 2). This phenomenon has been repeatedly and consistently observed in numerous experiments. Moreover, the increase in sensitivity to TNF in the resistant cells was dependent on the dose of genistein. Thus, at two nontoxic doses of genistein, there was a marked increase in TNF-mediated lysis of resistant cells and little effect on the sensitive cells. This indicates that the protein tyrosine kinase activity is part of the mechanism used by these cells to resist TNF-mediated lysis.

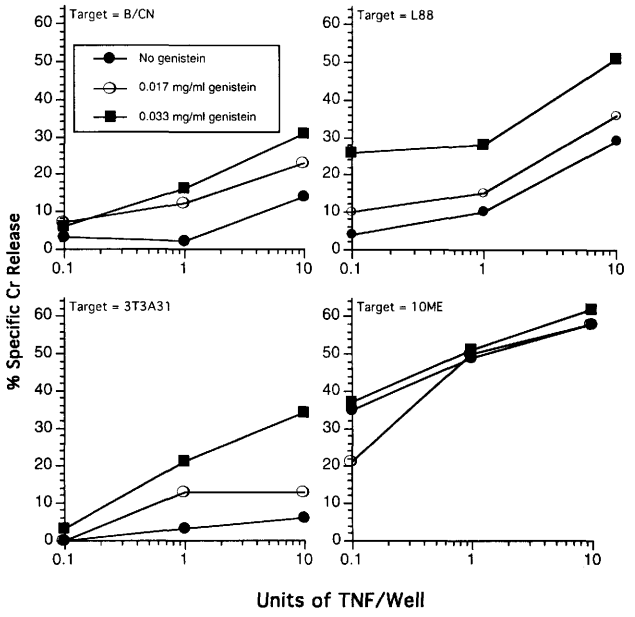

Figure 2. Genistein increases TNF-mediated lysis of TNF-resistant cell lines but not of TNF-sensitive cell line. TNF-resistant cell lines are B/CN, L88, and 3T3A31. TNF-sensitive cell line is 10ME.

It is important to note that several tyrosine kinase inhibitors other than genistein were also tested to ascertain whether they could increase TNF lysis of resistant cell lines. The inhibitors were herbimycin A, methyl2,5-dihydroxycinnamate, lavendustin A, RCAM-lysozyme, 2-hydroxy-5-(2,5-dihydroxybenzyl)aminobenzoic acid and tyrphostin. Of these, only herbimycin A had an effect comparable to genistein (Fig. 3). The inability of the other drugs to mimic the effects of genistein could be attributed to their selectivity of target protein kinase. Of the inhibitors used, only genistein and herbimycin A have been demonstrated to inhibit tyrosine kinases other than the EGF-receptor (19), and only these two inhibitors increased the levels of TNF-mediated lysis of TNF-resistant cell lines.

Genistein Was Effective Only When Administered to Cells During the First 3 hr of TNF Treatment. Genistein, the protein tyrosine kinase inhibitor, could increase the sensitivity of cells to TNF-mediated lysis by any of several mechanisms. These include: (i) increasing the number of TNF receptors per cells; (ii) increasing the amount of the lytic mechanism induced by the binding of TNF to its receptor; or (iii) decreasing mechanisms that protect cells from the TNF-induced lytic mechanism.

If the increase in TNF-sensitivity caused by genistein is the result of an increase in the number of

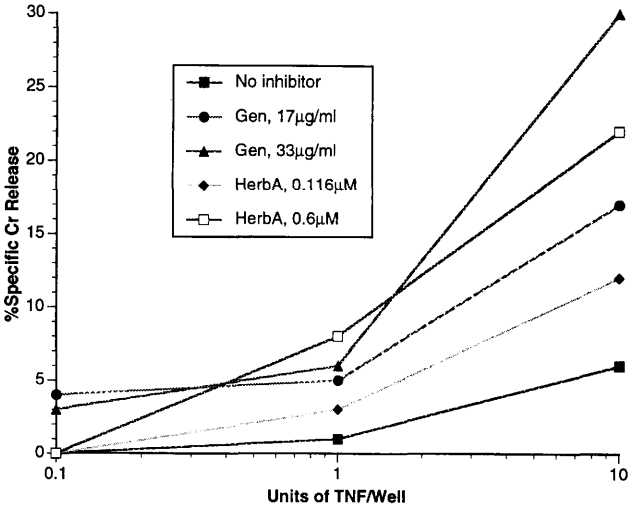

Figure 3. Herbimycin A, like genistein, increases TNF-mediated lysis of TNF-resistant cells. The target was B/CN. Gen, genistein, HerbA, herbimycin A.

TNF receptors, then pretreatment of cells with genistein should be effective in increasing TNF-sensitivity. This possibility was tested by incubating cells with 0.033 mg/ml of genistein for various length of time, washing out the genistein, and using these cells in the TNF assay. As shown in Table I, the addition of genistein to the TNF assay resulted in an increase of cytolysis of B/CN; however, pretreatment of B/CN for 0.5 or 2 hr did not have an effect on the sensitivity of these cells to TNF, and pretreatment for 4 or 6 hr had only a small effect at the highest dose of TNF. Likewise, pretreatment of B/CN for more than 6 hr did not increase TNF sensitivity (data not shown). Thus, it appears that genistein must be present along with TNF to have a significant effect on TNF-mediated lysis. This is consistent with our data showing protein tyrosine kinase activity was induced by TNF.

The preceding data suggest that TNF treatment activates protein tyrosine kinase activity in TNF-resistant cells. Accordingly, we did experiments to determine how long after TNF treatment it would be until the protein tyrosine kinase was active and able to maintain resistance to TNF lysis. After initiating the TNF assay, genistein (0.033 mg/ml) was added at various times during the ensuing 6 hr (Fig. 4). Genistein-induced TNF sensitivity was apparent when genistein was added to the targets within the first 3 hr after the addition of TNF. Interestingly, the addition of genistein 0.5 hr after TNF consistantly generated the maximum TNF sensitivity. In accordance with the pretreatment experiments, genistein appears to be inhibiting a resistance mechanism that is induced after TNF triggers the cytolytic mechanism. This suggests that a genistein-sensitive pathway, presumably involving tyrosine phosphorylation, is mediating the intracellular signaling required to resist TNF-mediated lysis; genistein does not appear to effect the lytic mechanism in either 10ME or in the TNF-resistant cell lines.

Table I. Genistein Pretreatment of B/CN, a TNF-Resistant Cell Line, Does Not Increase Cytotoxicity

	Percentage specific ^{51}Cr release		
	Units of TNF per well		
	0.1	*1*	*10*
Genistein (33 μg/ml)[a]			
–	4	9	20
+	8	19	39
Hours of pretreatment[b]			
0.5	6	9	16
2	3	8	18
4	2	12	27
6	3	10	25

[a] –, absent; +, present, during the assay period.
[b] B/CN was pretreated with 20 μg/ml genistein for various length of time before the start of the TNF assay. The cells were washed and used as targets in the absence of genistein.

Genistein-Induced TNF Sensitivity Was Similar to Cycloheximide-Induced Sensitivity. The TNF-resistant cell lines B/C-N and L88 have been shown to possess a protein synthesis-dependent TNF resistance mechanism, in that they become sensitive to TNF lysis when treated with a protein synthesis inhibitor such as cycloheximide (7). Moreover, maximum effect on these cells is seen when cycloheximide is admixed with the targets 2 hr after TNF exposure. Accordingly, genistein could be acting on a pathway that is functionally identical to the protein synthesis dependent resistance pathway blocked by cycloheximide. To ascertain whether the effect(s) of the protein tyrosine kinase inhibitor is independent of the protein synthesis inhibitor cycloheximide effect(s), the TNF cytotoxic assays were run in the presence of both genistein and cycloheximide. If they were operating on separate TNF resistance mechanism then it would be expected that their effects would be additive; if they blocked the same TNF resistance pathway then, at saturating doses of cycloheximide, genistein should not effect further increases in target lysis. As shown in Figure 5, TNF-mediated lysis of B/CN increased with increasing concentrations of cycloheximide. With low-dose cycloheximide, the effects of cycloheximide plus genistein were additive. However, as the maximum effective concentration of cycloheximide was approached, the effect of genistein was diminished. These data are consistent with the idea that genistein and cycloheximide function to make certain cells TNF-sensitive by inhibiting the same TNF resistance mechanism.

TNF-Sensitive Cells Were Not Lysed by TNF in the Presence of Vanadate, an Inhibitor of Protein Tyrosine Phosphatases. Phosphorylation occurring during signal transduction is usually followed by dephosphorylation. Thus, if tyrosine phosphorylation is

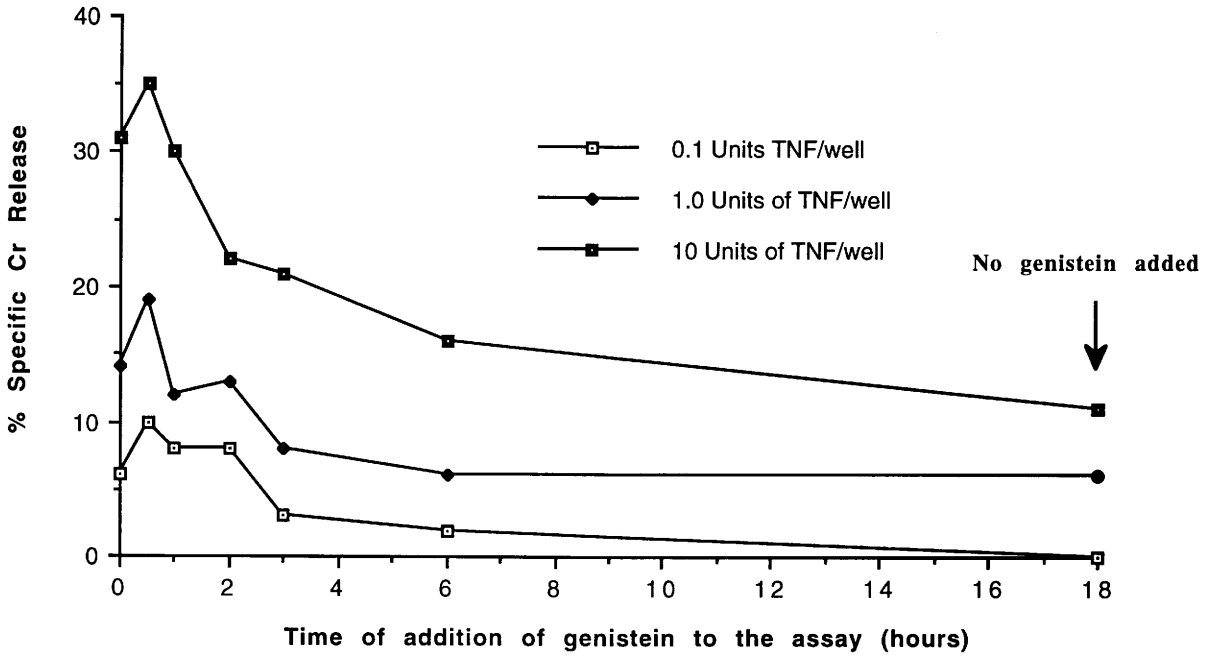

Figure 4. Genistein's effect in increasing TNF-mediated lysis of resistant cells is maximal when genistein is added 30 min after TNF treatment begins. The target was B/CN.

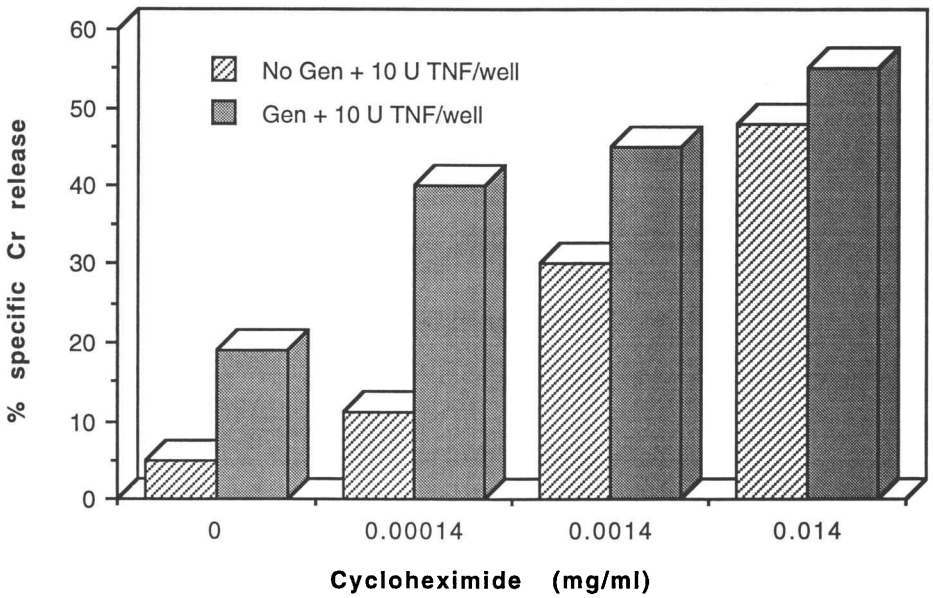

Figure 5. High doses of cycloheximide mitigate the effects of genistein (Gen). Genistein (0.033 mg/ml) was added at the start of the TNF assay; whereas cycloheximide was added 2 hr into the assay. The target was B/CN.

involved in TNF signaling, tyrosine dephosphorylation, via a phosphatase, is likely to be part of the signaling pathway. Under this scenario, an inhibitor of tyrosine phosphatase activity is expected to manifest the opposite effect of the protein tyrosine kinase inhibitors. In accordance, reports (20, 21) have recently indicated that protein phosphatases are involved in the TNF signaling mechanism. As shown in Figure 6, the addition of vanadate, a protein tyrosine phosphatase inhibitor, to the TNF assay resulted in the decrease in TNF-mediated lysis. From the data, it is not certain whether vanadate inhibits the TNF lytic pathway or increases TNF resistance. However, the findings that genistein, an inhibitor of protein tyrosine kinases, increased lytic activity, and vanadate, an inhibitor of dephosphorylation, decreased lytic activity are consistent with the idea that the level of protein tyrosine phosphorylation is an integral part of the cellular response to TNF.

Discussion

Although most cells possess TNF receptors, few are sensitive to TNF-mediated cytolysis. Clearly, cells that do not express TNF receptors will be resistant,

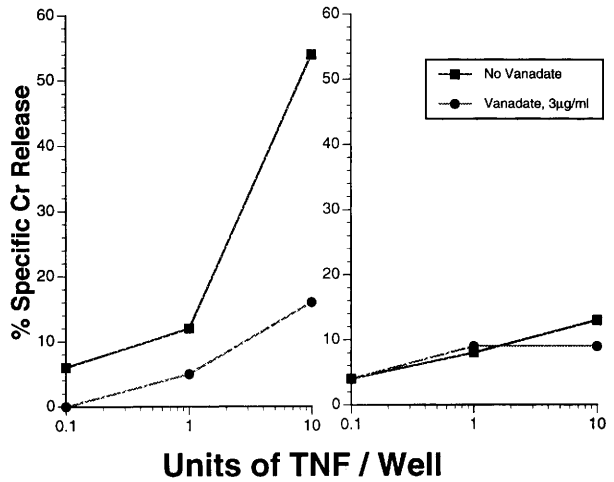

Figure 6. Vanadate decreases TNF-mediated lysis of TNF-sensitive cell line but not of TNF-resistant cell line. TNF sensitive cell line is 10ME, and TNF resistant cell line is L88.

but numerous studies have identified various other properties that contribute to TNF resistance. For instance, Tsujimoto (3) reported that the TNF-resistant cell line FS-4 will bind TNF, but these cells are incapable of internalizing the receptor-ligand complex. Some studies show that the expression of certain proteins correlates with TNF resistance (e.g., superoxide dismutase [22]). Additionally, there are reports of both protein synthesis-dependent and protein synthesis-independent TNF resistance mechanisms (5–7). Nonetheless, details of either the TNF lytic or resistance mechanisms remain unclear.

Herein, we report that the treatment of TNF-resistant cells with TNF resulted in an increase in protein tyrosine kinase activity. In addition, genistein, a tyrosine kinase inhibitor, caused these TNF-resistant cells to become sensitive to TNF-mediated lysis. Together these findings indicate that the TNF resistance mechanism involves the activation of protein tyrosine kinase activity after TNF binds to its receptor.

One concern in using genistein, a competitive inhibitor of ATP binding to tyrosine specific protein kinase, is the specificity of this drug. It is noteworthy that other tyrosine kinase inhibitors, usually used to study the EGF receptor-associated protein tyrosine kinase, do not increase TNF sensitivity in resistant cells. In our hands, methyl2,5-dihydroxycinnamate, lavendustin A, RCAM-lysozyme, 2-hydroxy-5-(2,5-dihydroxybenyzyl)aminobenzoic acid and tyrphostin did not increase TNF sensitivity (data not shown). Nonetheless, since these drugs are specific for the EGF receptor-associated protein tyrosine kinase and genistein is not, it was not entirely unexpected to observe dissimilar effects. Some of these drugs are competitive inhibitor of substrate binding to the EGF receptor-associated protein tyrosine kinase, whereas others are noncompetitive inhibitors (18). However, herbimycin, another tyrosine kinase inhibitor, was able to increase TNF-mediated lysis of TNF-resistant cell line but not of TNF-sensitive cell line. Herbimycin A, like genistein, has been demonstrated to inhibit a wide variety of tyrosine kinases (23). Unlike previous reports where EGF receptor influences TNF sensitivity (24, 25), this indicates that an EGF-receptor-like kinase is not involved in TNF resistance and that the substrate molecules of the TNF resistance mechanism is probably different from the substrates phosphorylated by the EGF receptor-associated protein tyrosine kinase.

It is also possible that genistein or herbimycin A affect other protein kinases. In particular, several reports have focused on the involvement of protein kinase C (PKC) in the TNF signaling pathway. However, our data show that the addition of staurosporine (26), a PKC inhibitor, did not affect the TNF resistance in the targets we used (data not shown). In addition, the TNF-mediated lysis of a sensitive cell line, 10ME, was not affected by staurosporine. These findings show that the inhibition of PKC does not increase TNF sensitivity in these resistant cells and, furthermore, supports the idea that genistein is not blocking PKC or other similar enzymes. We are currently trying to identify the target of the putative TNF-induced tyrosine kinase activity.

The data presented here also indicates that the protein tyrosine kinase inhibitor genistein inhibits a TNF resistance mechanism that is activated only after TNF binding by the target cell. Incubating cells with genistein prior to TNF exposure did not significantly increase TNF sensitivity (Table I). This suggests that the resistance mechanism is normally inactive and is induced by TNF. This is consistent with our data showing an increase in protein tyrosine kinase activity following TNF treatment (Fig. 1). Furthermore, genistein was effective in increasing lysis when added anytime within the first 3 hr of the TNF assay. However, the optimal effect of genistein was observed when added to the targets 0.5 hr after TNF addition (Fig. 4), again suggesting that the TNF resistance mechanism is induced only after exposure of targets to TNF.

Similarly, cycloheximide (an inhibitor of protein synthesis) increases TNF sensitivity of resistant cells. Moreover, the maximum effect of cycloheximide is observed when it is administered 2 hr after TNF addition (7). Genistein could act by inhibiting the same pathway that is blocked by cycloheximide or a pathway that is independent of the protein synthesis-dependent resistance mechanism. This investigation indicated that the addition of genistein and subsaturating doses of cycloheximide increased TNF-mediated lysis in an additive way; however, with high doses of cycloheximide, genistein did not substantially

increase the amount of cytotoxicity (Fig. 5). Since maximum genistein-induced TNF lysis was obtained only at subsaturating dose of cycloheximide, it suggests that genistein and cycloheximide inhibit the same TNF resistance mechanism.

The findings that the optimal effect of genistein was at $t = 0.5$ hr after TNF treatment whereas the optimal effect of cycloheximide was at $t = 2$ hr, along with the suggestion that genistein and cycloheximide are acting on the same resistance mechanism, is consistent with the hypothesis that the TNF resistance mechanism is initiated with the phosphorylation of a substrate which then induces protein synthesis. Such a sequence of events has been shown in other systems (reviewed in Ref. 27) where ligand-bound growth factor receptors phosphorylate, and thus activate, various substrates which eventually lead to the initiation of transcription. In accordance, we have shown that TNF treatment of resistant cells increased protein tyrosine kinase activity within 30 min of treatment. Although additional studies are necessary, it is conceivable that the TNF receptor, after binding to TNF, initiates phosphorylation of tyrosine residues on a transcription factor which then induces synthesis of proteins involved in the TNF resistance mechanism. Since genistein did not affect the TNF-mediated lysis of 10ME, it is presumed that such a factor is not involved in the 10ME (and B/CN) lytic mechanism.

Because the data suggest that a protein tyrosine kinase is involved in the TNF-resistance mechanism, and because dephosphorylation follows phosphorylation, it was considered that the addition of a tyrosine phosphatase inhibitor, such as vanadate, would produce the opposite effect of genistein. Our results are consistent with the hypothesis that high levels of tyrosine phosphorylation result in TNF resistance. It was observed that the phosphatase inhibitor vanadate decreased TNF-mediated lysis of a TNF-sensitive cell line but did not affect the lysis of the TNF-resistant cell line (Fig. 6). These data suggest that vanadate is either inhibiting the TNF-lytic mechanism or increasing TNF resistance. To be consistent with the evidence that genistein increases TNF-mediated lysis by inhibiting phosphorylation required for resistance, it is suggested that vanadate is increasing the level of a TNF-resistance mechanism by blocking dephosphorylation. Moreover, from the protein tyrosine kinase assay TNF treatment of TNF-sensitive cells resulted in a decrease in protein tyrosine kinase activity. It is conceivable that TNF treatment of TNF-sensitive cells either induces the activation or production of a protein tyrosine kinase inhibitor or a tyrosine phosphatase is activated which counteracts the protein tyrosine kinase activity. Since most kinases are themselves activated by phosphorylation, a phosphatase could directly inhibit kinase activity by inactivating the enzyme. Whatever the case, it seems that increased phosphorylation increases TNF resistance (i.e., reduces lysis), whereas decreased phosphorylation decreases TNF resistance (i.e., increases lysis).

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