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Lysosome Labilizers Potentiate the Antitumor Effects of Tumor Necrosis Factor-α

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Enhancement of *in vitro* cytotoxic activity of tumor necrosis factor-α (TNF-α) was observed in combination with lysosome labilizers, particularly with urokinase-type plasminogen activator (u-PA), tissue-type plasminogen activator (t-PA) and lipoprotein lipase (LPL). The concentration of TNF-α resulting in 50% cytotoxicity to L929 cells was only 20–30% of the value for TNF-α alone, when used in combination with a nontoxic dose of u-PA, t-PA or LPL. Furthermore, combined intravenous (i.v.) administration of TNF-α (3.5×10^5 U/mouse) and u-PA (300 IU/mouse) markedly increased the *in vivo* antitumor activity of TNF-α to Meth A tumors transplanted into BALB/c mice; the tumor weight in co-administered mice was about 40% of that in mice given TNF-α alone on day 6. The combination therapy of TNF-α (7.0×10^4 U/mouse, i.v.) and u-PA (300 IU/mouse, i.v.) was also effective for L929 tumors in Crj:CD-1(1CR)-nu nude mice compared with the conventional therapy with TNF-α alone. These results suggest that the combination of TNF-α and lysosome labilizers is a promising antitumor therapeutic regimen with clinical potential.

Key words: Tumor necrosis factor-α — Lysosome labilizer — Urokinase-type plasminogen activator — Combination therapy

Tumor necrosis factor-α (TNF-α[5]) induces hemorrhagic necrosis of transplanted tumors *in vivo* and is cytotoxic towards various tumor cell lines *in vitro*.[1] The mechanisms of necrotizing and cytotoxic actions of TNF-α are not understood, but some studies have suggested the involvement of lysosomal enzyme activation[2–5] and hydroxyl radical production.[3,6,7]

Recombinant human TNF-α has been subjected to clinical trials.[8–10] At this time, however, TNF-α appears to be of limited therapeutic value as a single agent. So attempts have been made to combine TNF-α with interferon-γ,[11,12] various antitumor agents[13] and hyperthermia.[14,15]

Lysosome labilizers are substances which interact with and labilize lysosomes, and activate lysosomal enzymes. Niitani and co-workers (for review, see ref. 16) reported a synergistic increase in the antitumor effects of mitomycin C and lysosome labilizers, proposing that both agents synergistically activate lysosomal enzymes, which then cause tumor cell lysis. Therefore, the combined use of lysosome labilizers was expected to increase the therapeutic potency of TNF-α. In this study, we demonstrated that the cytotoxic and antitumor effects of TNF-α were enhanced when TNF-α was used in combination with lysosome labilizers.

MATERIALS AND METHODS

TNF-α and other reagents The *Escherichia coli* strain C600 r^-m^- transformed by the plasmid pTNF401A[17] encoding human mature TNF-α was cultured by the method of Nakamura *et al.*[18] Recombinant human TNF-α was purified from the cell lysate as described previously.[19] The specific activity of the purified TNF-α was 1.4×10^7 U/mg protein, as determined from the cytotoxic activity towards actinomycin D-treated L929 cells.[17] The endotoxin content was less than 20 pg/mg protein by the *Limulus* amebocyte lysate assay with commercial reagents (Seikagaku Kogyo, Tokyo).

Human urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA) were purchased from Mochida Pharmaceutical (Tokyo) and Bio-Scot (Edinburgh, Scotland), respectively. Lipoprotein lipase (LPL) from *Pseudomonas* sp. (L-8634, Sigma Chemical, St. Louis, MO) was used in this study.

***In vitro* cytotoxicity assay** L929 cells were cultured, suspended in Eagle's minimal essential medium (Eagle's MEM, Nissui Pharmaceutical, Tokyo) containing 5% fetal calf serum (FCS, Flow Laboratories, Rockville, MD), and seeded into the wells of a 96-well microtiter plate (Costar, Cambridge, MA; 4×10^3 cells/100 μl/

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[5] Abbreviations used: TNF-α, tumor necrosis factor-α: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; LPL, lipoprotein lipase.

well). After incubation at 37°C for 24 h in 5% CO_2, 100 ml of phosphate-buffered saline [PBS(−), Nissui Pharmaceutical] containing TNF-α (2–2,000 U/well) and nontoxic doses of lysosome labilizers was added to the wells and culture was continued for 48 h. Cytotoxicity was then assessed by the dye uptake method using crystal violet.[20]

***In vivo* assay of tumor growth inhibition** Meth A cells were maintained in RPMI-1640 medium (Gibco Laboratories, New York, NY) containing 10% FCS and a cell suspension (5.0×10^5 cells) prepared in RPMI-1640 was implanted intradermally (i.d.) in the abdominal region of BALB/c mice (Charles River Japan, Tokyo; male, 6 weeks of age with an average weight of 19 g). A suspension of L929 cells (1.3×10^6 cells) in Eagle's MEM was also i.d. transplanted into Crj: CD-1(1CR)-nu (nu/+) nude mice (Charles River; male, 6 weeks, 18 g body weight). When the tumor had grown to a diameter of about 10 mm, TNF-α (3.5×10^5 U per BALB/c mouse; 7.0×10^4 U per nude mouse) and u-PA (300 IU/mouse) were intravenously (i.v.) injected. Control mice received PBS(−). The tumor weight was estimated by measuring the tumor in two dimensions and calculating the tumor weight as described previously.[19] Data were expressed as relative tumor weight (mean tumor weight at given time/initial mean tumor weight).

RESULTS

Cytotoxicity *in vitro* In initial studies, *in vitro* cytotoxic effects of TNF-α in combination with nontoxic doses of lysosome labilizers were assessed. L929 cells, which are widely accepted as standard cells for *in vitro* cytotoxicity assay of TNF-α, were used as target cells. Thirty kinds of lysosome labilizers, such as u-PA, t-PA, plasmin, trypsin, LPL, phospholipase A_2, lysozyme, lactic acid, streptolysin S, Tween 20, sodium dodecyl sulfate, vitamin A, cysteine, etc., were examined in this study. As shown in Fig. 1, u-PA, t-PA and LPL markedly enhanced TNF-α cytotoxicity towards L929 cells. The concentration of TNF-α alone resulting in 50% cytotoxicity against L929 cells was 100 U/ml; the concentration required to obtain the same cytotoxic effect was 30, 20 or 25 U/ml, when used in combination with u-PA at 300 IU/ml, t-PA at 330 ng/ml or LPL at 2.5 U/ml, respectively. A similar effect was observed with 0.15 U/ml of plasmin (data not shown). u-PA, t-PA, LPL and plasmin alone did not show cytotoxicity at the concentrations tested.

Antitumor effects *in vivo* The *in vivo* antitumor effect of TNF-α was evaluated in combination with u-PA. At first, Meth A cells, which are generally used for assay of the *in vivo* antitumor effect of TNF-α, were adopted as the target. As shown in Fig. 2A, the ratio between the tumor weight in the control group on day 6 after administration

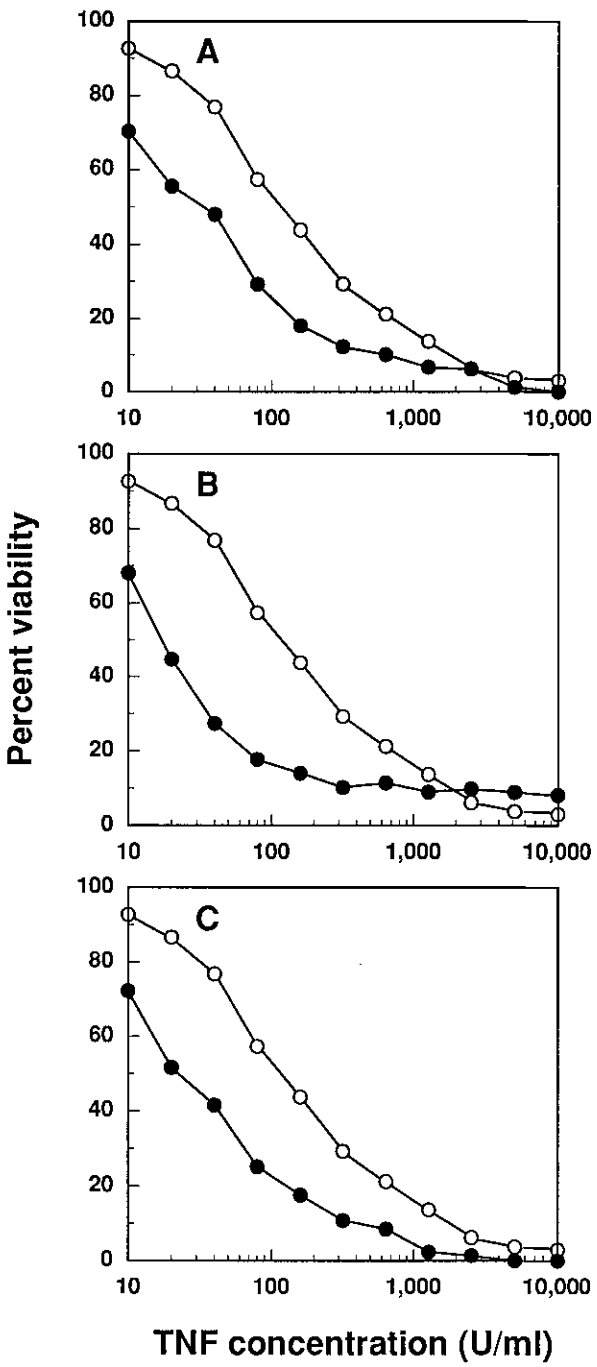

Fig. 1. *In vitro* cytotoxicity of TNF-α on L929 cells in the absence or presence of u-PA (A), t-PA (B) and LPL (C). L929 cells were seeded into 96-well microplates at 4.0×10^3 cells/well, and cultured at 37°C for 24 h, then various concentrations of TNF-α and u-PA (30 IU), t-PA (33 ng) or LPL (0.25 U) were added to the wells. After incubation for 48 h, the surviving cells were counted. Values shown are the means of four separate determinations. ○, TNF-α alone; ●, TNF-α and u-PA, t-PA or LPL. SDs were consistently $<5\%$ of means.

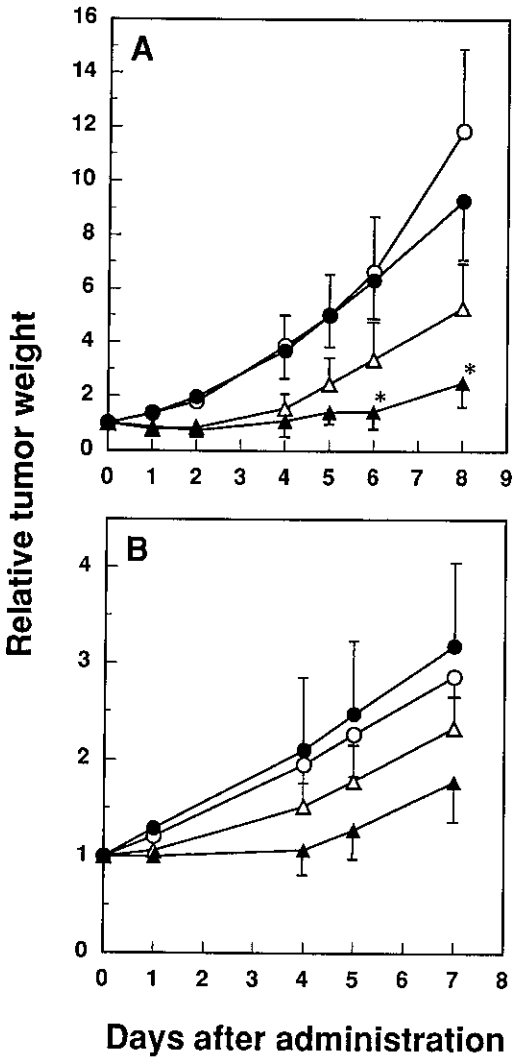

Fig. 2. *In vivo* antitumor effect of TNF-α, u-PA or the combination of both against Meth A cells transplanted into BALB/c mice (A) and L929 cells into Crj:CD-1(1CR)-nu nude mice (B). ○, control [PBS(−)]; ●, u-PA (300 IU/mouse); △, TNF-α [3.5×10^5 U/mouse (A); 7.0×10^4 U/mouse (B)]; ▲, TNF-α [3.5×10^5 U/mouse (A); 7.0×10^4 U/mouse (B)] and u-PA (300 IU/mouse). Groups consisted of 4–6 mice each. Values are the mean±SD (bars). The mean tumor weights were 191±51 mg (A) and 289±92 mg (B). Asterisks indicate $P<0.01$ (vs. control group) and $P<0.05$ (vs. TNF-α group).

and that on day 0 (day of injection) was 6.6. In the group receiving TNF-α alone (3.5×10^5 U/mouse), it was 3.4, thus indicating a mean growth inhibition of 49%. In the group receiving u-PA alone (300 IU/mouse), the ratio of the mean tumor weights on day 6 and day 0 was 6.3, suggesting negligible inhibition of tumor growth. On the other hand, the mean tumor weight ratio for the group receiving both TNF-α (3.5×10^5 U/mouse) and u-PA (300 IU/mouse) was 1.4, indicating growth inhibition of 79%. The enhanced antitumor effect of TNF-α with u-PA was also observed on day 8 (Fig. 2A) and thereafter (data not shown).

The results with Crj:CD-1(1CR)-nu nude mice bearing L929 tumors also showed that u-PA potentiated the *in vivo* antitumor effect of TNF-α (Fig. 2B). Administration of TNF-α (7.0×10^4 U/mouse) in combination with u-PA (300 IU/mouse) resulted in 38% inhibition of tumor growth as compared with the control on day 7. By the same criteria, injection of TNF-α alone resulted in only 19% inhibition, and that of u-PA alone resulted in no inhibition.

Total body weights showed about 10% reduction at the highest in both tumor-bearing mice (BALB/c and nude mice) given TNF-α alone. The decrease of mean total body weight in combination therapy was almost the same as that in the conventional therapy with TNF-α alone (data not shown).

DISCUSSION

Enhancement of the cytotoxicity of TNF-α towards L929 cells was observed when TNF-α was used in combination with lysosome labilizers, particularly u-PA, t-PA and LPL. These phenomena might be related to the putative mechanism of TNF-α cytotoxicity, involving lysosomal enzyme activation.[2-5] Furthermore, combined administration of TNF-α and u-PA markedly increased the *in vivo* antitumor activity of TNF-α. The decrease of body weight of tumor-transplanted mice in combination therapy was similar to that induced by TNF-α alone. Thus, u-PA appears to enhance the antitumor activity of TNF-α without amplifying the side-effects.

Our results suggest that the use of TNF-α and lysosome labilizers in combination is a promising antitumor therapeutic regimen with potential clinical application. On the other hand, many tumor cells have been shown to produce and release plasminogen activators, especially u-PA, which may contribute to invasion and metastasis of tumor cells.[21-23] So experiments are now under way to see whether the combined use of u-PA with TNF-α induces metastasis in tumor-bearing mice.

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