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ORIGINAL ARTICLE

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An antimelanoma immunotoxin containing recombinant human tumor necrosis factor: tissue disposition, pharmacokinetic, and therapeutic studies in xenograft models

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Abstract The ability of monoclonal antibody conjugates to re-direct plant or bacterial toxins, chemotherapeutic agents and radionuclides to selected target cells has been well-documented. Recombinant human tumor necrosis factor (TNF) is a macrophage-derived, non-glycosylated (17 kDa) peptide with a broad range of biological and immunological effects including antiviral activity, cytotoxic and cytostatic effects. A conjugate of the antimelanoma antibody ZME-018 and TNF in previous studies has shown melanoma-selective cytotoxic effects in vitro. Pharmacokinetic studies of the ZME-TNF immunotoxin showed that the agent cleared from plasma biphasically with α- and β-phase half-lives similar to that of ZME itself (72 min and 36 h compared to 84 min and 41 h respectively). In contrast, TNF itself was cleared rapidly from plasma with a terminal-phase half-life of only 2.7 h. The clearance rate of ZME-TNF from plasma (Cl_p) was almost tenfold more rapid than for ZME (1.1 versus 0.16 ml/kg × min) but was threefold slower than the clearance for TNF itself (3.4 ml/kg × min). Tissue distribution studies in nude mice bearing human melanoma xenografts showed similar tumor localization of the immunotoxin compared to the free antibody and slightly higher concentrations in liver and kidney compared to ZME itself. Treatment of nude mice bearing well-developed A375 tumors with the immunotoxin resulted in a statistically significant ($P < 0.002$) suppression in tumor growth rate (fivefold increase) compared to saline-treated controls, which increased 20-fold over the same period. These studies demonstrate the feasibility of this approach and suggest that TNF may represent a non-antigenic alternative to immunotoxins containing plant and bacterial toxins.

Key words TNF · Immunotoxins · Melanoma · Pharmacokinetics · Xenograft models

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Introduction

The use of monoclonal antibodies to impart selectivity to indiscriminately cytotoxic drugs, radionuclides, and toxins has intrigued a number of investigators and has been the focus of considerable attention, beginning in the late 1960s with studies describing polyclonal antibodies linked to diphtheria toxin [1–10]. Monoclonal antibodies have the potential to serve as targeting vehicles for the classes of agents above and can also be utilized for targeting proteinaceous therapeutic agents such as biological response modifiers, interleukins, lymphokines and cytokines.

Our group [11] and others [12, 13] have previously described studies with human interferon covalently linked to monoclonal antibodies. These studies have demonstrated that interferon-antibody conjugates can have superior in vitro antiviral and cytotoxic activity compared to that of interferon alone. In addition, interferon-antibody conjugates have been shown to localize within human tumor xenografts to a much greater degree than interferon alone, thus demonstrating the ability of these constructs to achieve a higher intratumor concentration after systemic administration than that obtained with the native molecule [14].

Tumor necrosis factor is a polypeptide secreted primarily by activated macrophages [15, 16] and shares some sequence homology (30%) with another peptide hormone, lymphotoxin (TNF-β) secreted by activated lymphocytes. Purified recombinant human tumor necrosis factor (TNF) is a single-chain, non-glycosylated polypeptide with a molecular mass of 17.1 kDa. In vitro, TNF is cytostatic or cytotoxic to a number of human tumor cells. Several groups have demonstrated that human tumor cells can display

between 100 and 5000 receptor sites/cell [17, 18]. However, there have been no apparent correlations made between receptor number (or affinity) and the cellular response to TNF cytotoxic effects, suggesting a mechanism beyond receptor signaling that can modulate TNF biochemical effects [19]. In vivo studies with recombinant human TNF have demonstrated that this molecule is cleared from circulation rapidly in both animal and human trials [20, 21].

Murine monoclonal antibody ZME-018 binds to an epitope of a high-molecular-mass antigen (gp-240) found on the surface of more than 80% of melanoma cell lines and fresh tumor samples [22, 23]. When labeled with ^{111}In and administered to patients with melanoma, ZME-018 was found to localize in 77% of soft-tissue melanoma lesions [23]. In a previous study [24], we have characterized an immunoconjugate of recombinant human TNF crosslinked to antibody ZME-018 and purified by gel-permeation and affinity chromatography. The ZME-TNF conjugate was found to contain biologically active TNF as assessed using murine L-929 cells. The conjugate was found to bind specifically to melanoma target cells in a manner identical to native ZME. Melanoma BRO cells were killed with the conjugate at a concentration of 10 units/ml while free TNF had no cytotoxic effect at concentrations up to 50,000 units/ml.

The purpose of this study was to evaluate the in vivo pharmacokinetic and therapeutic effects of ZME/TNF conjugate to determine how this material behaves in vivo and whether a potential exists for the use of this agent for the treatment of patients with melanoma.

Materials and methods

Materials

The reagents 2-iminothiolane and *N*-succimindyl 3-(2-pyridyldithio)-proprionate were all obtained from Sigma Chemical Co. Ethylenediaminetetraacetic acid (EDTA), disodium salt was purchased from Boehringer Mannheim. Triethanolamine hydrochloride was obtained from Kodak Chemical Co. and dimethyl formamide was purchased from Aldrich Chemical Co. TRIS(Trizma)/HCl was obtained from BioRad Laboratories. Recombinant human tumor necrosis factor (0.5 mg/ml, specific activity 3×10^7 units/ml) was obtained from Genentech Inc. Antibody ZME-018 was a generous gift of Hybritech Inc. Dulbecco's modified Eagle's medium was obtained from Cellgro (Washington, D. C.). Minimum essential medium (MEM) was obtained from Gibco Laboratories (Grand Island, N. Y.). Fetal bovine serum was purchased from Hyclone Inc. Horseradish-peroxidase-conjugated goat anti-(mouse IgG) antibody for enzyme-linked immunosorbent assay was purchased from BioRad Laboratories.

Cells

Human melanoma (A375-M) cells were obtained from Dr. I. J. Fidler of M. D. Anderson Cancer Center, Houston, Tex. The A375-M cells were routinely grown at a density of 7×10^6 cells/T-75 flask in Dulbecco's MEM 10% fetal bovine serum (FBS) containing gentamicin (0.05 mg/ml), added sodium pyruvate (100 mM), nonessential amino acids (10 mM), glutamine (200 mM) and MEM vitamins. The cells were routinely subcultured twice per week. Murine L-929 cells were purchased from the American Type Culture Collection and were grown in Dulbecco's MEM, 10% FBS containing gentamicin (0.05 mg/ml) and added glutamine (200 mM).

All cells were routinely tested and found to be free of *Mycoplasma* contamination using the Gen-Probe assay kit.

Methods

Conjugation of ZME-018 with TNF

The conjugation of recombinant TNF to antibody ZME was performed as previously described [24]. The purification and tests for binding were also performed as previously described.

The biological activity of the ZME-TNF immunoconjugate was determined using the standard bioassay for TNF activity examining cytotoxicity against murine L-929 cells in culture [25]. The specific activity of the ZME-TNF preparation was found to be 4.6×10^5 untis/mg protein.

In vitro cytotoxicity of TNF and ZME-TNF

To examine the cytotoxicity of ZME-TNF and TNF, antigen-positive human melanoma cells (A375-M) in MEM, 10% fetal bovine serum were plated into 96-well plates at a density of 5×10^3 cells well and allowed to adhere for 24 h at 37 °C in 5% CO_2. After 24 h, the medium was replaced with medium containing different concentrations of either TNF or ZME-TNF conjugate [8]. The effect of TNFα and ZME-TNF on the growth of tumor cells in culture was determined by crystal violet staining. After the plates had been incubated with TNF and ZME-TNF for 72 h at 37 °C, medium was aspirated, and the cell monolayers were rinsed three times with phosphate-buffered saline (PBS). Following the final rinse, cells were fixed and stained by the addition of 0.5% crystal violet in 20% methanol (0.1 ml/well). The plates were rinsed three times in deionized water and crystal violet was extracted from adherent cells by the addition of 0.2 ml Sorenson's buffer/well (0.1 M sodium citrate, pH 4.2, in 50% ethanol). Cell plates were vortexed for 30 min at room temperature and the absorbance was read at 540 nm (Bio-Tek Instruments, Winooski, Vt.) and compared with control wells (medium alone). Values shown are the mean of duplicate experiments performed in octuplicate.

Tissue distribution studies of radiolabeled ZME-TNF, TNF AND ZME antibody using p-iodobenzoate

One drawback in the use of ^{125}I- or ^{131}I-labeled protein in vivo is the potential for rapid and extensive dehalogenation. A novel procedure for radioiodination has been described and utilized for monoclonal antibodies, which incorporates iodine into protein via a metabolically stable linkage [25]. This method conjugates *N*-succinimidyl *p*-iodobenzoate to the protein. Briefly, 37.5 µl 1% HOAc/MeOH, 10 µl 1 mg/ml *N*-chlorosuccinimide in MeOH and 10 µl PBS were sequentially added to a reaction vial fitted with a rubber septum containing *N*-succinimidyl 4-tri-*n*-butylstannylbenzoate (Neorx Corp., Seattle, Wash.) (12.5 mg) in 12.5 µl HOAc/MeOH. A 1-mCi aliquot of ^{125}I (Dupont) was added to the reaction solution and, after 5 min, the reaction was quenched by addition of 10 µl 0.1 M $NaHSO_3$. The MeOH solvent was evaporated under a N_2 stream for 10 min. A 500-µg sample of protein in 100 µl PBS was mixed with 100 µl 0.5 M borate buffer (pH 9.3) and then added to the reaction vial. The conjugation was allowed to proceed for 5 min at room temperature. Unreacted radioiodine was removed by chromatography on a Sephadex G-25 (PD-10) column (Pharmacia LKB Biotechnology, Piscataway, N. J.). The radiochemical yield was 40%–60%. Incorporation of radiolabel into protein measured by trichloroacetic acid precipitation was greater than 90%. The specific activity of radiolabeled proteins ranged between 0.2 µCi/µg and 0.4 µCi/µg.

Immunoreactivity assay

The immunoreactivity of radiolabeled ZME-018 and ZME-TNF was evaluated using the Lindmo method [26]. Briefly, melanoma cells (2×10^6 A375-M) were incubated with various concentrations of ^{125}I-labeled antibody or immunoconjugate for 1 h at 4 °C. The cells were washed with PBS containing 1% bovine serum albumin, lysed with 2% NP-40 (Sigma) and counted in a gamma counter (Packard model 5360). The immunoreactivity values ranged from 40% to 60% for

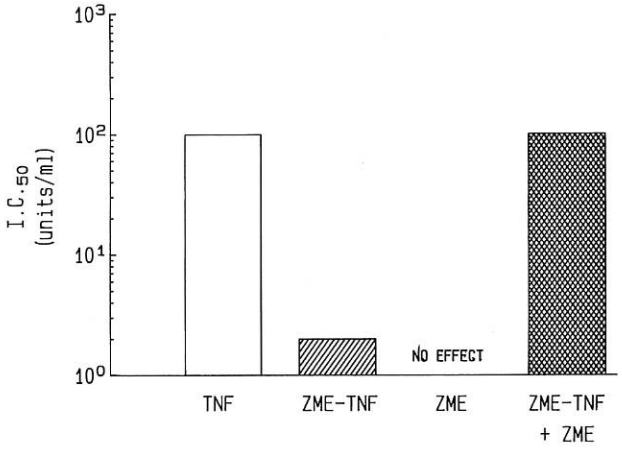

Fig. 1 Antiproliferative effect of tumor necrosis factor (*TNF*) and ZME-TNF on A375-M melanoma cells with and without addition of mAb ZME-018 (10 μg/ml). mAb ZME-018 (10 μg/ml) alone was not cytotoxic to A375-M cells. Various concentrations of TNF and ZME-TNF were added to log-phase cells and incubated for 72 h at 37 °C

both ZME-TNF immunoconjugate and ZME-018 monoclonal antibody.

Animal model studies

Tissue distribution study

Athymic (*nu/nu*) mice, 4–6 weeks old, were obtained from Harlan Sprague Dawley, Indianapolis, Ind. The animals were maintained under specific-pathogen-free conditions and were used at 6–8 weeks of age. Animals were injected subcutaneously, (right flank) with 2×10^6 log-phase A375-M melanoma cells and tumors were allowed to establish for 3 weeks. Monoclonal antibodies and immunoconjugates were labeled with ^{125}I 24 h prior to injection at a specific activity 0.3 μCi/μg protein. After the immunoreactivity of the antibody and immunoconjugate had been examined, mice were injected (i.v. tail vein) with 5 μCi label and 10 μg total protein in 200 μl normal saline. Mice were sacrificed by cervical dislocation 24 h and 72 h following injection. Samples of blood, tumor, heart, lung, liver, spleen, kidney, intestine and muscle were removed, weighed and assayed for radioactivity in a Packard gamma counter (model 5360). The percentage of injected mAb per gram of tissue (%ID/g) in tumor and normal organs was calculated. Tumor-to-blood or tumor-to-organ ratios were also calculated by dividing the mAb (%ID/g) in tumor by the mAb (%ID/g) in the respective organ.

Pharmacokinetic study

BALB/c mice, 4–6 weeks old, were injected with 0.3 μCi (5 μg) either labeled mAb ZME-018 or ZME-TNF immunoconjugate; 15, 30, 45, 60, 75, 90, 105, 120, 240 min and 24 h after injection, two mice at each assay time were sacrificed by cervical dislocation. Blood samples were removed (chest cavity), weighed and counted to determine total radioactivity in a gamma counter (Packard, model 5360). The blood samples were also centrifuged and plasma was decanted and counted to determine radioactivity. Results from plasma determinations of radioactivity were analyzed by a least-square nonlinear regression (RSTRIP, from MicroMath, Inc.) program to determine pharmacokinetic parameters.

In vivo efficacy study

BALB/c nude (*nu/nu*) mice, 4–6 weeks old, were injected with 2×10^6 A375-M log-phase melanoma cells subcutaneously in the right flank. The tumors were allowed to establish for 3 weeks prior to the start of therapy and the mice were divided into three groups. Each treatment group had five mice with 100- to 200-mm^3 established tumors. The mice were injected (i.v. tail vein) with either saline, ZME-018 or ZME-TNF immunoconjugate (10000 units/injection into each mouse) daily for 5 days followed by 10 days off therapy and another course of therapy for 5 days. At the end of therapy, the mice were monitored for an additional 30 days.

Results

Cytotoxicity of ZME-TNF in vitro

The antiproliferative effect of TNF and ZME-TNF against cells in log phase is shown in Fig. 1. The concentrations required to inhibit cell growth by 50% of control values were 100 U/ml free TNF but only 2 U/ml ZME-TNF conjugate. Co-administration of ZME-018 antibody (50 μg/ml) with ZME-TNF conjugate shifted the IC_{50} values of the ZME-TNF conjugate to that of free TNF alone, suggesting that the augmented cytotoxicity observed with the antibody conjugate may be due to its interaction with the gp240 antigen on the cell surface. Since addition of ZME-018 alone to these cells had no appreciable cytotoxic or cytostatic effects, the TNF component of the conjugate appears to be solely responsible for the cytotoxic events observed.

In vivo pharmacokinetics of ZME, TNF and ZME-TNF conjugate

As described in Materials and methods, ZME, TNF, and the ZME-TNF immunoconjugate were radiolabeled using the *p*-iodobenzoate method described above and previously [25]. Figure 2 shows the plasma clearance of both radiolabeled TNF and ZME-TNF conjugate. The clearance of both agents as well as that of native ZME-018 (not shown) was biphasic and closely fit ($r^2 > 0.94$) an open, two-compartment mathematical model. As shown in Table 1, the half-lives for ZME-018 and ZME-TNF were similar in this model with α-phase half-lives of 83.5 min and 72 min respectively. In addition, the β-phase half-lives were also similar at 41.3 h and 36.1 h respectively. In contrast, the clearance of free TNF in this model was relatively rapid with α- and β-phase half-lives of 27.1 min and 2.7 h respectively. The immediately apparent volume of distribution (V_d) for ZME-018 alone approximated the blood volume (1.9 ml) while TNF alone had a somewhat larger V_d, suggesting a greater distribution outside the vasculature. The ZME-TNF conjugate displayed a higher V_d than either ZME-018 or TNF, suggesting a more extensive extravascular disposition than either of its component agents. The area under the concentration curve ($c \times t$) for TNF was substantially lower than that of ZME-018 alone (3.51 compared to 139.6 μCi ml^{-1} min) because of its relatively short plasma half-life. THE-$c \times t$ for ZME-TNF was substantially larger than that of TNF (approx. 3-fold) and approximately 10-fold lower than that of ZME-018 because of its relatively greater distribution outside the vasculature.

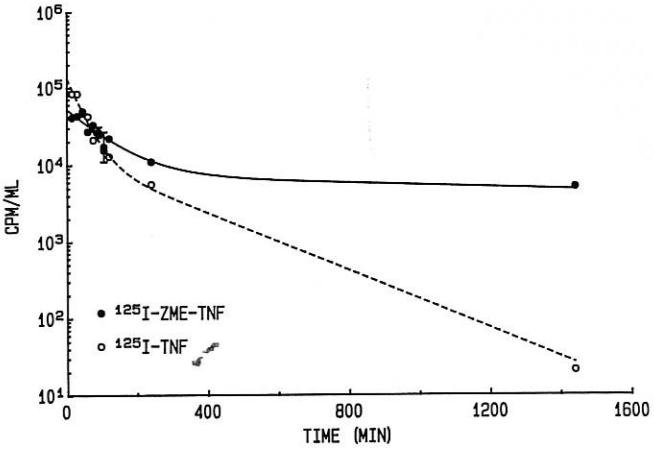

Fig. 2 Plasma clearance of ^{125}I-labeled TNF and ZME-TNF conjugate in BALB-C mice. The figure shows the data points and best-fit least-square line through the data points. Both curves are biphasic with TNF clearance significantly faster than that of the ZME-TNF conjugate

Tissue disposition of ZME-018 and ZME-TNF in a melanoma xenograft model

Radiolabeled ZME-018 or ZME-TNF were spearately administered to groups of five nude mice bearing subcutaneous, well-developed human melanoma (A375-M) xenografts. The animals were sacrificed 72 h after administration, and tissue and blood samples were analyzed for radioiodine content as described above. As shown in Fig. 3, uptake of ^{125}I ZME-018 was highest in tumor tissue with smaller amounts found in lung, spleen, kidney, heart and liver. After administration of ^{125}I ZME-TNF, the uptake of radiolabel was highest in tumor tissue followed by kidney, liver heart spleen and lung. Compared to the tissue distribution found for ZME, the conjugate displayed an increased concentration primarily in liver and kidney, suggesting that these sites may be primarily responsible for the increased V_d of the conjugate compared to that of native ZME.

In vivo antitumor effects of TNF and ZME-TNF

Groups of mice bearing well-developed (100 mm^3) A375-M xenografts were treated (i.v. tail vein) with either saline, TNF (500 000 U mouse^{-1} day^{-1}) or ZME-TNF (500 000 U mouse^{-1} day^{-1}) daily for 5 days followed by no treatment for 10 days and then another course of daily injections for 5 days. Mice were observed and their tumors were measured daily. As shown in Fig. 4, tumors in the saline-treated mice had increased over 20-fold by day 40 and stabilized in size thereafter. The TNF-treated mice showed a delay in growth increasing by 12-fold over the initial tumor volume by day 53. At the termination of the experiment, tumors in all animals in this group were clearly increasing in volume. In contrast, the group of mice treated with the ZME-TNF conjugate showed only a modest 5-fold increase in tumor volume by day 40 and were clearly decreasing in size at the end of the observation period. One mouse in this group showed complete disappearance of the tumor nodule. The ZME-TNF treatment group demonstrated a statistically significant ($P < 0.002$) decrease in tumor size compared to the saline-treated control group.

Table 1 Pharmacokinetic studies of ^{125}I ZME vs ZME-TNF vs TNF

Parameter	ZME	ZME-TNF	TNF
Plasma Half Life (α) (min)	83.50	71.95	27.08
Plasma Half Life (β) (hrs)	41.30	36.05	2.68
Vd (ml)	1.91	11.68	3.95
Cxt (μ Ci/ml × min)	139.60	12.61	3.51
Clp (ml/kg × min)	0.16	1.09	3.38

Discussion

Immunoconjugates made with monoclonal antibodies and a variety of chemotherapeutic agents or protein toxins have been extensively studied over the past few years [27–30]. Monoclonal antibodies provide not only for enhanced localization to tumor tissue after in vivo administration, they also have the potential to increase the plasma half-life of therapeutic agents [31]. As shown in this study, TNF has a relatively short serum half-life (2 h) compared to monoclonal antibodies (typically 40 h). The ZME-TNF immunoconjugate also demonstrates a much longer serum half-life thereby increasing the circulating time of biologically active TNF. In addition to these properties, the incorporation of a tumor-cell-binding domain to TNF appears to increase the cytotoxic properties of TNF in culture.

The explanation for these observations is unclear since TNF is known to operate through interaction with a cell-surface receptor [19, 32]. One possibility is that ZME-018 may have a higher affinity for the gp240 surface antigen than TNF has for its receptor. The ZME-TNF conjugate may then provide for a prolonged surface contact thus holding active TNF at the cell surface for protracted interaction with its surface receptor compared to free TNF. Another possibility is that there are far more gp240 surface sites per melanoma cell (100 000–1 000 000) compared to TNF receptor sites per cell (1000–2000). Therefore, more TNF may be bound per cell after ZME-TNF than after TNF addition.

Our initial studies with native TNF demonstrated that this agent requires a sustained (18 h) contact with target cells in vitro in order to generate a cytotoxic effect [33]. Studies with the ZME-TNF conjugate, on the other hand, suggest that only 1–2 h contact is required to produce cytotoxicity. Therefore, some of the improved performance of the conjugate appears to be simply due to its increased number of cell binding sites and increased affinity for cells compared to native TNF.

Studies by Mujoo et al. [34] have examined the effect of TNF administration on the biodistribution of both relevant and irrelevant monoclonal antibodies in a mouse/human-tumor xenograft model. While TNF administration was

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