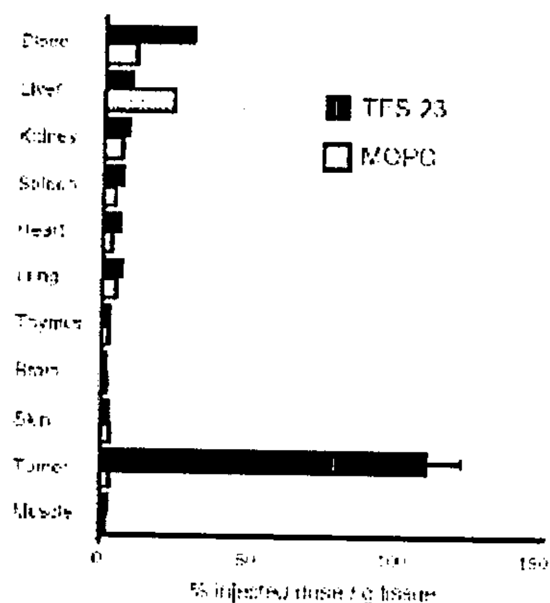

Fig. (6). Tissue distribution of ^{125}I-labelled TES-23 in tumor-bearing mice.
Tissue distribution experiments were performed in BALB/c mice bearing Meth-A Fibrosarcoma. A total of 20ng of radiolabelled antibodies were injected intravenously into mice. One hour later the mice were anaesthetized and 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.

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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,

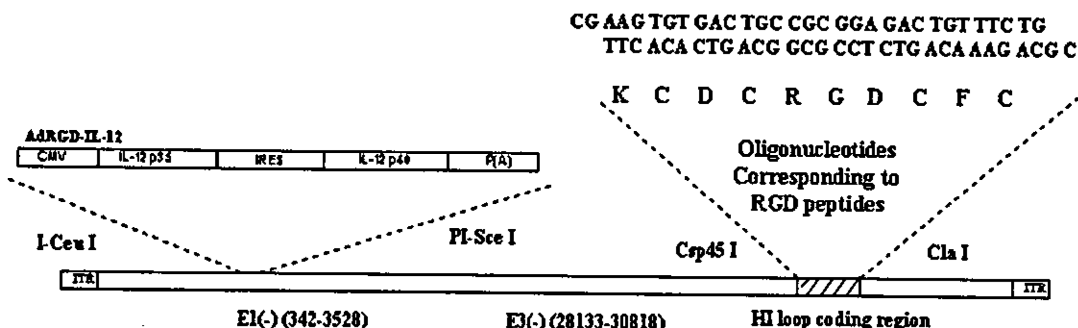

Fig. 1. Construction of IL-12 encoding fiber-mutant adenoviral vector.

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

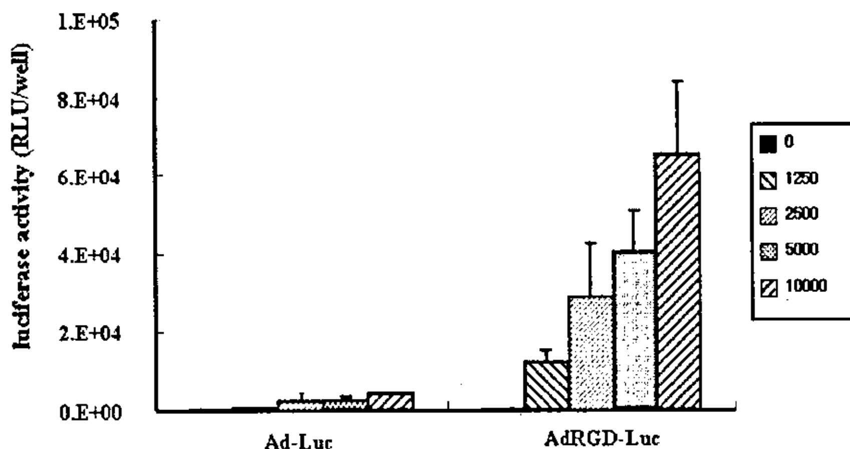

Fig. 2. Gene expression by AdRGD-Luc or conventional Ad-Luc in Meth-A cells. Meth-A cells (2×10^3/well) in 96-well plates were treated with Ad-Luc or AdRGD-Luc at the indicated numbers of viral particles/cell for 1.5 h. Cells were washed and cultured for an additional 48 h. Subsequently, the cells were 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.

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).

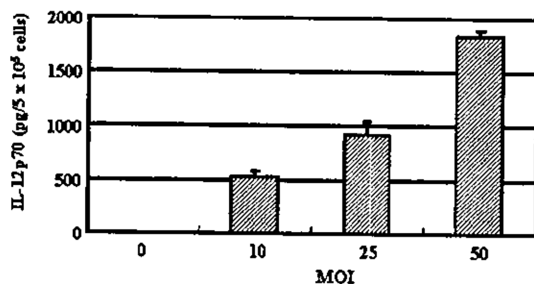

Fig. 3. Production of IL-12 p70 from Meth-A cells transfected with AdRGD-IL-12. We transfected 5×10^5 Meth-A cells with AdRGD-IL-12 for 24 h at the indicated multiplicities of infection (MOIs). Then the cells were cultured for a further 24 h with fresh medium. The supernatants were collected and the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

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.

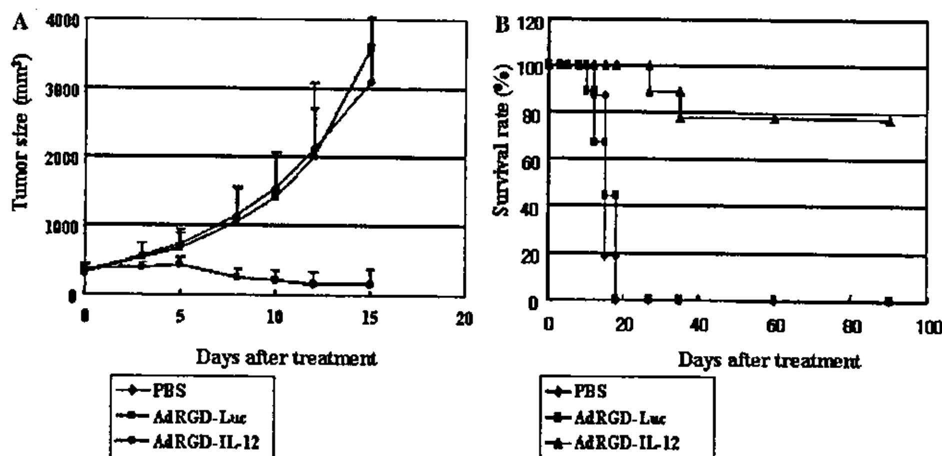

Fig. 4. Growth in BALB/c mice of established Meth-A tumor cells injected intratumorally with IL-12-encoding adenoviral vector. Mice were inoculated intradermally in the flank with 2×10^6 Meth-A cells (100 μl in RPMI 1640). They were then intratumorally injected with 2×10^7 PFU AdRGD-IL-12, AdRGD-Luc, 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.

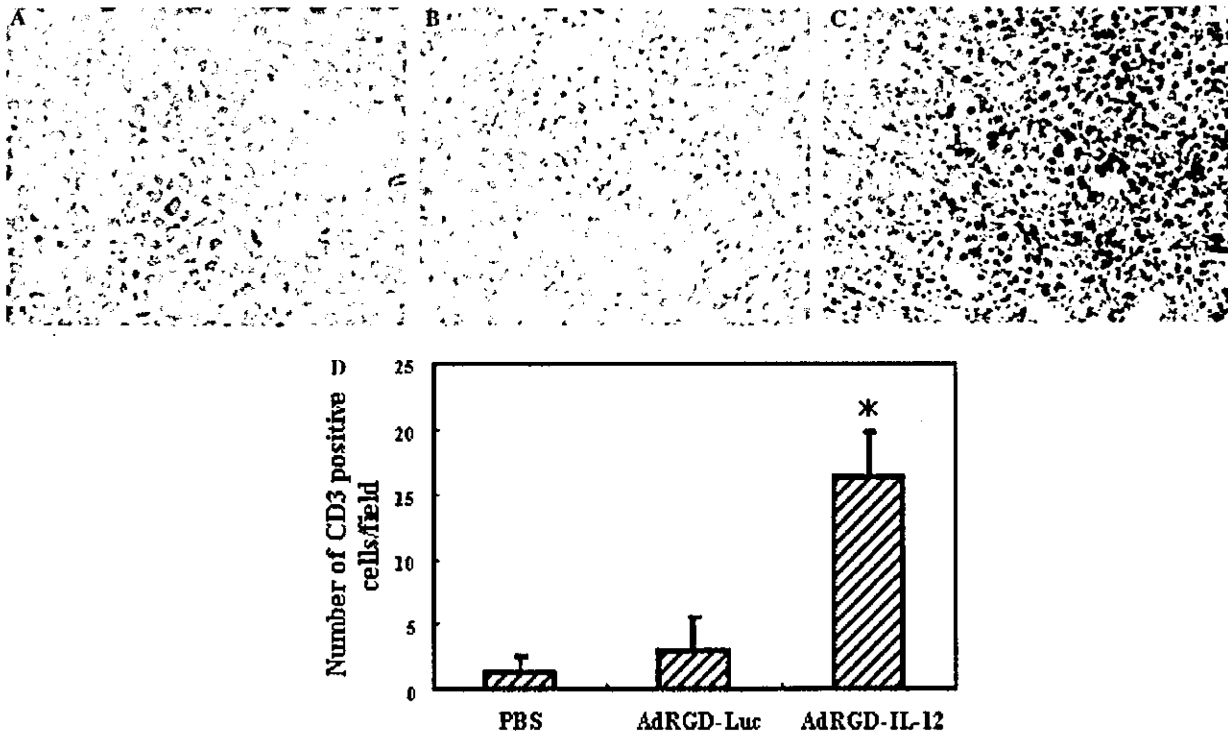

Fig. 5. Intratumoral injection of AdRGD-IL-12 induced the infiltration of $CD3^+$ T cells into Meth-A tumors. Representative views of tumor nodules from mice, harvested 6 days after intratumoral injection of the indicated vectors and controls, and stained for CD3. (A) PBS, (B) AdRGD-Luc, (C) AdRGD-IL-12. 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	0/8	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 (Figs. 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.

All the results of our present study indicate that a single intratumoral injection of an IL-12-encoding fiber-mutant Ad vector induces T-cell infiltration into stroma-deficient Meth-A fibrosarcoma and is effective in the treatment of, and protection against challenge with, syngeneic tumors. Our results also suggest that a single intratumoral administration of AdRGD-IL-12 can induce a curative immune response in the face of a micrometastasizing tumor.

Acknowledgments

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Review

Optimization of Protein Therapies by Polymer-Conjugation as an Effective DDS

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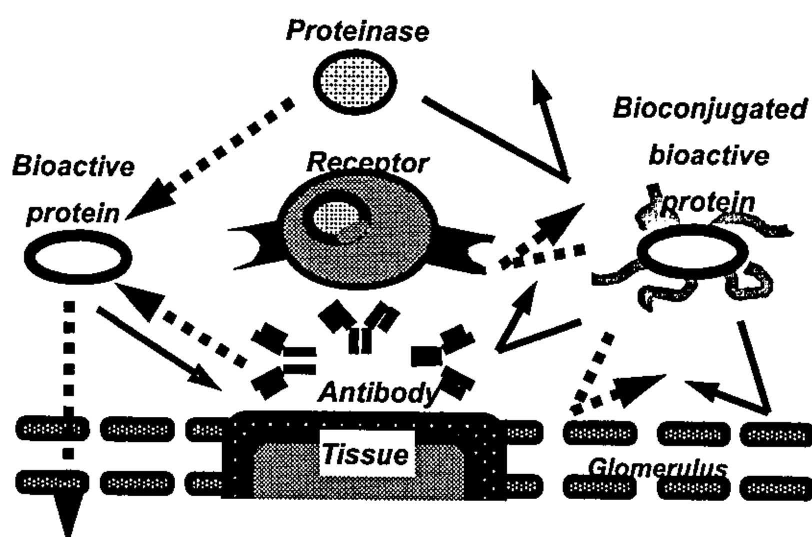
Abstract: Due to recent advances in disease proteomics, many disease-related proteins have been found. It is expected that there will be therapeutically useful proteins among them. However, it is clinically difficult to use most proteins as effective and safe drugs because of their very low stability and pleiotropic actions *in vivo*. To promote disease proteomic based drug development for protein therapies, we have attempted to develop an optimal polymer-conjugation system for improving the therapeutic potency of proteins. In this review, we introduce this innovative protein-drug system.

Keywords: PEGylation, polyvinyl pyrrolidone, Tumor necrosis factor-alpha, Interluekin-6.

Introduction

With the success of the human genome project, the focus of life science research has shifted to the functional and structural analyses of proteins, such as disease proteomics. Therapeutic application of bioactive proteins, such as newly identified proteins and cytokines, are also promising [1-7]. However, because these proteins are generally quite unstable *in vivo*, their clinical application requires frequent administration at high dosages. This administration markedly destroys homeostasis, resulting in unexpected side effects. In addition, since bioactive proteins exhibit diverse pharmacological actions in various tissues, it is difficult to obtain selectively only the favorable *in vivo* actions. For these reasons, clinical applications of bioactive proteins have been limited [7-11].

Figure 1. Characteristics of bioconjugated proteins.



In recent years, to overcome these problems, the conjugation of proteins with water soluble polymeric modifiers has been developed (Figure 1), especially, the conjugation with polyethylene glycol (PEG), often called "PEGylation". Bioconjugation of proteins with water-soluble polymeric modifiers increases their molecular size and steric hindrance, both of which are dependent on the polymeric modifiers attached the proteins. These effects improve the plasma half-lives of proteins and their stability against proteolytic cleavage, and also decrease their immunogenicity. This allows the therapeutic dose and frequency to be decreased.

In fact, PEGylated interleukin-2, PEGylated interferon and PEGylated adenosine deaminase have demonstrated markedly improved therapeutic efficacy over the native forms, and clinical applications have already been realized [12-16]. However, clinical use of polymer-conjugated proteins has been limited yet. This is due to the conflicting effects of polymer-conjugation of bioactive proteins;

conjugation with a polymeric modifier inhibits the transport from blood to tissues and the binding to their receptors. In addition, specific activities of proteins are decreased by the attachment of polymeric modifiers to active sites. Therefore, determination of the relationships among the degree of modification, molecular size, and specific activity is very important to optimize the modification-condition, which enable designing of polymer-conjugated proteins applicable to clinical use.

On the other hand, for further enhancement of the therapeutic potency and safety of polymer-conjugated proteins, more precise control of the *in vivo* behavior of each protein is necessary for selective expression of their therapeutic bioactivities. We found that polymer-conjugated proteins can be greatly affected by the properties of the polymeric modifiers attached to the surface of the proteins. Therefore, it is necessary to identify appropriate polymeric modifiers for design of conjugated proteins with desirable *in vivo* behavioral characters. PEG is a low toxicity and low antigenicity polymeric modifier that has been used frequently for conjugation of proteins. From the viewpoint of a drug delivery system, PEG, however, also has some disadvantages as a drug carrier, principally the fact that PEG only has a functional group at the end of the chain, limiting the possibilities of adding new functions to the drugs to control more precisely their pharmacokinetics and tissue distribution. Therefore, alternative water-soluble polymeric modifiers in which new functions, such as targeting and release control of drugs, can be added are required for further development of polymer-conjugated drugs.

In this review, we, at first, show the fundamental information enabling us to design the conjugated bioactive proteins applicable to therapeutic use, taking tumor necrosis factor-alpha (TNF-alpha) and interleukin-6 (IL-6) as examples [17-19], and a novel method using the reversible amino-protective reagent dimethylmaleic anhydride (DMMAn) to prevent the decrease of bioactivity by attachment of polymeric modifiers [20]. Next, we show the usefulness of PVP in achieving long plasma half-lives and for application to the tissue-targeting polymer [21-23].

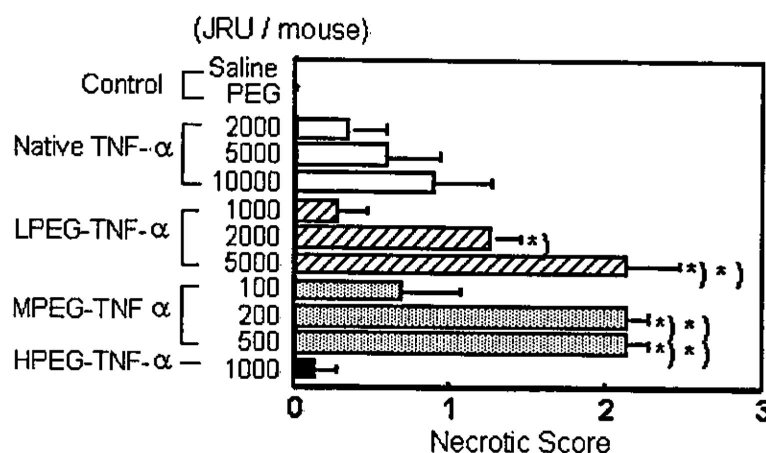
PEGylation of bioactive proteins

a) PEG-TNF-alpha

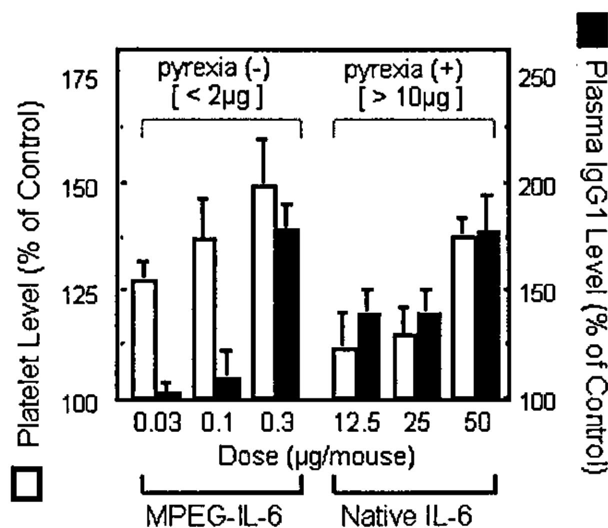
TNF-alpha is a cytokine that was discovered to specifically injure tumors and was thus highlighted as a novel anti-tumor agent [24, 25]. However, due to its very low *in vivo* stability, the continuous-infusion or frequent administration at high doses of TNF-alpha was needed [26, 27]. Intravenous TNF-alpha administration has caused marked side effects such as fever, nausea, vomiting, a decrease in blood pressure, and endotoxin-like shock [10, 28]. The anti-tumor effects of TNF-alpha result not only from its direct cytotoxic action against various tumor cells, but also from activation of

anti-tumor effector immune cells in the blood and specific damage to the tumor vessels. In addition, in the process of bleeding necrosis in the tumor vessels, the vascular permeability of the tumor vessels is selectively increased, promoting transport from blood to the tumor tissue. Therefore, improvement in blood stasis may enhance all anti-tumor action mechanisms of TNF-alpha increasing its bioavailablity. Therefore, we performed conjugation of the lysine amino residues of TNF-alpha using PEG [18]. In PEGylation of TNF-alpha, the specific activity of PEGylated TNF-alpha decreased with the PEG modification rate. Additionally, when the PEG modification rates (degree of PEG-modification) are the same, the bioactivity of PEGylated TNF-alpha decreased with an increase in the molecular size of the attached PEG [17]. Our other studies on PEGylated IL-6 and leukemia inhibitory factor (LIF) yielded similar results [18]. Thus, in bioactive proteins such as TNF-alpha, IL-6 and LIF that require binding to a receptor for the expression of activity, consideration should be given to inhibition of activity derived from inhibition of binding to receptor molecules caused by steric hindrance by the polymeric modifier, in addition to a decrease in the specific activity due to modification of the lysine residues. On the other hand, the *in vivo* anti-tumor effects of PEGylated TNF-alpha was the most marked for MPEG- TNF-alpha (molecular weight; Mn=108,000) obtained by PEGylation using PEG with a molecular weight of 5,000 (PEG5000) (Figure 2a).

Figure 2. PEGylation of TNF-α and IL-6 selectively increase their therapeutic effects. a) Tumor necrosis effects of native TNF-α and PEG-TNF-αs on Meth-A solid tumors. Each value represents mean ± S.E. *P<0.001, **P<0.05 significantly different from the group treated with 10,000 JRU of native TNF-α.



b) Effect of PEG5000-IL-6 and Native IL-6 on platelet production (favorable effect) or plasma IgG1 depressions (side effect) were compared. On day 9, blood was taken form tail vein of mice injected of native IL-6 or PEG5000-IL-6 Fr.4 every 2 days for a week.



Its anti-tumor activity was 100 times higher than that of the unmodified TNF-alpha without increasing the toxic side effects. No marked enhancement of anti-tumor effects were observed for slightly modified LPEG-TNF-alpha (Mn=84,000) with a high specific activity or excessively modified HPEG-TNF-alpha (Mn=122,000) with a high molecular size, though they were also obtained after conjugation of PEG with a molecular weight of 5,000. Conjugation using PEG with a molecular weight of 2,000 or 12,000 did not produce PEGylated TNF-alpha s comparable to MPEG-TNF-alpha. These results suggest that PEGylation of TNF-alpha does not always produce marked *in vivo* anti-tumor effects, but there is an optimal molecular weight of the polymeric modifier and an optimal modification rate - molecular size - activity correlation of PEGylated TNF-alpha.

b) PEG-IL-6

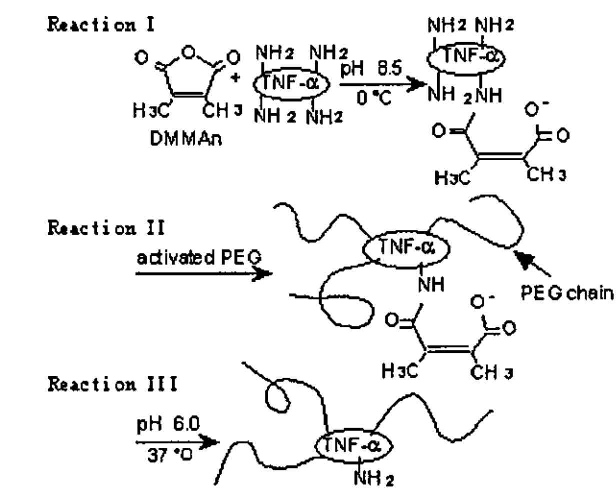
When IL-6 is utilized therapeutically to promote the formation of platelets, its targets are the megakaryocytes [29-31]. It is known that megakaryocytes express large amounts of high affinity IL-6 receptors (IL-6 receptor-gp130 complex) [32, 33], and that they are abundant on the pulmonary vascular lumens and on the outer surfaces of marrow veins. Therefore, if IL-6 is modified to remain longer in the blood and thus smaller dose levels are required for therapeutic use, it will be possible to make the *in vivo* distribution and receptor affinity of IL-6 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 cause adverse reactions [34-36], 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 conjugation of IL-6 with PEG, to increase in the activity of IL-6 in the promotion of platelet production and to reduce its side effects. When IL-6 was subjected to PEGylation under optimum conditions, selected by consideration of the relationships between specific activity, degree of PEG-modification, molecular size, etc., the resultant PEG-modified IL-6 (MPEG-IL-6) showed plasma half-life more than 100 times greater than that of native IL-6. MPEG-IL-6 showed more than 500 times the thrombopoietic potency of native IL-6 (Figure 2b). Furthermore, strong adverse reactions such as fever, IgG production and acute protein production observed following administration of native IL-6, were seldom seen after administration of MPEG-IL-6. These results suggested separation of therapeutically favorable targeted actions from side effects by means of PEGylation has been successful. It was found that PEGylation allows exertion of selected favorable actions of cytokines via the following mechanisms: (1) improved *in vivo* stability reduces the dose level and thus the blood level of cytokines, making it possible for a given cytokine to exert its selected actions on the basis of 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 differences between its distribution in different tissues. We have thus succeeded in making cytokines useful as therapeutic agents by improving their stability *in vivo* and increasing in 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 suggested that polymer-conjugation is a pragmatic approach to successful therapies with various bioactive proteins and peptides.

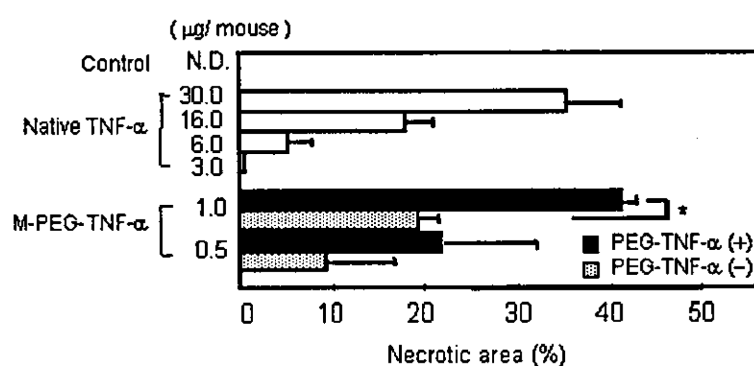
A novel polymer-conjugation technique

Lysine amino groups of proteins are often used as binding sites of PEG because they are highly reactive and the PEGylation reaction is mild enough to minimize disruption of the protein structure [37]. This PEGylation, however, is nonspecific and occurs at the N-terminus as well as internal lysine residues, some of which may be in or near protein active sites, resulting in loss of specific bioactivity. Therefore, conjugation methods which avoid modification of amino groups around the receptor binding region of proteins are needed. We attempted to control modification by using the reversible amino-protective reagent dimethylmaleic anhydride (DMMAn) [20]. PEG-TNF-alpha(+) was prepared according to the reactions presented in Figure 3a.

Figure 3. A novel polymer-conjugation technique with a reversible amino-protective reagent. a) Schematic protocol of PEGylation of TNF-α using DMMAn: Reaction I, protection of partial amino groups by DMMAn; Reaction II, PEGylation to remaining lysine amino groups; Reaction III, regeneration of amino groups by releasing DMMAn.



b) Tumor necrotic effects of PEG-TNF-αs on Meth-A solid tumor in mice. Meth-A-bearing BALB/c mice were given native TNF-α, PEG-TNF-α(-),or PEG-TNF-α(+) i.v. The control group was given saline. The area of hemorrhagic necrosis was measured 24h after injection. Each value is the mean ± S.E. of four animals. $^{*}P < 0.01$, statistical significance compared with PEG-TNF-α(-)s.



The protection of partial amino groups in TNF-alpha by DMMAn was confirmed by fluorimetric analysis. Then, to examine whether the active core of TNF-alpha can be protected from PEGylation using DMMAn, changes in specific activity were examined in vitro. Specific activities improved for all fractions of PEG-TNF-alpha(+) compared with the similar molecular size fractions of PEG-TNF-alpha(-) tested. This result indicates that the use of DMMAn improves cytokine receptor

binding. We found similar results for PEGylation of IL-6 and granulocyte macrophage colony-stimulating factor. To examine the influence of improvements in specific activity *in vivo*, Meth-A fibrosarcoma bearing mice were given native and PEG-TNF-alphas (Figure 3b). The antitumor effects of MPEG-TNF-alpha(+) were 2-fold more potent than those of MPEG-TNF-alpha(-), which was the most potent TNF-alpha not treated with DMMAn, and 30-fold more potent than those of native TNF-alpha. Significantly, improvements in the specific activity of MPEG-TNF-alphas were only about 50%, but improvements in antitumor effects were more than 2-fold *in vivo*. These results suggested that our method is easy and useful for the clinical application of polymer-conjugated cytokines.

Screening of polymeric drug carriers

It is well known that the fate and distribution of the conjugates can be attributed to the physicochemical properties of polymeric modifiers such as molecular weight, electric charge, and hydrophilic-lipophilic balance [38]. The increase of therapeutic effects of drugs conjugated with polymeric modifiers is attributed to the pharmacokinetics of conjugated drug. Therefore, selecting the polymeric modifier by considering the influence of physicochemical characteristics on the pharmacokinetics of the polymeric modifier is markedly important. As mentioned above, sequential and multiple strategies are needed for optimization of drug therapy based on polymer-conjugation: i) optimum selection of polymeric modifier considering the disposition of drugs and objective such as targeting or controlled release, ii) polymer-conjugation based on estimation of characterization such as molecular size, modification site, degree of modification, and specific activity, and iii), assessment of therapeutic effect and pharmacokinetics of conjugated drug.

As mentioned, PEG is a low toxicity and low antigenicity polymeric modifier that has been frequently used for polymer-conjugation. PEG is a polyether diol of general structure $HO\text{-}(CH_2CH_2O)_n\text{-}H$, where functionalization of PEG is restricted to the utilization of the terminal primary OH groups [39]. From this viewpoint, modifiable polymeric modifiers are needed to control the biopharmaceutical characteristics of conjugated drugs. Therefore, we assessed the pharmacokinetic profile of various water-soluble polymers with molecular sizes of about 5,000, and compared their pharmacokinetics to PEG5000 [40].

First, the elimination profile of ^{125}I-labeled various polymers with the same molecular size after i.v. injection in mice bearing S-180 solid tumors was studied. The polymer formulations used to evaluate these are: PEG, polyvinylpyrrolidone (PVP), polyacrylamide (PAAm), polydimethylacrylamide (PDAAm), polyvinyl alcohol (PVA), and dextran. PVP, PAAm, and PDAAm are functionalized by introduction of various comonomers on radical polymerization. PVA and dextran have many primary OH groups that can be used for conjugation on the side chain.

Figure 4. Plasma clearance of various water-soluble polymers in mice bearing S-180 solid tumors after i.v. injection. Mice were intravenously injected with 125I-labeled polymer. After administration, blood was collected from the tail vein at indicated times and the radioactivity was measured by a γ-counter. Mice were used in groups of five. Each value is mean ± SD.

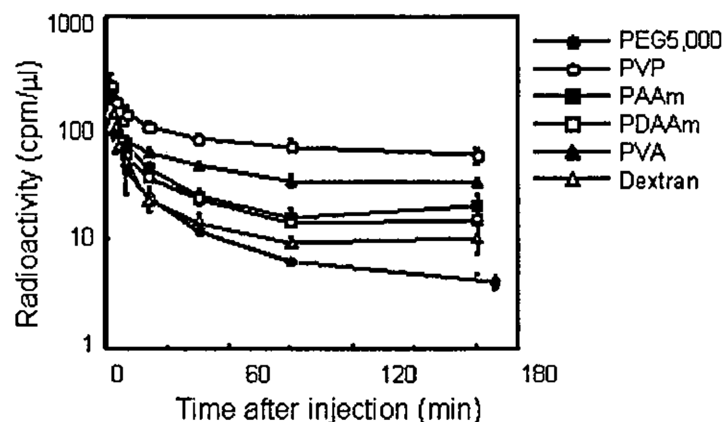


Figure 4 illustrates the plasma clearance of various polymers. All polymers showed biphasic elimination patterns. PEG5000 and dextran, which are used frequently as drug carriers, were eliminated rapidly from the blood circulation. On the other hand, PVA and PVP circulated longer than the other polymers, while these nonionic polymers had the same molecular size of PEG5000. PVP exhibited the longest residence of all the polymers in this study, and 25% of the injected dose remained after 180 min. Pharmacokinetic analysis revealed definite differences among each polymer with respect to plasma clearance and tissue distribution. PVP showed the longest MRT of all polymers examined. The total clearance of PVP was about 10-fold lower than that of PEG5000. The distribution volume of dextran was the highest of all these polymers; its volume was double that of PVP. We next studied the tissue distribution of polymers 3 h after i.v. injection. Although all polymers with the same molecular weight dispercity in this study were nonionic and water-soluble, each polymer showed a characteristic distribution. PEG and PVP did not exhibit specific tissue accumulation, on the other hand, dextran was accumulated in the liver and spleen. PVA and PAAm also had no specific distribution, but PDAAm tended to accumulate in the kidney. As demonstrated clearly, dextran is not appropriate for prolonging the circulation time of drugs.

PVP has the longest circulation time and, its tissue distribution was extremely restricted. In addition, it is easy to introduce various comonomers on radical polymerization to PVP. These results suggest that PVP is the most feasible polymeric modifier for localizing the conjugated drug in blood.