Tumor necrosis factor (TNF) stimulates the production of nerve growth factor in fibroblasts via the 55-kDa type 1 TNF receptor

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Abstract The role of the two types of tumor necrosis factor (TNF) receptors, TNF-R1 and TNF-R2, in mediating the capacity of TNF to stimulate nerve growth factor (NGF) production in fibroblasts has been investigated. Although Swiss 3T3 cells express both the TNF-R1 and TNF-R2, an agonistic anti-TNF R1 antibody, but not an agonistic anti-TNF-R2 antibody, increases the NGF mRNA level and stimulates the production of NGF protein in the cells. Treatment of the cells with a combination of anti-TNF-R2 antibody and interleukin-1β (IL-1β) does not increase the NGF production induced by IL-1β alone, although TNF's activity to stimulate NGF production is markedly enhanced by IL-1β. Furthermore, simultaneous addition of the both antibodies does not increase the NGF production above that observed with the anti-TNF-R1 antibody, indicating that TNF-R1 alone mediates the TNF's activity to stimulate NGF production in fibroblasts.

Key words: Tumor necrosis factor; Nerve growth factor; 55-kDa type 1 TNF receptor; 75-kDa type 2 TNF receptor; Fibroblast

1. Introduction

Tumor necrosis factor-α (TNF), a macrophage/monocyte-derived cytokine, was originally identified as a factor with anti-tumor activity in vitro and in vivo, but is now known to be implicated in a wide range of biological processes including inflammation, immunoregulation, antiviral defense, endotoxic shock, cachexia, angiogenesis, and mitogenesis [1–3]. We have recently shown that TNF is also involved in modulating neuronal cell function through an indirect mechanism by which it stimulates the synthesis and secretion of NGF in fibroblasts and glial cells [4].

TNF exerts its diverse effects through two distinct cell surface binding sites, the type 1 TNF receptor (TNF-R1, ~55-kDa in size) and the type 2 TNF receptor (TNF-R2, ~75-kDa) [5,6]. These two types of receptors bind TNF with high affinity but differ in their intracellular domains and thus initiate distinct signaling pathways that result in the induction of different cellular responses [7]. A number of studies have investigated the importance of each of the two TNF receptors in signaling the different activities of TNF. Several polyclonal and monoclonal antibodies directed against human and mouse TNF-R1/TNF-R2 have been shown to behave as receptor agonists and to elicit the biological activities of TNF. Analysis of the results obtained using these antibodies has indicated that TNF-R1 is implicated in eliciting the major effects of TNF such as cytotoxicity, fibroblasts proliferation, and induction of protective activity [8–10], whereas TNF-R2 initiates signals for thymocyte and cytotoxic T cell proliferation [11]. On the other hand, several reports have suggested that there is a redundancy in the function of these two types of receptors [12,13]. In this study, we have investigated whether TNF-R1 or TNF-R2, or both are involved in mediating a newly described activity of TNF that stimulates NGF production in fibroblasts.

2. Materials and methods

2.1. Materials

Recombinant murine TNF-α (muTNF), the agonistic polyclonal anti-muTNF-R1 antibody (lot. 12571-18B) [11], and the agonistic polyclonal anti-muTNF-R2 antibody (lot. 12074-26B) [11] were kindly provided by the Genentech, Inc. manufacturing group (South San Francisco, CA). Recombinant human TNF-α (hTNF) was a kind gift from Dr. M. Tsujimoto (Suntory Institute for Biomedical Research, Osaka). Recombinant human IL-1β was purchased from Toyobo (Osaka), and recombinant murine interferon-γ (IFN-γ) was from Genzyme (Cambridge). Other chemicals and reagents were of the purest grade available.

2.2. Cell culture

Swiss albino mouse fibroblasts (Swiss 3T3; American Type Culture Collection, CCL92) were obtained through the Japanese Cancer Research Resources Bank. Subconfluent cultures of Swiss 3T3 cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum were rendered quiescent by incubation in serum-free medium (DMEM containing 5 mg/ml of bovine serum albumin, 1 μg/ml insulin, 2 μg/ml transferrin, 20 nM Na_2SeO_3, and 10 mM HEPES, pH 7.4) for 24 h; TNF, other cytokines, or the anti-TNF receptor antibodies were then added [4,14].

2.3. Two-site enzyme-linked immunosorbent assay of NGF

The NGF level in the conditioned media of Swiss 3T3 cells, treated with cytokines or the anti-TNF receptor antibodies, was assayed by the two-site enzyme-linked immunosorbent assay (ELISA) specific for murine submaxillary gland β-NGF as described previously [4,14].

2.4. RNA isolation and Northern blot analysis

Total RNA was isolated from anti-TNF receptor antibodies/cytokines-stimulated Swiss 3T3 cells according to the method of Chomczynski and Sacchi [15]. Twenty μg of total RNA was fractionated on 1.2% agarose gels containing 2.2 M formaldehyde and then transferred to a Zeta-probe membrane (Bio-Rad). The filter-bound RNA was hybridized with the randomly primed, ^{32}P-labeled mouse β-NGF cDNA probe [4] for 16 h at 42°C, then the filters were washed under stringent conditions following the instruction manual (Bio-Rad).

2.5. Iodination of muTNF and binding assay

Recombinant muTNF was radioiodinated by using the Bolton and Hunter reagent as described previously [16]. The specific radioactivity of the ^{125}I-labeled muTNF was 4.17×10^7 cpm/μg. Binding of ^{125}I-labeled muTNF to Swiss 3T3 cells was performed as described [16] with slight modification. Briefly, Swiss 3T3 cells were seeded at 5×10^5 cells/well in 6-cm dishes and allowed to growth for 24 h. Cells were then incubated in binding buffer (DMEM containing 10% calf serum and

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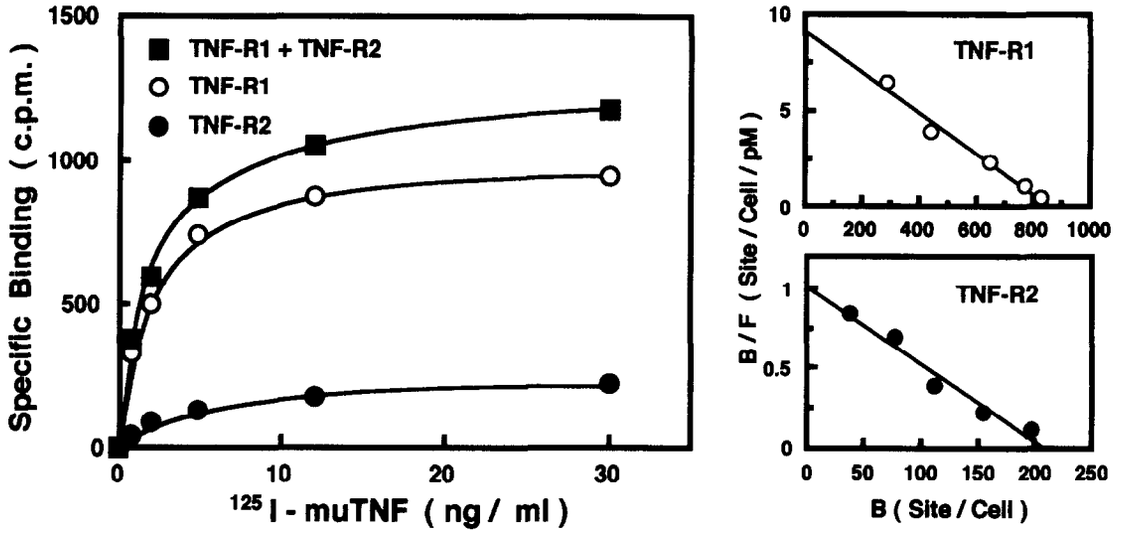

Fig. 1. Binding of ^{125}I-labeled muTNF to Swiss 3T3 cells. Increasing concentrations of ^{125}I-labeled muTNF were incubated with 6×10^5 Swiss 3T3 cells for 2 h at room temperature, and the cell associated radioactivity was determined (total binding). The specific binding activity of the receptors (total binding minus nonspecific binding) for ^{125}I-labeled muTNF was the binding activity observed in the presence of a 200-fold excess of unlabeled muTNF or hTNF. When unlabeled muTNF was employed, the binding activity was considered to be due to the sum of TNF-R1 and TNF-R2 specific binding activity (■). When unlabeled hTNF was used, the binding activity was considered to be due solely to TNF-R1 (○). The difference between the two specific binding activities gave the TNF-R2 specific binding activity (●) (left). The data are also presented in Scatchard plots (right). Two independent experiments yielded similar results.

25 mM HEPES, pH 7.4) containing 0.30–30 ng/ml of [^{125}I]muTNF for 2 h at room temperature on a rocker platform. After washing with the binding buffer, the cells were solubilized with 0.75 ml of solubilization buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES, pH 7.5, 0.05% bovine serum albumin) for 20 min at 37°C. Radioactivity was determined in aliquots (0.6 ml) in a Packard gamma counter. Nonspecific binding was determined by using a 200-fold excess of unlabeled muTNF or hTNF. The number of cells in replicate wells was determined by counting in a hematocytometer. Each data point was determined in triplicate, and binding data were analyzed according to the method of Scatchard [17].

3. Results

3.1. Swiss 3T3 cells express both the 55-kDa type 1 TNF receptor and the 75-kDa type 2 TNF receptor

Human TNF can bind murine 55-kDa type 1 TNF receptor (TNF-R1) but not murine 75-kDa type 2 TNF receptor (TNF-R2), whereas murine TNF binds both TNF-R1 and TNF-R2 [18]. We utilized this TNF species specificity to analyze the expression of TNF receptors on Swiss 3T3 cells. Thus, the specific binding (total binding minus nonspecific binding) of [^{125}I]muTNF to Swiss 3T3 cells observed in the presence of a 200-fold excess of unlabeled muTNF was considered to be the sum of specific binding to TNF-R1 and TNF-R2, while the specific binding observed using a 200-fold excess of unlabeled hTNF was considered to be that due to only TNF-R1.

Radiolabeled muTNF specifically bound to Swiss cells in a dose-dependent manner; a typical set of specific binding curves and corresponding Scatchard analyses are shown in Fig. 1. These results clearly showed that the Swiss 3T3 cells expressed both the TNF-R1 (870 receptors/cell with a dissociation constant of 95 pM) and TNF-R2 (210 receptors/cell with a dissociation constant of 200 pM). The expression of both types of the TNF receptors on Swiss 3T3 cells was also shown by FACS analysis using agonistic polyclonal anti-muTNF-R1 and anti-muTNF-R2 antibodies which were identical to those used in the experiments described below (data not shown).

3.2. An agonistic anti-muTNF-R1 antibody, but not an agonistic anti-muTNF-R2 antibody, stimulates the production of NGF in Swiss 3T3 cells

Both hTNF and muTNF were found to stimulate the production of NGF in Swiss 3T3 cells, when analyzed by the two-site ELISA for the murine submaxillary gland β-NGF [4].

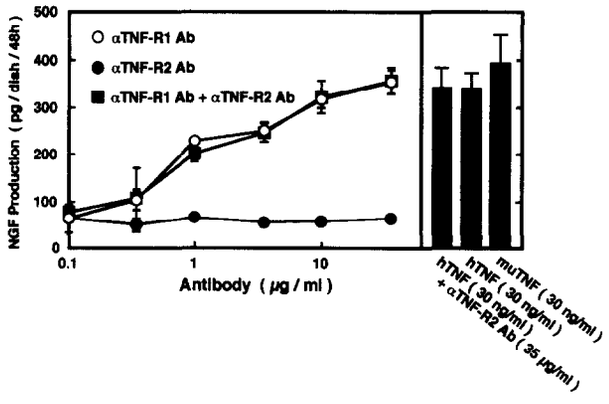

Fig. 2. Dose–responses of anti-muTNF-R1 and anti-muTNF-R2 antibodies to stimulate NGF production in Swiss 3T3 cells. Swiss 3T3 cells were cultured for 48 h with varying dose of anti-muTNF-R1 antibody, anti-muTNF-R2 antibody, or both antibodies in combination. The cells were also treated with hTNF (30 ng/ml), muTNF (30 ng/ml), or the combination of hTNF (30 ng/ml) and anti-muTNF-R2 antibody (35 μg/ml). The NGF level in the culture media was assayed by the two-site ELISA system. Means ± S.E. of 8 determinations using two 3.5-cm dishes for each experimental condition are indicated. Results shown are representative of three experiments that gave essentially the same results.

However, muTNF was more effective than hTNF when the cells were treated at the maximally effective concentration (30 ng/ml) (Fig. 2). These results suggested that the stimulatory effect of TNF on NGF production in Swiss 3T3 cells might be mediated through the activation of both TNF-R1 and TNF-R2, since muTNF can bind to both the muTNF-R1 and muTNF-R2, whereas hTNF binds only to muTNF-R1 [18]. To examine more directly this point, we stimulated the cells with agonistic antibodies directed against muTNF-R1 and muTNF-R2 [11].

Treatment of Swiss 3T3 cells with an agonistic anti-muTNF-R1 antibody increased the level of a transcript migrating at about 1.3 kilobases (Fig. 3), which is consistent in size with the NGF mRNA detected in murine submaxillary glands [19]. The increase in the NGF mRNA level was observed after 3 h, and became maximal within 12 h of incubation with the antibody. This result is essentially identical to that observed in hTNF-stimulated Swiss 3T3 cells [4]. On the other hand, treatment of Swiss 3T3 cells with an agonistic anti-muTNF-R2 antibody did not increase the NGF mRNA level at all. Furthermore, the NGF mRNA level in Swiss 3T3 cells stimulated with both receptor antibodies or a combination of the anti-muTNF-R2 antibody and hTNF was essentially identical to that in the cells treated with the anti-muTNF-R1 antibody or hTNF alone (data not shown).

The anti-muTNF-R1 antibody also stimulated the production of NGF protein in Swiss 3T3 cells. As shown in Fig. 2, the response was dose-dependent, and the amount of NGF produced by the cells stimulated with 35 μg/ml of anti-muTNF-R1 antibody was essentially identical with that produced by the cells stimulated with the maximally effective concentration (30 ng/ml) of hTNF. On the contrary, the anti-muTNF-R2 antibody did not stimulate the production of NGF in Swiss 3T3 cells up to concentration of 35 μg/ml, and furthermore, treatment of the cells with both receptor antibodies or a combination of anti-muTNF-R2 antibody and hTNF did not increase the amount of NGF compared to cells stimulated with the anti-muTNF-R1 antibody or hTNF alone.

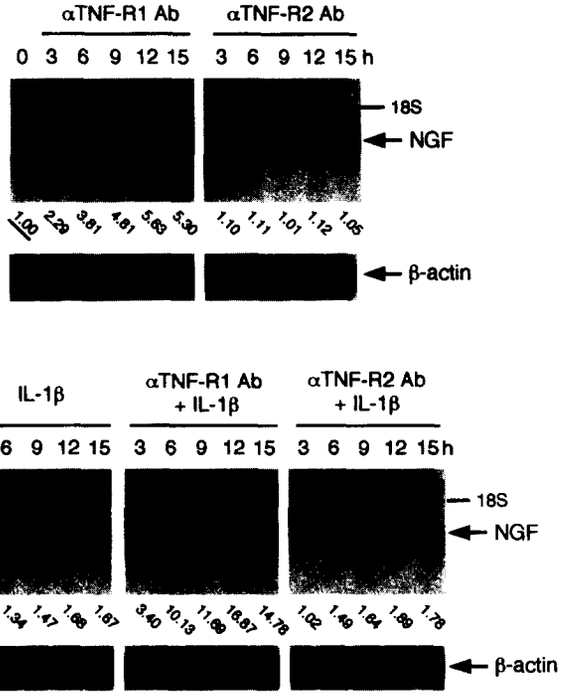

Fig. 3. Northern blot analysis. Total RNA (20 μg) was extracted from Swiss 3T3 cells that had been treated for varying times with the anti-muTNF-R1 antibody (35 μg/ml), anti-muTNF-R2 antibody (35 μg/ml), IL-1β (3 ng/ml), or combinations of these agents. The RNA was electrophoretically resolved in a 1.2% agarose/formaldehyde gel, blotted, and hybridized with ^{32}P-labeled mouse β-NGF cDNA probe, and, after stripping, with a β-actin probe. Blots were quantitated with a FUJIX Bio-imaging analyzer BAS 1500, and -fold induction of NGF mRNA normalized to β-actin mRNA is indicated. Position of 18S rRNA is indicated. Results shown are representative of three similar experiments.

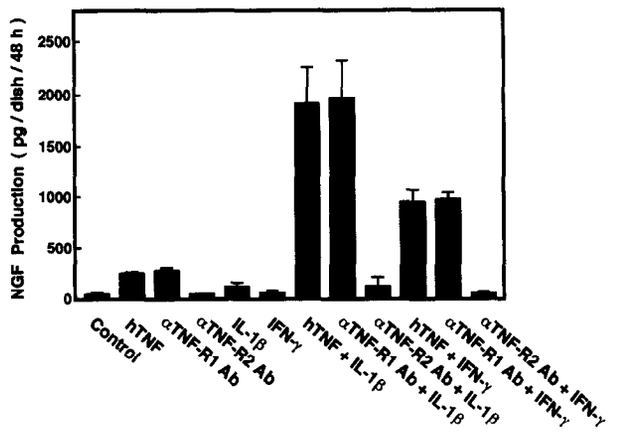

Fig. 4. Stimulation of NGF production in Swiss 3T3 cells with combinations of anti-muTNF R1/R2 antibodies and various cytokines. The NGF level in the culture medium of Swiss 3T3 cells treated for 48 h with hTNF (30 ng/ml), anti-muTNF R1 antibody (35 μg/ml), anti-muTNF R2 antibody (35 μg/ml), IL-1β (3 ng/ml), or IFN-γ (30 U/ml), separately or in combination, was assayed by the two-site ELISA system as described in the legend of Fig. 2. Results shown are representative of two independent experiments.

We have recently shown that the stimulatory activity of TNF on NGF production in fibroblasts is markedly synergized by IL-1α/β and IFN-γ [14]. Likewise, stimulation of Swiss 3T3 cells with combinations of anti-muTNF-R1 antibody and IL-1β/IFN-γ apparently increased the NGF mRNA level and also stimulated the NGF production in the cells to a much greater degree than did each agent alone (Figs. 3 and 4). However, the simultaneous addition of anti-muTNF-R2 antibody and IL-1β or IFN-γ did not increase the NGF production above the level induced by IL-1β or IFN-γ alone.

4. Discussion

The present studies have clearly demonstrated that TNF-R1 is involved in mediating the recently described activity of TNF that stimulates NGF production in fibroblasts [4]. Although Swiss 3T3 cells express both TNF-R1 and TNF-R2, an agonistic anti-muTNF-R2 antibody did not stimulate the production of NGF in these cells up to a concentration of 35 μg/ml. Stimulation of Swiss 3T3 cells with a combination of anti-muTNF-R2 antibody and IL-1 β or IFN-γ did not increase the NGF production induced by IL-1β or IFN-γ alone, even though TNF's activity to stimulate NGF production is markedly enhanced by IL-1 α/β and IFN-γ [14]. Furthermore, the simultaneous addition of both antibodies or anti-muTNF-R2 antibody

and hTNF did not increase the production of NGF above that observed with the anti-muTNF-R1 antibody or hTNF alone.

These results indicate that TNF-R1 alone mediates the TNF's activity to stimulate NGF production in Swiss 3T3 cells. However, muTNF (which has access to both muTNF-R1 and muTNF-R2) was slightly more effective than hTNF and anti-muTNF-R1 antibody (which binds only to muTNF-R1) for the stimulation of NGF production. A similar higher efficacy of muTNF compared to hTNF in stimulating the production of IL-6 and granulocyte macrophage-colony stimulating factor in mouse fibroblasts has been recently reported [20]. A combination of anti-muTNF-R2 antibody with anti-muTNF-R1 antibody or hTNF did not show any increased activity compared to anti-muTNF-R1 antibody or hTNF alone, suggesting that TNF-R2 by itself does not generate signals which potentiate TNF activity. Rather, TNF-R2 contributes to elicit the maximal activity of TNF through an indirect mechanism that facilitates the association of TNF with TNF-R1 which is the receptor responsible for all signal generation. The possible utilization of such a 'ligand passing' mechanism by TNF-R2 has recently been proposed [21].

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