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Enhancement of Lysosomal Enzyme Activity by Recombinant Human Tumor Necrosis Factor and Its Role in Tumor Cell Killing *in vitro*

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We investigated the effect of recombinant human tumor necrosis factor (TNF) on the lysosomal enzyme activity of various established cell lines *in vitro*. Incubation of 1×10^6 TNF-sensitive mouse tumorigenic fibroblasts (L-M cells) in the presence of TNF (100 U/ml) for 48 h increased the total (the sum of the enzyme activities in the lysosomes and the cytoplasm) acid phosphatase and β-glucuronidase activities by 3.7- and 4.2-fold, respectively. The same increase was observed even when 1 U/ml of TNF was added to some cultures and no further augmentation occurred at 10 or 100 U/ml. Measurement of total and free enzyme activities showed that TNF stimulation not only enhanced the total intracellular enzyme activity but also accelerated the conversion into free (cytoplasmic) enzyme activity. Addition of a lysosomotropic agent (methylamine) suppressed both the enhancement of lysosomal enzyme activity and the cytotoxicity of TNF. A similar enhancement of lysosomal enzyme activities was also detected in various TNF-sensitive tumor cell lines, and a strong correlation (acid phosphatase: $r=0.836$, β-glucuronidase: $r=0.910$) was observed between the enhancement of enzyme activity and sensitivity to TNF. No such increase was detected in TNF-resistant human diploid cells. These results show that TNF induces the activation and release of lysosomal enzymes in TNF-sensitive cells, and suggest that such events may play an important role in TNF-mediated cytotoxicity.

Key words: Tumor necrosis factor — Lysosomal enzyme — Tumor cell killing

Tumor necrosis factor (TNF) is a monocyte/macrophage-derived cytokine[1–3] that shows strong cytotoxicity for some types of tumor cells *in vitro*.[4–12] The mechanism of its lethal effect on tumor cells, however, remains largely unexplained.

We have already reported that TNF induces increased hydroxyl radical generation in TNF-sensitive cells, and that the iron chelator 2,2′-bipyridine (which inhibits the iron-catalyzed Fenton-reaction and so inhibits hydroxyl radical generation) can suppress both this increase of hydroxyl radicals and the cytotoxicity of TNF.[13] These results suggest that intracellular hydroxyl radical production may play an important role in the mechanism of tumor cell killing by TNF.[13, 14] However, hydroxyl radical scavengers cannot completely inhibit TNF-dependent cytotoxicity, so it appears that mechanisms other than hydroxyl radical generation also play a part.

Various studies have shown that TNF-mediated cytotoxicity is suppressed in the presence of lysosomotropic agents.[15, 16] Such studies are methodologically limited, however, as a reduction in cytotoxicity does not provide a means of determining the kinetics of lysosomal enzyme activation in TNF-sensitive cells, or of detecting any changes in lysosomal enzymes in resistant cells. It has thus not been possible so far to demonstrate a direct relationship between the cytotoxic effect of TNF and its stimulation of lysosomal enzyme activation.

We therefore measured the lysosomal enzyme activity (using acid phosphatase and β-glucuronidase as indicators) in various cell lines following their exposure to TNF and investigated the relationship between changes in enzyme activity and the cytotoxic effect of TNF.

MATERIALS AND METHODS

Materials Human recombinant TNF-α was produced in *Escherichia coli* and purified (99.9%),[17] and generously provided by Asahi Chemical Ind. Co., Ltd. (Tokyo). The TNF used had a specific activity of 2.3×10^6 U/mg protein as determined by its cytotoxicity for murine L-M cells,[18] and had a molecular mass of 51,000 as a trimer. The *p*-nitrophenyl phosphoric acid disodium salt and *p*-nitrophenyl-D-glucuronide used were purchased from Sigma (St. Louis, MO), while methylamine came from Kishida Chemical Co., Ltd. (Osaka).

Cell culture The 10 cell lines used in this experiment are listed in Tables I and II. L-M cells (mouse tumorigenic fibroblasts, ATCC CCL 1, 2) and HEL cells (human embryonic fibroblasts, ATCC CCL 137) were obtained from the American Type Culture Collection (Rockville, MD). HET cells (human embryonic fibroblasts) were obtained from Commonwealth Serum Laboratories (Victoria, Australia). L-R cells (TNF-resistant) were derived from L-M cells by repeatedly exposing the cells to TNF

at gradually increasing doses (1 U/ml to 5×10^5 U/ml).[16] Cultures were performed in Eagle's minimal essential medium (Nissui Pharmaceutical Co. Ltd., Tokyo) with 10% heat-inactivated fetal bovine serum (FBS, Flow Laboratories, Inc., North Ryde, Australia), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 incubator.

KYM (human myosarcoma) cells were kindly provided by Dr. M. Sekiguchi (Institute of Medical Science, University of Tokyo) and were cultured in DM-160 medium (Kyokuto Pharmaceutical Industries, Tokyo) with 10% FBS, antibiotics, 10 m*M* HEPES (pH 7.2), and the other conditions as mentioned above. PC-10 (mouse lung cancer) cells, HMV (human melanoma) cells and B-16 (mouse melanoma) cells were kindly provided by Dr. K. Kikuchi (Department of Pathology, Sapporo Medical College). RPMI (human colon cancer) cells and HeLa (human uterine cervical cancer) cells were provided by Fujisawa Pharmaceutical Co. Central Institute (Osaka) and Dr. K. Fujinaga (Cancer Research Institute, Sapporo Medical College), respectively.

These cells were cultured in RPMI-1640 medium (Gibco Laboratories, New York) with 10% FBS, antibiotics, 10 m*M* HEPES (pH 7.2) and the other conditions described above.

Assay of enzyme activity Cells (1×10^6/ml) were lysed by pipetting in a hypotonic buffer (0.25 *M* sucrose/1 m*M* EDTA/10 m*M* acetic acid/10 m*M* triethanolamine, pH 7.4) using the method of Miskimins and Shimizu.[19] The resulting solution was centrifuged at 800*g* for 10 min to obtain a supernatant.

The total acid phosphatase and β-glucuronidase activities were determined by modifications of the method of Shibko and Tappel[20] and that of Kato *et al.*,[21] respectively. An aliquot of the supernatant (100 μl) was incubated for 90 min with 200 μl of 0.5 *M* sodium acetate buffer (pH 5.0 for acid phosphatase, pH 4.0 for β-glucuronidase), 100 μl of 0.2% Triton X-100, and 100 μl of 100 m*M* substrate (*p*-nitrophenyl phosphate for acid phosphatase, *p*-nitrophenyl-β-glucuronide for β-glucuronidase).

The reaction was stopped by the addition of 500 μl of 1% (w/v) phosphotungstic acid in 0.1 *N* HCl. Then the mixture was centrifuged at 10,000*g* for 10 min to remove the precipitated protein. After the addition of 200 μl of 1 *N* NaOH to 800 μl of the resulting supernatant, *p*-nitrophenol release was measured using a Hitachi spectrophotometer (Model U-3200, Tokyo) at a wavelength of 405 nm. The free activity was determined by the identical procedure except that Triton X-100 was not added. Results were expressed as milliunits (mU) per 1×10^6 cells or a percentage versus respective controls. One unit means the amount of enzyme necessary to release 1 μmol (μM) of the product per min.

RESULTS

TNF-induced lysosomal enzyme activation Highly TNF-sensitive L-M cells and TNF-resistant HEL cells were incubated in the presence of TNF (10 U/ml) for 0–48 h and the changes in enzyme activity were studied (Fig. 1). In the L-M cells, both acid phosphatase and β-glucuronidase activities increased by approximately 1.9-fold after 24 h, and by 3.7-fold and 4.2-fold, respectively, after 48 h. There was no increase in the activity of either enzyme in the HEL cells.

Influence of TNF concentration on lysosomal enzyme activity Enzyme activities were measured after the incubation of L-M cells for 24 h with TNF at 1, 10, and 100 U/ml. Acid phosphatase and β-glucuronidase activities were increased 1.9-fold and 1.6-fold, respectively, by TNF at 1 U/ml and thereafter reached a plateau at 10 or 100 U/ml (Fig. 2).

Changes in lysosomal enzyme activity in L-M and L-R cells incubated with TNF We also investigated whether the increase in enzyme activity observed in L-M cells occurred in L-R cells, which are derived from the same original cell line but are TNF-resistant. In the L-M cells, the activities of both acid phosphatase and β-glucuronidase increased by 1.9-fold and 1.8-fold, respectively,

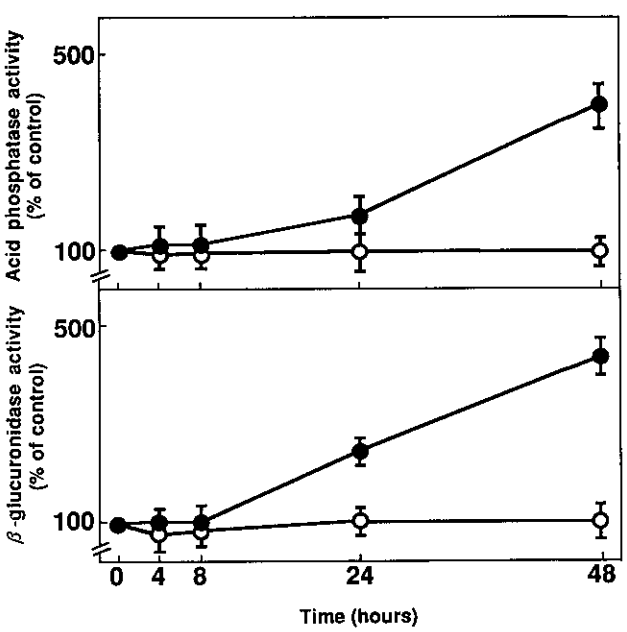

Fig. 1. Time course of changes in lysosomal enzyme activity in L-M (●) and HEL (○) cells incubated with TNF (10 U/ml). Cells were incubated in the presence of TNF for 0–48 h and enzyme activity was measured as described in "Materials and Methods." Values are the mean±SD of three separate experiments.

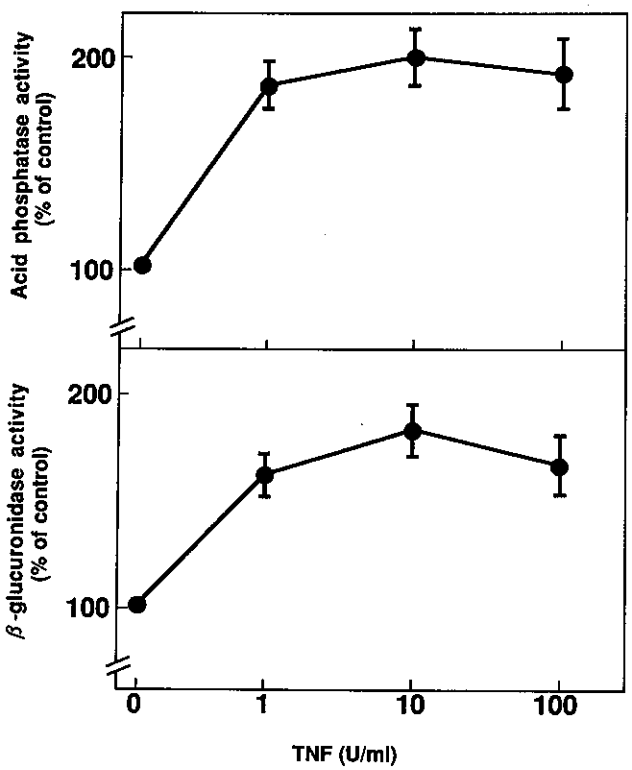

Fig. 2. Lysosomal enzyme activity in L-M cells as a function of TNF concentration. Enzyme activity was measured after a 24 h incubation with TNF at 1, 10, and 100 U/ml as described in "Materials and Methods." Values are the mean±SD of three separate experiments.

after 24 h of incubation with TNF (10 U/ml), but L-R cells showed no increase in the activity of either enzyme (Tables I and II).

Effects of a lysosomotropic agent on the lysosomal enzyme activity and TNF sensitivity of L-M cells The inhibitory effect of methylamine (10 μM) on the activation of lysosomal enzymes and the TNF sensitivity of L-M cells after 48 h of incubation with TNF (10 U/ml) is shown in Fig. 3. In the presence of methylamine, the acid phosphatase and β-glucuronidase activities were inhibited by 36% and 49%, respectively, in comparison with the addition of TNF alone. The cytotoxicity of TNF for L-M cells incubated without the addition of methylamine was 90.5%, but it was reduced to 61.7% in the presence of methylamine.

Changes in lysosomal enzyme activity induced by TNF in various tumor cells and normal diploid cells We then investigated whether the increase in lysosomal enzyme activity induced by TNF in L-M cells also occurred in other cell lines, using human and murine tumor cells with various degrees of TNF sensitivity and normal human diploid fibroblast cells (Tables I and II). Cells were incubated with TNF (10 U/ml) for 24 h and the acid phosphatase and β-glucuronidase activities were measured.

An increase in enzyme activity was observed in all the TNF-sensitive cells, amounting to 1.1- to 2.9-fold for acid phosphatase and 1.1- to 2.4-fold for β-glucuronidase. In KYM cells, which are highly sensitive to TNF, the acid phosphatase and β-glucuronidase activities increased by 2.9-fold and 2.4-fold, respectively, showing

Table I. Effect of TNF on the Acid Phosphatase Activity of Various Cell Lines

Cell lines	Percent cytotoxicity[a]	Acid phosphatase activity (mU)[b]		TNF (+)/TNF (−) ratio
		TNF (−)	TNF (+)	
Human				
KYM	54.1	6.93±0.10	19.96±0.30	2.88
PC-10	29.3	7.82±0.02	14.38±1.05	1.83
HMV	10.1	7.48±0.03	8.22±1.23	1.09
RPMI	9.5	6.58±0.46	9.90±0.63	1.38
HeLa	7.0	8.39±0.16	13.07±1.76	1.56
HEL	−2.2	7.63±0.19	7.54±0.63	0.99
HET	−6.9	6.45±0.03	6.38±0.52	0.99
Murine				
L-M	44.6	8.25±0.04	15.70±1.16	1.90
B-16	15.4	6.89±0.28	13.37±0.59	1.94
L-R	0	7.67±0.03	7.64±0.68	1.00

a) Percent cytotoxicity was measured by means of dye uptake assay after incubation with TNF (10 U/ml) at 37°C for 24 h.

b) Cells were incubated with or without TNF (10 U/ml) at 37°C for 24 h. Enzyme activity was expressed as mU/1×10^6 cells. Values are the mean±SD of three separate experiments.

Table II. Effect of TNF on the β-Glucuronidase Activity of Various Cell Lines

Cell lines	β-Glucuronidase activity (mU)[a]		TNF (+)/TNF (−) ratio
	TNF (−)	TNF (+)	
Human			
KYM	1.41±0.02	3.40±0.06	2.41
PC-10	2.05±0.17	3.75±0.47	1.83
HMV	1.23±0.01	1.32±0.01	1.41
RPMI	1.45±0.10	1.86±0.12	1.28
HeLa	1.46±0.01	1.58±0.01	1.08
HEL	1.38±0.10	1.39±0.06	1.00
HET	1.22±0.05	1.20±0.02	0.98
Mouse			
L-M	1.09±0.06	1.98±0.09	1.82
B-16	2.18±0.39	3.37±0.21	1.71
L-R	1.28±0.04	1.24±0.06	1.00

a) Cells were incubated with or without TNF (10 U/ml) at 37°C for 24 h. Enzyme activity was expressed as mU/1×10^6 cells. Values are the mean±SD of three separate experiments.

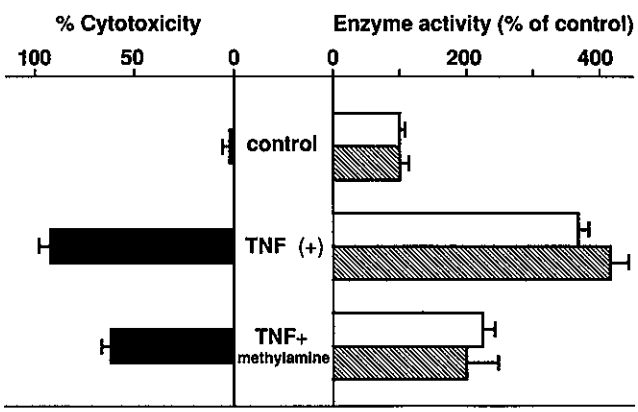

Fig. 3. Effects of a lysosomotropic agent on the lysosomal enzyme activity and TNF sensitivity of L-M cells. The percent cytotoxicity and lysosomal enzyme activity were measured after L-M cells were incubated with or without TNF (10 U/ml) and in the presence or absence of methylamine (10 μ*M*) at 37°C for 24 h as described in "Materials and Methods." Values are the mean±SD of three separate experiments.

similar changes to those noted in L-M cells. There was no increase in the activity of either enzyme in the TNF-resistant HEL and HET cells, similarly to the case for L-R cells.

A strong correlation was noted between TNF-mediated cytotoxicity and the rate of increase of enzyme activity in the TNF-sensitive tumor cells (acid phosphatase, $r=0.836$; β-glucuronidase, $r=0.910$) (Fig. 4).

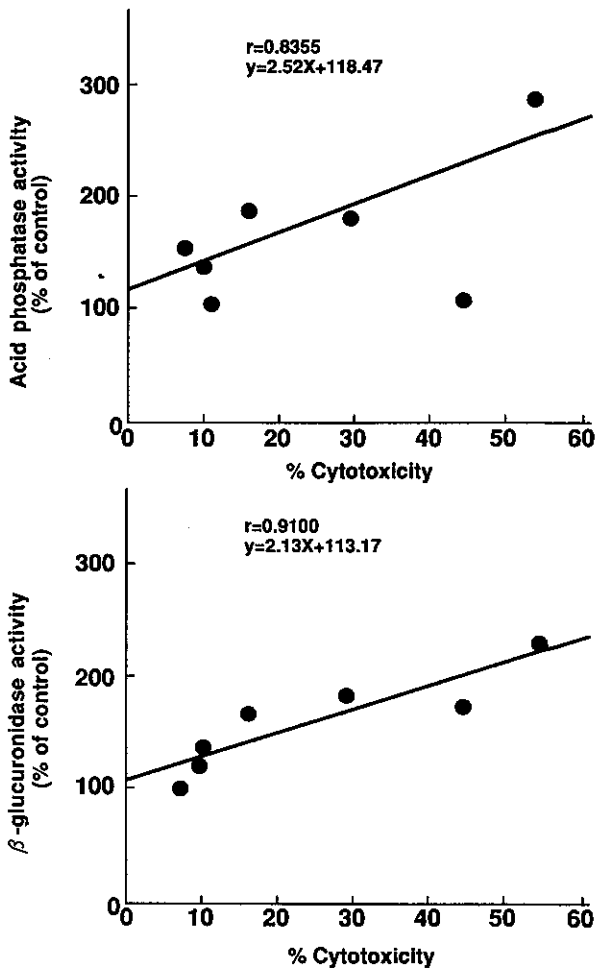

Fig. 4. Correlation between lysosomal enzyme activity and TNF sensitivity in various tumor cell lines. Cells were incubated with TNF (10 U/ml) at 37°C for 24 h. Enzyme activity and percent cytotoxicity were determined by the methods described in Tables I and II.

Effects of TNF on the intracellular distribution of lysosomal enzymes To study the effects of TNF (10 U/ml for 24 h) on the intracellular distribution of acid phosphatase and β-glucuronidase activity in L-M cells, the total activity (the sum of the enzyme activity in the lysosomal granules and the cytoplasm) and the free activity (the activity in the cytoplasm alone) were measured separately.

For acid phosphatase, the total and free activities increased by 1.9-fold and 3.1-fold, respectively, in the presence of TNF (Fig. 5). For β-glucuronidase, there was also an increase in the total and free activities after the addition of TNF (1.8-fold and 2.4-fold, respectively) (Fig. 5).

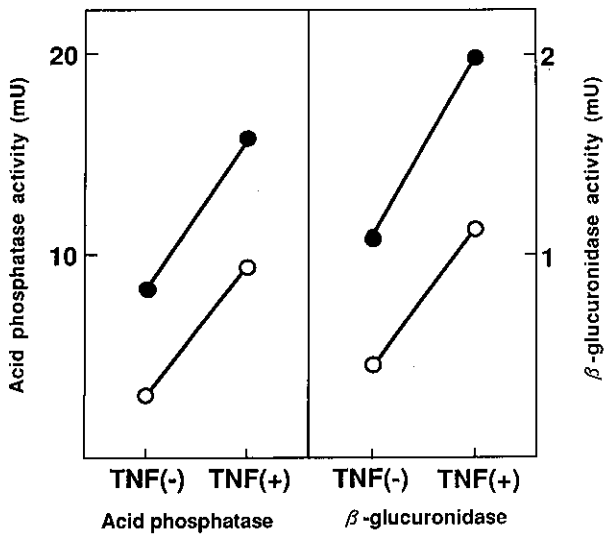

Fig. 5. Effects of TNF on the intracellular distribution of lysosomal enzymes. Cells were cultured with or without TNF (10 U/ml) for 24 h. Total (●) and free (○) enzyme activities were measured as described in "Materials and Methods."

DISCUSSION

We have previously reported that intracellular hydroxyl radical production plays an important role in TNF-mediated cytotoxicity.[13, 14] In addition, the inhibition of TNF cytotoxicity by lysosomotropic agents, such as chloroquine and leupeptin,[15, 16] suggests that the activation of lysosomal enzymes is also rather a case than a result of cell death. It has therefore been proposed that after TNF binds to specific receptors on the target cell membrane, intracellular hydroxyl radical production is induced and lysosomal enzymes are released into the cytoplasm as one of the mechanisms of autolysis.

Liddil *et al.* measured six lysosomal enzymes in L-929 (TNF-sensitive) and L-929R (TNF-resistant) cells.[22] They found that L-929R cells had a lower β-glucuronidase activity when compared with L-929 cells and suggested that a decrease in lysosomal enzyme activity was related to TNF resistance. We also measured lysosomal enzyme activity (using acid phosphatase and β-glucuronidase activities) in 11 cell lines, but observed no significant correlation between TNF sensitivity and lysosomal enzyme activities (Tables I and II). Therefore, in this study we elucidated the relationship of changes in lysosomal enzyme activity and cytotoxicity in response to the direct action of TNF on various cell lines.

We initially incubated TNF-sensitive L-M cells and TNF-resistant HEL cells in the presence of TNF, and found that the acid phosphatase and β-glucuronidase activities increased according to the duration of incubation in L-M cells, while no changes were seen in the HEL cells.

Next, the effect of changes in the TNF concentration on the increase in lysosomal enzyme activity was studied. Acid phosphatase and β-glucuronidase activities were increased by 1.9- and 1.6-fold, respectively, at 24 h after TNF addition at 1 U/ml, and thereafter (10,100 U/ml) reached a plateau. When this increase in enzyme activity was inhibited by a lysosomotropic agent (methylamine), suppression of TNF-mediated cytotoxicity was also seen, which suggested a close relationship between the two.

We further investigated the relationship between TNF sensitivity and changes in lysosomal enzyme activity in various tumor cell lines and normal diploid cells. An increase in enzyme activity was seen in all of the TNF-sensitive cell lines, but no such change was seen in the TNF-resistant cells. In addition, a close correlation between TNF sensitivity and the rate of increase of enzyme activity was found. These findings suggest that TNF-mediated lysosomal enzyme activation may play an important role in the mechanism of tumor cell killing by this cytokine.

Furthermore, we assessed the intracellular total and free enzyme activity to clarify the mechanism of lysosomal enzyme activation. Acid phosphatase and β-glucuronidase showed increases in both total and free activity in the presence of TNF, suggesting that treatment with TNF not only increased the release of lysosomal enzymes into the cytoplasm but also raised the actual enzyme activity inside the lysosomes.

It is known that intracellular hydroxyl radical production is induced in tumor cells by TNF,[13, 14] and that hydroxyl radicals themselves cause DNA fragmentation[23, 24] and membrane damage due to lipid peroxidation.[25, 26]

Therefore, it could be suggested that TNF induces an increase in the lysosomal enzyme activity in TNF-sensitive cells and that these enzymes are then released due to lysosomal membrane damage caused by hydroxyl radicals, the production of which is also promoted by TNF. Such a mechanism may have an important role in tumor cell killing by TNF.

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