Preferential Activity of Wild-type and Mutant Tumor Necrosis Factor-α against Tumor-derived Endothelial-like Cells

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Tumor-derived endothelial-like cells (tEC) were prepared by culturing human umbilical vein endothelial cells (HUVEC) in the presence of HT1080 human fibrosarcoma-conditioned medium. tEC showed higher permeability and less cell-adhesion activity than normal HUVEC (nEC). Tumor necrosis factor-α (TNF) is known to have tumor-vasculature disrupting activity. tEC showed higher cytotoxicity to recombinant human TNF (rhTNF) than nEC, and was not observed using HUVEC cultured with WI38 human diploid cell-conditioned medium as a medium-control. These results demonstrate that tEC acquire physiological properties of tumor-associated vasculature, and may be a useful model system for the study of the mechanisms of TNF antitumor action. The TNF-mutant RGD-V29 (code No. F4614), which has an inserted 4Arg-Gly-Asp sequence and an 29Arg→Val replacement, was found to induce greater preferential destruction of tEC compared to rhTNF. When the preferential activities were evaluated in terms of 30% cytotoxicity (IC30) ratio (nEC/tEC), the ratio was 460 for RGD-V29 compared to 4.2 for rhTNF. RGD-V29 also exhibited cell-adhesive function and bound preferentially to the p55 TNF-receptor. Both these properties of RGD-V29 contributed to the tEC selective cytotoxicity, indicating that the RGD ligands and selective p55 receptor binding on the cells, although uncharacterized, are involved in tEC targeting. Therefore, the TNF mutant RGD-V29 may show greater selectivity toward tumor vasculature than wild-type TNF.

Key words: Tumor-derived endothelial cells — Tumor vascular targeting — TNF-α — TNF-mutant RGD-V29 — F4614

Targeted disruption of tumor vasculature has the potential to become a highly effective strategy to fight cancer. A single administration of tumor necrosis factor-α (TNF) can cause rapid disruption of the tumor vasculature, and can lead to necrosis of tumor tissues.10 While TNF clearly displayed preferential activity toward tumor-associated vasculature in angiographic studies,2 3 intravenously administered TNF also induced destruction of normal tissue microvasculature.4 5 Thus, TNF is not administered to patients due to the severe systemic side effects: the maximum tolerated dose (MTD) is only 350–500 µg/m2, at least 10-fold less than the effective dose in animals.6 Eggermont et al. demonstrated that raised local TNF levels using the isolated limb perfusion (ILP) technique induced tumor-endothelial cell damage that resulted in a 90% complete response rate in melanoma and 60% in sarcoma cancer therapies.2 3 7 Tsutsumi et al. reported tumor accumulation of TNF by modifying TNF with polyethylene glycol (PEG-TNF) and found that this resulted in marked anti-tumor activity without toxicity in tumor-bearing mice.8 9 Also, we have previously reported that a human TNF mutant V29 (29Arg→Val), which preferentially binds to the TNF receptor p55, was less toxic in mice, while retaining its antitumor efficacy.11 The p55 receptor is one of two types of TNF receptor (p55 and p75), and contains an intracellular death domain12 capable of triggering cell death (apoptosis). Another mutant, RGD-T (F4618), containing the cell-adhesive sequence Arg-Gly-Asp (RGD), has also been shown by histopathology to have decreased gastrointestinal toxicity compared with recombinant human TNF (rhTNF; SSSRTPSDK...155L).5 It is thought that the RGD sequence may be involved in tumor targeting. Indeed, the combined mutant RGD-V29 (S5SSRGDSDK...29VR...155L; F4614) has been shown to have potent anti-tumor activity with lower lethal toxicity in mice.13 Tumor blood vessels are distinct from blood vessels in normal tissue in several important functional respects. First, the permeability of tumor blood vessels and tumor vasculature is higher than that of normal blood vessels, both in vitro and in vivo.14 16 Second, less interaction between blood vessels and leukocytes is observed in tumor compared to normal microvessels.14 17 18 In previous studies, we have shown that tumor-derived endothelial cells (TEC), isolated from a rat KMT-17 fibrosarcoma, pas-
saged in normal culture medium lost the hyperpermeability and leukocyte adhesion-deficient properties, while TEC in the primary culture retained these properties. Conversely, the phenotype of normal tissue-derived endothelial cells can be changed to a tumor-derived endothelial cell phenotype by culturing the endothelial cells in tumor cell-conditioned medium. In this paper, we report that human umbilical vein endothelial cells (HUVEC) cultured in the presence of human tumor cell (HT1080)-conditioned medium (tEC; tumor-derived endothelial-like cells) acquired preferential TNF-sensitivity compared with rhTNF.

Materials and Methods

Endothelial cells

HUVEC used in this study were isolated from freshly obtained umbilical cords by 0.2% (w/v) collagenase digestion according to Jaffe et al. Cells were maintained in gelatin-coated dishes in MCDB107 medium supplemented with 10% FBS (fetal bovine serum, Gibco BRL, Grand Island, NY), 100 μg/ml BPE (bovine pituitary extract, Becton Dickinson, Lincoln Park, NJ), 100 μg/ml heparin (heparin sodium salt type IA, Sigma), 100 U/ml penicillin, and 100 μg/ml streptomycin. Endothelial phenotypes were identified using Factor VIII-related antigen, and routinely assayed using acetylated low density lipoprotein labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI-Ac-LDL, BTI, Stoughton, MA). HUVEC were maintained at split ratios of 1:2 or 1:3 and used up to 12 passages, with each harvest and split referred to as one passage. All cultures were maintained at 37°C in a humidified 5% CO2 atmosphere.

Assay media

To obtain conditioned medium (CM), HT1080 (a human fibrosarcoma cell line; for TEC) and WI38 (a human embryonic lung fibroblast cell line; for EC as cells cultured in a control medium) were seeded at 1×10⁵/well and further cultured for 4 days in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FBS, and adherent cells were fixed with 4% formaldehyde and counted under a microscope.

Preparation of rhTNF, RGD-V29, RGD-T and V29

Recombinant-hTNF (SSS’RTPSDK...29RR...155L) and mutant TNF RGD-V29 (SSS’RGDSDK...29VR...155L; F4614) were prepared by protein engineering. Specific activities of rhTNF and RGD-V29, determined by the L929 cell lytic assay, were 2.5×10⁷ and 2.6×10⁶ units/mg-protein, respectively. Other mutants RGD-T (SSS’RGDSDK...29RR...155L; F4168) and V29 (SSS’RTPSDK...29VR...155L) were also prepared in our laboratory. The specific activities of RGD-T and V29 were 2.8×10⁶ and 2.3×10⁶ U/mg-protein, respectively. One laboratory unit was equivalent to 2.7 international units (uTNF; international reference WHO87/650, was a gift from the National Institute for Biological Standards and Control, South Mimms, UK). Endotoxin content of the samples was less than 3.8 ng/mg-protein (Limulus amebocyte lysate assay, Wako, Osaka).

Measurement of permeability

To assess the permeability of tEC and nEC monolayers, the amount of fluorescein-isothiocyanate (FITC)-labeled dextran (FD70, Sigma) passing across an endothelial monolayer was measured using a permeation chamber. Briefly, TEC and nEC were cultured in the upper compartments of permeation chambers (Intercell, Kurabo, Osaka) coated with collagen type IA (CellmatrixIA, Nitta Gelatin, Osaka), and allowed to form monolayers in assay media. On day 6, 10 μM FITC-dextran was added to the upper compartments (0.2 ml), and the lower compartments were filled with 0.6 ml of HEPES-buffered Ringer’s solution (pH 7.4). After 0.5 h at 37°C, fluorescence in the lower compartments was measured (EX.495/EM.550 nm). Permeability coefficients were calculated from the following equation:

\[ P_{EC} = \frac{1}{P_{total}} - \frac{1}{P_{EC}} \]

where \( P_{EC} \), \( P_{total} \), and \( P_{M} \) represent the permeability coefficient of HUVEC monolayer alone, HUVEC monolayer over the membrane, and membrane alone, respectively.

Adhesion assay for EC preparations

Adhesive functions of TEC and nEC were evaluated using HL60 cells. EC preparations that had been cultured for 3 days in assay media were seeded at 1×10⁵/well and further cultured for 2 days in gelatin-coated microplates. EC monolayers and 1×10⁴/ml HL60 were pretreated with or without rhTNF for 4 h. Cells were washed with MCDB107 containing 10% FBS, and HL60 were suspended at 1.0×10⁷/ml in MCDB107 containing 10% FBS, 100 μg/ml BPE and 100 μg/ml heparin. HL60 cell suspensions (0.1 ml) were added to the EC monolayers and incubated for 30 min at 37°C, non-adherent cells removed by two rounds of aspiration, and adherent cells were fixed with 4% formaldehyde and counted under a microscope.

Cytotoxicity assay

For evaluation of the cytotoxic activity of rhTNF and RGD-V29 against tumor-associated endothelial cells, a human model cell system was constructed. HUVEC (1×10⁵/well) were cultured in appropri-
reate media to allow the generation of tEC, nEC, and cEC cultures in gelatin-coated microplates (Corning, Corning, NY) for 3 to 5 days. Various concentrations of rhTNF or RGD-V29 were then added in fresh medium containing 1 µg/ml cycloheximide (CHX). After 21 h at 37°C, the cell viability (%) was determined by MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added at 0.5 mg/ml and incubated for 3 h. After centrifugation at 2000g for 5 min, MTT-formazan deposits were dissolved with 0.1 ml of dimethyl sulfoxide (DMSO) and measured at 540 nm using an ELISA reader.

Cell-adhesion assay against immobilized RGD-V29 To determine whether RGD-V29 contained a functioning cell-adhesive sequence, an adhesion assay was carried out as described previously.24) Briefly, 50 µg/ml rhTNF, RGD-V29 or fibronectin (as a positive control), and phosphate-buffered saline (PBS) without Ca2+ and Mg2+ (PBS(−), i.e. non-coated) were coated onto 96-well microplate wells. After 2 h at room temperature, wells were washed and blocked with 100 µl/well 0.1% bovine serum albumin for 2 h at room temperature. Wells were washed 3 times with PBS(−) and immediately used for the adhesion assay. HUVEC (2×10⁴/ml) in serum-free MCDB107 were prepared and cells were seeded at 2×10⁴/well. Plates were incubated for 1.5 h at 37°C and agitated every 15 min. Non-adherent cells were removed by two rounds of aspiration with serum free-MCDB107 and adherent cells were observed under a phase-contrast microscope.

Radioligand binding analysis HEp-2 (a human epidermoid carcinoma cell line; expressing mainly TNF receptor p55) and HL60 (a human promyelocytic leukemia cell line; expressing mainly p75) were cultured in RPMI1640 containing 10% FBS. Expression ratios of the two receptors on HEp-2 and HL60 were confirmed using anti-p55
and anti-p75 monoclonal antibodies as 8:2 (p55:p75) and 2:8–3:7 (p55:p75), respectively. rhTNF and RGD-V29 were radioiodinated using Na[125I] (Amersham, Aylesbury, UK) and Enzymobeads (BioRad, Richmond, CA), with specific radioactivities of 9.0–10.2 and 5.4 µCi/µg, respectively. Competition binding assays using HEP-2 and HL60 cells were performed as described previously. Saturation binding assays using HL60 cells were performed by incubating 1×10⁶ cells with increasing concentrations (0.32 to 21 nM) of [125I]rhTNF or [125I]RGD-V29 with or without a 200-fold excess of unlabeled rhTNF or RGD-V29 in final volumes of 0.11 ml, followed by incubation for 2 h in RPMI1640 medium containing 2% FBS and 0.2% NaN₃ (incubation medium) with agitation every 15 min on ice. Cells were then washed 3 times in incubation medium, and radioactivity bound to the cells was determined with a γ counter.

RESULTS

Properties of tEC
HVECs cultured in the presence of 30% HT-CM formed tEC monolayers on gelatin-coated dishes. While HVECs cultured without conditioned medium (nEC) exhibited a cobblestone pattern at confluence, a typical morphological feature of endothelial cells (Fig. 1B), tEC did not exhibit such a pattern (Fig. 1A). Although the numbers of tEC or nEC within the monolayers were similar (data not shown), tEC exhibited a tubelike morphology.

In order to investigate the functional properties of tEC monolayers, permeability and cell-adhesive properties were measured. The relative permeabilities of tEC and nEC were measured using a membrane-culture chamber and the results indicated that the permeability of tEC was 1.7-fold higher than that of nEC (Fig. 1C).

While no significant differences in adhesion to HL60 cells were observed between the EC preparations under non-stimulatory conditions, pre-treatment with rhTNF altered the adhesion activity of both EC types (Fig. 1D). HVECs treated with TNF are known to show upregulated expression of ICAM-1 and E-selectin, resulting in increased leukocyte adhesion. The number of HL60 cells that adhered to nEC was markedly increased (3.7×10⁴/cm²) after treatment with 1 ng/ml of rhTNF. In contrast, we observed a loss of adhesion function by tEC (2.2×10⁴/cm²) even after the addition of 10 ng/ml rhTNF.

Cytotoxicity of rhTNF and TNF mutants towards tEC and nEC
After treatment with 1 µg/ml CHX, rhTNF showed cytotoxic activity towards all the EC preparations in a dose-dependent manner. However, compared to non-treatment, approximately 50% of nEC survived treatment even at 1000 U/ml, whereas the survival rate of tEC was decreased by 30% (Fig. 2A). Similar to nEC, HVECs cultured in the presence of 30% of WI-CM as a CM-control (cEC) also showed less sensitivity to rhTNF. Thus, tEC showed preferential sensitivity to rhTNF. Interestingly, the survival rate of nEC after treatment with RGD-V29 was increased compared to rhTNF, while the survival of tEC remained similar to that of nEC.
remained the same (Fig. 2B). The RGD-V29 concentration required for 30% cytotoxicity (IC₃₀) was 5.6 U/ml for tEC, compared to 2600 U/ml for nEC (Table I). The IC₃₀ ratio (nEC/tEC) for RGD-V29 was 460, in contrast to rhTNF which had IC₃₀ values of 22 and 5.2 U/ml for nEC and tEC, respectively, resulting in an IC₃₀ ratio (nEC/tEC) of 4.2 (Table I). The relative increase of the RGD-V29 IC₃₀ ratio was therefore due to the loss of cytotoxicity to nEC (Fig. 2B and Table I). Table I also shows the cytotoxic activities of TNF mutants RGD-T and V29, which contain the 4R 5G 6D and 29R→V mutations, respectively, towards EC preparations. The IC₃₀ values of RGD-T for nEC and tEC were 1000 and 8.6 U/ml, respectively, and those of V29 for nEC and tEC were 1300 and 4.6 U/ml, respectively. Both mutants showed a loss of cytotoxicity towards nEC, while retaining activity towards tEC.

Properties of TNF-mutant RGD-V29

The N-terminal end of the mutant TNF RGD-V29 contains the RGD cell-adhesion sequence. To confirm the cell-adhesive properties of the mutant TNF, HUVEC adhesion to RGD-V29 immobilized onto microplate wells was tested (Fig. 3). Fibronectin, which also contains an RGD sequence, was used as a positive control (Fig. 3B). While HUVEC exhibited adhesion to immobilized RGD-V29, with adherent cell spreading, HUVEC adhesion was not observed to immobilized rhTNF (Fig. 3, C and D).

RGD-V29 contains a second mutation, 29R→V. This amino-acid position is involved in the interaction with the

| IC₃₀ (U/ml) values were obtained from means of 3 or 4 experiments performed in duplicate using 2 individual HUVEC cultures. nEC/tEC-ratios were then calculated from the IC₃₀ values obtained. |

|          | nEC  | RGD-T | V29  | RGD-V29 |
|----------|------|-------|------|---------|
| rhTNF    | 22   | 1000  | 1300 | 2600    |
| tEC      | 5.2  | 8.6   | 4.6  | 5.6     |
| nEC/tEC | 4.2  | 120   | 280  | 460     |

Fig. 3. Adhesion of HUVEC to immobilized RGD-V29. Microplate wells were coated with the indicated proteins (A) non-coated, (B) fibronectin, (C) RGD-V29, and (D) rhTNF.
Fig. 4. Receptor binding activity of RGD-V29. Displacement curves showing inhibition of specific binding of $[^{125}\text{I}]$rhTNF by unlabeled rhTNF (△) or RGD-V29 (●) to HEp-2 (A) and HL60 (B) cells. Results are the means±SD of 2 experiments performed in triplicate. The relative binding constants (%) of RGD-V29 to HEp-2 (○) and HL60 (■) cells were calculated as (IC$_{50}$ of rhTNF)/(IC$_{50}$ of RGD-V29 or rhTNF)$\times$100 (C). Scatchard analysis of $[^{125}\text{I}]$rhTNF (D) and $[^{125}\text{I}]$RGD-V29 (E) binding to HL60 cells. Specific binding was determined in duplicate for each concentration. B/F, bound/free. (Inset) Saturation curves. Specific binding (●), total binding (○) and non-specific binding (△). Results were from a single experiment repeated twice, performed in duplicate.
TNF receptors. To characterize this mutation, affinities to the TNF receptors p55 and p75 were determined using two cell lines, HEp-2 (which expresses mainly p55) and HL60 (which expresses mainly p75). Competitive binding assays were performed. For HEp-2 cells, the concentrations required to inhibit 50% binding (IC\textsubscript{50}) of rhTNF and RGD-V29 were 0.063 and 0.15 \( \mu \text{g/ml} \), respectively (Fig. 4A). For HL60 cells, the IC\textsubscript{50} of RGD-V29 was 2.2 \( \mu \text{g/ml} \), compared to 0.051 \( \mu \text{g/ml} \) for rhTNF (Fig. 4B). Thus, the binding affinities of RGD-V29 to both cell lines were lower than for rhTNF; the relative binding constants for RGD-V29 being 42% on HEp-2 and 2.3% on HL60 compared to rhTNF. However, the ratio of the relative binding constants for RGD-V29 (HEp-2/HL60) was 18 (Fig. 4C) compared to 1.0 for rhTNF. This suggested that RGD-V29 bound preferentially to p55. Saturation binding assays were then performed on HL60 cells. Scatchard analysis of rhTNF showed a curvilinear profile characteristic of two binding sites: a high-affinity (p75; \( K_d = 4.8 \times 10^{-11} \) \( M \)) component with 4500 sites/cell and a low-affinity (p55; \( K_d = 0.63 \times 10^{-9} \) \( M \)) component with 1100 sites/cell (Fig. 4D). Scatchard analysis of RGD-V29 indicated a single binding site corresponding to the low-affinity (\( K_d = 1.0 \times 10^{-9} \) \( M \)) binding site with 1700 sites/cell (Fig. 4E). Taken together, these results demonstrate that RGD-V29 binds preferentially to the p55 receptor.

**DISCUSSION**

It is clear that targeting anticancer drugs, including TNF, to tumor tissues is a highly advantageous therapeutic intervention for cancer. Our results demonstrated that a model human cell system that mimicked tumor-derived endothelial cells could be useful in the development of tumor-associated endothelial cell targeting therapies, and that the TNF mutant RGD-V29 exhibited more preferential cytotoxicity than rhTNF towards tEC. As shown in Fig. 1, tEC prepared by culturing HUVEC in the presence of 30% HT-CM acquired two characteristic properties of tumor vascular endothelial cells; hyperpermeability and deficient cell-adhesive function. This latter property was further shown by the decreased interaction between HL60 and tEC after stimulation by rhTNF (Fig. 1C). HUVEC treated with TNF are known to show up-regulated expression of E-selectin, VCAM-1 and ICAM-1, leading to increased leukocyte adhesion. Accordingly, the diminished adhesive function of tEC suggests that adhesion molecules were not properly expressed. While there is little direct evidence to support a mechanism for the decreased adhesion function of tEC compared with that of nEC, it is clear that TNF can directly increase the cell-adhesion properties of tEC. Such an effect of TNF would promote increased adhesion to tumor vasculature and could result in an indirect antitumor effect through increasing the number of cytotoxic effector cells that become localized in tumor vessels.

In accordance with several previous studies, including ours, HUVEC acquired a tumor-derived endothelial cell phenotype after culture in human tumor (HT1080)-conditioned medium. However, as described previously, intact TEC isolated from the rat fibrosarcoma KMT-17 lost hyperpermeability and leukocyte adhesion-deficient properties after passage in normal culture medium. Thus, alterations in endothelial cell phenotype, at least in terms of permeability and adhesive function, are reversible in vitro and can be induced by tumor cell-conditioned media alone.

Furthermore, tEC showed higher sensitivity than nEC to direct TNF cytotoxicity (Fig. 2A). In contrast, HUVEC cultured in the presence of 30% WI-CM as a CM-control (cEC) did not show strong sensitivity to rhTNF. These results are consistent with the physiological properties of tumor-associated vasculature in the presence of TNF, and support, at least quantitatively, other data on TNF-related phenomena in clinical and animal model studies.

Like rhTNF, RGD-V29 showed preferential cytotoxicity towards tEC (Fig. 2B). However, the cytotoxicity of RGD-V29 towards nEC was much less than that of rhTNF, with an IC\textsubscript{50} ratio (nEC/tEC) of 460 for RGD-V29 compared to 4.2 for rhTNF (Table I and Fig. 2B). Clearly, RGD-V29 had greater preferential activity towards tEC compared to rhTNF. Some phase I trials using TNF have indicated that the MTD is \( \leq 350 \mu \text{g/m}^2 \) intravenously, with peak plasma TNF levels of 90–900 pg/ml, corresponding to 2.3–23 U/ml. While the IC\textsubscript{50} of both rhTNF (5.2 U/ml) and RGD-V29 (5.6 U/ml) for tEC, and the IC\textsubscript{50} of rhTNF (22 U/ml) for nEC, were associated with the plasma TNF levels, that of RGD-V29 (2600 U/ml) to nEC was not. Therefore, where intravenously administered TNF would lead to destruction of normal tissue-endothelial cells, the same concentration of RGD-V29 may have the ability to induce significant destruction of tumor-associated endothelial cells with little toxicity to normal tissue-endothelial cells.

The TNF mutant V29 contains the amino acid Val at position 29 of the TNF protein, which leads to preferential binding to the p55 receptor, while the RGD-T mutant has a cell-adhesive function through the RGD sequence. As RGD-V29 retained both preferential binding to the p55 receptor (Fig. 4), and cell-adhesive function (Fig. 3), our results demonstrated that both mutations of RGD-V29 apparently contributed to preferential cytotoxicity towards nEC (Table I). Therefore, it is possible that as no signal was delivered to the p75 receptor, RGD-V29 could only transduce the cell-death signal through p55, resulting in the selective destruction of tEC with less toxicity toward nEC compared to rhTNF. The RGD sequence is known to be a ligand of integrin \( \alpha_v \beta_3 \), which is expressed on angiogenic endothelial cells, including during tumor angiogene-
sis, but is not, or is only weakly, expressed on quiescent cells. Antagonists of αβ4, such as cyclic RGD peptide, have been shown to promote tumor regression by inducing apoptosis of angiogenic blood vessels.26,28

In our model cell system, the presence of CHX was required in order to observe TNF-induced cell-death. Although CHX alone did not affect cell viability at 1 µg/ml, cytotoxicity of rhTNF and RGD-V29 towards the EC preparations was not observed in the absence of CHX (data not shown). TNF elicits its multiple biological effects through two distinct cell surface receptors, p55 and p75. The p55 receptor is not only involved in triggering effects through two distinct cell surface receptors, p55 and p75. The p55 receptor is not only involved in triggering the cell-death signal, but also can mediate the activation of nuclear factor-κB (NF-κB) that induces various inflammatory reactions. Several independent studies have reported that the inhibition of NF-κB function (through the use of CHX, for example) potentiated TNF-induced apoptosis, and that the use of agents such as glucocorticoids that block NF-κB function may prove to be highly beneficial in the treatment of tumors when combined with standard cancer therapies. Accordingly, the use of CHX would inhibit NF-κB function, resulting in induction of TNF-induced cell death in tEC. However, the mechanism of TNF specificity for tEC remains unknown. Further study is required, including investigation into the control of the preferential activity of TNF towards tEC, identification of the factors in HT-CM that mediate the conversion of nEC to tEC, and further elucidation of the differences between tEC and nEC, such as p55/p75 TNF-receptor expression. RGD-V29 and related mutant TNF molecules should be useful tools to study the characteristics of tEC.

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REFERENCES

1) Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. and Williamson, B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc. Natl. Acad. Sci. USA, 72, 3666–3670 (1975).

2) Eggermont, A. M. M., Koops, H. S., Lienard, D., Kroon, B. B. R., Van Geel, A. N., Hoekstra, H. J. and Lejeune, F. J. Isolated limb perfusion with high-dose tumor necrosis factor-α in combination with interferon-γ and melphalan for nonresectable extremity soft tissue sarcomas: a multicenter trial. J. Clin. Oncol., 14, 2653–2665 (1996).

3) Renard, N., Nonnen, P. T. G. A., Schalkwijk, L., DeWaal, R. M. W., Eggermont, A. M. M., Lienard, D., Kroon, B. B. R., Lejeune, F. J. and Ruiter, D. J. VWF release and platelet aggregation in human melanoma after perfusion with TNFα. J. Pathol., 176, 279–287 (1995).

4) Tracey, K. J., Butler, B., Lowry, S. F., Merryweather, J., Wolpe, S., Mills, I. W., Hariri, R. J., Fahey, T. J., III, Zentella, A., Albert, J. D., Shires, G. T. and Cerami, A. Shock and tissue injury induced by recombinant human cachectin. Science, 234, 470–474 (1986).

5) Shikama, H., Miyata, K., Sakae, N., Kuroda, K., Nishimura, K., Yotsuya, S. and Kato, M. A novel mutein of TNFα containing the Arg-Gly-Asp sequence shows reduced toxicity in intestine. Mediators Inflammation, 3, 111–116 (1994).

6) Spriggs, D. R., Sherman, M. L., Michie, H., Arthur, K. A., Imamura, K., Wilmore, D., Frei, E., III and Kufe, D. W. Recombinant human tumor necrosis factor administered as a 24-hour intravenous infusion. A phase I and pharmacologic study. J. Natl. Cancer Inst., 80, 1039–1044 (1988).

7) Renard, N., Lienard, D., Lespangnard, L., Eggermont, A., Heimann, R. and Lejeune, F. Early endothelium activation and polymorphonuclear cell invasion precede specific necrosis of human melanoma and sarcoma treated by intra-vascular high-dose tumour necrosis factor alpha (rTNFα). Int. J. Cancer, 57, 656–663 (1994).

8) Tsutsui, Y., Kihi, T., Tsunoda, S., Kanamori, T., Nakagawa, S. and Mayumi, T. Molecular design of hybrid tumour necrosis factor alpha with polyethylene glycol increases its anti-tumor potency. Br. J. Cancer, 71, 963–
Properties of tEC and RGD-V29

968 (1995).
9) Tsutsumi, Y., Tsunoda, S., Kamada, H., Kihira, T., Nakagawa, S., Kaneda, Y., Kanamori, T. and Mayumi, T. Molecular design of hybrid tumour necrosis factor-α. II. The molecular size of polyethylene glycol-modified tumour necrosis factor-α affects its anti-tumour potency. Br. J. Cancer, 74, 1090–1095 (1996).
10) Tsutsumi, Y., Kihira, T., Tsunoda, S., Kamada, H., Nakagawa, S., Kaneda, Y., Kanamori, T. and Mayumi, T. Molecular design of hybrid tumour necrosis factor-α. III. Polyethylene glycol-modified tumour necrosis factor-α has markedly enhanced antitumour potency due to longer plasma half-life and higher tumour accumulation. J. Pharmacol. Exp. Ther., 278, 1006–1011 (1996).
11) Kuroda, K., Miyata, K., Shikama, H., Kawagoe, T., Nishimura, K., Takeda, K., Sakae, N. and Kato, M. Novel muteins of human tumor necrosis factor with potent antitumor activity and less lethal toxicity in mice. Int. J. Cancer, 63, 152–157 (1995).
12) Tartaglia, L. A., Ayres, T. M., Wong, G. H. W. and Goeddel, D. V. A novel domain within the 55 kd TNF receptor signals cell death. Cell, 74, 845–853 (1993).
13) Shikama, H., Miyata, K., Sakae, N., Mitsuishi, Y., Nishimura, K., Kuroda, K. and Kato, M. Novel mutein of tumor necrosis factor α (F4614) with reduced hypotensive effect. J. Interferon Cytokine Res., 15, 677–684 (1995).
14) Uotoguchi, N., Dantakean, A., Makimoto, H., Wakai, Y., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. Isolation and properties of tumor-derived endothelial cells from rat KMT-17 fibrosarcoma. Jpn. J. Cancer Res., 86, 193–201 (1995).
15) Woolcock, J. C. E. and Carr, P. The ultrastructure and permeability characteristics of the blood vessels of a transplantable rat sarcoma. J. Pathol., 107, 157–166 (1972).
16) Dvorak, H. F., Nagy, J. A., Dvorak, J. T. and Dvorak, A. M. Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. Am. J. Pathol., 133, 95–109 (1988).
17) Wu, N. Z., Klitzman, B., Dodge, R. and Dewhirst, M. W. Diminished leukocyte-endothelium interaction in tumor microvessels. Cancer Res., 52, 4265–4268 (1992).
18) Fukumura, D., Salehi, H. A., Wittwer, B., Tuma, R. F., Melder, R. J. and Jain, R. K. Tumor necrosis factor α-induced leukocyte adhesion in normal and tumor vessels: effect of tumor type, transplantation site and host strain. Cancer Res., 55, 4824–4829 (1995).
19) Uotoguchi, N., Mizuguchi, H., Dantakean, A., Makimoto, H., Wakai, Y., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. Effect of tumour cell-conditioned medium on endothelial macromolecular permeability and its correlation with collagen. Br. J. Cancer, 73, 24–28 (1996).
20) Uotoguchi, N., Mizuguchi, H., Saeki, K., Ikeda, K., Tsutsumi, Y., Nakagawa, S. and Mayumi, T. Tumor-conditioned medium increases macromolecular permeability of endothelial cell monolayer. Cancer Lett., 89, 7–14 (1995).
21) Jaffe, E. A., Nachman, R. L., Becker, C. G. and Minick, C. R. Culture of human endothelial cells derived from umbilical veins. J. Clin. Invest., 52, 2745–2756 (1973).
22) Miyata, K., Kato, M., Shikama, H., Nishimura, K., Sakae, N., Kawagoe, K., Nishikawa, T., Kuroda, K., Yamaguchi, K., Aoyama, Y., Mitsuishi, Y. and Yamada, N. A YIGSR-containing novel mutein without the detrimental effect of human TNF-α of enhancing experimental pulmonary metastasis. Clin. Exp. Metastasis, 10, 267–272 (1992).
23) Stiflinger-Birnboim, A., Der Vecchio, P. J., Cooper, J. A., Blumenstock, F. A., Shepard, J. M. and Malik, A. B. Molecular sieving characteristics of the cultured endothelial monolayer. J. Cell. Physiol., 132, 111–117 (1987).
24) Miyata, K., Mitsuishi, Y., Shikama, H., Kuroda, K., Nishimura, K., Sakae, N. and Kato, M. Overcoming the metastasis-enhancing potential of human tumor necrosis factor α by introducing the cell-adhesive Arg-Gly-Asp sequence. J. Interferon Cytokine Res., 15, 161–169 (1995).
25) Bevilacqua, M. P. Endothelial-leukocyte adhesion molecules. Annu. Rev. Immunol., 11, 767–804 (1993).
26) Watanabe, N., Niitsu, Y., Umeno, H., Kuriyama, H., Neda, H., Yamauchi, N., Maeda, M. and Urushizaki, I. Toxic effect of tumor necrosis factor on tumor vasculature in mice. Cancer Res., 48, 2179–2183 (1988).
27) Sherman, M. L., Spriggs, D. R., Arthur, K. A., Imamura, K., Frei, E., III and Kufe, D. W. Recombinant human tumor necrosis factor administered as a five-day continuous infusion in cancer patients: phase I toxicity and effects on lipid metabolism. J. Clin. Oncol., 6, 344–350 (1988).
28) Brooks, P., Clark, R. A. F. and Cheresh, D. A. Requirement of vascular integrin αvβ3 for angiogenesis. Science, 264, 569–571 (1994).
29) Brooks, P. C., Montgomery, A. M. P., Rosenfeld, M., Reisfeld, R. A., Hu, T., Klier, G. and Cheresh, D. A. Integrin αvβ3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. Cell, 79, 1157–1164 (1994).
30) Wang, C.-Y., Mayo, M. W. and Baldwin, A. S., Jr. TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-κB. Science, 274, 784–787 (1996).
31) Van Antwerp, D. J., Martin, S. J., Kafri, T., Green, D. R. and Verma, I. M. Suppression of TNF-α-induced apoptosis by NF-κB. Science, 274, 787–789 (1996).
32) Liu, Z.-G., Hsu, H., Goeddel, D. V. and Karin, M. Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell, 87, 565–576 (1996).