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. Local skin necrosis and tissue neutrophil infiltration were seen after subcutaneous injections of TNF and p55TNF. 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, chemoattraction, 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 nonfuctional p75 TNF receptor (17).

Recently, human TNF mutants have been created that selectively bind only one of the two TNF receptors (18).

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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 Arg^{32} replaced by Trp, and Ser^{86} replaced by Thr (18). The p75TNF mutant was generated by replacing Asp^{143} with Asn, and Ala^{145} 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 p75-TNF 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 coated with affinity-purified anti–human p55 and p75 TNF receptor polyclonal antibodies. Binding of human ^{125}I-TNF (10 ng/ml) to the receptors was measured in the absence and presence of 20 μg/ml recombinant human TNF and p75TNF. Bound radioactivity was quantitated in a PhosphorImager® (Molecular Dynamics, Inc., Sunnyvale, CA).

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 in 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^{-5}, 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 [^{3}H]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 or 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, 5 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 *Papio* 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)[1] 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 ^{125}I-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

[1] *Abbreviations 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 ^{125}I-TNF to these samples was low and, as a consequence the nonspecifically 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 [^{3}H]thymidine uptake by baboon thymocytes in a dose-dependent manner when costimulated with Con A (Fig. 2). Peak incorporation of [^{3}H]thymidine with TNF was seen with concentrations of 40 ng/ml. Further increasing the concentration of TNF led to decreased incorporation of [^{3}H]thymidine with a return to baseline stimulation by Con A alone at a dose of 10 μg/ml. Similarly, p75TNF increased [^{3}H]thymidine uptake, but as expected, higher concentrations were required. [^{3}H]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 [^{3}H]thymidine uptake with a return to baseline values of Con A alone at a concentration of 33 μg/ml.

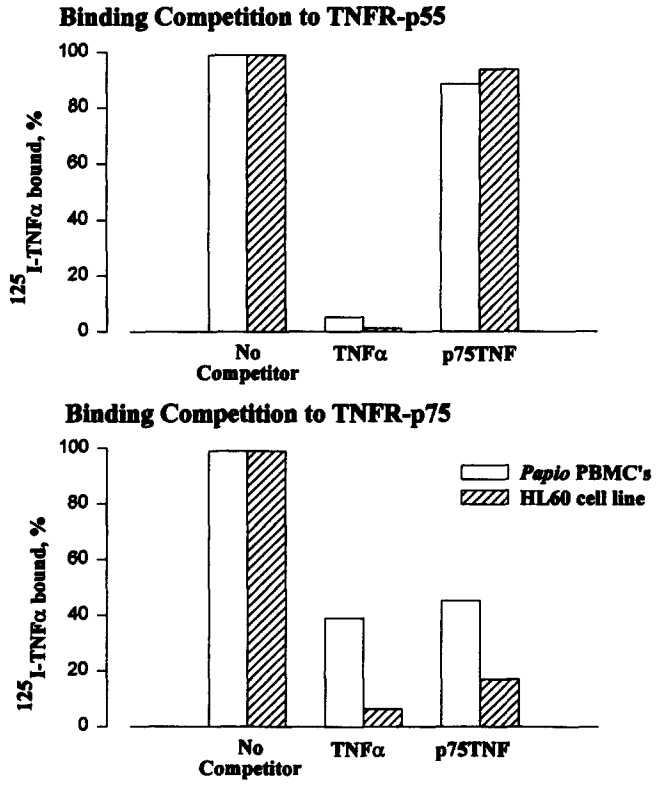

Figure 1. TNF receptor-type selectivity of p75TNF in baboons and humans. Solubilized HL60 and baboon leukocyte TNF receptors were captured on microtiter plates coated with affinity-purified polyclonal antibodies specific for human p55 and p75. Binding of human recombinant ^{125}I-TNF (10 ng/ml) was determined in the presence or absence of excess unlabeled recombinant human TNF or p75TNF (2 μg/ml). The bound radioactivity in the absence of competitor is taken as 100%; it was 1.35×10^5 and 1.05×10^6 PhosphorImager® counts (p55TNF receptor; *top*), and 14,850 and 121,380 counts (p75 TNF receptor; *bottom*) for baboon peripheral blood mononuclear and HL60 cells, respectively. (*Top*) Binding competition to p55; (*bottom*) binding competition to p75.

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

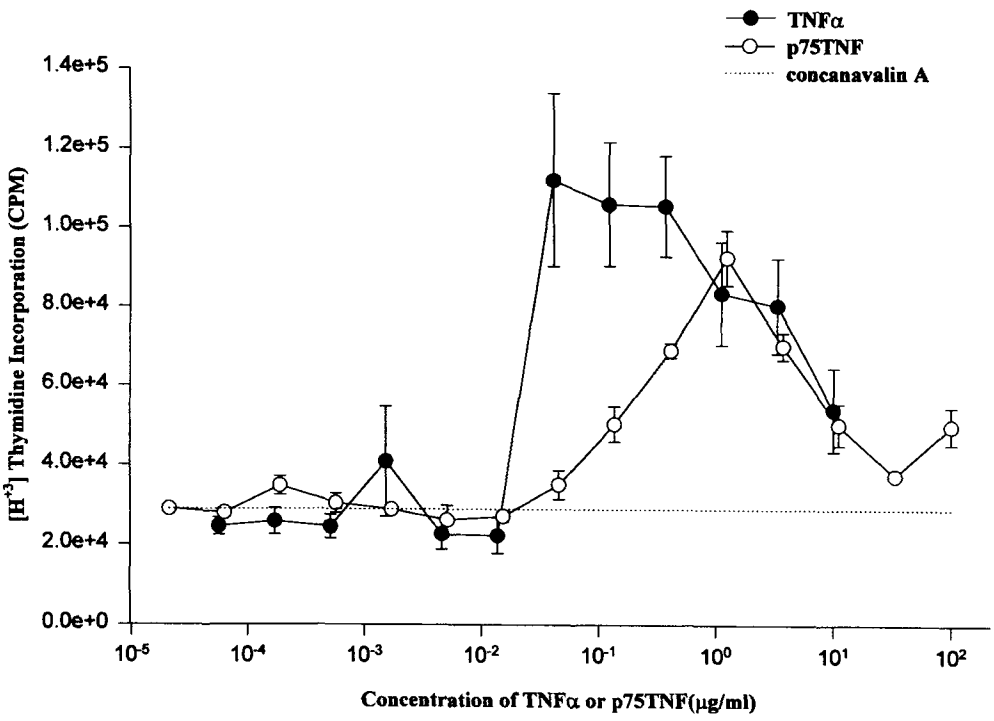

Figure 2. Thymocyte proliferation with recombinant human TNF and p75TNF in the presence of Con A. The thymus was removed from a healthy juvenile baboon after euthanasia and cells dispersed mechanically. 3×10^6 cells/well were incubated in 96-well microtiter plates with complete medium containing 1 μg/ml of Con A and increasing concentrations of recombinant human TNF or p75TNF for 60 h, pulsed with 1 μCi of [^{3}H]thymidine for 18 h, and harvested as described in Materials and Methods. Values are presented as mean cpm/well.

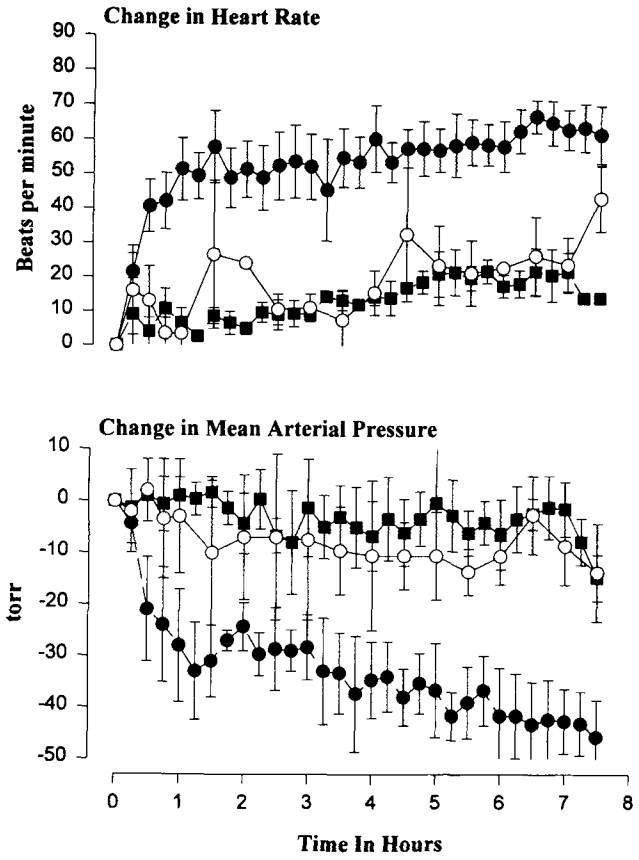

Figure 3. Hemodynamic changes in baboons treated with 100 μg/kg BW recombinant human TNF, 1,000 μg/kg BW of p75TNF, or saline. (*A*) Changes in heart rate. (*B*) Changes in mean arterial pressure. Baboons treated with wild-type TNF (*nv* = 4; ●-●); animals treated with p75TNF (n = 3; ■-■), and baboons receiving only saline (n = 2; ○-○). mean values ± SEM, except for the saline group (n = 2) which is standard deviation.

the soluble p75, but the response to p75TNF was dramatically lower than that of wild-type TNF (Table 1). There was no significant increase in the plasma concentration of soluble p75 or p55 in the animals infused with saline alone.

Subcutaneous Inflammatory Response. The sequelae of subcutaneous injections of TNF, p55TNF, and p75TNF at two doses were studied by histologic analysis. 6 h after the subcutaneous injection of TNF or p55TNF, neutrophil infiltration was noted (Fig. 4). At the low dose, p55TNF (0.1 μg) led to margination of moderate numbers of neutrophils in the venules with perivenular aggregation and edema. In contrast, administration of the low doses of TNF (0.1 μg) and p75TNF (1 μg), or saline, resulted in no significant lesion after 6 h. Injections of the high doses of p55TNF and TNF (each 10 μg) resulted in more pronounced histological changes 6 h after treatment. High dose TNF led to margination of neutrophils with perivenular aggregates of neutrophils, similar to the low dose p55TNF injections. p55TNF at the high dose caused a similar but more severe lesion than that seen after administration of the low dose p55TNF. The administration of high dose p75TNF (100 μg) did not result in significant histological changes 6 h after treatment (Fig. 4).

The tissue reaction to repeated subcutaneous administration was more pronounced. After daily injections of the low dose of TNF (0.1 μg), no histological changes were

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.

detected, but low dose (0.1 μg) repeated administration of p55TNF (0.1 μg) caused multifocal, low density aggregates of mononuclear cells and neutrophils. The repeated administration of low dose p75TNF (1 μg) resulted in minimal multifocal low density aggregates of lymphocytes and macrophages in the superficial dermis. There were no significant lesions associated with the injection of saline alone. The repeated high dose administration of p55TNF and TNF (each 10 μg) resulted in clinically apparent, indistinguishable macroscopic ulcerations of the overlying epidermis and dermis with eschar formation. The ulcerations appeared to penetrate partially through the dermis with extensive fibrosis of the underlying subcutaneous tissue. Repeated high dose administration of p75TNF (100 μg) and saline resulted in no macroscopic tissue changes. Histologically, repeated high dose p55TNF and TNF administration resulted in both superficial dermal coagulation and necrosis with surrounding areas of hemorrhage and dense surrounding aggregates of both macrophages and neutrophils (Fig. 4). The biopsy of skin treated after repeated high dose administration of p75TNF (100 μg) revealed no significant histological changes.

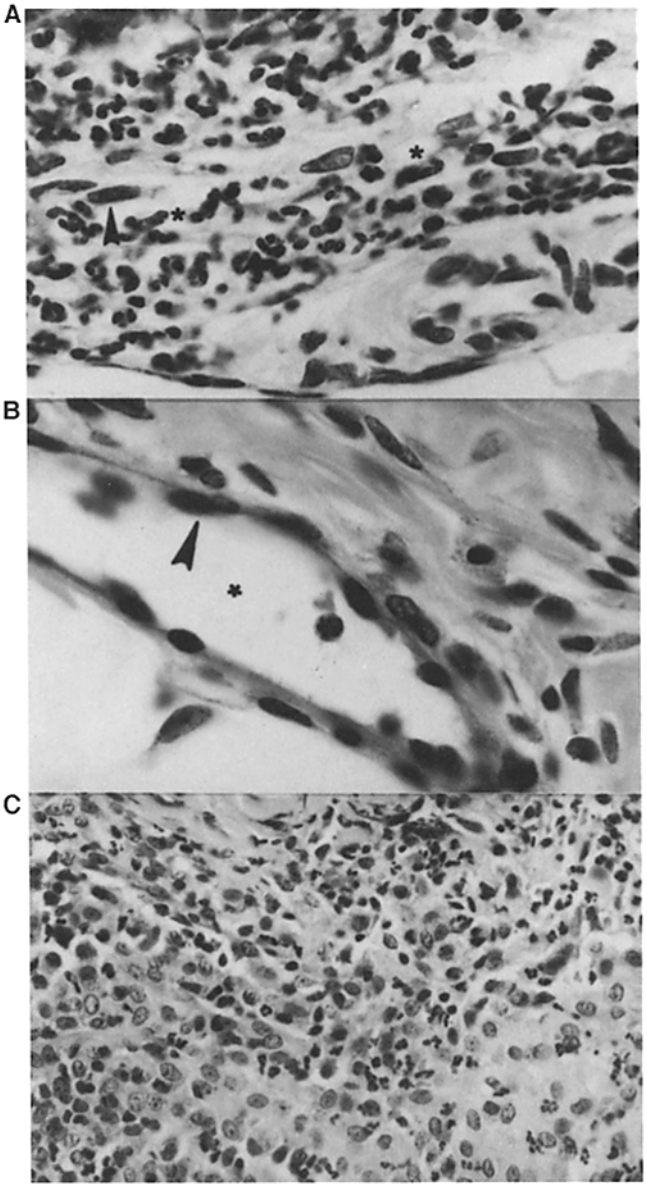

Figure 4. Histological changes after acute or chronic TNF or p75TNF administration. (*A*) Dermis from baboon having received 10 μg p55TNF 6 h before collection of the biopsy sample. A dermal venule is surrounded by and the wall infiltrated by dense aggregates of neutrophils. (*) Venule lumen; (▶) a lining endothelial cell. Hematoxylin and eosin stained paraffin section. (*B*) Dermis from baboon having received 100 μg p75TNF 6 h before collection of biopsy sample. A dermal venule has an open lumen (*) and easily identified endothelial lining (▶) with no inflammatory cell infiltrate. (*C*) Dermis from baboon having received 10 μg p55TNF daily for 5 d before collection of biopsy sample. The dermis contains dense aggregates of macrophages with scattered collections of neutrophils. A focus of necrotic cells is presented in the lower left corner of the field. (*A–C*) ×675.

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-

tered systemically at doses >200 $\mu g/m^2$ causes significant toxicity including fever, rigor, nausea, diarrhea, and hypotension (20). However, regional administration of higher dose TNF in combination with chemotherapy and hyperthermia more recently has shown high response rates in patients with melanoma and sarcoma of the extremities (22). In addition, local administration of TNF has recently shown promising response rates in Kaposi's sarcoma, plasmacytomas, ovarian adenocarcinomas, and various metastatic tumors in the liver (20, 21). Unfortunately, systemic leakage of regionally administered TNF has occasionally resulted in life-threatening complications including shock (20).

Previous studies have suggested that p55 plays the primary role in the proinflammatory properties of TNF (3, 4), and, conversely, the p75 TNF receptor promotes in vitro thymocyte proliferation as well as the proliferation of peripheral T cell populations (13, 14). The results of the present study confirm that p75TNF, at doses equivalent to those of wild-type TNF or of p55TNF mutants that produce significant systemic toxicity (3), lacks systemic toxicity in the baboon. Because this study did not evaluate pharmacokinetic properties of p75TNF when compared with p55TNF and wild-type TNF, and because a dose–response was not investigated, we cannot conclude that p75TNF is without any systemic toxicity. However, at doses of TNF and p75TNF that are equivalent based upon their binding affinities (18), only TNF produced significant systemic toxicity and skin inflammation.

Previous investigations of the local inflammatory response of skin to subcutaneous and intradermal injection of TNF had demonstrated that TNF leads to acute (within 8 h) neutrophil margination and edema formation (23–25). More recent studies have demonstrated that when injected subcutaneously daily over 5–7 d, TNF causes skin necrosis and intense neutrophil infiltration (16, 17). Two previous studies suggested that skin inflammation is partially dependent on TNF binding to the p75 TNF receptor, because mice treated with antibodies against murine p75 showed attenuated TNF-induced skin inflammation (16). Similarly, transgenic mice lacking a functional p75 had attenuated skin lesions after subcutaneous TNF administration (17). Those studies clearly show that TNF binding to a functional p75 receptor promotes the complete manifestation of an inflammatory response elicited by p55 TNF receptor activation. The present study addresses the question of whether TNF binding to the p75 TNF receptor alone is sufficient to elicit a skin inflammatory lesion. Clearly, subcutaneous administration of p55TNF in the baboon resulted in similar histological changes as seen with TNF, such as hemorrhage, necrosis, and dense aggregates of both macrophages and neutrophils. In contrast, p75 activation merely produced a modest degree of mononuclear cell infiltration. Beyond that cellular recruitment phenomenon, however, the manifestations of acute and chronic tissue reaction required p55 activation.

This study cannot rigorously establish whether p55 TNF receptor activation alone is sufficient to elicit a local skin inflammatory response, because it had been found in a previous study (3) that systemic administration of p55TNF elicited an endogenous baboon TNF response that activated both baboon p55 and p75. By analogy, subcutaneous administration of p55TNF may induce local baboon TNF production that would also bind to p75. Further studies thus are required to establish whether manifestation of skin inflammatory responses is an exclusive p55-dependent function or is dependent on TNF binding to both receptors.

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