A Human Tumor Necrosis Factor p75 Receptor Agonist Stimulates In Vitro T Cell Proliferation But Does Not Produce Inflammation or Shock in the Baboon

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Summary

Tumor necrosis factor (TNF) is a potentially useful adjunct to anticancer therapies. However, the clinical utility of TNF has been limited by generalized toxicity and hypotension. Recently, studies have begun to dissect the individual proinflammatory and immunologic responses that result from TNF binding to its two cellular receptors, p55 and p75, in an attempt to develop TNF receptor agonists with reduced systemic toxicity. To evaluate a p75 receptor selective TNF mutant (p75TNF), TNF and p75TNF were administered to healthy anesthetized baboons. Intravenous infusion of the p75TNF produced none of the hemodynamic changes seen after the infusion of TNF. Infusion of p75TNF also failed to induce the plasma appearance of interleukins 6 and 8. However, p75TNF enhanced in vitro baboon thymocyte proliferation to concanavalin A, and infusion of p75TNF resulted in increased soluble p55 and p75 receptor plasma concentrations. Subcutaneous injection of TNF and p55TNF resulted in local skin necrosis and tissue neutrophil infiltration. Subcutaneous injection of p75TNF did not result in skin necrosis but did result in a modest dermal infiltration of lymphocytes and macrophages. The findings suggest that p75TNF may stimulate T cell proliferation without the systemic and local toxicity seen with TNF.

TNF was first identified as the macrophage-derived product responsible for the hemorrhagic necrosis of several murine solid tumors after endotoxin administration (1). More recently, TNF has been shown to mediate several diverse biologic effects including macrophage and PMN activation, chemotaxis, induction of endothelial adhesion molecules, apoptosis of certain tumor cells lines, lymphocyte proliferation, and the release of other proinflammatory cytokines, most notably IL-1β, IL-6, and IL-8 (1-4). TNF acts by binding to two cell surface receptors, identified as p55 (type I) and p75 (type II). The two receptors share similar cysteine-rich extracellular domains but dissimilar intracellular structures (5). TNF binding to the p55 receptor induces apoptosis in some tumor cell lines, the expression of endothelial cell adhesion molecules, fibroblast proliferation, and neutrophil activation (4, 6-10). Transgenic mice with a nonfunctional gene coding for the p55 receptor have been shown to be resistant to endotoxemic shock but are more susceptible to Listeria infection (11, 12).

TNF binding to the p75 receptor promotes in vitro thymocyte and circulating lymphocyte proliferation (13, 14). Binding to the p75 receptor mediates lysis of certain tumor cell lines in vitro alone and in conjunction with binding to the p55 receptor (2, 14, 15). However, knowledge of the in vivo function of the p75 TNF receptor is limited. Sheehan et al. (16) demonstrated that antibodies specific for the p75 TNF receptor protect mice from the development of skin necrosis after the subcutaneous administration of murine TNF. Similar results have been obtained in transgenic mice with a nonfunctional p75 TNF receptor (17).

Recently, human TNF mutants have been created that selectively bind only one of the two TNF receptors (18).
The present study investigates the effects of systemic and subcutaneous administration of a p75 TNF mutant (p75TNF) in healthy baboons, and its comparison to wild-type TNF.

Materials and Methods

TNF, p55, and p75 TNF Receptor Agonists. p55TNF, p75TNF, and wild-type TNF were prepared as previously reported (18). The p55TNF mutant was generated by site-specific mutagenesis of the human TNF cDNA with Arg20 replaced by Thr, and Ser36 replaced by Thr (18). The p75TNF mutant was generated by replacing Asp143 with Asn, and Ala145 with Arg. This p75TNF mutant has no affinity for the p55 TNF receptor while binding to the human p75 TNF receptor with about one tenth the affinity of wild-type TNF (18).

Solid-phase Radioligand Binding Assay. The specificity of p75TNF for human and baboon p75 TNF receptors was confirmed in competitive radioligand binding assays as described (18). Briefly, TNF receptors were extracted with Triton X-100 from baboon buffy coats and HL60 cells, and immobilized to microtiter plates by the human p75 TNF receptor with about one tenth the affinity of wild-type TNF (18).

Thymocyte Proliferation Assay. The thymocyte proliferation assay was adapted from Tartaglia et al. (14). Briefly, thymic tissue was obtained at necropsy from a young healthy Papio sp. baboon. Baboon thymocytes were isolated by Dounce homogenization, and cells were plated at 96-well flat-bottomed plates at a density of 3 × 10^6 cells/ml to a final volume of 0.2 ml/well. The cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated FCS, 1% glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin in the presence of 2 µg/ml purified Con A (Sigma Chemicals, Co., St. Louis, MO). Serial dilutions of recombinant human TNF and p75TNF were added in a concentration range of 1 to 10^−3, and 10 to 10^−4 µg/ml, respectively. The cells were incubated for 60 h at 37°C and pulsed with 1 µCi/well of [3H]thymidine (Amersham Ltd, Norwalk, CT) for a further 18 h. The cells were then harvested from the 96-well plate and precipitated with 10% TCA. Cell pellets were washed, solubilized in normal NaOH, and counted in a liquid scintillation counter.

Treatment Protocol of Baboons. Juvenile (5–10-yr-old) Papio sp. animals were quarantined for at least 4–6 wk to confirm that they were healthy and free of pathogens. The animals were housed at the Research Animal Resource Center of Cornell University Medical College, the Health Science Center Animal Resource Department at the University of Florida. The experimental protocol was approved by the Institutional Animal Care and Use Committees of Cornell University Medical College and the University of Florida.

In the studies of intravenous administration of TNF and p75TNF, the baboons were anesthetized and instrumented as previously described (3).

After 1 h of equilibration, baseline blood samples were obtained. Recombinant human TNF, p75TNF, or saline (control injection) was administered intravenously in a blinded fashion. TNF was infused at a dose of 100 µg/kg body weight. p75TNF was administered at a 10-fold-higher dose (1 mg/kg) to compensate for its ~10-fold-decreased binding affinity for the p75 receptor (18). Four baboons received TNF, three received p75TNF, and two baboons received placebo injections of saline.

Arterial blood sampling was carried out at the time of treatment and after 2 min, 10 min, 30 min, 1 h, 1.5 h, 2 hr, 2.5 h, 3 h, 4 h, 5 h, 6 h, 7 h, and 8 h. Heparinized blood was centrifuged at 2,500 rpm for 15 min at 4°C and the plasma fraction aliquoted and stored at −70°C. Plasma IL-6, IL-8, and IL-1β concentrations were measured by ELISA as previously described (19). Plasma concentrations of the soluble p55 and p75 TNF receptors were also measured by ELISA using polyclonal rabbit antibodies (19). Endogenous TNF bioactivity was determined by incubating baboon plasma with the murine WEHI 164 clone 13 fibroblast cell line and determining cytotoxicity (3, 19), taking advantage of the fact that neither p55TNF nor p75TNF is bioactive on the murine WEHI cell line (data not shown). Thus, WEHI cytotoxic activity in plasma samples from baboons treated with p75TNF can be attributed to an endogenous Peroxidase TNF response (3). This strategy cannot be used with plasma samples from baboons that have received recombinant human TNF, since both recombinant human TNF and endogenous baboon TNF are cytotoxic to WEHI 164 clone 13 cells.

In two additional baboons, subcutaneous injections of recombinant human TNF, p55TNF, p75TNF, or saline, were performed. TNF, p55TNF, p75TNF, and a saline control in a volume of 200 µl were injected subcutaneously into different regions on the medial aspect of each hind leg. In one baboon, TNF and p55TNF were injected at a dose of 0.1 µg, and p75TNF was injected at 10-fold-higher doses (1.0 µg) in one leg. In the second baboon, TNF and p55TNF were given subcutaneously at a dose of 10 µg and the p75TNF was given at a dose of 100 µg in one leg. Injection sites were identified with a permanent dye marker and were spaced at least 5 cm apart to avoid any diffusion of proteins between injection sites. Skin biopsies were taken 6 h after the injection. The tissue was fixed in buffered formalin, embedded, sectioned, and stained with hematoxylin and eosin. On the contralateral hind leg of each of the two animals, TNF, p55TNF, p75TNF, and saline were administered subcutaneously at the high and low dose as before, but repeated daily for 4 d. Biopsy samples were obtained at the end of the fifth day and processed in an identical fashion as for the 6-h samples. Hematoxylin and eosin stained sections were evaluated without knowledge of the treatment groups.

Statistical Analysis. Statistical analyses of the values obtained were carried out by one-way analysis of variance (ANOVA) with posthoc analysis by Dunnett's method, and two-way ANOVA with posthoc analysis by the Student-Newman-Keuls method. Statistical significance was determined with a P value of <0.05. All values are expressed as mean ± standard error of the mean.

Results

Receptor-type Specificity of p75TNF in Papio. p75TNF binds exclusively human p75 with an affinity of about one tenth of recombinant human TNF (18). To analyze whether the receptor-type selectivity of p75TNF is maintained in baboons, binding of p75TNF to human and baboon TNF receptors was compared in a solid-phase competitive binding assay. As shown in Fig. 1, p75TNF inhibited binding of human 125I-TNF to p75 TNF receptor, but not to p55, regardless of whether the receptors were derived from human HL60 cells or Papio leukocytes. Recombinant human TNF competitively blocked binding to both receptors.

1Abbreviations used in this paper: ANOVA, analysis of variance.
from both species. Because of the relatively low number of p75 present on the *Papio* leukocyte, the binding of 125I-TNF to these samples was low and, as a consequence the non-specifically bound radioactivity in these wells became more apparent (Fig. 1, bottom). Nevertheless, the competitive binding assays clearly demonstrate a p75 selectivity of p75TNF in *Papio*, analogous to the earlier described p55TNF which also maintained receptor-type selectivity in *Papio* (3). Recombinant human TNF binds to both *Papio* TNF receptors, p55 and p75.

**Thymocyte Proliferation.** Previous studies have suggested that TNF proliferative signals in lymphoid cells can be mediated independently via the p75 (13, 14). To determine if human p75TNF was active in *Papio*, thymocytes were isolated and proliferation was measured in vitro in response to p75TNF and TNF in the presence of suboptimal quantities of Con A. It was found that both p75TNF and TNF increased [3H]thymidine uptake by baboon thymocytes in a dose-dependent manner when costimulated with Con A (Fig. 2). Peak incorporation of [3H]thymidine with TNF was seen with concentrations of 40 ng/ml. Further increasing the concentration of TNF led to decreased incorporation of [3H]thymidine with a return to baseline stimulation by Con A alone at a dose of 10 μg/ml. Similarly, p75TNF increased [3H]thymidine uptake, but as expected, higher concentrations were required. [3H]Thymidine incorporation started to increase at 137 ng/ml, with maximal proliferation occurring at a concentration of 1,235 ng/ml. Stimulation with higher doses of the p75 agonist again led to decreased [3H]thymidine uptake with a return to baseline values of Con A alone at a concentration of 33 μg/ml.

**Hemodynamic Response.** Baboons treated with p75TNF developed only a mild tachycardia with a maximum peak increase in heart rate (22 ± 7 beats/min) 1.5 and 2 h after treatment (Fig. 3). The tachycardic response thus was much shorter and less pronounced than in the animals treated with TNF, but it was significantly different (P <0.05) from the heart rate immediately before the p75TNF infusion. The difference between the animals treated with p75TNF and TNF was statistically significant.

Administration of p75TNF did not produce hypotension. The changes in mean arterial blood pressure were not significantly different from baseline or measurements from saline control animals. The maximal fall in mean arterial blood pressure in the animals treated with p75TNF, 15 ± 5 torr, was significantly less (P <0.05) in extent and duration than what had been seen in the animals receiving TNF. Animals receiving saline as a parallel placebo control developed a mild tachycardia and a fall in mean arterial blood pressure that was not significantly different from values obtained immediately before infusing the saline, or from p75TNF-treated baboons, by one-way ANOVA.

**Core Body Temperature Response.** Administration of TNF and p75TNF produced fever in the baboons (Table 1). In contrast, control animals administered saline had only a modest elevation. The fever curves of the animals treated with TNF and p75TNF were not significantly different from each other by two-way ANOVA (data not shown), but were different from the control animals.

**Proinflammatory Cytokine Response.** IL-1β was not detected in any of the plasma samples after either TNF, p75TNF, or saline infusions (data not shown). No significant release of circulating IL-6 and IL-8 (Table 1) or endogenous TNF response (i.e., WEHI cytotoxicity) was detected after infusion of p75TNF or saline (data not shown). In contrast, wild-type TNF produced a significant appearance of IL-6 and IL-8 that peaked after 6 and 7 h, respectively.

Administration of TNF and p75TNF both resulted in increased plasma concentrations of soluble p55 and p75, but the magnitude of the response was much less in the p75TNF–treated animals (Table 1). The administration of TNF caused a sustained increase of soluble p55 throughout the 8-h study period, whereas administration of p75TNF also resulted in the transient increase in soluble p55 concentrations with the maximum being reached 1 h after infusion (P <0.05 vs. baseline). Administration of TNF and p75TNF also resulted in increased plasma concentrations of...
Figure 2. Thymocyte proliferation with recombinant human TNF and p75TNF in the presence of Con A. The thymus was removed from a healthy juvenile baboon after euthanasia and cells dispersed mechanically. 3 × 10^6 cells/well were incubated in 96-well microtiter plates with complete medium containing 1 μg/ml of Con A and increasing concentrations of recombinant human TNF or p75TNF for 60 h, pulsed with 1 μCi of [3H]thymidine for 18 h, and harvested as described in Materials and Methods. Values are presented as mean cpm/well.

Figure 3. Hemodynamic changes in baboons treated with 100 μg/kg BW recombinant human TNF, 1,000 μg/kg BW of p75TNF, or saline. (A) Changes in heart rate. (B) Changes in mean arterial pressure. Baboons treated with wild-type TNF (n = 4; 0-0); animals treated with p75TNF (n = 3; ■■■■), and baboons receiving only saline (n = 2; O-O). Mean values ± SEM, except for the saline group (n = 2) which is standard deviation.
Table 1.  Febrile Response and Peak Plasma Cytokine Appearance in Baboons Treated with Wild-type TNF, p75TNF, or Saline

|                      | Wild-type TNF (n = 4) | p75TNF (n = 3) | Saline (n = 2)* |
|----------------------|-----------------------|----------------|---------------|
| Change in body       |                       |                |               |
| temperature (°C)     | +2.7 ± 0.1*           | +2.0 ± 0.2*    | +1.2 ± 0.4*   |
| IL-1β, pg/ml         | nd                    | nd             | nd            |
| IL-6, ng/ml          | 48.5 ± 8.0*           | 0.3 ± 0.03     | 0.2 ± 0.07    |
| IL-8, ng/ml          | 14.3 ± 2.0*           | 0.2 ± 0.2      | 0             |
| Change in p55, pg/ml | 1,025 ± 373*          | 553 ± 57*      | -8 ± 74       |
| Change in p75, pg/ml | 6,061 ± 2,477*        | 537 ± 132*     | 89 ± 93       |

*With an n = 2 in the saline group, the variance reported is standard deviation.

**Statistical difference from baseline measurements (i.e., time = 0 h) by one-way ANOVA and Student-Newman-Keuls multiple range test.

Not detected with a sensitivity of 11 pg/ml.

Values represent a change in baseline measurement obtained before protein administration.

Discussion

The present studies clearly show that a p75 TNF selective agonist is not nearly as toxic as wild-type TNF with regard to its capacity to produce hypotension and shock when administered systemically, and to produce inflammation and tissue necrosis in the skin after subcutaneous injection. However, the p75 TNF receptor selective agonist stimulates thymocyte proliferation in vitro, elicits a febrile response in vivo, and produces a modest transient mononuclear cell infiltration when administered intradermally.

Attempts to use TNF as an antitumor agent in humans have met with little success, due at least in part to dose-limiting toxicity (20–22). Recombinant human TNF adminis-
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