In Vitro Cytotoxicity of Recombinant Human-TNF-α and Actinomycin D on Canine Normal and Tumor Cells

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ABSTRACT. The in vitro cytotoxicity of recombinant human tumor necrosis factor-α (rh-TNF-α) and actinomycin D (ACT-D) on canine normal and tumor cells was investigated. rh-TNF-α showed dose-dependent cytotoxic and cell-growth inhibitory effects on cultured canine kidney carcinoma cells (CKCa-1). rh-TNF-α alone produced little cytotoxic effect on canine normal cells. However, combined with ACT-D, it showed moderate cytotoxicity on normal canine cells from the kidney medulla, spleen, heart muscle and lung. When the effects on the spontaneous tumor cells were examined, the combination of rh-TNF-α and ACT-D produced substantial cytotoxic effect on 60% of the tumor cells. All mammary mixed tumors and perianal gland tumors tested were susceptible to this combination. The data indicated the combination of rh-TNF-α and ACT-D have in vitro cytotoxicity on certain canine tumor cells. — KEY WORDS: ACT-D, canine, cytotoxicity, rh-TNF-α, tumor cell.

Tumor necrosis factor (TNF) is a multifunctional cytokine produced mainly by activated macrophage [6]. TNF has been known to show marked anticancer effects on certain transplantable tumors [4]. The potential mechanisms of TNF are the vascular changes in tumor tissues [22, 30], the modulation of host immunity against tumors [13, 24] and the direct cytotoxicity on tumor cells by inducing apoptosis [5, 29].

However, when administrated intravenously, TNF may cause the side effects such as vomiting, fever, hypotension, anemia and hepatic dysfunction in human patients [7, 12]. To achieve the effective dose of TNF in tumors without these side effects, TNF should be administered intratumorally [10] or through isolation perfusion [8, 16].

The dose of TNF could be reduced by promoting its efficacy with other chemotherapeutic drugs [14, 25], which was another way to alleviate the side effects of TNF. Actinomycin D (ACT-D), an antitumor antibiotic, has been experimentally used to treat lymphoma and malignant melanoma in small animal practice [9, 23]. We previously demonstrated that incubation of canine tumor cells with ACT-D increased their susceptibility to the cytotoxic effect of recombinant human TNF-α (rh-TNF-α) [1]. Moreover, in in vivo study using tumor-bearing mice, the combined treatment of rh-TNF-α and ACT-D was reported to produce synergistic antitumor effect [15].

This study was conducted to evaluate the in vitro cytotoxic and growth-inhibitory effects of rh-TNF-α in combination with ACT-D on subcultured canine kidney carcinoma cells (CKCa-1), primary-cultured normal canine cells and tumor cells surgically removed from canine tumor patients.

Reagents: Dulbecco’s modified eagle medium (DMEM) and Roswell Park Memorial Institute medium (RPMI)-1640 were purchased form Nissui Co. (Tokyo, Japan). Non-essential amino acids and pyruvate solution were obtained from GIBCO laboratories (Grand Island, NY, U.S.A.). rh-TNF-α (specific activity 2.55 × 10⁶ U/mg protein) was kindly given from Dainippon-Seiyaku (Osaka, Japan) [26]. ACT-D was purchased from Banyu Pharmaceutical Co. (Tokyo, Japan). 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl terazolium bromide (MTT) and dimethyl sulfoxide (DMSO) was obtained from Nacalai Tesque Inc. (Kyoto, Japan).

Canine tumor and normal cells: CKCa-1 cells were originally taken from a canine kidney carcinoma patient at the Veterinary Teaching Hospital in Osaka Prefecture University [1]. The cells were cultured in Dulbecco’s modified eagle medium (DMEM) (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum, 5% non-essential amino acids solution and 5% sodium pyruvate solution (GIBCO BRL, Gaithersburg, MD, U.S.A.) at 37°C in a humidified 5% CO₂ incubator.

Primary-cultured normal cells were taken from a normal disease-free Beagle dog. Fresh cell samples of the kidney medulla, kidney cortex, liver, spleen, urinary bladder, heart muscle and lung were prepared as follows. A block of each organ was resected immediately after euthanasia. These blocks were washed and minced with scissors as finely as possible and passed through a stainless-steel mesh. The filtrates were washed in RPMI-1640 followed by centrifugation for 10 min at 3,000 rpm [2, 27] and suspended in RPMI-1640 with 10% FBS.
Primary tumor cells were obtained from 18 canine tumor patients admitted to our Veterinary Teaching Hospital. The characteristics of the patients were listed in Table 1. During surgery, tumor specimens were aseptically resected and prepared by the same method described above.

Assay for rh-TNF-α and ACT-D cytotoxicity: A hundred microliters per well of the cell suspension in medium were dispensed into 96-well microtiter plates (Iwaki, Chiba, Japan). After 24 hr incubation, cells were exposed to rh-TNF-α with or without 1 µg/ml of ACT-D for 24 hr. Cell viability in each well was measured by MTT assay [18]. Briefly, 10 µl per well MTT dissolved in 5 mg/ml phosphate-buffered saline, pH 7.4 (PBS) was added to each well and incubated at 37°C for 4 hr. The medium was removed and replaced with 100 µl of DMSO to solubilize the crystals of MTT formazan. The absorbance (OD) were read on an microplate reader (Bio-Rad, CA) at 550 nm and the relative viability (% of control cell cultures) was calculated as follows:

\[
\text{Relative viability} = \frac{\text{OD test well} - \text{OD blank}}{\text{OD control well} - \text{OD blank}} \times 100
\]

Assay for cell growth inhibition: The CKCa-1 cells were plated on 96 well cultured plates at a density of 5 x 10^3 cells in 100 µl per well. After 24 hr incubation, rh-TNF-α with or without 0.01 µg/ml of ACT-D were added (day 0). The relative cell numbers were measured by MTT assay daily till day 5. The cell growth inhibition was defined as the reduction in OD of treated cells compared with untreated controls.

Statistical analysis: Comparison between different groups was performed by a one way analysis of variance (ANOVA). The Mann-Whitney test was used to compare differences in quantitative variables within 2 groups. The mean and standard deviation (SD) were calculated from 4 determinations.

RESULTS

Effects of rh-TNF-α and ACT-D on CKCa-1: The exposure to different doses of rh-TNF-α had significant (p<0.001) effects on cell viability of CKCa-1 (Fig. 1). When ACT-D was added to various dose of rh-TNF-α, cell viability was lowered by 30–40% at any dose of rh-TNF-α.

The inhibitory effect on rh-TNF-α on CKCa-1 cell growth in vitro were shown in Fig. 2. The cell growth was dose-dependently inhibited by rh-TNF-α on days 4 and 5. When ACT-D was added, CKCa-1 cell growth was inhibited as shown in Fig. 3. Cell number was significantly lowered on days 3, 4 and 5.

Effects of rh-TNF-α and ACT-D on primary-cultured cells from a normal dog: Cells from the kidney medulla and spleen showed mild (30 and 16%, respectively) but significant reduction of cell viability with the addition of rh-TNF-α alone (Table 2). ACT-D showed cytotoxicity on the cells from the kidney medulla, kidney cortex, spleen, heart muscle and lung. By exposure to the combination of
rh-TNF-α and ACT-D, the cell viability was significantly reduced on the kidney medulla, spleen, heart muscle and lung cells. The most sensitive cells to this combination was those from the kidney medulla. On the contrary, this combination did not have significant cytotoxicity on the cells from the kidney cortex, liver and urinary bladder.

Effects of rh-TNF-α and ACT-D on primary-cultured tumor cells from canine spontaneous tumors. Out of 20 tumors, 3 malignant mixed tumors of the mammary gland and 1 perianal gland carcinoma, had mild (12 to 36%) but significant decrease in cell viability by the exposure to rh-TNF-α alone (Table 3). ACT-D alone also showed cytotoxic effect on 9 tumors. When treated with both rh-TNF-α and ACT-D, 12 tumors showed reduction in cell viability. The cytotoxic effect of the combination varied among the cells (17 to 99%), in most of which (n=9), the cell viability reduced to less than 60%. rh-TNF-α significantly enhanced the cytotoxicity of ACT-D on 6 tumors.

Of 9 mammary gland tumors (5 malignant mixed tumors, 3 adenocarcinomas and 1 benign mixed tumor), all mixed tumors both malignant and benign were sensitive to the cytotoxic effect of rh-TNF-α and ACT-D, but any of the adenocarcinomas were not. The exposure to rh-TNF-α and ACT-D significantly decreased the cell viability of both perianal gland tumors. None of the mast cell tumors (n=3) had the response to rh-TNF-α alone.

**DISCUSSION**

rh-TNF-α had dose-dependent cytotoxic and growth-inhibitory effects on CKCa-1. ACT-D enhanced the effects of rh-TNF-α probably by inhibiting the synthesis of protective proteins induced by rh-TNF-α [29]. Either in a clinical study of human patients or in *in vivo* studies using tumor-bearing mice, the effective concentration of rh-TNF-α and ACT-D in tumor or even in plasma have not been reported. However, murine transplantable tumor cells, which showed a correlation between the results in *vivo* and *in vitro*, were sensitive to *in vitro* cytotoxic effect of rh-TNF-α at the range of concentration from 0.01 to 10 ng/ml combined with ACT-D at the dose of 1 μg/ml [15]. These concentrations of the agents were the same as those used in this study, which suggests that the concentrations we used were suitable to evaluate the antitumor effect.

The cell growth inhibition of rh-TNF-α may be attributed to the combination of cytostatic and cytotoxic effects on the cells. It was described that the small cell number in each wells and the long duration of exposure, such a condition as the long-term assay, increased the sensitivity of the cultured cells to the *in vitro* cytotoxicity of rh-TNF-α [11]. Both in *in vivo* study using mice bearing tumors and in clinical trials for human patients, rh-TNF-α was administered...
intratumorally for 4 to 7 days in one cycle [10, 15, 26]. For these reasons, the growth-inhibition assay seems to reflect the effect of intratumoral injection of these agents to rapidly growing tumors. However, the characteristics of the primary cells such as short life and slow proliferation in vitro make this assay difficult on these cells and only the cytotoxicity assay were carried out on the primary-cultured cells. Further studies are necessary to clarify the relation between the results of the cell growth inhibition and cytotoxicity assay.

Milsark and Cerami [19] reported that the radioiodinated TNF is cleared from the plasma with a half-life of 6 to 7 min after intravascular injection and distributed to the liver, kidney, spleen, gastrointestinal track, lung, and skin. To predict the damage on normal canine tissues with rh-TNF-α, we tested the cytotoxicity on primary-cultured normal canine cells. The kidney medulla cells were most sensitive to the cytotoxic effect of rh-TNF-α, and ACT-D on canine tumor cells. The overall response rate of the combination of rh-TNF-α and ACT-D to primary cultured canine tumor cells were 60% and the additive effect of the agents were observed on a half of the sensitive cells (30%). The different responses of the agents on adenocarcinomas and malignant mixed tumors of mammary gland were thought to be caused by their cellular components. Mueller et al. [21] demonstrated that TNF-α inhibited the growth of hormone-dependent cell lines of human breast cancer. The sensitivity of each tumor cell types and its characters should be examined in these canine mammary tumors. The results of our study suggested the potent efficacy of the combination of rh-TNF-α and ACT-D on mixed tumors of mammary gland and perianal gland tumors, which are common in canine tumors [17, 31]. Consequently, our results confirm the in vitro effect of rh-TNF-α and ACT-D on canine tumor cells.

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REFERENCES


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<th>No.</th>
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<th>Tumor type</th>
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<th>ACT-D</th>
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The cells were incubated with rh-TNF-α and/or ACT-D for 24 hr. Cell viability was determined in MTT assay and expressed as % of untreated controls. * P<0.05 vs. untreated controls. ** P<0.05 vs. ACT-D treated cells.