Vol. 199, No. 1, 1994
February 28, 1994
BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 70-77

Lymphotoxin Lacks Effects on 75-kDa Receptors in Cytotoxicity on U-937 Cells

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Received December 13, 1993

Summary; We examined differences in cytotoxic activity between human lymphotoxin (LT) and tumor necrosis factor (TNF) as functions of their interaction with two types of TNF receptors, 55-kDa (p55R) and 75-kDa (p75R). Cytotoxic activity of LT was much lower than that of TNF on a human monocytic cell line, U-937, on which p75R was predominant. Monoclonal antibodies specific for p55R (htr-5 and htr-9) and p75R (utr-1) significantly diminished TNF cytotoxicity, whereas, utr-1 was only slightly inhibitory to LT cytotoxicity, and htr-5 reduced it significantly. TNF individual binding to p75R increased cytotoxic activity when p55R was occupied by htr-9 and a mutein of TNF which significantly lost affinity to p75R. However, LT binding to p75R did not increase. Scatchard analysis with [^{125}I]LT and [^{125}I]TNF showed that LT still had approximately half of the affinity to p75R and slightly less affinity to p55R than TNF. These results indicate slight cytotoxicity of LT compared to TNF, due to inability of LT to signal through p75R on U-937 cells without significant loss of affinity to p75R.

Lymphotoxin (LT), originally identified as a T cell-derived protein that can mediate cellular cytotoxicity on neoplastic cells (1), mimics the behavior of tumor necrosis factor (TNF) in various kinds of biological activity (2,3). TNF and LT bind to two types of specific surface membrane receptors, 55-kDa (p55R) nad 75-kDa (p75R), and compete for binding to both receptors (4.5). Although some comparative studies of their biological activity have exposed similarities, many significant differences between them, such as low activity of LT compared to TNF, have been described in many reports (2, 6-8).

Human LT is about 30% homologus to human TNF in amino acid sequence, but it is 14 amino acids longer and N-glycosylated (9). X-ray

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Abbreviations: LT, lymphotoxin. TNF, tumor necrosis factor. p55R, TNF 55-kDa receptor. p75R, TNF 75-kDa receptor.

0006-291X/94 $5.00

crystalography revealed that LT and TNF have the same β-sandwich motif conformation, but they are suspected to have alternative receptor binding conformation due to different sequences in their receptor binding lesions (10).

Human p55R and p75R have similar protein sequences in their extracellular domains, characterized as repeating cysteine rich sequences, but they are quite different in their intracellular domains (11). Tartaglia, *et al.* reported that T cell proliferation was mediated though p75R, whereas cytotoxicity and induction of manganese superoxide dismutase were activated through p55R in murine cell lines (12). Monoclonal antibodies specific for p55R and p75R mediate cytotoxicity and T cell proliferation, respectively (13,14). Another role of p75R is amplification of the activity generated through p55R (15,16). Synergism between p55R and p75R was also reported (13,17). Although cytotoxicity mediated through p75R was reported (18,19), this activity should be examined only in a condition that completely deletes the effects of p55R. Therefore, the role of each of the TNF receptors is not yet well understood.

Though many studies of the ability of TNF to signal through the receptors have been published, much less is known about how the activity of LT is mediated by the two TNF receptors. We investigated different roles of p75R with the aid of monoclonal antibodies for the two TNF receptors, and propose that there are major differences between TNF and LT in their actions, due to inability of LT to signal through p75R.

MATERIALS and METHODS

Materials: Recombinant TNF (3×10^6 U/mg protein) was a gift from Dainippon Pharmaceutical Co., Ltd., Osaka, and natural LT derived from human lymphocytes (7.47×10^5 U/mg protein) was a gift from Hayashibara Biochemical Laboratories Inc. Recombinant LT (2×10^7 U/mg protein) was perchased from R&D Systems, Inc., Minneapolis, MN, USA. Three monoclonal antibodies: htr-9, which can mediate various TNF activities through human p55R (13,20,21); another monoclonal antibody for human p55R, htr-5 (13,21), which was inactive; and a monoclonal antibody for p75R, utr-1 (13,18,22), were generously provided by Dr. Brockhaus, Hoffman-La Roche Co. A mutein of rhTNF, TNF(^{29}Val), in which Arg was replaced by Val at the 29th amino acid from the N-terminal by the method of slide-direct mutagenesis (23), was generously provided by Ishihara Sangyo Kaisha, Ltd, Shiga, Japan. [^{125}I]rhTNF (1,000 Ci/mmol) was perchased from Amersham International plc., Buckinghamshire, England.

Cells and culture: Cells used for experiments were U-937 cells, a human monocytic leukemia cell line. They were cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% heat inactivated FCS (Filtron, Pty., Ltd., Victoria, Australia) in a humid atmosphere of 5% CO_2 at 37 °C.

Assay for cytotoxic activity: U-937 cells (3×10^5 /ml) were cultured for 2 days in 200 μl of culture medium, in 96 well culture plates with appropriate concentrations of materials. Viability and death of the cells were estimated by trypan blue dye exclusion, and percent cytotoxicity was calculated by counting at least 200 cells in triplicate samples.

Radiolabeling of natural LT: [^{125} I]LT was prepared with Bolton Hunter reagent (Amersham). A specific radioactivity of approximately 150 Ci/mol was routinely achieved and its biological activity, normally between 50-100% of the starting material, was assessed in the cytotoxic assay on L-929 cells.

[^{125}I]TNF or [^{125}I]LT binding to the receptors: Cells were preincubated with monoclonal antibodies for TNF receptors, utr-1 and/or htr-9, for 1 h at 4°C before binding to radiolabeled TNF or LT. Serial dilution of labeled TNF and LT was added to 2 x 10^6 cells to a final volume 200 µl (PBS without $CaCl_2$ and $MgCl_2$, 0.1% BSA) and left for 3 h at 4°C. Background was measured in the presence of more than 100 times molar excess of unlabeled ligands with cold PBS containing 0.1% BSA. Radioactivity of the precipitate of the cells was measured by a γ-counter.

RESULTS

The dose responses of cytotoxic activity of human rTNF and natural LT on U-937 cells are shown in Fig. 1. The proportion of p75R contained in total specific binding to TNF receptors on U-937 cells used in the experiments was 60-70% (data not shown). Cytotoxic activity of rTNF increased dose-dependently and most cells were killed in the culture at the highest dose after 2 days. Cytotoxic activity mediated by natural LT was limited to 13% even at the highest dose (10 µg/ml). The LT specific activity was estimated from the dose response curves to be 0.01-0.03 times that of rTNF.

The reduction of rTNF and natural LT cytotoxic activity by utr-1 (10 µg/ml), htr-9 (10 µg/ml), and htr-5 (25 µg/ml) are shown in table 1. Utr-1 alone had no activity and htr-5 had only slight activity. However, htr-9 alone or combined with utr-1 had cytotoxic activity equivalent to about 0.1

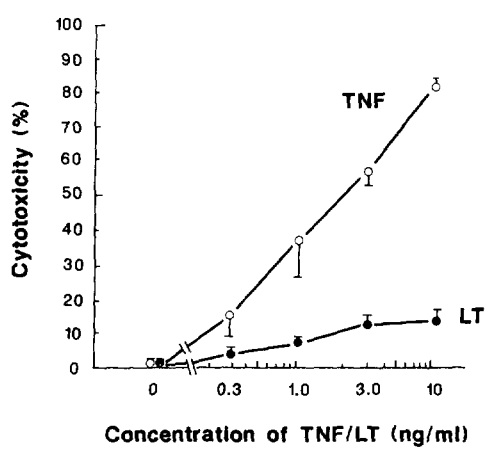

Figure 1. Dose response curves of rhTNF and nhLT in cytotoxic activity on U-937 cells. Cells (3 x 10^5 cells/ml) were cultured for 2 days at 37°C in the presence of a serial concentration of rhTNF (closed circles), or nhLT (open circles). Percent cytotoxicity was determined by trypan blue dye exclusion. Values are mean ± S.D. of triplicate samples.

Table 1. Neutralization of Cytotoxic Activity of rhTNF and nhLT by Monoclonal Antibodies for Human p55R and p75R on U-937 Cells

Pretreatment	Control	% Cytotoxicity	
		rhTNF (3 ng/ml)	nhLT (3 ng/ml)
None	0.4 ± 0.1	52.6 ± 1.6	7.5 ± 1.0
utr-1 (10 μg/ml)	0.6 ± 0.2	13.8 ± 2.6	7.0 ± 0.5
htr-5 (25 μg/ml)	2.4 ± 0.4	16.3 ± 1.3	2.2 ± 1.0
htr-9 (10 μg/ml)	6.5 ± 2.2	23.0 ± 2.5	6.4 ± 1.6
utr-1 and htr-5	1.9 ± 1.0	5.6 ± 1.1	2.5 ± 0.2
utr-1 and htr-9	6.9 ± 1.7	7.1 ± 2.0	6.8 ± 1.8

U-937 cells were pretreated with 10 μg/ml of monoclonal antibody for human p75R (utr-1), 25 μg/ml of monoclonal antibody for p55R (htr-5), and/or 10 μg/ml of monoclonal antibody for p55R (htr-9) at 4 °C for 1 h before addition of rhTNF (3 ng/ml) or nhLT (3 ng/ml). Cells were cultured at 37 °C for 48 h, and percent cytotoxicity was estimated by trypan blue dye exclusion. Values are mean ± S.D. of triplicate samples.

ng/ml of rTNF. The cytotoxic activity of rTNF was reduced significantly by all of the antibodies, and the rTNF activity was blocked completely by htr-9 combined with utr-1. The data suggest that cytotoxicity mediated by rTNF may be caused by the synergistic effects between p55R and p75R. On the other hand, natural LT cytotoxicity was reduced significantly by htr-5, whereas utr-1 reduced the activity only slightly. Cytotoxic activity of natural LT combined with htr-9 was equal to that of htr-9 alone, but htr-9 reduced the activity of higher dose of natural LT (data not shown). The data suggest that most natural LT cytotoxicity may be mediated through p75R only. Espevik *et al.* reported that htr-5 minimally blocked rLT binding to p55R (21). We observed that 25 μg/ml of htr-5 alone, or combined with utr-1, respectively blocked, partially or completely, radiolabeled natural LT specific binding to the cells. Furthermore, cytotoxic activity of rLT was also neutralized significantly by htr-5, and only slightly by utr-1 (data not shown). Our results indicate that both natural and recombinant LT had similar effects on the TNF receptors.

Fig. 2 shows TNF- and LT-induced cytotoxicity in response to independent occupation of two TNF receptors. Affinity of TNF(^{29}Val) to p55R was equivalent to that of wild type rTNF, but its affinity to p75R was about 1/200th that of wild type TNF. Its cytotoxic activity, which was much less than that of TNF, was neutralized significantly by htr-9 or htr-5, but was only slightly reduced by utr-1 (unpublished data). Activity of 3 ng/ml

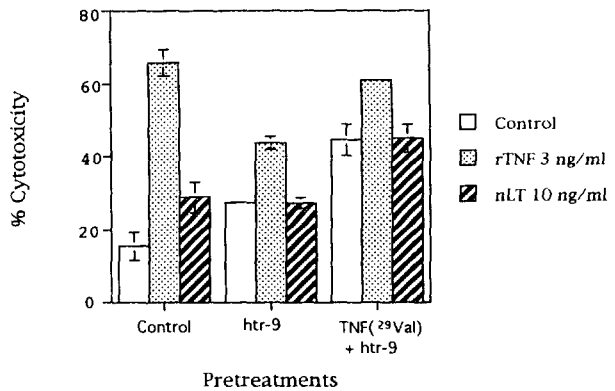

Figure 2. TNF- and LT-induced cytotoxic activity in response to independent occupation of two TNF receptors. Cells were treated with or without 10 μg/ml of htr-9 at 4°C for 1 h after pretreatment with 1 ng/ml of TNF(^{29}Val) at 4°C for 30 min. The cells were incubated with 3 ng/ml of rTNF or 10 μg/ml of natural LT at 37°C for 24 h. Percent cytotoxicity was determined by trypan blue dye exclusion. Values are mean ± S.D. of triplicate samples.

of TNF was completely neutralized by treatment with 10 μg/ml of htr-9 at 4 °C for 1 h (Table 1). Viability of control cells was decreased by cold injury during pretreatment. Activity of rTNF combined with the htr-9 pretreatment was greater than that of htr-9 alone, perhaps, because of the effects of p75R. However, natural LT activity combined with htr-9 was equivalent to that of htr-9. Cytotoxicity of rTNF combined with htr-9 and TNF(^{29}Val), which additionally stimulated p55R, was also somewhat greater than that of controls. Natural LT combined with the treatment also had the same activity as the controls. These data indicate that TNF has accessory activity through p75R in cytotoxicity is individually stimulated by other ligands. However, natural LT did not have activity through p75R.

Affinities of TNF and LT to p55R and p75R at 4°C were estimated by Scatchard analysis (Fig. 3). Data of the radiolabeled rTNF and natural LT specific binding to the receptors on U-937 cells without treatment yielded straight line Scatchard plots that were consistent with a single class of TNF and LT binding sites. However, pretreatment with utr-1 and htr-9 completely blocked specific binding of radiolabeled TNF and LT to the receptors on U-937 cells. Thus, TNF and LT binding to each p55R or p75R site was accomplished by treatment with utr-1 or htr-9, respectively. TNF affinity for total binding and p75R on U-937 cells were about 2-3 times the LT affinity, and the affinity of TNF to p55R was slightly greater than that of LT.

DISCUSSION

Our data clarify the different roles of p55R and p75R in cytotoxicity triggered by TNF and LT. In cytotoxicity mediated by TNF, the activity

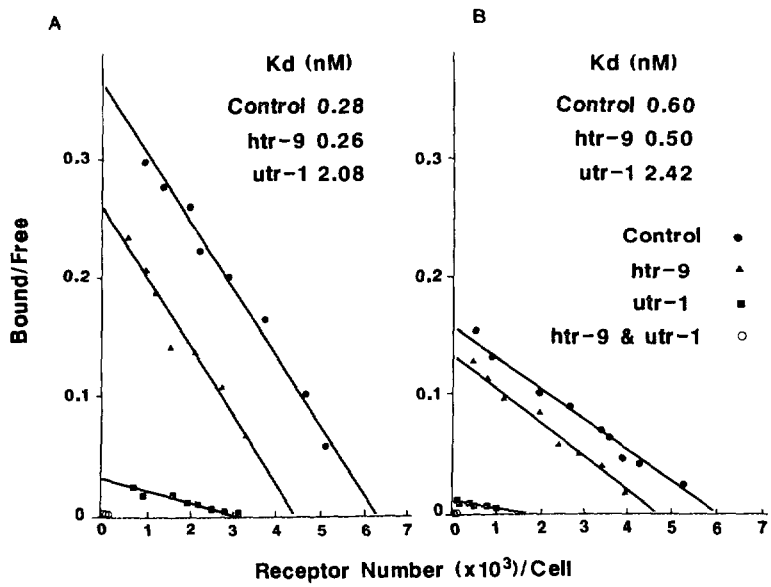

Figure 3. Scatchard analysis of [^{125}I]TNF (A), or [^{125}I]LT (B) specific binding to two TNF receptors, p55R and p75R, on U-937 cells. Cells without treatment (closed circles); cells treated with 10μg/ml of monoclonal antibodies for p55R, htr-9, (closed triangles); with 10 μg/ml of monoclonal antibody for p75R, utr-1, (closed squares); and with both antibodies (open circles) at 4°C for 3 h, and then washed 4 times with cold PBS containing 0.1% BSA. Radioactivity associated with the cells was counted by a γ-counter. Background was measured in the presence of more than 100-times molar excess of unlabeled ligands. The Kd values were determined from Scatchard analysis.

triggered at p55R and the effect of p75R in enhancing it may be important. Cytotoxicity mediated through p75R alone might be small, but LT lacked any effect on p75R, so the great difference between TNF and LT in their cytotoxicity on U-937 cells must be due mostly to their different effects on p75R.

Many reports describe the slight activity of LT compared to TNF in human cell lines (2,6-8). However, the similarity has been reported in murine cells (7,24,25) in which human TNF could not affect p75R (12). Exceptionally, in human cells, Browing *et al.* reported only that TNF had one- to two-times the activity of LT in inhibition of proliferation on BT-20 cells, that had only low affinity receptors (suggesting p55R), whereas in cell lines that have high affinity receptors (suggesting p75R), TNF activity was 20 to 320 times that of LT (26). Thus, the major reason why LT showed little activity could be its the lack of effect on p75R in certain biological activity. However, LT might lose some of its effectiveness through p75R, since it still had binding activity to p75R. Practically, LT has a proliferative effect on certain human lymphocytic cell lines through p75R (27). Some unexplained differences between LT and TNF, indicated by the inability of

LT on p75R, must be recognized (28), although there may be other different processes involved in the cellular responses to these cytokines.

TNF had accessory cytotoxic activity through p75R when p55R was individually stimulated by other ligands. These results suggest that there might be enhancement of the activity by p75R at the post receptor level, or the activity triggered only through p75R. Recently, Tartaglia *et al.* proposed the possibilty of enhancing p55R activity by p75R, if p75R recruits TNF for signaling by p55R (16). However, our results disagreed with some of their results that were based on their theory in individual stimulation of the two TNF receptors. Thus, there might be other mechanisms of enhancement of p55R activity through p75R.

Signaling mechanisms of the diverse biological activities of TNF/LT are still being considered. There may be a key to resolving the complicated signal transduction in the roles of the two TNF receptors, and comparison between TNF and LT could be an available tool for the study of functions of the two TNF receptors.

ACKNOWLEDGMENTS

We thank Dr. M. Brockhaus (hoffmann-La Roche, Co., Switzerland) for kindly providing the TNF receptor antibodies, htr-9, htr5, and utr-1; Dr. Kurimoto (Hayashibara Biochemical Laboratories Inc. Japan) for providing the natural human lymphotoxin; Dr Simpson for carefully reading the manuscript.

This work was supported by Grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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