An Apoptosis-inducing Gene Therapy for Pancreatic Cancer with a Combination of 55-kDa Tumor Necrosis Factor (TNF) Receptor Gene Transfection and Mutein TNF Administration

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ABSTRACT

Intratumoral injection of recombinant human tumor necrosis factor (TNF) for inoperable pancreatic cancer has shown some efficacy in suppressing tumor growth or decreasing tumor markers. However, complete regression has not yet been achieved, possibly due to a lack of TNF receptors on tumor cells or an abundance of intracellular resistance factors. Recently, two distinct types of TNF receptors, R55 and R75, were identified, which are responsible for signaling of cytotoxicity and of proinflammatory activity, respectively. In this study, a novel type of suicide gene therapy is proposed that is based on transfection of the R55 gene into human pancreatic cancer cells (AsPC-1 and PANC-1) and subsequent administration of TNF. The transfectants from both cell lines showed higher TNF susceptibility than their parental cells. In vivo tumor formation of an AsPC-1 clone (clone 10) inoculated in nude mice was substantially suppressed by administration of TNF.

For practical use of this strategy, however, the adverse effects of TNF may become an obstacle. We previously produced mutein TNF 471, which had a higher affinity for R55, superior antitumor activity, and fewer adverse effects. This mutein TNF 471 manifested greater antitumor activity against clone 10.

Because the R55 receptor is known to be involved in augmentation of cellular immunity by TNF, mutein TNF 471 is also expected to be highly potent in this function. In fact, the mutein TNF 471 induced higher splenic natural killer cell activity in nude mice inoculated with clone 10 than did native TNF. This property of augmenting cellular responses may be advantageous in the eradication of viable tumor cells left untransfected in practical gene therapy regimens in which 100% transfection of the R55 gene into tumors is not feasible.

Thus, gene therapy combining transfection of the TNF-R55 gene with administration of mutein TNF 471 may provide a new modality for the treatment of pancreatic cancer.

INTRODUCTION

Pancreatic cancer is a highly aggressive neoplasm that readily invades neighboring tissues and metastasizes to distant organs at early stages of the disease. Most current therapeutic approaches, including extensive surgery combined with radiation therapy or chemotherapy, therefore remain palliative, and the prognosis is still extremely poor. Thus, the introduction of a new modality such as gene therapy is desperately needed to overcome this malignancy.

We have previously reported that intratumoral injection of recombinant human TNFα for inoperable cases of pancreatic cancer brought about regression of the tumor or a decrease in tumor markers (1). However, a complete response had not been achieved in any of these cases, and the overall outcome was not sufficient, possibly because cytoprotecting factors such as enTNF and MnSOD are abundant in pancreatic cancer cells (2, 3), or because TNF receptors were hardly expressed. This refractoriness to TNF may be overcome by transfection of a gene for the TNF receptor, which transduces cytotoxic (apoptotic) signals.

TNF is known to exert pleiotropic activities that are mediated through two high-affinity receptors: the TNF-R55, which mainly transduces cytotoxic signals (4), and the TNF-R75, which is related to proinflammatory activity (i.e., side effects; Ref. 5). The gene encoding TNF-R55, therefore, may be of practical use in a gene therapy of this type.

Alternative approaches to enhance the cytotoxicity of TNF and/or to dissociate it from systemic toxicity could include utilization of genetically engineered mutein TNFs.

In this context, some mutein TNFs were designed to bind selectively to R55 receptors and were proven to exhibit lower proinflammatory activity or less adverse effects than native TNF. Their antitumor effects or tumoricidal effects, however, were not substantially greater than those of native TNF (5, 6). Another set of mutein was reported to exert much higher antitumor effects than native TNF, although their adverse effects in vivo were not elucidated (7, 8).

We have engineered mutein TNF 471, which exhibited superior antitumor activity and caused fewer adverse effects in vivo (9, 10).

In the present study, we have attempted to establish a new strategy of gene therapy for pancreatic cancer by transfection of the TNF-R55 gene and subsequent administration of a mutein TNF 471. This strategy is comparable to gene therapy using suicide genes that render tumor cells susceptible to a nontoxic prodrug. In this type of gene therapy, not only gene-transduced cells, but also neighboring nontransduced cells, are known to undergo cytology through a bystander effect (11, 12) and an immune response induced by the antigens of extinct cancer cells (13, 14), although the effect is relatively limited to the localized tumor. To enhance the effectiveness of suicide gene therapy, combinations with other modalities such as immunomucocytokine gene therapy have recently been used, and long-term survival was attained by preventing tumor recurrence (15).

Because TNF is known to play some essential roles in cellular immunity, such as in the generation of lymphokine-activated killer activity (16), activation of NK cells (16), induction of human lymphocyte antigen on tumor cells (17), and production of IL-12 by macrophages (18) through signals via R55, the present strategy to use mutein TNF 471, which has a higher affinity for R55, is expected to exert a combined effect of suicide gene therapy and immunomucocytokine gene therapy.

The results obtained indeed demonstrated that with R55 gene-transfected pancreatic cancer cells, mutein TNF 471 was more potent than native TNF in inducing apoptosis in vitro as well as in vivo tumor regression in conjunction with enhancement of NK cell activity in nude mice. Thus, the strategy of transduction of the R55 gene and
MATERIALS AND METHODS

Native and Mutein TNF. Recombinant human TNF (native TNF) was provided by Asahi Chemical Industry (Tokyo, Japan). Mutein TNF 471 has seven NH₂-terminal amino acids deleted and is substituted with Pro⁴ Ser⁹ Asp¹⁸ with Arg¹ Lys⁴ Arg¹⁰, was engineered as described earlier (9, 10).

Cell Culture. The human pancreatic adenocarcinoma cell lines AsPC-1 and PANC-1 and mouse lymphoma cell line YAC-1 were obtained from the American Type Culture Collection. AsPC-1, YAC-1, and PANC-1 cells were each cultured in RPMI 1640 and DMEM supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, North Ryde, Australia) at 37°C under 5% CO₂.

Expression Vector of TNF-R55. The 2.1-kb EcoRI-EcoRI TNF-R55 cDNA was provided kindly by Hoffmann-La Roche Ltd. (Basel, Switzerland; Ref. 19). The TNF-R55 expression vector pCDNA3-R55 was constructed from the mammalian expression vector pCDNA3 (Invitrogen, San Diego, CA), which contains the human cytomegalovirus immediate-early promoter, SV40 ori, and neomycin resistance marker. The pCDNA plasmid was digested by EcoRI (TaKaRa, Kyoto, Japan) and ligated to TNF-R55 cDNA.

Transfection. AsPC-1, and PANC-1 cells were transfected with pCDNA3-R55 by a lipofection method (20), and then the transfectants were selected by incubation with 300 μg/ml G418 (Life Technologies, Inc., Gaithersburg, MD) for 14 days. The parental cells transfected with pCDNA3 were used as controls (mock transfectants).

Flow Cytometric Analysis. The expression of TNF-R55 and R75 on the cell surface was analyzed by FACScan flow cytometer (Becton Dickinson) using anti-TNF-R55 monoclonal antibody (ht-9) and anti-TNF-R75 monoclonal antibody (utr-1) provided by Hoffmann-La Roche (21).

Southern Blot Analysis. Cellular DNAs purified from parental tumor cells and their transfectants were digested with EcoRI, electrophoresed on 0.7% agarose gel, and transferred to a nylon filter membrane (Schleicher & Schuell, Inc., Keene, NH), hybridized, and washed. The blots were probed with the upstream region of an internal EcoRI site of R55 cDNA with the size of 1.4 kb, excised from the agarose gel, purified using Prep-A-Gene DNA Purification Systems (Bio-Rad, Richmond, CA), and labeled with [α-³²P]dCTP using the BcaBEST Labeling Kit (TaKaRa).

In Vitro Cytotoxic Activity. Cells (5 x 10⁶ cells/100 μl) were incubated for 12 h in a 96-well microculture plate (Coster, Tokyo, Japan). To these cells, 10⁻² to 10⁻⁵ ng/ml native TNF or mutein TNF 471 was added and incubated either for 48 h or for 6 h plus another 42 h without TNF. Cytotoxic activity was determined by a dye-uptake method, as described earlier (2).

Receptor Binding Competition Assay. AsPC-1 cells (2 x 10⁵) were treated with 10 μg of anti-TNF-R75 monoclonal antibody (utr-1) for 30 min at 4°C to block R75 and were then incubated with 100 ng/ml native TNF labeled with ¹²⁵I using IODO-GEN (Pierce Chemical Co., Rockford, IL). The binding of [¹²⁵I]labeled native TNF to TNF receptors was competed with various concentrations of mutein TNF 471 or native TNF in 0.3% Tween 20 at 4°C. After washing with PBS, the cells were solubilized in a 0.1% SDS solution, and radioactivity was determined by a gamma counter.

DNA Fragmentation Assay. Parental tumor cells (1 x 10⁷) and their transfectants were cultured in 5 ml of RPMI 1640 with 10% fetal bovine serum with 100 ng/ml native TNF or mutein TNF 471 for 48 h in a six-well culture plate (Coster). The cells were harvested, centrifuged, and lysed in Triton X-100/Tris buffer. The lysates were centrifuged at 13,000 x g for 30 min, and the supernatants were electrophoresed in a 2.5% agarose gel containing 1 μg/ml ethidium bromide. The ladder formation was visualized under UV light.

In Vivo Antitumor Activity. Fifty BALB/c athymic nude mice (6 weeks old) were s.c. inoculated with either an AsPC-1 clone (5 x 10⁶ cells) or mock transfectants (5 x 10⁶ cells) into the lateral abdominal wall and grouped into 10 (each group consisted of 5 mice). Two groups bearing R55 transfectants were given daily i.v. injections of 5 μg of native or mutein TNF 471 on days 10, 12, 14, 16, and 18, for a total of five injections. Similarly, two groups of mice with mock transfectants were injected with TNF on the same schedule. Another set of R55 transfectants was injected with 1 μg of mutein TNF on the same regimen mentioned above, and one of these groups of mice was additionally treated with 20 μg of anti-asialo GM1 antibody (Pascal GmbH, Frankfurt, Germany) on day 9. One group of animals with R55 transfectant was given 1 μg of native TNF in a similar manner. The other three groups, which were inoculated with parental cells, mock transfectants, or R55 transfectants, were given PBS instead of TNF. The diameter of the tumors was measured with a caliper and their volume was calculated with the following formula: Volume = α² x b/2, where α represents the shortest diameter, b, the longest.

Assay for NK Cell Activity. Spleen cells from nude mice inoculated with R55 transfectants and treated with 1 μg of native TNF or mutein TNF 471 were used as effectors, and YAC-1 cells (NK cell-sensitive mouse lymphoma cell line) were used as target cells. NK cell activity was determined by a 5¹ Cr-release assay (24).

RESULTS

Transfection of the R55 Gene into Pancreatic Cancer Cells. To examine the effects of the TNF-R55 gene on susceptibility to TNF, we chose two pancreatic cancer cell lines that were previously found to exhibit low susceptibility to TNF because of abundant intracellular resistant factors, such as enTNF or MnSOD (3), and expressed R55 and R75 at very low levels as determined by a receptor binding competition assay for that receptor was not feasible.

Enhanced Susceptibility of R55 Gene-transfected Clones to Native TNF. Susceptibility of R55 gene-transfected clones to native TNF was investigated (Table 1). All clones of AsPC-1 R55 and PANC-1 R55 showed greater susceptibility to 10 ng/ml of native TNF as compared to parental cells or mock transfectants. Clones 10 and 12 of AsPC-1 cells and clones 3 and 5 from PANC-1 cells, of which expression of R55 was higher than in other clones, are shown in Fig. 1, A and B. To confirm R55 gene transfection, Southern blot analysis of clone 10 from AsPC-1 cells and clone 3 from PANC-1 cells was carried out. As shown in Fig. 2, 1.4-kb bands of the transfected R55 gene were observed for both clones. No significant difference was observed between the growth curves of the parental cells and the R55 gene-transfected clone (data not shown).

Binding Affinity of Native TNF and Mutein TNF 471 to TNF-R55. To compare the affinity of mutein TNF 471 to TNF-R55 with that of native TNF, a receptor binding competition assay using R55 clone 10 of AsPC-1 cells was performed. The affinity of mutein TNF to R55 was 6-fold greater than that of native TNF based on molar ratios of 50% binding inhibition (Fig. 4). Because the R75 was very poorly expressed on the R55 clone (data not shown), a competitive assay for that receptor was not feasible.

Cytotoxic Effects of Mutein TNF 471 on R55 Gene-transduced AsPC-1 Clone. Because mutein TNF 471 had higher affinity to TNF-R55 than native TNF (Fig. 5), cytotoxicity of the former to the R55 clone was expected to be greater than that of the latter. When the R55 clone 10 of AsPC-1 cells was incubated with mutein TNF 471 or
TNF-R55 GENE AND MUTEIN TNF FOR CANCER THERAPY

Fig. 1. Enhanced expression of TNF-R55 in R55 gene-transduced cells. AsPC-1 (A) and PANC-1 (B) cells were transfected with pcDNA3-R55 or pcDNA3. Flow cytometric analysis was performed on parental cells, mock transfectants, and R55 clones. Fluorescence was obtained with anti-R55 or R75 monoclonal antibody (—). Control fluorescence was obtained with a second antibody alone (—). Significant increments of intensity were consistently observed in clones 10 and 12 from AsPC-1 and in clones 3 and 5 from PANC-1.

native TNF for 48 h, cytotoxicity of mutein TNF surpassed that of native TNF at lower dosages (10^{-2}–1 ng/ml), whereas the cytotoxic curves at higher dosages (10^{-1}–10^{3} ng/ml) were quite similar (Fig. 5A), presumably due to the fact that apoptotic signals induced by both TNFs were equally saturated after 48 h of incubation as discussed below. Therefore, to enhance the difference in cytotoxicity of these TNFs, the incubation period was shortened to 6 h. As shown in Fig.

Table 1 Enhanced TNF susceptibility induced by transduction with the R55 gene

<table>
<thead>
<tr>
<th>Cells</th>
<th>AsPC-1 (%)</th>
<th>PANC-1 (%)</th>
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<tbody>
<tr>
<td>Parental cells</td>
<td>76 ± 3</td>
<td>95 ± 4</td>
</tr>
<tr>
<td>Mock transfectants</td>
<td>75 ± 3</td>
<td>97 ± 2</td>
</tr>
<tr>
<td>R55-transfectant clones</td>
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<td></td>
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<tr>
<td>1</td>
<td>37 ± 2</td>
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</tr>
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<td>34 ± 3</td>
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<td>15</td>
<td>36 ± 3</td>
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*The percentages of cells that survived after treatment with 10 ng/ml native TNF for 48 h.

5B, with this incubation period, mutein TNF 471 exhibited clearly greater cytotoxicities than (native) TNF throughout the dosages examined. Because parental cells barely expressed R55 (Fig. 1 A, a), they exhibited very low susceptibilities against both native and mutein TNF (Fig. 5, A and B).

Induction of Apoptosis on R55 Gene-transduced AsPC-1 Clone by TNF. To confirm that TNF causes apoptotic death, DNA ladder formation of R55 clone 10 of AsPC-1 treated with 100 ng/ml of native and mutein TNF 471 for 48 h incubation was examined (Fig. 6). Without TNF treatment, no ladder formation was observed in either clone 10, parental cells, or mock transfectants, whereas clone 10, but not parental cells or mock transfectants, treated with both TNFs showed an apparent fragmentation of DNA. The apoptotic death was...
Fig. 3. TNF susceptibility of R55 gene-transduced AsPC-1 (A) or PANC-1 (B) cells. Panels represent the viability of parental cells (○), mock transfectants (△), and R55-transfectant clones (● and ▲) treated with $10^{-2}$ to $10^{3}$ ng/ml native TNF for 48 h. Viability was determined by a dye-uptake method as detailed in "Materials and Methods." The data represent the means (bars, SD) of triplicate wells. The clones from R55 transfectants from both AsPC-1 and PANC-1 showed dose-dependent sensitivities to TNF.

further verified by examining the expression of ICE mRNA by Northern blot analysis. As shown in Fig. 7, bands of ICE mRNA were clearly detected in R55 clone 10 treated with both TNFs. A corresponding band was not seen in either parental cells or mock transfectants with or without TNF treatment.

Fig. 4. Binding competition assay for mutein TNF and native TNF to R55 receptor. R55 gene-transduced AsPC-1 clone 10 cells were pretreated with anti-TNF-R75 monoclonal antibody utr-1. Binding of $^{125}$I-labeled native TNF to R55 was inhibited by unlabeled mutein 471 (●) or native TNF (○) added to the cells at the indicated molar ratio. The data represent the means (bars, SD; n = 3). Note that mutein TNF 471 competes with (replaces) the binding of $^{125}$I-labeled native TNF more strongly than native TNF on clone 10.

Antitumor Effects of Native and Mutein TNF on R55 Gene-transduced Tumor Inoculated in Nude Mouse. R55 clone 10 of AsPC-1 cells, parental cells, and mock transfectants were inoculated into nude mice, and either PBS, native TNF, or mutein TNF 471 was administered according to the regimen mentioned in "Materials and Methods" (Fig. 8). Among tumors of the R55 clone treated with PBS; parental cells treated with PBS; and mock transfectants treated with PBS, 5 µg of native TNF, or 5 µg of mutein TNF, there were no essential differences in growth curves. Although data are not shown in the figure, tumors of parental cells treated with 5 µg of native or mutein TNF also showed growth curves similar to those of the mock transfectants, whereas the growth curve of the clone 10 tumors was significantly suppressed by administration of 1 µg of native TNF. The suppression of the growth curve of the same clone became more evident when mice were treated with the equivalent dosage of mutein TNF. This suppression of tumor growth was recovered, to a certain extent (although not with statistical significance), by the pretreatment...
compared to native TNF in activating NK cells was also evident, with demonstrated that NK cell activity from nude mice injected with directed at YAC-1 cells (NK cell target) were measured. The results TNF is known to activate NK cells via R55. mutein TNF 471 is so great that no difference in growth curves was found between concentrations (5 μg), the antitumor effect of both native and mutein TNF were obtained, and their killing activities directed at YAC-1 cells (NK cell target) were measured. The results demonstrated that NK cell activity from nude mice injected with mutein TNF was significantly higher than that of the native TNF-injected mice (Fig. 9). This enhanced activity of mutein TNF 471 as compared to native TNF in activating NK cells was also evident, with nude mice bearing no tumors (data not shown).

NK Cell Activity Induced by Native and Mutein TNF. Because TNF is known to activate NK cells via R55, mutein TNF 471 is predicted to be more potent in augmenting NK cell activity. To verify this, spleen cells from BALB/c-nu/nμ mice bearing clone 10 tumor treated with TNFs were obtained, and their killing activities directed at YAC-1 cells (NK cell target) were measured. The results demonstrated that NK cell activity from nude mice injected with mutein TNF was significantly higher than that of the native TNF-injected mice (Fig. 9). This enhanced activity of mutein TNF 471 as compared to native TNF in activating NK cells was also evident, with nude mice bearing no tumors (data not shown).

DISCUSSION

There may be at least four strategies to enhance the tumoricidal activity of TNF. Apoptotic death induced by TNF is known to be counteracted by intracellular resistant factors, such as enTNF (2, 25, 26), MnSOD (27, 28), bcl-2 (29, 30), and nuclear factor κB (31–33). One of the strategies to enhance antitumor activities, therefore, could involve the use of antisense oligonucleotides to these resistant factors. However, a practical approach to using antisense technology for suppression of specific gene expression is presently considered to be complex because of the instability of antisense preparations, incomplete inhibition of the endogenous gene expression, and the requirement for highly efficient delivery systems.

A second strategy involves the augmentation of apoptotic cell death by administering agents such as various anticancer drugs that enhance its signaling (34).

A third strategy is a therapy using the gene involved in transduction of the apoptotic signal of TNF. Apoptotic cell death caused by TNF is known to be mediated by one of the TNF receptors (R55) that transmits death signals. It is initiated by association with TRADD (TNF receptor 1-associated death domain protein; Ref. 35) and FADD (Fas-associated protein with death domain; Refs. 36 and 37), which in turn connect the receptors to ICE-like cysteine proteases (38) via FLICE/MACH (39, 40). Another receptor (R75) transduces the signal of proinflammatory reactions causing adverse effects through the interaction with TRAF1 and TRAF2 (TNF receptor-associated factors 1 and 2, respectively; Ref. 41), which induces activation of NIK (MAP3K) and then nuclear factor κB (42, 43). The gene for TNF receptor R55, therefore, may be the most plausible candidate for this type of gene therapy.

A fourth strategy involves the use of mutein TNF, which exerts higher tumoricidal activity, having higher affinity for R55 than native TNF.

In the present investigation, the latter two strategies were combined for the treatment of pancreatic cancer, which is known to be one of the most aggressive malignancies, having a poor prognosis and being markedly refractive to any of the common current modalities. The results here clearly demonstrated the effectiveness of this approach. The transfection of the R55 gene into human pancreatic cell lines AsPC-1 and PANC-1, which had been shown to exhibit low TNF susceptibility presumably due to the low expression of R55 (Fig. 1) and the high concentration of intracellular TNF-resistant factors (2, 25–28), apparently enhanced their susceptibility to native TNF (Fig. 3). Differences in cytocidal effects with higher dosage or with long incubation times (48 h) between mutein and native TNF were not significant, possibly because apoptotic signals transduced with these with the NK cell-specific antibody antiasialo GM1. At higher concentrations (5 μg), the antitumor effect of both native and mutein TNF was so great that no difference in growth curves was found between them.

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TNFs were equally fully saturated, as revealed by DNA ladder formation. Conversely, the enhancement of susceptibility was more pronounced with mutein TNF 471, which has a higher affinity to R55 (Fig. 4) and a lower affinity to R75 than native TNF when lower dosages or shorter incubation times (6 h) are used (Fig. 5). These in vitro observations are compatible with the subsequent in vivo findings that growth of the R55 transfectant in nude mice was markedly and equally suppressed with native TNF and mutein TNF at a higher dosage (5 µg, five injections), and superiority of mutein TNF over native TNF in terms of growth suppression was apparent at a lower dosage (1 µg, five injections).

More importantly, with regard to adverse effects, mutein TNF 471 is much more beneficial than native TNF (9). Although nude mice, which are unavoidably used in the present study to deal with human pancreatic cancer, are rather insensitive to the signal induced through R75 (44), it has been already proven that mutein TNF 471 is less toxic than native TNF in C3H/HeJ mice pretreated with b-D-galactosamine, which activates a second pathway of mice to become sensitive to adverse effects of TNF with coadministration of lipopolysaccharide, IL-1, or a glucocorticoid antagonist (6). It therefore may be reasonable to use mutein 471 for considerations of both higher tumorcidal activity and fewer adverse effects.

In this context, a recently uncovered TNF family protein, TRAIL, which causes apoptosis only in tumor cells but not in normal cells (45, 46), also suggesting low adverse effects, may be considered. TRAIL activates an internal suicide program through the death receptors DR4 and DR5 in cancer cells but not in normal cells expressing an additional receptor (decay receptor 1/TRID), which counteracts the cytotoxic signal from the death receptor (47, 48). Therefore, in future trials similar to those of the present investigation, use of TRAIL and its cytotoxic signal gene may be warranted.

Nevertheless, the effectiveness and outcome of this therapy may largely depend on the transduction efficiency of R55 gene into target tumor cells. At present, however, no vector system that can transduce the gene into all of the targeted cells is available, and recurrence from the residual tumor cells after the therapy may be inevitable. In fact, suicide gene therapy, which shares a strategy in common with the present modality (i.e., simply replacing R55 gene with suicide gene and TNF with toxic produgs), invariably suffers from local recurrence. To overcome this obstacle in suicide gene therapy, immune-cytokine gene therapy has been combined with it (15).

The strategy of using TNF has an apparent theoretical advantage over suicide gene therapy, because TNF itself is capable of inducing an immune response in tumor cells. As the immune response is mainly mediated through R55, it may be even more logical to adopt mutein TNF 471, which has a higher affinity for this particular receptor in this strategy. Our data, in fact, indicated that NK cell activity of spleen cells of mice inoculated with tumors were enhanced more by administration of mutein TNF 471 than by native TNF. This difference in enhancement is not simply ascribed to the differences in direct activation of NK cells via R55 (16), because both native and mutein TNF were equally capable of activating NK cells in vitro (data not shown), but possibly involves indirect in vivo effects such as augmentation of IL-12 (NK cell activator) production from macrophages (18).

The present nude mouse model, however, is apparently unsuitable to evaluate full immune responses. We are currently undertaking experiments in which s.c. tumors inoculated in syngeneic mice are injected with adenovirus carrying the R55 gene and subsequently with native or mutein TNF to determine whether apoptotic regression of the tumor occurs at the same levels as in the present experiment, and whether the immune response is indeed sufficient to reject nontransduced residual tumor.

In conclusion, the present approach of using R55 gene in combination with mutein TNF 471 appears to be promising for the therapy of pancreatic cancer and is possibly applicable for other cancers as well.

Fig. 9. Effects of mutein TNF 471 on mouse spleen NK cell activity. Nude mice were inoculated with R55-transfectant clone 10 of AsPC-1 cells. Each mouse was injected five times with 1.0 µg of native TNF (○) or mutein TNF 471 (■). Nude mice injected with PBS alone were used as controls (□). One day after the last injection of TNF, mouse spleens were removed and NK cell cytotoxicity against YAC-1 cells was measured. One × 10⁴ cells/well of labeled targets were incubated with effectors in various ratios in 96-well plates. The plates were centrifuged after incubation for 4 h, and the supernatant was counted using a gamma counter. Percentage specific lysis was calculated as follows: 

\[ \text{Specific lysis} = \left( \frac{\text{Mean cpm from test supernatants} - \text{Mean cpm from supernatants of target cells alone (spontaneous release)}}{\text{Mean cpm after target cell lysis with lysis buffer (total release)}} \right) \times 100\% \]

The data represent the means (bars, SD) of the results obtained from three separate experiments. Similar results were obtained in three experiments. NK cell activity of nude mice injected with native TNF was higher than that of control mice. With mutein TNF, it was even higher than that with native TNF.

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*3 Manuscript in preparation.*
ACKNOWLEDGMENTS

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REFERENCES


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