and prepared as described.[7] S-180 cells were implanted intradermally (5×10^5 cells/200 μl/site) in 5-week-old female BALB/c mice. On day 7, when the tumor diameter reached 7 mm, PEG-TNF-α [500 Japan Reference Unit (JRU)/mouse] was injected i.v. into the mice. At 1, 1.5, 2, 3, 4, and 6 h later, various molecular weight of ^{125}I-Labeled PEG (1×10^6 cpm/200 μl) were administered i.v. Thirty minutes after the injection of ^{125}I-Labeled PEG, the mice were killed by abdominal arterial exsanguination. Then, the liver, kidney, spleen, heart, lungs, brain, inflammatory sites in which cotton had been implanted, skin and tumor were recovered and their radioactivity was measured by a γ-counter. The vascular permeability (VP) ratio was calculated according to the following equation.

$$\text{VP ratio} = \frac{\text{the radioactivity of tissue after the administration of PEG-TNF-}\alpha}{\text{the radioactivity of tissue without the administration of PEG-TNF-}\alpha}$$

RESULTS AND DISCUSSION

Recently, TNF-α has been clinically applied to loco-regional combination therapy with Melphalan, and this therapy showed a marked antitumor effect for patients in transit melanoma metastases.[11] This clinical approach, using TNF-α as a selective destruction agent against tumor endothelial cells and as a selective enhancer of tumor vascular permeability for effective accumulation of antitumor chemotherapeutic agents, is presently an attractive topics in the study of the optimization of cancer chemotherapy. Furthermore, it has also been approved by the European Agency for the Evaluation of Medicinal Products.[12] However, systemic administration of TNF-α was restricted due to its side effects. To use TNF-α for various therapeutic applications, we have created PEG-TNF-α with superior both in antitumor effectiveness and safety.

PEG-TNF-α at a dose of 1000 JRU showed marked hemorrhagic necrosis in S-180 tumors by a single i.v. injection without side-effects (Fig. 1). In mice treated with native TNF-α, a little hemorrhagic necrosis was caused at 10000 JRU. However, one of seven mice treated with native TNF-α at this dose died within 24 h, and the remaining mice developed piloerection and tissue inflammation (*e.g.*, erythma) and showed a decrease in body weight. Therefore, we considered that native TNF-α was not appropriate for examining the biological function and the application of therapy by systemic administration.

Figure 2 shows the vascular permeability (VP) induced in various tissues using PEG-TNF-α. The VP of tumor blood vessels formed in S-180 tumors was increased at 1—2 h, after an i.v. injection of PEG-TNF-α. At 3 h after the injection of PEG-TNF-α, the VP returned to the control-basal level, and it was lower than the control-basal level at 3 h. Furthermore, PEG with high molecular weight (molecular weight=500000) could accumulate in the tumor tissue as well as PEG with low molecular weight (molecular weight=12000). On the other hand, the permeability of the vessels in normal tissues, such as the brain, lung, liver, spleen, kidney, and skin, or inflammatory sites in which cotton had been implanted, changed slightly by i.v. injection of PEG-TNF-α.

Many researchers reported that TNF-α induces change of endothelial cytoskeletal actin and formation of intercellular gaps with increased permeability to macromolecules.[13,14] In addition, we previously reported that endothelial cells cultured in conditioned medium prepared from tumor cells, converted normal endothelial cells to have various character similar to tumor endothelial cells, and these cells had highly sensitivity to TNF-α.[15,16] Therefore, the increased permeability of the newly formed vessels in the system at 1—2 h after the administration of PEG-TNF-α would result from the increasing of the permeability of tumor endothelial cells and the cytotoxicity of PEG-TNF-α on tumor vascular endothelial cells. On the other hand, TNF-α is known to have procoagulant effects on tumor neovasculature, by causing fibrin deposition and localized thrombosis, which, in turn, leads to ischaemic necrosis of tumors.[17] Therefore, normalized and decreased permeability at 3 h would reflect the cessation

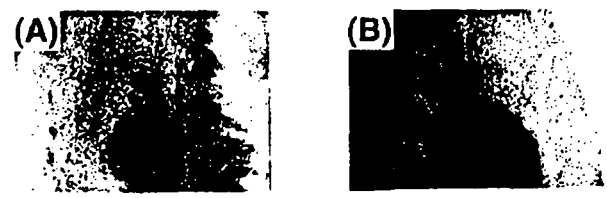

Fig. 1. Hemorrhagic Necrosis Effects of PEG-TNF-α

At 7 d after the tumor inoculation, mice were treated with i.v. injection of the (A) native TNF-α: 10000 JRU/mouse, (B) PEG-TNF-α: 1000 JRU/mouse. Tumor hemorrhagic necrosis was observed 24 h after the injection by macroscopic observation.

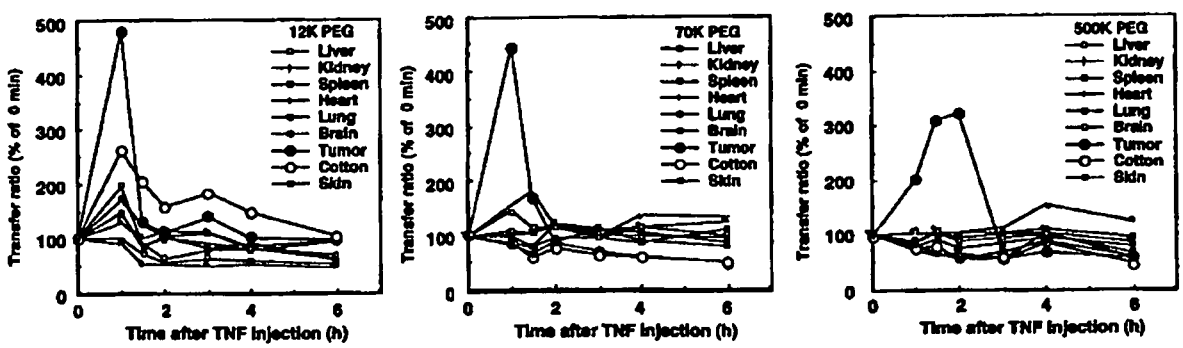

Fig. 2. Vascular Permeability in Various Tissues with PEG-TNF-α Treatment

Mice bearing S-180 tumor were killed at the indicated times after i.v. injection of PEG-TNF-α (500 JRU/ml). Thirty minutes before killing, each mouse was pulsed by an i.v. injection of ^{125}I-labeled PEG at a dose of 400000 cpm. Control levels without pre-injection of PEG-TNF-α are shown for time 0.

of the blood circulation because of the adsorption of fibrin-like substances on the luminal surface of the tumor vascular endothelial cells.

A matter of interest was why PEG with high molecular weight (molecular weight=500000) showed highest VP at 2 h when PEG with low molecular weight (molecular weight=12000 or 70000) could not accumulate in the tumor. The degree of accumulation into the tumor was decided by the balance between the penetration into the tumor tissue from the circulation and the leak from the tumor. Therefore, at 1 h after the administration of PEG-TNF-α, PEG with low molecular weight might accumulate in the tumor easily and accumulate in the tumor without leak. However, PEG with high molecular weight could not penetrate into the tumor. At 2 h after the administration of PEG-TNF-α, PEG with low molecular weight might penetrate into the tumor easily and leak from the tumor into the circulation. On the other hand, PEG with high molecular weight could penetrate into the tumor and accumulate in the tumor without leak.

Recently, many bioactive proteins such as immunotoxin, antibody-toxin conjugates, and cytokine has been attracted a highly expectation for tumor therapy. However, there has been no significant therapeutic response to solid tumors due to insufficient tumor accumulation. Therefore we consider that PEG-TNF-α would overcome these drawbacks. We are now examining the usefulness of PEG-TNF-α for combination therapy using chemotherapeutic drugs or immunotoxin. Thus PEG-TNF-α will open the new way to combination therapy with antitumor agents.

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Recent Progress on Tumor Missile Therapy and Tumor Vascular Targeting Therapy as a New Approach

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Abstract: Tumor targeting therapy, that is "Missile therapy", using a complex composed of a tumor suppressive drug and a whole antibody against tumor cells, is expected to become an attractive chemotherapy strategy. However, clinically convincing results have not yet been obtained mainly due to poor transport from the circulation to tumor tissue and marked toxicity. Recently, recombinant immunotoxins, composed of an Fv fragment of an antibody to a tumor-related antigen fused to various truncated toxins have been developed to overcome the distribution of immunotoxins in tumors. These recombinant immunotoxins have shown encouraging clinical results for some hematopoietic malignancies. However, there were no significant anti-tumor responses to many tumors, especially solid tumors, probably due to their rapid clearance from the circulation and their immunogenicity and antigenicity. More recently, PEGylation of recombinant immunotoxins has been attempted to overcome these drawbacks. It was found that PEGylation of recombinant immunotoxins improves their effectiveness. We discuss the recent progress in tumor missile therapy. In contrast to others, we developed "Missile therapy against tumor blood vessels" by using specific monoclonal antibodies against tumor endothelial cells rather than actual tumor cells. The complex between antibodies to tumor vascular endothelial cells and anti-tumor drugs can freely access the target cells without concern for their vascular permeability. These preparations have exhibited excellent anti-tumor effects for solid tumors. In this review, we also discuss this vascular targeting therapy as an attractive new strategy for tumor chemotherapy.

Keywords: Immunotoxin, PEGylation, Tumor vascular targeting, Antibody, Immunoconjugate, Tumor necrosis factor-alpha, Interleukin-6, Tumor endothelial cells.

1. INTRODUCTION

In modern tumor therapy, the paucity of efficient and target-specific anti-tumor drugs that are without serious side effects is a considerable problem. Therefore, drug delivery systems (DDS), such as immunoconjugates and immunotoxins, which are composed of anti-tumor agents (anti-tumor antibiotics and toxins) and monoclonal antibodies against a tumor-associated antigen (TAA), are being studied by many investigators [1-6]. However, this tumor missile therapy (Targeting Therapy) has not yielded satisfactory results [6,7], except in some limited cases, for the following reasons: (1) when a whole monoclonal antibody is used as a missile molecule, the complex of an antibody and an anti-tumor agent (immunoconjugate/ immunotoxin) has a very large molecular weight (over 150,000), resulting in poor selective transfer to the tumor tissue [8,9]; (2) the preparation of immunoconjugates or immunotoxins requires complex processes and it is not easy to produce them in adequate amounts for clinical use; (3) cross-linking monoclonal antibodies to anti-tumor agents often reduces the antibody titer or anti-tumor cytotoxic activity; and (4) monoclonal antibodies or toxins used as anti-tumor agents, which generally have stronger cytotoxicity to tumor cells than anti-tumor antibiotics, sometimes acquire antigenicity or immunogenicity, which makes their repeated administration difficult and elevates the risk of anaphylaxis. This reduces their anti-tumor effects while inducing unexpected adverse reactions. Recently, Pastan *et al.* have partially resolved these limitations [10,11]. They attempted to improve drug transfer to tumor tissue by using the variable region (Fv region) of antibodies as missile molecules. They succeeded in the large-scale production of recombinant immunotoxins that exhibit completely preserved antibody titers and toxin activity. Genetic engineering, using a fusion protein composed of the Fv portion of an antibody (that recognizes the tumor-specific antigens) and toxins, was used for this purpose. Excellent preclinical results of these recombinant immunotoxins *in vitro* and *in vivo* have led to the initiation of several clinical trials [12-14]. However, some immunotoxins have problems with stability, immunogenicity and lack of specificity. Thus, the elimination of these problems will elucidate a new approach to immunotoxin therapy.

As mentioned above, recombinant immunotoxins have been showing excellent anti-tumor responses to hematopoietic malignancies in Phase I trials but have not shown significant clinical effects to solid tumors (e.g. colon). This is probably due to their short plasma half-lives and

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other factors. Angiogenesis is a prominent feature of many physiological and pathological processes, including wound healing, luteinization and tumor growth [15,16]. Tumor angiogenesis is mediated by substances produced by tumor cells, and many previous studies have demonstrated that tumor growth is dependent upon it. Angiogenesis is also critical for tumor growth, since tumor vasculature not only supplies some nutrients but also mediates waste removal. Thus, selective inhibition of tumor angiogenesis and/or destruction of the tumor vascular -network seemed to be a more attractive approach for controlling neoplasms than direct anti-tumor therapies [17-20]. Tumor cells are heterogeneous [21,22]: therefore, a common antibody applicable to a wide range of tumor types does not exist. Conversely, almost all tumor tissue blood vessels exhibit various common features, such as enhanced permeability regardless of the tumor type or animal species (however, a whole antibody of molecular weight 150 kDa cannot rapidly access the solid tumor tissue from the circulation), suggesting the expression of common specific molecules in tumor tissue vascular endothelial cells (TEC), typified by the VEGF receptor. In addition, targeted TEC are directly exposed to blood, so their transfer from blood to the tumor tissue need not be considered.

In this review, we first, show the recent progress in tumor missile therapy using recombinant immunotoxins. We also summarize the essential technologies for ensuring the efficacy and safety of recombinant immunotoxins. Finally, we describe the usefulness of tumor vascular targeting therapy as a new approach to treatment.

2. RECOMBINANT IMMUNOTOXINS TO TUMOR CELLS

Recombinant immunotoxins are chimeric proteins in which a truncated toxin that serves as the cytotoxic moiety is fused to the Fv fragment of an antibody that serves as the targeting moiety. Pastan *et al.* produced a recombinant immunotoxin composed of an Fv fragment of an antibody to a tumor-related antigen fused to a 38-kDa mutant form of Pseudomonas exotoxin A (PE) [11.23-25]. Using several antibodies to various tumor-related antigens, they made recombinant immunotoxins by deleting the cell-binding domain of PE and replacing it with the Fv portion of an antibody. Some of these recombinant immunotoxins have recently been evaluated in Phase I trials. All the recombinant immunotoxins have been shown to cause complete regression of human tumor xenografts in nude mice and are relatively well tolerated by mice and monkeys.

For example, anti-Tac(Fv)-PE38 (LMB2, anti-CD25 recombinant immunotoxin), containing a single-chain Fv fragment of the anti-human Tac monoclonal antibody to the IL-2 receptor alpha subunit fused to a fragment of Pseudomonas exotoxin (PE38) with the translocation and ADP-ribosylation domains of PE, was recently found to be the first recombinant immunotoxin to induce major clinical responses in tumors [12,26]. Anti-Tac(Fv)-PE38 was administered to 35 patients with CD25+ hematologic malignancies, who failed standard and salvage therapies. As a result, one patient with hairy cell leukemia (HCL) had a complete remission [12], on going at 16 months, and seven partial responses were observed in HCL (n=3), cutaneous T-cell lymphoma (n=1), chronic lymphocytic leukemia (n=1), Hodgkin's disease (n=1), and adult T-cell leukemia (n=1).

However, anti-Tac(Fv)-PE38 exhibits, which limits the amount of immunotoxin that can be administered. These toxic were probably due to its non-specific distribution from the circulation to various normal organs, because it was found that the dose-limiting toxicity of anti-Tac(Fv)-PE38 in the Phase I trials was most often due to damage of liver cells that do not express IL-2 receptors. In addition, human anti-mouse antibodies and anti-PE antibodies were elevated in some patients treated with anti-Tac(Fv)-PE38, and this immunogenicity of recombinant immunotoxins may reduce their therapeutic potency. These problems may occur with other recombinant immunotoxins as well. Thus, if these side effects can be avoided, recombinant immunotoxins will show improved responses in human trials.

3. IMPROVEMENT OF THE NON-SPECIFIC TOXICITY OF RECOMBINANT IMMUNOTOXINS BY MUTATIONS IN THE FRAMEWORK REGIONS OF THE FV REGION

The toxic side-effects of recombinant immunotoxins are of two types. One type of toxicity results from the specific targeting of normal cells that express the same antigen as the tumor cells. Thus, Pastan *et al.* have been identifying more specific antigens present on prostate and ovarian tumor by using the latest technique in molecular biology such as DNA chip technology, phage display and DNA immunization as novel preparative methods for Fv polypeptides against tumor cells [5,27,28]. The other type of toxicity is non-specific and is usually characterized by damage to liver cells, although other toxic effects may also occur. Recombinant immunotoxins are foreign proteins for humans and mice; they may distribute to the reticuloendothelial system (RES), such as the Kupffer cells in the liver. In addition, these recombinant immunotoxins (Mw: 65,000) have a lower molecular weight than conventional antibody-toxin conjugates (Mw: 195,000) leading to a shorter survival in circulation and increased distribution to various normal tissues such as kidney and liver. For example, anti-Tac(Fv)-PE38 showed marked anti-tumor effects in leukemias and lymphomas in a Phase I trials. But it was found that one of the reasons for the limitation of dose escalation of anti-Tac(Fv)-PE38 was liver toxicity. If this liver toxicity of recombinant immunotoxins is overcome, the clinical results will be improved. Pastan *et al.* noted that the Fv of anti-Tac has an isoelectric point (pI) of 10.2 [29,30]. They hypothesize that the overall positive charge on the Fv portion of anti-Tac(Fv)-PE38 contributes to the non-specific binding to liver cells and results in dose-limiting liver toxicity. They found that lowering the pI of the Fv of anti-Tac, from 10.2 to 6.8 by selective mutation of surface residues, causes a 3-fold decrease in animal toxicity and hepatic necrosis in mouse models. This change in pI did not significantly alter the CD25 binding affinity, the cytotoxic activity towards target cells, or anti-tumor activity *in vivo*. These mutations in the framework regions of the Fv, which lower the pI, were found to improve the toxicity of other recombinant immunotoxins, such as SS1(dsFv)-PE38 targeting ovarian tumor and B3(dsFv)-PE38 targeting colon and breast tumors. If this

decreased toxicity also occurs in humans, it should markedly enhance the clinical effects.

4. PEGYLATION OF BIOACTIVE PROTEINS

As mentioned above, recombinant immunotoxins often exhibit immunogenicity and antigenicity, and this reduces their therapeutic usefulness. In addition, it was found that the plasma half-lives of these recombinant immunotoxins were unexpectedly short. Preclinical studies have shown that anti-tumor activity in hematopoietic malignancies is enhanced if recombinant immunotoxins survive longer in the circulation [31]. One of the most common ways to increase the blood-residency of proteins is to modify them with polyethylene glycol (PEG) [32-40] (Fig. 1). Many studies have reported that chemical modification of proteins with PEG (PEGylation) increases the molecular size and steric hindrance of the protein [41-44]. This results in improved plasma half-life and proteolytic-stability, and decreased immunogenicity and hepatic uptake. PEGylation of bioactive proteins decreases their renal excretion rate due to the increased molecular size. In addition, since the PEG chain covers the protein surface, attack from proteinases is blocked by steric hindrance, resulting in prolongation of the *in vivo* half-life. Similar steric hindrances cause a decrease in antigenicity and immunogenicity, resulting in the prolongation of *in vivo* clearance and stability. Due to these comprehensive effects of PEGylation, the doses of bioactive proteins can be decreased, resulting in reduced toxic side effects. Thus, the optimal molecular designs of proteodrugs are considered to be developed by PEGylation. For example, PEGylation of interleukin-2 has been reported to increase its anti-tumor potency *in vivo* and PEGylation of an F (ab') 2, derived from monoclonal antibody A7 has improved its localization to tumors. Recently, we also attempted the PEGylation of some bioactive proteins, such as tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6) and superoxide dismutase (SOD) to improve their therapeutic potencies. In this section, we show, using TNF-alpha and IL-6 as examples, the possibility that PEGylation of bioactive proteins, such as recombinant immunotoxins, overcomes their drawbacks, allowing their clinical application.

The recent marked advances in molecular biology and genetic engineering have enabled the large-scale production of a number of bioactive proteins, such as cytokines and recombinant immunotoxins. Attempts at applying these recombinant bioactive proteins to the treatment of intractable diseases such as tumors have been receiving close attention. However, since almost all of these recombinant bioactive proteins are quite unstable *in vivo*, their clinical use as a therapeutic agent requires frequent administration and high doses, which can impair homeostasis and cause severe adverse effects [46]. Furthermore, since cytokines such as TNF-alpha and IL-6, have diverse biological actions on various tissues, it is not easy to selectively obtain some particular favorable action (therapeutic effects) among their diverse actions and minimize side effects. These disadvantages markedly limit the clinical use of cytokines [46-48]. These problems concerning cytokines also apply to many other bioactive proteins typified by recombinant immunotoxins, whose large-scale production has become possible in recent years. Therefore, it is essential not only to develop a DDS that can overcome their instability *in vivo* but also to establish DDS technology that selectively exerts some particular beneficial action among their diverse

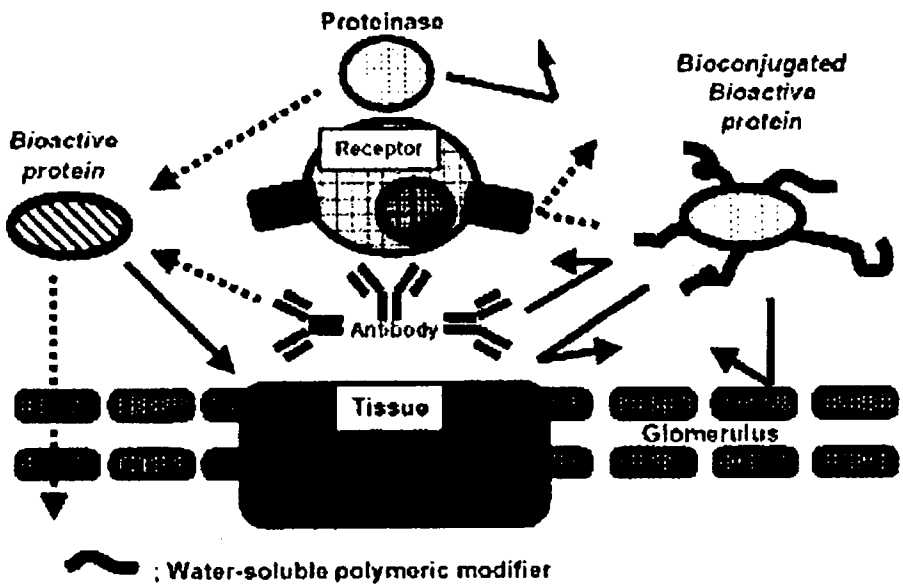

Fig. (1). Characteristics of hybrid bioactive proteins.
Hybrid bioactive proteins with water-soluble polymeric modifiers increase their molecular size and steric hindrance, resulting in augmented plasma half-lives and stability. For example, PEGylation enables the therapeutics dose and frequency to be decreased.

biological actions, i.e., a technology, which allows the exertion of only the targeted therapeutic action, without being accompanied by side effects. Keeping these points in mind, we attempted the bioconjugation of bioactive proteins using water-soluble polymeric modifiers such as PEG, to improve the stability of these proteins *in vivo*, and to achieve a more selective exertion of their therapeutic actions.

The behavior of bioconjugated bioactive proteins is expected to be greatly affected by the features of the polymeric modifiers, which cover the surface of these proteins. Therefore, it is necessary to first identify the particular polymeric modifiers, which provide desirable behavioral characteristics *in vivo* to individual bioactive proteins, taking into consideration the mechanisms by which these proteins exert their favorable actions. To prepare an index for selecting optimum polymeric modifiers, we evaluated the behaviors and pharmacokinetics of various polymeric modifiers *in vivo* and their interactions with vascular endothelial cells *in vitro*. This evaluation revealed that polymeric modifiers that possess charged functional groups or hydrophobic residues selectively accumulate in certain tissues such as kidney, liver and spleen, depending on the type and density of the functional groups. Additionally, non-ionic water-soluble polymers typified by PEG do not show a marked transfer to tissue and are suitable for the use in bioconjugation with proteins to improve their retention in blood.

When IL-6 is utilized therapeutically to promote the formation of platelets, its targets are the megakaryocytes [49,50]. It is known that megakaryocytes express large amounts of high affinity IL-6 receptors (IL-6 receptor-gp130 complex) [51] and that they are abundant in the pulmonary vascular lumens and on the outer surface of marrow veins [49]. Therefore, if IL-6 is modified to remain longer in circulation and thus smaller doses will be required for therapeutic use, then it will be possible to make the distribution and receptor affinity of IL-6 *in vivo* such that a selective and efficient action of IL-6 on megakaryocytes can be achieved. Furthermore, since the transfer of IL-6 into the liver and spleen causes adverse reactions [52], the improved retention of IL-6 in the blood and the resultant decrease in the transfer and accumulation of IL-6 to these tissues is expected to reduce the side effects of IL-6 therapy. Thus, we attempted the bioconjugation of IL-6 with PEG (PEGylation) to increase the activity of IL-6 in the promotion of platelet production and reduce its side effects. When IL-6 was subjected to PEGylation under optimum conditions that were selected by considering the relationships between specific activity, degree of PEG-modification, molecular size, etc., the resultant PEG-modified IL-6 (MPEG-IL-6) had a plasma half-life over 100 times greater than that of native IL-6. MPEG-IL-6 showed more than 500 times the thrombopoietic potency of native IL-6 (Fig. 2). Furthermore, strong adverse reactions such as fever, IgG production and acute protein production observed following the administration of native IL-6 were seldom seen after the administration of MPEG-IL-6. Separation of therapeutically favorable targeted actions from side effects, using PEGylation, has been successful not only for IL-6 but also for TNF-alpha. Our study demonstrates that the PEGylation of TNF-alpha, under optimum conditions, remarkably improved the stability of

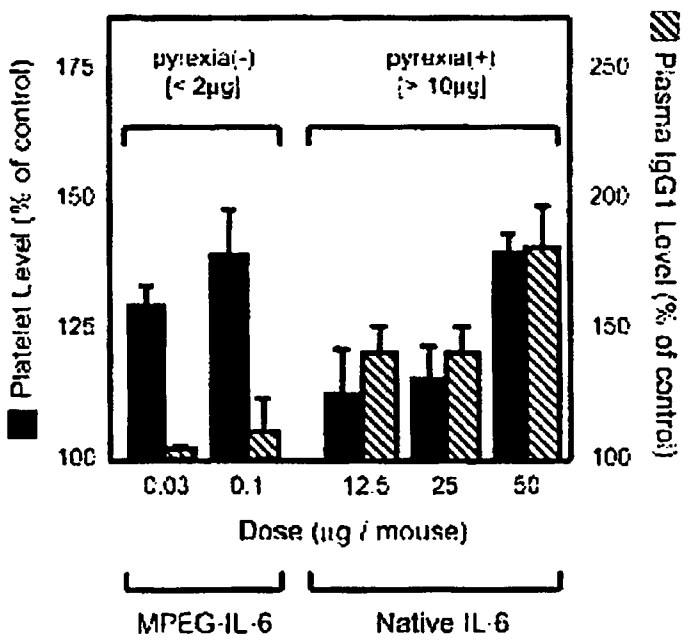

Fig. (2). Effects of PEG-IL-6 on platelet production and serum IgG1 level.
Blood samples of mice on day 9 after injection were used every 2 days. Serum IgG1 concentration was measured by ELISA. (Mean ± SE, N=4.)

TNF-alpha in blood and its transfer to tumor tissue resulting in a greater therapeutic efficacy of TNF-alpha (about a 100-fold increase in anti-tumor activity, compared to native TNF-alpha), while reducing the adverse reactions. It was found that PEGylation allows the exertion of selected favorable actions of cytokines via the following mechanisms: (1) improved stability *in vivo* reduces the dosage and thus the blood level of cytokines, making it possible for a given cytokine to exert its selected actions on the basis of the differences in the affinity of the cytokine for various receptors; and (2) regulation of the behavioral characteristics (blood retention and tissue transfer) of a given cytokine enables the cytokine to exert selected actions, depending on the differences in its distribution among various tissues. We have thus succeeded in making cytokines useful as therapeutic agents by improving their stability *in vivo* and increasing their selected favorable actions (anti-tumor activity in the case of TNF-alpha and the promotion of platelet production in the case of IL-6). These results suggest that optimal PEGylation will also be useful in improving the clinical problems of recombinant immunotoxins.

5. SITE-SPECIFIC PEGYLATION OF RECOMBINANT IMMUNOTOXINS AND THE IMPROVEMENT OF THEIR PLASMA HALF-LIVES, STABILITY AND IMMUNOGENICITY.

Pastan *et al.* reported that a PEGylated chimeric toxin composed of transforming growth factor-alpha and PE, showed an improved blood-residency time and a decrease in immunogenicity resulting in enhanced *in vivo* anti-tumor potency and reduced *in vivo* toxicity [58]. However, PEGylation was accompanied by a significant loss of specific cytotoxic activity of PE. Unlike PEGylation of enzymes, which act on small substrates, the PEGylation of recombinant immunotoxins may cause a decrease in activity due to loss of antigen-binding, ADP-ribosylation, or the ability to translocate to the cytosol, which are based on macromolecular interactions that are easily sterically hindered by the attached PEG. We also obtained similar results. The specific activity of PEGylated TNF-alpha decreased with the PEG modification rate (degree of PEG-modification). Lys11 is considered to play an important role in the development of TNF-alpha activity. Thus, a decrease in the specific activity may be partly due to the modification of the lysine residues involved in the development of TNF-alpha activity. Additionally, the bioactivity of PEGylated TNF-alpha decreased with an increase in the molecular weight of the attached PEG. Our other studies on PEGylated IL-6 and leukemia inhibitory factor (LIF) yielded similar results. In our previous study on PEGylated SOD, SOD-substrate complex formation was possible without steric hindrance of the PEG attached to SOD because of the low molecular weight of the substrate (superoxide anion). As a result, the specific activity of the PEGylated SOD was not associated with the molecular weight of PEG and was only determined by the modification rate of the lysine residues. On the other hand, bioactive proteins such as TNF-alpha, IL-6 and LIF require binding to macromolecular receptors for the exertion of activity. In such bioactive proteins, in addition to a decrease in the specific activity due to modification of the lysine residues, consideration should be given to the inhibition of activity caused by the steric hindrance of PEG. In most cases, the PEGylation of proteins is non-specific and targets all lysine residues in the protein, some of which may be in or near the active site. To overcome this drawback, Pastan *et al.* attempted to carry out site-specific PEGylation of mutant PE molecules that were engineered to contain one or two cysteine residues on their surface [54,55]. PEG was attached to these residues by free thiol chemistry. However, this approach proved to be unsuccessful due to a low yield of PEGylated immunotoxin and a significant loss in activity. Recently, different approaches to site-specific PEGylation have been chosen to overcome these problems of PEGylation using anti-Tac(Fv)-PE38 as a model recombinant immunotoxin. To keep the antigen-binding, translocation and ADP-ribosylation activities intact, a mutant anti-Tac(Fv)-PE38, containing a single cysteine in the peptide connector that attaches the Fv moiety to the toxin, was prepared. Subsequently, site-specific modification of the mutant LMB-2 with PEG-maleimide, via the formation of a thioether bond, was carried out.

In anti-Tac(Fv)-PE38, the Fv portion of the anti-Tac antibody is linked to PE38 by a peptide connector (ASGGPE). To prevent loss of the antigen-binding, translocation and ADP-ribosylation functions of anti-Tac(Fv)-PE38 that are necessary for its specific cytotoxic activity against CD25+ tumor cells, we prepared a mutant form of anti-Tac(Fv)-PE38 with one cysteine in the peptide (ASGCPE) that connects the Fv to PE38. Site-specific PEGylation was carried out at this cysteine using 20-kDa and 5-kDa of PEG. Both types of PEGylated anti-Tac(Fv)-PE38 had similar cytotoxic activities compared to the unmodified anti-Tac(Fv)-PE38. The anti-tumor activities of both types of PEGylated mLMB-2s *in vivo* were markedly higher than the native anti-Tac(Fv)-PE38. Additionally, the plasma half-life of the unmodified anti-Tac(Fv)-PE38 was about 13 min. In contrast, the serum concentration profiles of both PEGylated anti-Tac(Fv)-PE38 showed monoexponential elimination curves. The plasma half-life of PEG5K-anti-Tac(Fv)-PE38 and PEG20K-anti-Tac(Fv)-PE38 increased by about 5-fold and 8-fold, respectively. Native anti-Tac(Fv)-PE38 markedly induced anti-(anti-Tac(Fv)-PE38) IgG antibodies in mice. In contrast, the immunogenicity of both PEGylated LMB-2s was found to be much lower. Therefore, it has been shown that site- specific PEGylation of recombinant immunotoxin increases its stability, blood-residency time and anti-tumor activity, while decreasing its non-specific toxicity and immunogenicity. The overall therapeutic window increased over 20-fold. These results have important clinical implications for the use of immunotoxins in patients. The approach used for improving the action of anti-Tac(Fv)-PE38 should be applicable to other recombinant immunotoxins as well and may increase their activity.

6. STRATEGY OF TUMOR VASCULAR TARGETING

Targeting therapy using immunoconjugates and immunotoxins composed of a monoclonal antibody to tumor cells and an anti-tumor antibiotics/toxin is attractive. However, the use of a monoclonal antibody against tumor cells themselves has often been ineffective due to their poor

penetration into tumor tissue [6]. As mentioned above, recombinant immunotoxins, have shown great anti-tumor effects to hematopoietic malignancies in Phase I trials, but there has been no significant therapeutic response to solid tumors. This can be attributed to their short plasma half-lives and insufficient tumor accumulation whereas, by using whole monoclonal antibodies, it is known that the distribution of recombinant immunotoxins in tumors is much higher than that of immunoconjugates and immunotoxins [8,56]. Furthermore, targeting tumor cells requires monoclonal antibodies against different types of tumor cells, because the antigens of tumor cells are heterogeneous. Tumor growth is dependent on new blood vessel formation to supply nutrients and oxygen [57,58]. The vasculature that is created by angiogenesis, as the tumor develops, is reported to be similar among various tumor types. Additionally, tumor vasculature has properties that differ from those of normal vasculature, such as enhanced permeability, suppressed leukocyte adhesion and high sensitivity to TNF-alpha [59-61]. These anatomical, morphological and behavioral differences between blood vessels in tumor and in normal tissues suggest that antigenic differences would be induced on endothelial cells by the tumor microenvironment. Recent reports indicate a higher expression of some molecules on tumor vascular endothelium than on normal endothelium [62-64]. These molecules include endoglin [62], endosialin and aminopeptidase N [63,64] and are considered suitable candidates for tumor antibody therapy, since the antibody can freely access the target regardless of vascular permeability. Furthermore, destruction of the tumor vascular endothelium can cause irreversible clotting, resulting in the formation of an occlusive thrombus that would halt blood flow and cause effective tumor regression (Fig. 3). However, a specific monoclonal antibody against TEC without some monoclonal antibodies to overexpressed antigens, such as VEGF-receptor, had not been previously prepared. Furthermore, neither isolation nor culture of TEC, which may be an immune source, has been performed. In the next section, in order to develop the tumor targeting therapy, we initially described the method of isolation of vascular endothelial cells from tumor tissues, and then indicate its usefulness as tumor therapy.

7. ISOLATION AND PROPERTIES OF TUMOR-DERIVED ENDOTHELIAL CELLS

It is believed that tumor and stroma cells, directly or indirectly, affect the properties of endothelial cells in tumor tissues. It is now well-established that great heterogeneity exists between not only endothelial cells in macro and microvessels but also among microvessels and endothelial cells from different tissues [65-73]. Brain microvessel endothelial cells form a blood-brain barrier through which macromolecules cannot pass. Liver and spleen microvessels, known as discontinuous capillaries, possess open cellular junctions that allow the passage of macromolecules. Blood vessels in tumors also show significant differences from those of normal tissues, in terms of structure and function. Many investigators have reported that tumor vessels are more permeable than normal tissue vessels. The various tissue-derived endothelial cells that have been cultured and examined to date include those from the human umbilical vein, human adrenal capillary, rat cerebral microvessels and bovine retinal microvessels. However, a method to isolate and culture TEC had not yet been developed.

We isolated TEC from KMT-17 fibrosarcoma-bearing rats by Percoll's density gradient centrifugation method. We examined whether the isolated TEC maintained the *in vivo* properties of tumor-derived vasculature. Tumor vessels are more permeable than normal tissue vessels *in vivo*. TEC monolayers showed greater permeability than the aortic, venous and epididymal fat-derived endothelial monolayers by FITC-dextran (molecular weight 70,000) diffusion (Table 1). Leukocyte adhesion to tumor vessel endothelium is known to be often lower than that to normal tissue vessel endothelium (Table 2). We examined this feature in the isolated TEC, and found that leukocyte adhesion to TEC was reduced compared to that of rat epididymal-fat pad derived capillary endothelial cells (FCEC).

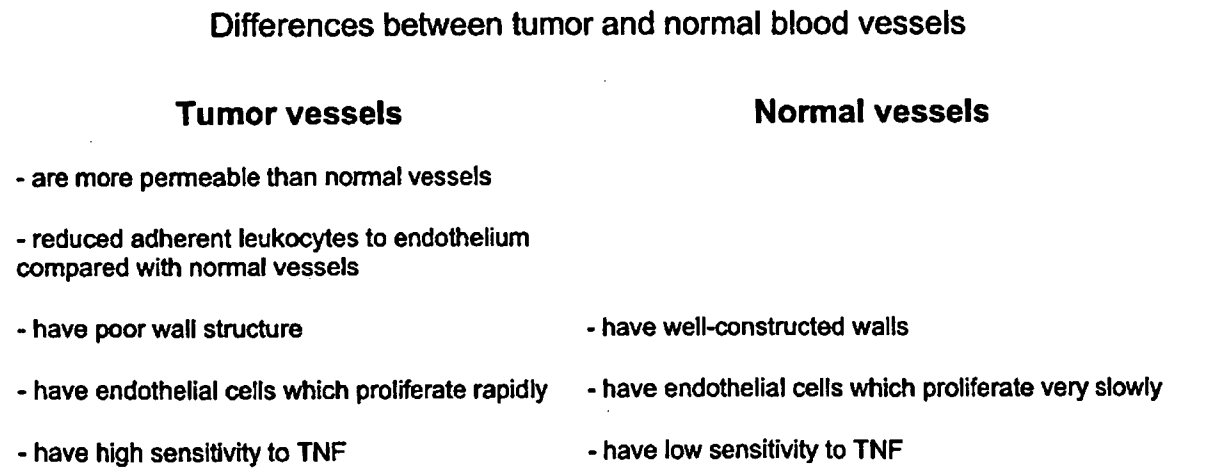

Fig. (3). Targeting therapy of cancer with monoclonal antibody against tumor derived endothelial cells.

Table. 1. Permeability of Rat Endothelial Monolayers to FITC-dextran. Rat Aortic, Vena Cava, Epididymal Fat Capillary and Tumor-derived Endothelial Cells were Cultured on the Permeation Chamber. When the Cells were Confluent, the Permeability of FITC-dextran (Molecular Weight 70,000) was Determined. Mean±SD. N=4

Source	Permeability coefficient ($\times 10^{-3}$cm/h)
Aorta	17.23 ± 2.52
Vena cava	8.43 ± 1.96
Fat capillary	19.49 ± 3.57
Tumor capillary	41.10 ± 3.99

Table. 2. Number of Leukocytes Adhering to Endothelial Cells. Rat Epididymal Fat-derived Endothelial Cells (FCEC) and Tumor-derived Endothelial Cells (TEC) were Cultured on a 96-well Tissue Culture Plate. When the Cells were Confluent, Rat Leukocyte Adhesion to Endothelial Cells was Assayed. a) P<0.001 Compared from FCEC by Students' t-Test. Mean±SD. N=4

	$\times 10^5$cells/cm^2	X10^5cells/10^5cells
FCEC	1.58 ± 0.12	2.83 ± 0.22
TEC	0.86 ± 0.14[a)]	1.75 ± 0.28[a)]

Various tissue-derived endothelial cells have been cultured, and it is very important to determine whether the isolated cells retain the properties of the tissues from which they were derived. Endothelial cells present not only tissue characteristics but also properties specific to the type of vessel from which they are derived i.e., aorta, vena cava or capillaries. *In vivo*, histamine induces plasma protein leakage in the venous and capillary vessels; it also increases venous and capillary-derived endothelial permeability in culture systems. In contrast, histamine has been shown to decrease bovine aortic endothelial cell permeability. These findings suggest that it is necessary to study endothelial cells derived from the specific tissue of interest to obtain valid results. Primary cultures of TEC showed hyperpermeability to macromolecule diffusion and low leukocyte adhesion, properties similar to tumor vascular endothelium *in vivo*.

It is known that the *in vivo* features of cells obtained by primary culture gradually disappear after repeated cell passages. Therefore, we examined the number of cell passages during which the TEC maintained the features of tumor vessels, using permeability and leukocyte adhesion as indices. As a result, TEC maintained the features of tumor vasculature until the first passage, but the features disappeared after the fourth passage. This may be due to the discontinuation of various factors obtained from tumor cells. Therefore, we used cells obtained by primary culture or the first cell passage for all our experiments. Thus, it is very likely that the TEC isolated in this study can be utilized as an immune source for preparing a monoclonal antibody against tumor-tissue endothelium.

8. PREPARATION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES TO TUMOR-DERIVED ENDOTHELIAL CELLS

To develop antibody-based tumor vascular targeting therapy for solid tumors, mice were immunized with TEC by passive and active immunization methods using TEC in which the *in vivo* features of tumor vasculature was maintained [74-76]. The mice were initially immunized with membrane protein components extracted from normal FCEC. After the antiserum collected from these mice was administered to other mice, these mice were immunized with vesicles from TEC to prepare hybridomas that produce a specific antibody against TEC. The specificity of the antibody to TEC was evaluated by ELISA using TEC as a solid antigen and immunostaining using frozen tissue sections of the parent rat tumor (KMT-17) tissue. As a result, five kinds of tumor tissue blood vessel-specific monoclonal antibodies were obtained. Of these, we will describe a monoclonal antibody that was named TES-23.

First, we examined tissue distribution, 1 h after intravenous administration of TES-23, using KMT-17 tumor-bearing rats. A negative antibody, MOPC, did not accumulate in tumor tissues. However, TES-23 markedly accumulated in tumor tissues within a short period; only 1 h (Fig. 4). Generally, when an antibody against tumor cells

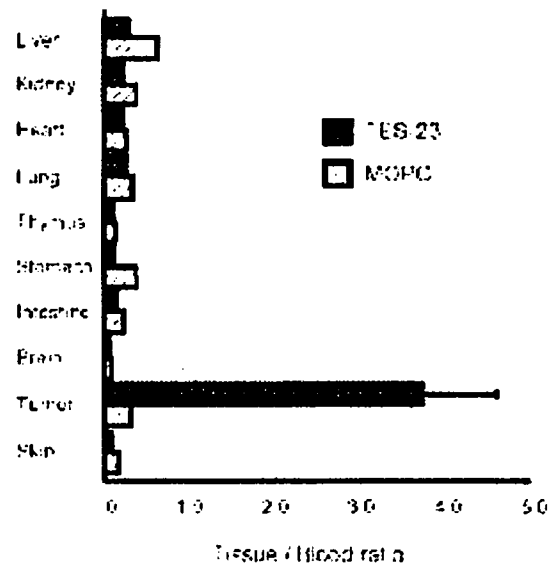

Fig. (4). Tissue distribution of ^{125}I-Labelled TES-23 in QKAH rats bearing KMT-17 fibrosarcoma.
100ng of radiolabelled TES-23 of MOPC were injected intravenously into tumor-bearing rats. One hour later the rats were anaesthetized and exsanguinated via the abdominal aorta. Each organ was removed and its radioactivity was counted by an auto gamma counter. Each value shown is the Mean ± SE for four animals.

themselves is used, antibodies do not accumulate in tumor tissues within 1 h of administration, and only a 2 or 3-fold amount of antibodies transfer to tumor tissues compared to that of negative antibodies 24 h after administration. Considering this finding, our results strongly suggest the efficacy of missile therapy targeted against tumor vasculature. Additionally, we evaluated, in detail, the immunostaining of KMT-17 tumor tissue sections with TES-23. It was suggested that TES-23 might recognize endothelial cells of nourishing blood vessels and neogenetic blood vessels in tumor tissues. Therefore, the specificity of TES-23 to TEC was suggested.

Subsequently, we examined the *in vivo* antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC) -mediated anti-tumor effects of administrating TES-23 antibodies alone. KMT-17 fibrosarcoma was intracutaneously inoculated in rats. One mg/rat (about 5 mg/kg) of TES-23 antibodies alone were intravenously administered for 5 days starting from 4 days after tumor inoculation. Cisplatin, which was used as a positive control, markedly inhibited tumor proliferation. Marked anti-tumor effects were observed in the TES-23-treated group compared to those in the control group. Furthermore, in the group treated with MOPC, no anti-tumor effects were observed. In contrast, marked weight loss was observed in the group treated with 1.2 mg of cisplatin. However, no weight loss was observed in the TES-23-treated group. These findings suggest that TES-23 alone inhibits tumor proliferation without serious side effects.

Hematoxylin and eosin staining of tumor tissue sections, 24 h after intravenous injection of 1 mg/rat of TES-23, was performed to clarify the usefulness of TES-23 as a missile molecule. In rats treated with the control antibody (MOPC), spontaneous necrosis was observed only at the center of the tumor focus. However, in the TES-23-treated group, extensive tissue necrosis was observed. In addition, degeneration, necrosis and exfoliation of TEC was observed in the TES-23-treated group. Furthermore, degeneration of tumor cells was observed in the TES-23-treated group. From the result of Elastica Van Gieson's staining, swelling of TEC was observed whereas no changes were observed in the control antibody-treated group. Additionally, fibrin thrombus formation was observed in the TES-23-treated group by phosphotungstic acid hematoxylin staining. No marked changes were observed in normal tissues after TES-23 administration. These results suggest that the anti-tumor effects of TES-23 administration involved the following mechanism: CDC or ADCC was induced by specific binding of TES-23 to the TEC, causing TEC injury and promoting thrombus formation and thus cutting off the lifeline of tumor cells. Therefore, TES-23 is expected to be a useful missile molecule.

9. PREPARATION AND CHARACTERIZATION OF THE IMMUNOCONJUGATE CONSISTING OF TES-23 AND NEOCARZINOSTATIN

We prepared an immunoconjugate (TES-23-NCS) consisting of TES-23 and a protein anti-tumor drug, neocarzinostatin (NCS), and applied TES-23-NCS to tumor vascular targeting therapy [77-79]. The TES-23-NCS conjugate was prepared by cross-linking the Fc site of TES-23 with the apoprotein site that was not associated with the site of NCS activity. Therefore, the immunoconjugate (TES-23-NCS) synthesized by this method completely sustained both the antibody titer and NCS activity. As described above, TES-23 was obtained using rat KMT-17-derived TEC as an immune source. Therefore, we initially examined the effects of the TES-23-NCS conjugate on KMT-17 solid tumors for primary screening. TES-23-NCS conjugate was administered through the tail vein. In the group treated with the TES-23-NCS conjugate, marked anti-tumor effects on rat KMT-17 solid tumor were observed. A conjugate consisting of MOPC and NCS did not show any efficacy. Administration of the TES-23-NCS conjugate resulted in tumor hemorrhagic necrosis, suggesting that the anti-tumor effects of this conjugate were associated not only with improved blood retention but also with tumor regression related to the decay of blood vessels. Subsequently, the *in vivo* distribution was examined to clarify the mechanism involved in the anti-tumor effects. One hour after intravenous administration, TES-23 and the TES-23-NCS conjugate markedly accumulated in rat KMT-17 tumor tissues. .

TES-23 will be a highly favorable missile molecule if specific molecules that are common among animal species or tumor types appear on TEC and can be recognized by TES-23. To evaluate the efficacy of TES-23 on various tumors derived from several animal species, we initially examined the anti-tumor effects of the TES-23-NCS conjugate on murine Meth-A fibrosarcoma (Fig. 5 and Table 3). The administration of a 10-fold dose (500 μg/kg) of NCS alone did not achieve tumor retraction, although tumor proliferation was slightly inhibited. Furthermore, efficacy was not obtained in groups treated with TES-23 alone (320 μg/kg) or a simple mixture of TES-23 (320 μg/kg) and NCS (50 μg/kg), whereas 5 mg/kg of TES-23 alone showed anti-tumor activity to rat solid tumor, as mentioned above. However, in the group treated with the TES-23-NCS conjugate (TES-23: 320 μg/kg, NCS: 50 μg/kg), marked anti-tumor effects with tumor hemorrhagic necrosis were obtained without any serious side effects. However, the efficacy disappeared when free TES-23 was simultaneously administered. A conjugate consisting of MOPC and NCS did not show any efficacy. Therefore, we found that the TES-23-NCS conjugate showed potent anti-tumor effects not only on rat KMT-17 but also on murine Meth-A without causing serious side effects. Next, we examined the pharmacokinetics of TES-23 in Meth-A tumor-bearing mice after intravenous administration (Fig. 6). Within a short period of 1 h, TES-23 showed tumor tissue accumulation that was 50-fold that of MOPC. TES-23 did not accumulate in normal tissues. Additionally, tumor vasculature accumulation of TES-23 was completely inhibited by pre-treatment with unconjugated TES-23. Therefore, the anti-tumor effects of the TES-23-NCS conjugate on murine Meth-A may have been obtained because the TES-23-NCS conjugate specifically accumulated in tumor vasculature, as demonstrated in the case of rat KMT-17. These findings suggest that TES-23 recognizes not only rat tumor vasculature but also murine Meth-A tumor vasculature. In addition, murine tumors other than Meth-A were similarly

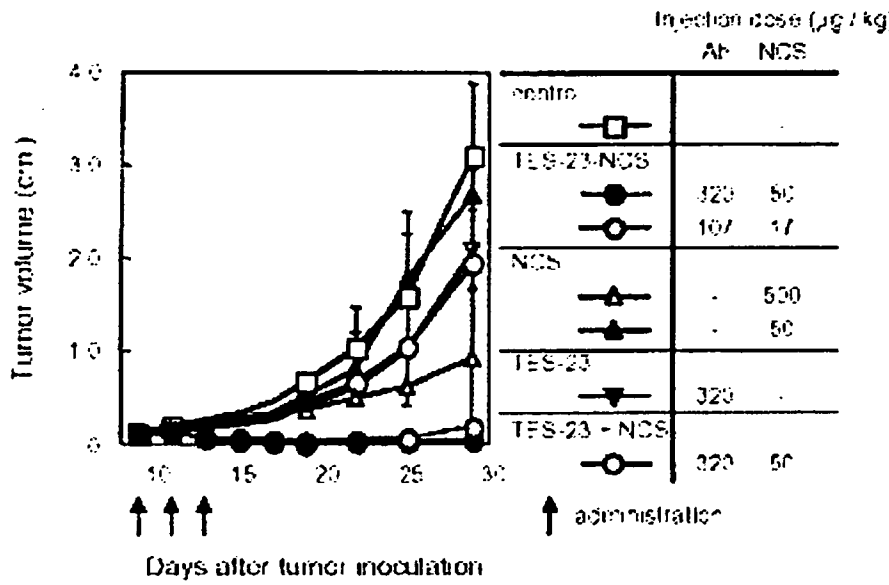

Fig. (5). Antitumor effects of TES-23-NCS on Meth-A Solid Tumor in BALB/c Mice.
Meth-A cells were implanted intradermally in the abdomen of BALB/c mice. TES-23-NCS conjugates or control was given intravenously on day9, 11, and 13 after tumor inoculation. Treatments are indicated by the arrows. Each value shown is the Mean ± SE for four animals.

Table. 3. Antitumor Effects of TES-23-NCS in Terms of Survival Days after Meth-A Tumor Inoculation

a) Groups of Four Mice Received Each Treatment Intravenously at day 9, 11, and 13

b) Days After Tumor Inoculation (Mean±SE).

c) Complete Regression was Defined as No Tumor Regrowth for More than 120 Days

d) Significant Difference from Control Group ($p<0.05$)

	Injection dose (μg/kg)[a]		Survival time (days)[b]		Complete regression[c]
	Ab	NCS			
control	-	-	53± 6	(39, 48, 59, 65)	0/4
TES-23-NCS	320 107	50 17	103±19[d] 95±17[d]	(53, 120<, 120<, 120<) (67, 72, 120<, 120<)	3/4 2/4
TES-23-NCS	107	17	46± 5	(34, 46, 50, 55)	0/4
+TES-23 (1070 μg/kg) MOPC-NCS	320 107	50 17	50± 3 45± 3	(45, 49, 50, 57) (38, 46, 47, 48)	0/4 0/4
NCS	- -	500 50	63±25 49± 6	(15, 48, 68, 120<) (31, 45, 51, 52)	1/4 0/4
TES-23	320	-	45± 4	(39, 39, 46, 53)	0/4
TES-23+NCS	320	50	51± 3	(46, 49, 50, 57)	0/4

examined. We examined tissue accumulation of TES-23 in Colon 26 adenocarcinoma or S-180 sarcoma-bearing mice. As observed in Meth-A tumor-bearing mice, TES-23 specifically accumulated in these murine tumors. In a human tumor-implanted model, TES-23 also specifically accumulated in tumor tissues at a level 30-fold that of MOPC. In this experimental system, tumor tissue blood vessels were derived from mice. However, this result suggests that common specific molecules recognized by TES-23 may also be induced in TEC in humans. Therefore, it is suggested that there may be specific molecules that are common among various animal species or tumor types on

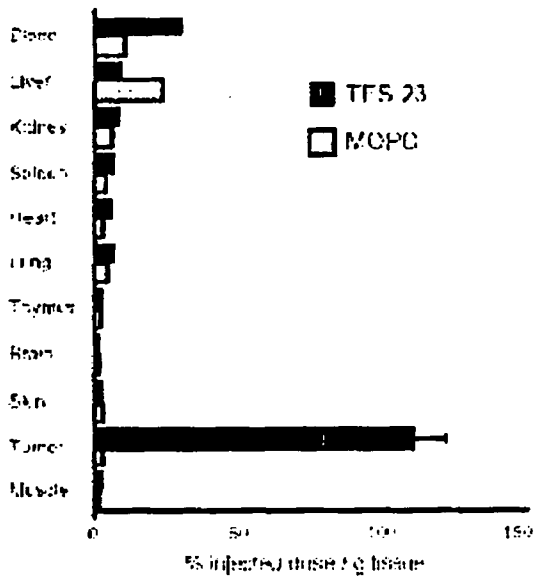

Fig. (6). Tissue distribution of ^{125}I-labelled TES-23 in tumor-bearing mice.

Tissue distribution experiments were performed in BALB/c mice bearing Meth-A Fibrosarcoma. A total of 20ng of radiolabelled antibodies were injected intravenously into mice. One hour later the mice were anaesthetized and exsanguinated via the abdominal aorta. Each organ was removed and its radioactivity was counted by an auto gamma counter. Each value shown is the Mean ± SE for four animals.

TEC and that TES-23 can recognize these specific molecules. Subsequently, immunostaining of tumor tissue sections was performed to confirm the expression of a common antigen on human TEC.

To clarify whether the tumor vascular antigen recognized was expressed on human tumor tissue by TES-23, sections of esophageal tumor tissue were prepared for immunostaining. Endothelium stained by an antibody to Factor VIII, an endothelial marker, was similarly stained with TES-23. The cross-reactivity of TES-23 in other types of tumors was investigated in specimens of esophagus, stomach, colon and breast tumor tissues. The endothelium in one of two esophageal tumor specimens was positively stained, as well as in one of two stomach tumor specimens both colon tumor specimens and both breast tumor specimens were also positively stained. In contrast, normal tissue from around the tumor was very weakly stained with TES-23 in two stomach tissue specimens whereas the other five normal tissue specimens, including two esophagi and two colon and one breast tissue specimen, were all negative. Thus, TES-23 may be a missile molecule that can be applied to many types of tumors. We are now investigating the antigen recognized by TES-23.

CONCLUSIONS

With the help of recent remarkable progress in molecular and cellular biology, it has gradually become clear that endothelial cells within tumor tissue, because of their close systemic relationship with tumor cells, express high levels of characteristic functional molecules commonly identified in a variety of tumor types. Most of the properties of these molecules are different from those of similar molecules found on endothelial cells within normal tissue. As a next-generation strategy, to replace chemotherapy against tumor cells, blocking of tumor angiogenesis by targeting a specific molecule has been recently devised. This therapy involves the regulation of functional molecules commonly present in tumor tissue endothelial cells. However, early angiogenesis blocking therapy merely attempted to impede proliferation of tumors by selectively inhibiting new construction and development of tumor tissue blood vessels. However, these approaches are not necessarily endothelial-cell specific. An innovative molecule-targeting therapy that not only inhibits angiogenesis in tumor tissue but also starves tumor cells by injuring existing tumor-feeding blood vessels has been long awaited. Molecular targeting therapy with TES-23, an antibody against a functional molecule present on tumor endothelium but not tumor cells, may overcome the problems associated with angiogenesis blocking therapy and earlier versions of targeted therapy against tumor cells. With this improved therapy variations in the diversity of tumor antigenicity need not be considered and both neo-vasculature and feeder vessels in the tumor tissue can be attracted. Our future research will attempt to isolate antibodies that selectively target tumor vasculature by *in vivo* screening with a phage-scFv naive library. We hope to obtain an even more useful antibody to tumor vasculature.

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A single intratumoral injection of a fiber-mutant adenoviral vector encoding interleukin 12 induces remarkable anti-tumor and anti-metastatic activity in mice with Meth-A fibrosarcoma☆

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Abstract

Cytokine-encoding viral vectors are considered to be promising in cancer gene immunotherapy. Interleukin 12 (IL-12) has been used widely for anti-tumor treatment, but the administration route and tumor characteristics strongly influence therapeutic efficiency. Meth-A fibrosarcoma has been demonstrated to be insensitive to IL-12 treatment via systemic administration. In the present study, we developed an IL-12-encoding fiber-mutant adenoviral vector (AdRGD-IL-12) that showed enhanced gene transfection efficiency in Meth-A tumor cells, and the production of IL-12 p70 in the culture supernatant from transfected cells was confirmed by ELISA. In therapeutic experiments, a single low-dose (2×10^7 plaque-forming units) intratumoral injection of AdRGD-IL-12 elicited pronounced anti-tumor activity and notably prolonged the survival of Meth-A fibrosarcoma-bearing mice. Immunohistochemical staining revealed that the IL-12 vector induced the accumulation of T cells in tumor tissue. Furthermore, intratumoral administration of the vector induced an anti-metastasis effect as well as long-term specific immunity against syngeneic tumor challenge.

Keywords: Interleukin 12; Meth-A fibrosarcoma; Recombinant adenoviral vector; Anti-tumor; Anti-metastasis; Intratumoral administration; IL-12 insensitive

The immunostimulating cytokine interleukin 12 (IL-12), a heterodimeric protein composed of two disulfide-linked subunits, is secreted by dendritic cells as well as macrophages and is a key mediator of immunity [1,2]. A variety of studies have focused on the use of IL-12 in cancer therapy and, in these experiments, IL-12 has exhibited potent anti-tumor activity in a number of tumor models [3–5]. IL-12 acts on T and natural killer (NK) cells by enhancing the generation and activity of cytotoxic T lymphocytes and inducing the proliferation and production of cytokines, especially interferon-γ

☆ *Abbreviations:* Ad vector, adenoviral vector; AdRGD, RGD fiber-mutant Ad vector; FBS, fetal bovine serum; IL-12, interleukin 12; MOI, multiplicity of infection; PBS, phosphate-buffered saline; PFU, plaque-forming units; $TCID_{50}$, tissue culture infectious $dose_{50}$.

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[6]. In addition, IL-12 inhibits tumor angiogenesis mainly through IFN-γ-dependent production of the chemokine interferon-inducible protein-10 (IP-10) [7].

Several mechanisms of the anti-tumor activity of IL-12 have been identified, and each contributes differently to the overall therapeutic outcome in a given tumor model [8–10]. Further, some tumor models, such as Meth-A and MCH-1A1 cells, are resistant to treatment with systemically administered IL-12 [11,12]. For example, intraperitoneal administration of murine recombinant IL-12 failed to inhibit the growth of Meth-A fibrosarcoma, even at a dosage of 500 ng daily for 3 days [11]. Compared with so-called IL-12-sensitive tumor cells such as OV-HM ovarian carcinoma and CSA1M fibrosarcoma, which both exhibited notable tumor regression after IL-12-stimulated T-cell infiltration into tumor tissues, Meth-A and MCH-1-A1 tumors lacked similar accumulation of immune cells [12]. Furthermore, otherwise exciting tumor regression results from preclinical studies were moderated by the severe adverse effects that occurred after systemic administration of IL-12 in murine models [13]. The clinical development of IL-12 as a single recombinant protein for systemic therapy has been tempered by pronounced toxicity and disappointing anti-tumor effects [14].

Intratumoral administration of IL-12 may offer several potential advantages over systemic dosing, such as delivery of the gene directly to the tissue of interest and avoidance of the drawbacks of systemic delivery, including the induction of toxicity, acute allergic reactions, and other adverse effects due to the encoded gene [15]. The results of one clinical trial suggest that intratumoral injection of $\leqslant 3 \times 10^{12}$ viral particles of an IL-12-encoding adenoviral vector in patients with advanced gastrointestinal malignancies is feasible and well tolerated [16].

In the present study, we constructed a recombinant adenovirus (Ad) vector that encoded IL-12 (AdRGD-IL-12); the gene transfection efficiency of AdRGD-IL-12 was higher than that of a conventional Ad vector. We also investigated the feasibility of using a single intratumoral injection of AdRGD-IL-12 to provide effective cancer treatment for primary and metastatic Meth-A fibrosarcoma. Furthermore, immunostaining was used to measure the postinjection infiltration of immune cells into tumor tissue.

Materials and methods

Cell lines and animals. Meth-A fibrosarcoma cells (BALB/c origin) were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Osaka, Japan) and were maintained by intraperitoneal passage in syngeneic BALB/c mice. Human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. BALB/c female mice were obtained from SLC (Hamamatsu, Japan) and used at 6–8 weeks of age. All of the experimental procedures were performed in accordance with the Osaka University guidelines for the welfare of animals in studies of experimental neoplasia.

Vector construction. The replication-deficient AdRGD vector was based on the adenovirus serotype 5 backbone with deletions of E1/E3 region. The RGD sequence for αv-integrin targeting was inserted into the HI loop of the fiber knob by using a two-step method, as previously described [17]. AdRGD-Luc, which is identical to the AdRGD-IL-12 vectors but with the substitution of the luciferase gene expression cassette for the cytokine, was used as negative control vector in the present study. The replication-deficient AdRGD-IL-12, which carries the murine IL-12 gene derived from mIL-12 BIA/pBluescript II KS(−) [18] (kindly provided by Prof. Hiroshi Yamamoto, Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Japan), was constructed by an improved in vitro ligation method using pAdHM15-RGD [19,20]. The expression cassette, which was designed to be transcribed in order from the IL-12 p35 cDNA through the internal ribosome entry site sequence to the IL-12 p40 cDNA under the control of the cytomegalovirus promoter, was inserted into the E1-deletion region of the E1/E3-deleted Ad vector (Fig. 1). All vectors were propagated in HEK293 cells, purified by two rounds of CsCl gradient centrifugation, dialyzed with phosphate-buffered saline (PBS) containing 10% glycerol, and stored at −80 °C. The number of viral particles in vector stock was determined spectrophotometrically by the method of Maizel et al. [21]. Titers (tissue culture infectious dose$_{50}$; TCID$_{50}$) of infective AdRGD particles were evaluated by the end-point dilution method using HEK293 cells and expressed as plaque-forming units (PFU).

Gene expression by AdRGD-Luc or conventional Ad-Luc in Meth-A cells. Meth-A cells were plated in 96-well plates at a density of 2×10^3 cells/well and incubated with Ad-Luc or AdRGD-Luc at concentrations of 1250, 2500, 5000, or 10,000 viral particles/cell for 1.5 h. Cells were then washed with PBS and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and lysed with Luciferase Cell Culture Lysis buffer (Promega, USA), and their luciferase activity was measured by the Luciferase Assay System (Promega,

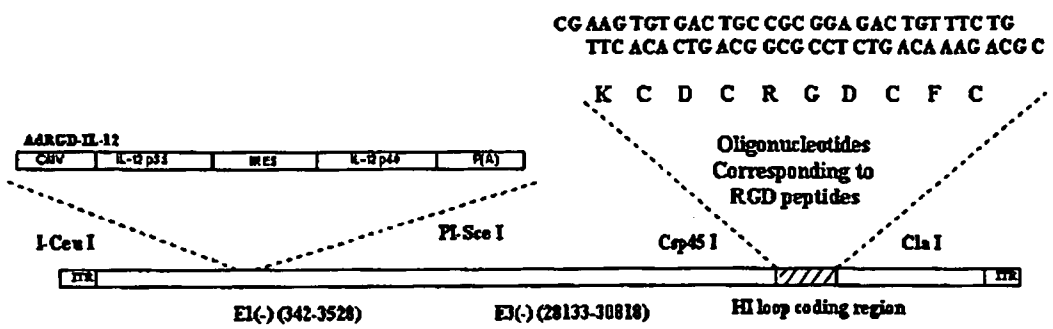

Fig. 1. Construction of IL-12 encoding fiber-mutant adenoviral vector.

USA) and Microlumat Plus LB96 (Perkin-Elmer) according to the manufacturer's instructions.

Analysis of gene transduction of AdRGD-IL-12 in vitro. Meth-A cells were plated in six-well plates at a density of 5×10^5 cells/well and transfected with AdRGD-IL-12 for 24 h at various multiplicities of infection (MOIs) in 2 ml RPMI 1640 medium containing 10% FBS. After three washes of the transfected cells with PBS, a 1.5-ml aliquot of culture medium was added to each well. The supernatants were collected after 24 h, and the amount of IL-12 p70 in each sample was measured with a murine IL-12 p70 ELISA kit (Biosource International, Camarillo, CA, USA) according to the manufacturer's instructions.

Tumor inoculation and intratumoral administration of vectors in animal experiments. Meth-A cells were inoculated intradermally into the flanks of BALB/c mice at 2×10^6 cells/mouse. After 7 days, established tumors (diameter, 9–10 mm) were injected with each vector at 2×10^7 plaque-forming units (PFU) in 50 μl PBS. Tumor size (length and width in mm) was measured twice weekly; animals were euthanized when either of the two parameters exceeded 20 mm. At 3 months after complete regression of the primary tumors, mice were challenged with freshly isolated Meth-A tumor cells or CT26 cells by intradermal injection of 1×10^6 cells into the flank.

Immunohistochemical staining. T-cell infiltration into the Meth-A tumors after intratumoral injection of AdRGD-IL-12 was determined by immunohistochemical analysis. Tumor-bearing mice were euthanized 6 days after administration of AdRGD-IL-12 or the control vector. The tumor nodules were harvested, embedded in OCT compound (Sakura, Torrance, CA, USA), and stored at −80 °C. Frozen thin (6-μm) sections of the nodules were fixed in 4% paraformaldehyde solution, washed with Tris-buffered saline (TBS), and incubated in methanol containing 0.3% hydrogen peroxide for 30 min at room temperature to block endogenous peroxidase activity. The sections were incubated with the optimal dilution of the primary antibody—either rabbit anti-human CD3 antibody (DakoCytomation) or normal rabbit IgG (Santa Cruz Biotechnology)—for 60 min at room temperature. Bound primary antibody was detected after incubation with the secondary antibody from the EnVision+ System (DakoCytomation) for 30 min, followed by a 15-min wash in TBS. The sections were stained with DAB (DakoCytomation) and finally counterstained with hematoxylin (DakoCytomation). We randomly selected six fields from different tumor sections and counted the immunostained cells under a light microscope at 400× magnification.

Experiments on metastatic tumor. We intradermally inoculated mice with 2×10^6 Meth-A cells as described earlier and, 5 days later, injected 8×10^4 cells intravenously. Two days after the intravenous injection, intratumoral injection of AdRGD-IL-12 (2×10^7 PFU) was carried out. The size of the primary tumor was measured twice weekly, and the lungs were harvested 2 weeks after the intravenous injection. The lungs were weighed, sectioned for histology, and stained with hematoxylin and eosin. Metastases in the lungs were identified under a light microscope.

Statistical analysis. Student's *t* test was used for statistical comparison when applicable. Differences were considered statistically significant at $P < 0.05$.

Results

Meth-A tumor cells transfected with the fiber-mutant adenoviral vector induce higher luciferase gene expression than do those induced with the conventional vector

To evaluate the gene transfection efficiency of the fiber-mutant Ad vector developed for this study, Meth-A cells were transfected with either the conventional Ad-Luc vector or the fiber-mutant AdRGD-Luc vector at various MOIs and the luciferase activity was measured. The luciferase gene expression due to transfection of the fiber-mutant vector was much higher than that from the conventional vector (Fig. 2). For example, at 5000 and 10,000 viral particles/cell, 16.8-fold and 15.7-fold greater gene expression, respectively, was obtained in response to AdRGD-Luc than to Ad-Luc. These results show that insertion of the RGD peptide into the viral fiber enhanced the transfection efficiency of the Ad vector into Meth-A cells.

Expression of IL-12 p70 in Meth-A cells via transfection of AdRGD-IL-12

The IL-12-encoding fiber-mutant adenoviral vector AdRGD-IL-12 was developed as shown in Fig. 1. To confirm the biological activity of AdRGD-IL-12, we used an ELISA to measure the amount of IL-12 in the

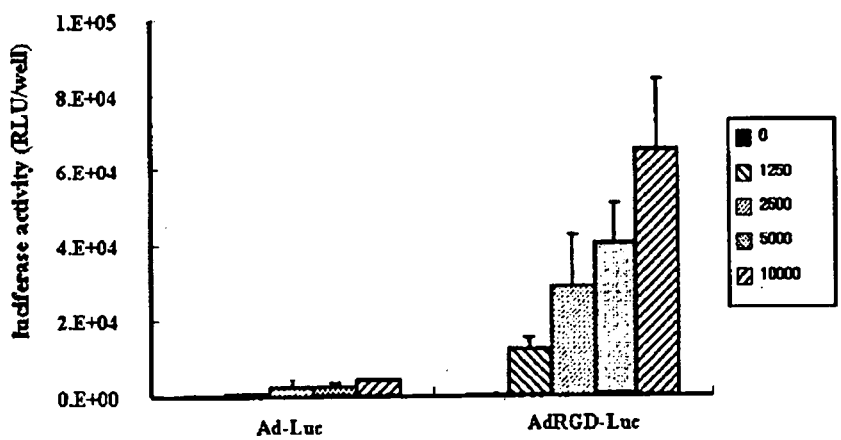

Fig. 2. Gene expression by AdRGD-Luc or conventional Ad-Luc in Meth-A cells. Meth-A cells (2×10^3/well) in 96-well plates were treated with Ad-Luc or AdRGD-Luc at the indicated numbers of viral particles/cell for 1.5 h. Cells were washed and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured. Data are presented as means ± SE of relative light units (RLUs)/well from three experiments.

supernatants of transfectants. Meth-A cells transfected with AdRGD-IL-12 showed dose-dependent concentrations of IL-12 p70 in the supernatants. In contrast, no detectable IL-12 p70 was present in the culture media of cells that had not been transfected (Fig. 3).

Anti-tumor activity and long-term specific immune response are induced by intratumoral injection of AdRGD-IL-12

The growth of Meth-A tumors was suppressed dramatically, and complete regression occurred in about 70% of the tumor-bearing mice after a single intratumoral injection of 2×10^7 PFU of AdRGD-IL-12. In contrast, the AdRGD-Luc group showed no apparent anti-tumor effect (Fig. 4A). In addition, the relative survival rates further demonstrated prolonged survival after treatment with IL-12 (Fig. 4B). In the rechallenge experiment, mice showing complete regression were reinoculated intradermally with Meth-A or CT26 cells 90 days after the initial injection of tumor cells. All of the mice challenged with Meth-A cells remained tumor-free for at least 2 months (Table 1). In contrast, 100% of the mice challenged with CT26 developed palpable tumors within 2 weeks. These results indicate the generation of specific immunity against Meth-A tumor cells in those mice that rejected Meth-A upon treatment with IL-12.

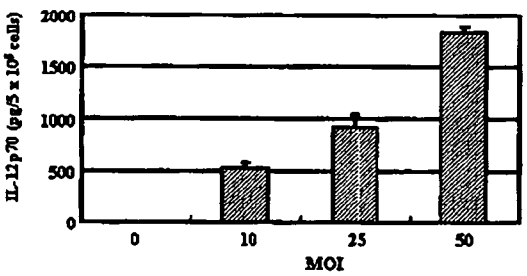

Fig. 3. Production of IL-12 p70 from Meth-A cells transfected with AdRGD-IL-12. We transfected 5×10^5 Meth-A cells with AdRGD-IL-12 for 24 h at the indicated multiplicities of infection (MOIs). Then the cells were cultured for a further 24 h with fresh medium. The supernatants were collected and the IL-12 p70 level was measured by ELISA.

Intratumoral administration of AdRGD-IL-12 induces the infiltration of T cells into Meth-A tumors

To investigate the anti-tumor mechanism of AdRGD-IL-12, tumor tissues were subjected to immunohistochemical staining for CD3 six days after treatment with AdRGD-IL-12 or AdRGD-Luc. Tissues from mice that received AdRGD-IL-12 demonstrated significantly increased accumulation of $CD3^+$ T cells compared
with animals injected with either AdRGD-Luc or PBS (Fig. 5).

Table 1
Specific long-term anti-tumor immune response to IL-12 treatment

Groups	Challenging cell	Tumor rejected mice/challenged mice
Intact mice	Meth-A[a]	0/5
Meth-A rejected[c]	Meth-A[a]	5/5
Meth-A rejected[d]	CT26[b]	0/3

[a] Challenged with 1×10^6 cells.
[b] Challenged with 3×10^5 cells.
[c] Meth-A cured; Meth-A rechallenged.
[d] Meth-A cured; CT26 rechallenged.

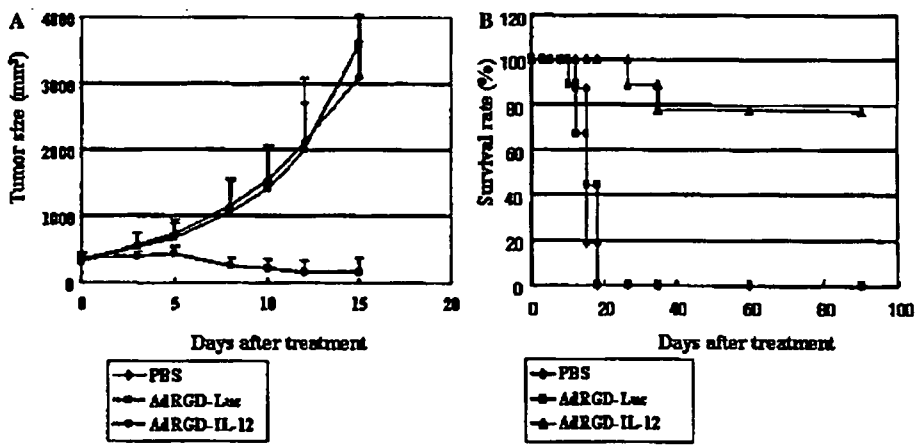

Fig. 4. Growth in BALB/c mice of established Meth-A tumor cells injected intratumorally with IL-12-encoding adenoviral vector. Mice were inoculated intradermally in the flank with 2×10^6 Meth-A cells (100 μl in RPMI 1640). They were then intratumorally injected with 2×10^7 PFU AdRGD-IL-12, AdRGD-Luc, or PBS. Tumor volume was calculated after measuring the length and width of tumors at the indicated time points, and data are expressed as means ± SE of results obtained from at least eight mice. Animals were euthanized when either the length or width of the tumor exceeded 20 mm. (A) Average tumor size. (B) Survival rate (%) of mice.

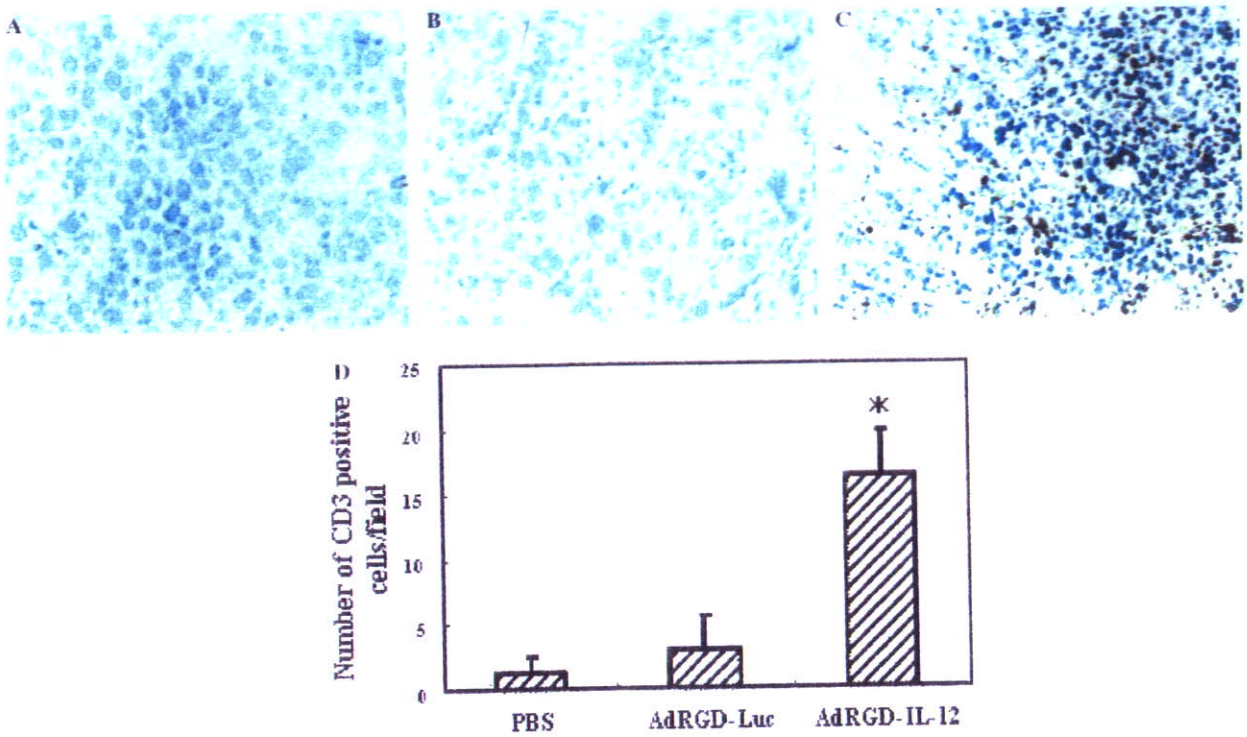

Fig. 5. Intratumoral injection of AdRGD-IL-12 induced the infiltration of $CD3^+$ T cells into Meth-A tumors. Representative views of tumor nodules from mice, harvested 6 days after intratumoral injection of the indicated vectors and controls, and stained for CD3. (A) PBS, (B) AdRGD-Luc, (C) AdRGD-IL-12. The photographs were obtained under light microscopy at 400× magnification. (D) Six fields from different tumor sections were randomly selected and positive cell number infiltrated into tumor tissue was counted. $*P < 0.05$ with Student's t test in groups between treated with AdRGD-IL-12 and AdRGD-Luc or PBS.

A

Group	Metastasis-free mice/mice in group	Occurrence of Metastasis (%)
PBS	0/3	100%
AdRGD-Luc	1/9	89%
AdRGD-IL-12	8/9	11%

B

Fig. 6. Anti-metastatic activity due to intratumoral injection of AdRGD-IL-12 into Meth-A fibrosarcoma. (A) Incidence of metastasis in each group. (B) Photomicrographs of lung tissue harvested 2 weeks after treatment and stained with hematoxylin and eosin. The photographs were obtained under light microscopy at 10× magnification. The arrows indicate micrometastasizing tumor.

Anti-metastatic activity is induced by intratumoral injection of AdRGD-IL-12

We then sought to evaluate whether intratumoral injection of AdRGD-IL-12 would induce anti-tumor effects against both the primary and metastatic tumors. Our results showed that single intratumoral injection of AdRGD-IL-12 induced pronounced anti-metastasis activity (Fig. 6A and B) while maintaining tumor-suppressive activity toward the primary tumor, similar to

that shown in Fig. 4 (data not shown). Compared with the control group treated with AdRGD-Luc, in which about 90% of the mice had readily discernable lung metastasis, only one of nine animals treated with AdRGD-IL-12 demonstrated metastasis.

Discussion

Viral vector-encoded chemokines and cytokines are used widely in cancer gene therapy [22,23]. IL-12 has demonstrated remarkable anti-tumor activity when used directly as a recombinant protein or after various viral and non-viral vectors have been used to transfer its genes [24–26]. The development of an efficient vector is pivotal for gene therapy. Because of its high transfection efficiency and because it can transfect both dividing and quiescent cells, Ad vectors are used widely in gene therapy protocols: about 26% of gene therapy clinical trials use Ad vectors as gene carriers [27,28]. However, the lack of Coxsackie adenovirus receptor (CAR), which is an important receptor for conventional Ad vector infection, in many types of malignant cells impairs the tranfection efficiency with Ad vector [29]. Meth-A fibrosarcoma has been confirmed by RT-PCR to be deficient in expression of CAR but with expression of integrin (data not shown). Our previous reports have also shown that insertion of the RGD peptide into the fiber sequences of Ad vectors induces enhanced gene transfection in CT26 and A2058 cells [30,31]. The results of our present study also demonstrate that the fiber-mutant Ad vector induced enhanced expression of the encoded luciferase gene in Meth-A fibrosarcoma cells compared with the expression due to conventional vector (Fig. 2). Furthermore, we confirmed the presence of IL-12 p70 in the supernatant of Meth-A cells transfected with AdRGD-IL-12 (Fig. 3).

Systemic administration of recombinant IL-12 at high doses induces adverse effects associated with high systemic peak concentrations [32,33]. Therefore, gene transfer methods are designed to confine IL-12 production to the tumor environment, thereby preventing systemic toxicity. Tumor cells, dendritic cells, and autologous fibroblasts have been transfected with recombinant adenoviruses or retroviruses to secrete IL-12 locally and have shown favorable efficacy and safety profiles [34,35]. Several groups have shown that intratumoral injection of an Ad vector encoding IL-12 efficiently eradicates experimental gastrointestinal cancer [36,37]. Disadvantages of direct topical administration include tissue damage, and some tumor sites may be inaccessible even to computed tomography-guided percutaneous injection and radiographically directed delivery [38]. However, these limitations favor those types of gene therapy that do not require all tumor cells or tumor masses that express the gene.

Meth-A has shown that it is an IL-12-insensitive tumor cell, in that established tumors could not be treated efficiently via systemic administration of IL-12 and could not even be suppressed effectively (i.e., only 42.5% of mice rejected the tumor) after transfection of an IL-12-containing retroviral vector [12,39]. In our present study, however, a single intratumoral injection of a relatively low dose of AdRGD-IL-12 (2×10^7 PFU) elicited strong anti-tumor activity against established tumors (i.e., diameter of about 10 mm at the beginning of treatment; Fig. 4A). Treatment induced complete tumor regression in about 70% of tumor-bearing mice, and the growth rates of the remaining tumors seem to have been retarded (individual data not shown). Treatment also prolonged the survival of the mice significantly compared with that of the group injected with AdRGD-Luc, a control vector (Fig. 4B). Meanwhile, no detectable IL-12 and IFN-γ existed in the sera after treatment (data not shown)—findings that are consistent with those other reports [40]. Furthermore, intratumoral injection of AdRGD-IL-12 induced a profound long-term specific anti-tumor immunity in mice with complete regression of the initial Meth-A lesion (Table 1).

Studies have shown that IL-12 elicits tumor regression after induction of T-cell migration to tumor sites [41]. The failure of IL-12 therapy in Meth-A via systemic administration is thought to be due to the inability to recruit immune cell migration into tumor cells, and further investigation has indicated a key role of the peritumoral stroma/stromal vasculature in the acceptance of the tumor-infiltrating T cells that are a prerequisite for IL-12-induced tumor regression [12]. Our results similarly demonstrated the accumulation and uniform distribution of $CD3^+$ T cells in the tumor after intratumoral injection, thus supporting the notion that the pronounced anti-tumor effect is related to immune cell infiltration (Fig. 5). However, it remains unclear why intratumoral injection but not systemic administration induces immune cell accumulation in tumor tissue.

We also evaluated the anti-metastasis activity associated with a single intratumoral injection of AdRGD-IL-12. Metastasis is a challenge for cancer treatment, especially because almost all immunotherapy performed in the clinical setting is adjuvant treatment given after surgical reduction of the primary tumor mass for controlling recurrence and metastasis. Interestingly, the single intratumoral injection of AdRGD-IL-12 did induce anti-tumor activity toward disseminated tumors in the lung: histopathology confirmed the complete absence of metastatic tumors in eight of the nine mice tested (and only sporadic residual tumor in the remaining animal). In contrast, all mice that received intratumoral injection of the control vector developed metastases, suggesting that local expression of IL-12 also stimulates the systemic immune response to subsequently affect distant malignant cells.