ORIGINAL ARTICLE

Studies on in vitro Mechanisms of Anti-tumor Activity of the Tumor Necrosis Factor α against Human Renal Carcinoma Cell Line (KU-2)

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Abstract

To clarify the mechanisms of anti-tumor activity of human recombinant tumor necrosis factor α (rTNFα), an established cell line KU-2, derived from a patient with human renal cell carcinoma (RCC), was treated with rTNFα alone or in combination with anti-cancer agents: actinomycin-D (ACD), vinblastine sulfate (VLB), nimustine hydrochloride (ACNU), and methotrexate (MTX). Cytotoxic assay by crystal violet dye exclusion test showed that 21.0 ± 4.0% and 34.8 ± 4.7% of the cells were killed by 72 hours incubation with 100 ng/ml of rTNFα alone, and 1 ng/ml of ACD alone, respectively. Synergistic cytotoxicity of 75.0 ± 0.3% was observed at 72 hours when 100 ng/ml of rTNFα and 1 ng/ml of ACD were added simultaneously. Furthermore, additive cytotoxicity of 48.5 ± 1.1% was observed by 0.1 ng/ml of VLB and 100 ng/ml of rTNFα. However, when KU-2 was treated in conjunction with both 100 ng/ml of rTNFα and 3 μg/ml of ACNU or 2.5 ng/ml of MTX, no significant increase in cytotoxicity was demonstrated. When KU-2 was pretreated with 1 ng/ml of ACD for 24 hours, followed by adding 100 ng/ml of rTNFα, a synergistic cytotoxicity by ACD and rTNFα was observed at 24 hours. On the other hand, when KU-2 was pretreated with 100 ng/ml of rTNFα for 24 hours, followed by adding 1 ng/ml of ACD, no significant increase in cytotoxicity was demonstrated. In clonogenic assay studies, the colony forming efficiency (CE) of the control cultures was 31.8 ± 8.1%. A 92.3 ± 1.8% reduction in CE was observed when 100 ng/ml of rTNFα was added to the cultures. No significant synergistic or
additive effects were demonstrated between rTNFα and chemotherapeutic agents in clonogenic assay studies. The effects of rTNFα on exponentially growing KU-2 cells were analyzed by studying the distribution of cells in the cell cycle. No cell cycle specific effect of rTNFα was demonstrated, regardless of whether or not chemotherapeutic agents were added. These results indicated that the cytotoxic and cytostatic activities of rTNFα may be mediated by separate mechanisms of action. Moreover, it was postulated that rTNFα may more significantly affect KU-2 cells having clonogenic potentials. rTNFα was concluded to have significant anti-tumor effects on renal cell carcinoma cells based on clonogenic assay studies. The combination therapy with rTNFα and ACD or VLB can be used in the management of patients with advanced renal cell carcinoma, resistant to current therapy based on biological response modifiers (BRMs).

Key words: rTNFα, anti-tumor activity, renal cell carcinoma

Introduction

Renal cell carcinoma is one of the most common cancers in the field of urology, but the effectiveness of radiation therapy or chemotherapy as a treatment for advanced renal cell carcinoma is discouraged. Although it has been shown in various kinds of cancers that the use of chemotherapeutic agents in combination may be more effective than the treatment with a single agent, this has not been demonstrated in renal cell carcinoma. In recent years, considerable attention has been paid to BRM therapy for renal cell carcinoma using agents such as interferons. Their effectiveness, however, has not necessarily satisfied urologists. Based on various studies, the tumor necrosis factor (TNF) may be a very attractive agent for combating against cancer, but its mechanisms of anti-tumor action are not clearly understood. Only few investigations concerning its effectiveness against renal cell carcinoma have been reported.

This study was thus designed to clarify the mechanisms of in vitro anti-tumor activity of rTNFα against the established cell line, KU-2, derived from human renal cell carcinoma. In the study, KU-2 was treated with rTNFα together with the other anticancer agents, the action mechanisms of which have already been established.

Materials and Methods

Cells

KU-2 cell line used in the present study was derived from a pulmonary metastasis of renal cell carcinoma. It was originally established as a cell line in September 1975 in the Department of Urology, School of Medicine, Keio University. The KU-2 cells were grown in a monolayer and maintained in Eagle's minimum essential medium
(MEM; Chiba Serum, Chiba, Japan) supplemented with 10% fetal bovine serum (FBS; Flow Lab., Virginia, USA). It was maintained in a humidified atmosphere of 5% CO₂ and 95% air at 37°C.

**rTNFα**

Highly purified human recombinant TNFα (FK 516) expressed in E. coli was supplied by Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). The molecular weight of rTNFα is approximately 17,000. The mean endotoxin level for all lots of rTNFα was ≤ 0.12 ng/mg as determined by Limulus amebocyte lysate assay.

**Anti-cancer Agents**

The following anti-cancer agents were purchased and used in the present study: actinomycin-D (ACD; Banyu Pharmaceutical Co., Ltd., Tokyo, Japan), vinblastine sulfate (VLB; Shionogi Pharmaceutical Co., Ltd., Osaka, Japan), nimustine hydrochloride (ACNU; Sankyo Pharmaceutical Co., Ltd., Tokyo Japan), and methotrexate (MTX; Japan Lederle Pharmaceutical Co., Ltd., Tokyo, Japan).

**In vitro rTNFα Assay**

The *in vitro* cytotoxic activity of rTNFα was measured by seeding portions (150 μl) of cells (2×10⁴ cells/ml of culture medium) in 96-well microtiter plates having U shaped bottoms (Sterilin, Middlesex, England) with 0, 1, 10 and 100 ng/ml of rTNFα and/or various concentrations of anti-cancer agents. The final concentration of the anti-cancer agents in each well was 1 ng/ml of ACD, 0.1 ng/ml of VLB, 3 μg/ml of ACNU and 2.5 ng/ml of MTX. The final volume of each well was 200 μl.

The wells were divided into four groups: wells without any agents, with rTNFα alone, with anti-cancer agents alone, and with both rTNFα and each of the anti-cancer agents. They were incubated at 37°C in 5% CO₂ in air for 24, 48 and 72 hours.

At the end of a culture period, the medium was drawn out and each well was stained with 0.2% crystal violet in 2% ethanol. Twenty minutes later, the wells were rinsed three times with phosphate buffered saline (PBS), and then dried. The absorbance value at 414 nm was measured for each well using a microplate photometer (MPT-02, CORONA, Ibaraki, Japan).

A linear relation was observed between the cell number and absorbance value obtained by crystal violet staining (R=0.93). All experiments were performed in triplicate. The percent cytotoxicity was calculated using the formula:

\[
\% \text{ cytotoxicity} = (1 - B/A) \times 100,
\]

where A was the average absorbance value of a control group, and B was the average absorbance of the treatment group.
The same number of KU-2 cells in the same amount of culture medium was pre-
treated for 24 hours with either 1 ng/ml of ACD or 100 ng/ml of rTNFα. After the
culture medium was discarded and the wells were rinsed three times, either 100 ng/ml of
rTNFα or 1 ng/ml of ACD was added to each well and incubated for 24, 48 and
72 hours, respectively. At the end of the culture periods, the wells were stained in the
same manner, the absorbance value was measured, and the percent cytotoxicity was
calculated. This study was also performed in triplicate.

Colony forming assay: KU-2 was harvested from the culture flasks and rinsed twice
with Eagle’s MEM. After the cells were irrigated using a tuberculin syringe fitted with
a 26-gauge needle to prevent cell aggregation, 1×10³ cells were seeded in 10 ml of
culture medium in polystyrene tissue culture flasks (25 cm², Falcon 3013, Becton,
Dickinson Lab., Oxnard, USA) with final concentrations of 0, 1, 10 and 100 ng/ml of
rTNFα, respectively, and/or with the same concentrations of each anti-cancer agent
previously described. The flasks were incubated at 37°C in 5% CO₂ and 95% air with
full humidity for 14 days without change of the medium. The cells were then fixed
with methanol, rinsed twice with PBS, and stained with Giemsa solution.

Aggregates of more than 50 cells were considered to be colonies. The colonies
were counted in triplicate microscopically. Colony forming efficiency (CE) was calcu-
lated using the formula:

\[
CE \, (\%) = \frac{B}{A} \times 100,
\]

where A was the number of cells plated, and B was the number of colonies formed.
The percent reduction rate of CE was determined using the formula:

\[
(1 - CE \, \text{in rTNFα treated medium} / CE \, \text{in control medium}) \times 100.
\]

Flow cytometric DNA analysis: Exponentially growing cells were prepared in poly-
styrene tissue culture flasks (25 cm², Falcon 3013, USA). Initially, the cultures were
treated with rTNFα alone (final concentrations of 100 ng/ml, and 50 µg/ml), ACD or
VLB alone (the same concentrations as in the previous studies), or a combination of
both 100 ng/ml of rTNFα and each of the anti-cancer agents, respectively. Cells treated
with neither rTNFα nor anti-cancer agents were also prepared as a normal control.

Twenty-four hours later, the cells were trypsinized from the culture flasks and
rinsed twice with PBS. Single cell suspensions were made using the mechanical dis-
aggregation. The cells were centrifuged at 900 rpm for five minutes and the cellular
DNA was stained according to a modification of the method of Vindelov. The
fluorochrome stain was performed by adding 25 mg of propidium iodide, 500 mg of
sodium citrate, and 0.5 ml of Triton X-100 to 500 ml of distilled water. One × 10⁵
cells were resuspended in 1 ml of the staining solution at 4°C for 30 minutes. Samples
were kept on ice and were filtered through a 30-µm nylon mesh immediately before
analysis. An Ortho System 50-H multiparameter flow cytometer equipped with an Ortho computer (Ortho Diagnostic Inc., Westwood, MA., USA) was used to determine the DNA content and the cell cycle distribution of the fluorescing cells.

Results

In vitro cytotoxic assay: The effect of rTNFα against the human renal cell carcinoma cell line, KU-2, was studied by in vitro cytotoxic assay. The maximal percent cytotoxicities were observed at 72 hours, and were 9.4 ± 1.8%, 13.9 ± 3.3% and 21.0 ± 4.0%, when the cells were treated with 1, 10 and 100 ng/ml of rTNFα, respectively (Fig. 1a). The maximal percent cytotoxicity was observed at 72 hours, and was 34.8 ± 4.7%, when the cells were treated with 1 ng/ml of ACD alone (Fig. 1a). When 1 ng/ml of ACD and various concentrations of rTNFα were added together, a synergistic cytotoxic response was observed (Fig. 1a). When the amount of rTNFα was increased, the synergistic effect was augmented. The increased percent cytotoxicity was statistically significant from 24 to 48 hours in all concentrations of rTNFα (p<0.01). However, the observed synergistic effect from 48 to 72 hours was not statistically significant. The maximal percent cytotoxicity was 75.0 ± 0.3%
a. Cytotoxic activity of rTNFα and ACD against KU-2. KU-2 reacted poorly even 72 hours after the treatment of 100 ng/ml of rTNFα alone. The synergistic cytotoxic response was observed when 1 ng/ml of ACD and various concentrations of rTNFα were added simultaneously.

b. Cytotoxic activity of rTNFα and VLB. The additive cytotoxic response between 0.1 ng/ml of VLB and 100 ng/ml of rTNFα was observed. The maximal percent cytotoxicity was observed at 72 hours when the cells were treated with both 1 ng/ml of ACD and 100 ng/ml of rTNFα. When KU-2 was treated with 0.1 ng/ml of VLB and 100 ng/ml of rTNFα, additive cytotoxic effects were noted (Fig. 1b). The maximal percent cytotoxicity was observed at 72 hours, and was 48.5 ± 1.1%, when the cells were treated with both 0.1 ng/ml of VLB and 100 ng/ml of rTNFα simultaneously. The maximal percent cytotoxicity was 26.2 ± 7.6% and 30.6 ± 4.2%, when the cells were treated with 3 μg/ml of ACNU alone and both 3 μg/ml of ACNU and 100 ng/ml of rTNFα, respectively. No significant increase in the cytotoxicity by rTNFα was observed. Likewise, when KU-2 was treated with 2.5 ng/ml of MTX and rTNFα, no significant increase was noted in the cytotoxicity. The maximal percent cytotoxicity was observed at 48 hours, and was 10.6 ± 2.5%, when the cells were treated with both 2.5 ng/ml of MTX and 100 ng/ml of rTNFα.

When KU-2 was pretreated with 1 ng/ml of ACD for 24 hours followed by adding 100 ng/ml of rTNFα, synergistic cytotoxic effects between ACD and rTNFα were noted.
Cytotoxic activity after pretreatment by ACD. Pretreatment by 1 ng/ml of ACD. The synergistic cytotoxic effects between ACD and rTNFα were observed on 24 hours.

on 24 hours (Fig. 2). The observed maximal percent cytotoxicity was 38.3 ± 2.9%. However, 72 hours after incubation, this synergism was not observed. When KU-2 was pretreated with 100 ng/ml of rTNFα for 24 hours followed by adding 1 ng/ml of ACD, on the other hand, no synergistic or additive effects were demonstrated (data not shown).

Colony forming assay: The CE of control cultures was 31.8 ± 8.1%. A significant reduction in the number of colonies was observed following the treatment with rTNFα, as shown in Fig. 3a. There was a 28.7 ± 3.5% and a 48.2 ± 2.9% reduction in CE, compared to the control cultures when 1 ng/ml and 10 ng/ml of rTNFα were added to the cultures, respectively. When KU-2 was cultured with 100 ng/ml of rTNFα, the percent reduction in CE was a 92.3 ± 1.8%. These reductions in CE were all statistically significant (P<0.01). When KU-2 was treated with both rTNFα and ACD, no synergistic effects were demonstrated (Fig. 3b). Similarly, no significant augmentation of the effects was demonstrated between rTNFα and VLB (Fig. 3c), ACNU, or MTX (data not shown).

Flow cytometric DNA analysis: Effects of rTNFα on exponentially growing KU-2 cells were analyzed by studying the distribution of cells in the cell cycle. DNA histogram patterns of the cells at 24 hours after the addition of 100 ng/ml of rTNFα alone were not different from the patterns in the control cells. Similarly, no differences in the DNA histogram patterns were observed when the dose of rTNFα was pushed up
Fig. 3a. The analysis of the cell cycle distribution by flow cytometry disclosed no obvious difference between the KU-2 cells treated with 1 ng/ml of ACD alone and the cells treated with both 1 ng/ml of ACD and 100 ng/ml of rTNFα. Likewise, no significant changes in the DNA histogram patterns were observed between 0.1 ng/ml of VLB alone and both 0.1 ng/ml of VLB and 100 ng/ml of rTNFα (data not shown).

Discussion

A remarkable number of in vitro investigations of the cytotoxic effects of TNF on cancer cell lines have been performed. Many of them have reported that TNF showed non-uniform cytotoxic effects on cancer cell lines, where the sensitivity of the cancer cell lines to TNF varied significantly from one another. However, the mechanisms of the anti-tumor activity of TNF still remained unclear. Hence, to clarify the anti-tumor activity of TNF, KU-2 was treated by both rTNFα and anti-cancer agents, the action mechanisms of which had already been well elucidated. Anti-cancer
agents selected in this study were such that their mechanisms of action were different from each other. The utilized anti-cancer agents were: the antibiotics, ACD, as an inhibitor of RNA synthesis; the nitrosourea, ACNU, as an inhibitor of DNA synthesis; the vinca alkaloid, VLB, as an inhibitor of cell division; and the antimetabolite, MTX, as an inhibitor of folic acid metabolism. Nitrosourea, particularly CCNU and VLB are also known as chemotherapeutic agents for advanced renal cell carcinoma.\(^1\)

The concentrations of the anti-cancer agents utilized in this study were determined by preliminary investigations, in which 0.01, 0.1, 1 and 10 ng/ml of ACD, 30, 300 ng/ml, 3 and 30 \(\mu\)g/ml of ACNU, 0.01, 0.1, 1 and 10 ng/ml of VLB, and 0.25, 2.5, 25 and 250 ng/ml of MTX were examined. The optimal concentration in each chemotherapeutic agent was chosen such that the cytotoxicity of a single anti-cancer agent was not remarkable because a reciprocal action was demonstrated between the anti-cancer agent and rTNF\(\alpha\).

Some cancer cell lines, especially L929, were highly sensitive to the cytotoxic effects of TNF.\(^5\,15\,16\) However, some were scarcely affected by the addition of TNF.\(^14\) It was
reported that the sensitivity of TNF in vitro did not correlate with the tumorigenicity of the cell lines. KU-2 reacted with rTNFα poorly even 72 hours after the treatment of 100 ng/ml of rTNFα alone with 21.0 ± 4.0% of cytotoxicity. Therefore, KU-2 was considered to be a cell line that showed a null response to rTNFα in vitro according to Sugarmann's classification. However, when KU-2 was treated with both rTNFα and ACD, synergistic cytotoxic effects were observed compared to those of the cultures treated with ACD alone or rTNFα alone with 75.0 ± 0.3% of cytotoxicity.

To confirm that rTNFα emphasized the cytotoxic effects of ACD, or, conversely, that ACD emphasized the effects of rTNFα, the cells were pretreated with rTNFα or ACD for 24 hours. The results indicated that the pretreatment with ACD enhanced the cytotoxic effects of rTNFα. The internal changes in cells treated by ACD could be the reason for the emphasized activity of rTNFα. It was reported that accelerated RNA syntheses in TNF-sensitive cells have been observed when these cells were treated with TNF. Inhibitors of RNA syntheses, such as ACD, enhanced the cytotoxic effect of TNF, and normal and tumorigenic cell lines resistant to TNF became...
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Fig. 4 DNA histogram patterns of KU-2.

Fig. 4a.

- G₀-G₁ 32.7%
- S 41.3%
- G₂-M 26.0%

Relative fluorescence intensity

Fig. 4b.

- G₀-G₁ 31.8%
- S 45.2%
- G₂-M 24.0%

Relative cell counts

Relative fluorescence intensity

Fig. 4 DNA histogram patterns of KU-2.

a. Control.

b. Twenty four hours after addition of 50 μg/ml of rTNFα. No cell cycle specific effects of rTNFα were obvious in this study.
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sensitive by treating them concomitantly with TNF and ACD.\textsuperscript{18,19} This was also confirmed in this study using KU-2.

The reason for this accelerated RNA syntheses still remains to be solved. One hypothesis, proposed by Ostrove \textit{et al.},\textsuperscript{17} suggests that protein synthesis reflected the repair process of damaged cells caused by TNF and that the inhibition of this repair process by using ACD showed a remarkable increase in the cytotoxic effect of TNF.

Only few chemotherapeutic agents have been available against advanced renal cell carcinoma including nitrosourea and VLB. However, their effectiveness was reported to be under 20\% for the treatment of advanced renal cell carcinoma.\textsuperscript{1} Therefore, the additive effects between VLB and rTNF\alpha demonstrated in this study deserve further investigations for the purpose of clinical application.

Meanwhile, when KU-2 was treated with 3 μg/ml of ACNU or 2.5 ng/ml of MTX in combination with rTNF\alpha, no significant increase in cytotoxicity was demonstrated. The major mechanisms of action of ACNU are thought to be the inhibition of DNA syntheses. Similarly, as the primary intracellular effects of MTX are thought to be the inhibition of dihydrofolate reductase, inhibition of this target enzyme results in a decrease of intracellular FH\textsubscript{4}, and consequently, an inhibition of DNA syntheses. The result in this study suggested that the inhibition of DNA syntheses did not emphasize the effects of rTNF\alpha.

Not so much studies have appeared concerning the cell cycle specificity of TNF. Darzynkiewicz \textit{et al.}\textsuperscript{20} reported that when 4.1 μg/ml of TNF was added to L929 cells, cell arrest in G\textsubscript{2}M was observed to last for approximately 4 to 6 hours, and was then followed by cell death. However, they also reported that there were no obvious effects of the cell cycle when the TNF-resistant subline of L929 cells was treated with TNF. Naomoto \textit{et al.}\textsuperscript{21} reported that L929 cells treated with TNF accumulated in the S phase of the cell cycle. The effects of TNF on the cell cycle are still controversial. In this study, using KU-2 cells showed no cell cycle specific effects of rTNF\alpha even though the dose of rTNF\alpha was pushed up to 50 μg/ml, regardless of whether or not ACD or VLB was added.

Unlike the cytotoxic assay, the significant reduction in the number of colonies of KU-2 was observed when the cultures were treated with 100 ng/ml of rTNF\alpha. The synergistic effects of rTNF\alpha and ACD were not demonstrated in clonogenic assay. The discrepancy in the consequences of these two \textit{in vitro} studies is explained by the following three principal points.

1. Clonogenic assay is essentially a cytostatic examination.\textsuperscript{12} The cytostatic and cytotoxic activities of TNF may be mediated by separate action mechanisms.\textsuperscript{12,19} Nakano \textit{et al.}\textsuperscript{19} proposed two possibilities for the mechanisms of rTNF\alpha. One is that cytostatic action is a prerequisite for the cytotoxic effects. rTNF\alpha first inhibits tumor cell growth and this is followed by killing the tumor cells. Another possibility is that rTNF\alpha may
act in two different manners on tumor cells in vitro. One is the inhibition of the growth of tumor cells without affecting viabilities. The other is the direct killing of the tumor cells.

This study may indicate that the cytostatic activity of rTNFα is not a prerequisite for the cytotoxic action.

2. rTNFα may show its anti-tumor effects more significantly against KU-2 when incubated for longer periods than 72 hours. However, this hypothesis also could not be explained why ACD did not enhance the anti-cancer activity of rTNFα in clonogenic assay. Based on this study, the remarkable cytostatic effect of rTNFα on KU-2 cells might not be merely the long-term effect of rTNFα but may indicate the versatile action of rTNFα.

3. The target cells for cytotoxic assay are all types of cell populations, while those for clonogenic assay are only cells having clonogenic potentials. Thus, from this investigation, it is postulated that rTNFα may more significantly effect KU-2 cells having clonogenic potentials.

It has been reported that an increase in the clonogenicity correlates with the recurrence or the malignant potential of the cancer.22,23 If this is so, and the hypotheses presented above are true, then based on this study, rTNFα may be expected to be a potent anti-cancer agent for combating against renal cell carcinoma.

Heterogeneity of KU-2 was also studied by Nagakura.24 It was reported that KU-2 has expressed biologically different clones.24 Therefore, effectiveness of BRMs against clones of KU-2 may differ from the clone to clones. In this point, further investigation should be considered.

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