medium for 10 min at 37 °C with FL containing pCMV-script/OVA (1 μg), Lipofectin (Invitrogen) complexed with 1 μg plasmid as described in the manufacturer's protocol. After 24 h cultivation, total RNA was extracted from the transfected cells and used as a template for RT-PCR analysis.

Antigen presentation assay. The in vitro assay for antigen processing and presentation was performed as described in other studies [26]. In brief, IC21 cells (10^5 cells/well in 96-well plate) were incubated with various concentrations of antigens-encoding plasmids entrapped or complexes with various vectors at 37 °C for only 10 min. The cells were washed and subsequently incubated at 37 °C for 24 h. Then the cells were fixed with 0.05% glutaraldehyde, washed three times, and cultured with CD8OVA1.3 T hybridoma cells (10^5 cells/well). The response of the CD8OVA1.3 T cells was determined by the level of IL-2 secretion using a CTLL-2 proliferation assay. Results are expressed as means ± SD for triplicate/group.

Detection of OVA-specific antibody production by ELISA. OVA-specific antibody was detected as described previously. ELISA plates were coated with 10 mg/ml OVA in 50 mM bicarbonate buffer. Wells were blocked with 2-fold diluted Block Ace (Dai-Nippon Pharmaceutical) for 16 h at 4 °C. After washing four times with PBS containing 0.05% Tween 20 (PBS-T), each diluted serum was added (50 μl/well) and incubated for 2 h at 37 °C. Serums from non-immunized mice were included as controls. HRP conjugated anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was used as the detection Ab. Following 2 h incubation at 37 °C, plates were washed, and the reaction was developed by 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA), and color development was terminated after a 15-min incubation by addition of 2 N H_2SO_4. Endpoint titers were expressed as the reciprocal log2 of the last dilution, which gave an OD at 450 nm of 0.1 greater than non-immunized mice.

Cytokine analysis by ELISA. Cytokine levels in culture supernatants of Ag-stimulated splenocytes were determined by a cytokine-specific ELISA. Briefly, splenocytes from immunized mice were cultured with 1 mg/ml OVA. Culture supernatants were harvested 96 h after incubation, and the levels of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-6)-type cytokines were determined by cytokine-specific ELISA kit (Amersham–Pharmacia Biotech). The concentration of cytokines was calculated by standard curves obtained according to the instruction provided by the manufacturer.

In vitro CTL induction and cytotoxic assay. C57BL/6 mice (7 weeks old, male, H-2b) were immunized twice at two-week intervals with 50 μg of naked DNA, cationic lipid/plasmid complex, and plasmids entrapped in a conventional liposome or FL. Spleen cells from immunized or non-immunized mice were recovered 14 days after the last immunization and were stimulated in vitro with mitomycin C-treated EG7 cells for 5 days. The cytotoxic activity of these effector cells was tested on ^{51}Cr-labeled target cells, OVA-expressed EG7 cells, and EL4 as a control, at various effector/target ratios. The cytotoxicity assay was completed in triplicate. The maximum release was determined by adding 1% Triton X-100 to the target cells. A spontaneous release was obtained in target cells incubated without effector cells. EL4 cells were used as control for specificity. The released radioactivity was measured in the supernatant. The specific lysis was determined as follows: percentage of specific lysis = [(percentage of lysis of positive target) − (percentage of lysis of negative target)].

Tumor rejection assay. C57BL/6 mice were immunized twice s.c. at the tail base using three doses of naked DNA, cationic lipid/plasmid complex, and plasmids entrapped in a conventional liposome or a FL at two-week intervals. Fourteen days after the last immunization (day 0), 1×10^6 OVA-expressing EG7 cells or 1×10^6 EL4 cells were intradermally injected. Six to thirteen mice were used for each experimental group. Tumor growth was monitored by calculating the tumor volume and was individually plotted. The tumor volume was calculated as follows: $V = (\text{length} \times \text{width}^2)/2$. Tumor measurements were determined until they exceeded 1000 mm^3.

Results and discussion

Initially, using RT-PCR analysis, we verified the expression of OVA mRNA transcribed from the plasmid, pCMV-script/OVA (Fig. 1), used for DNA vaccination (Fig. 2, lane a). We also performed in vitro transfection studies to assess the OVA proteins expressed in a transfected mouse macrophage cell line (IC-21) and fibroblasts (L cells). It appears that a majority of the DNA injected in vivo is rapidly degraded by extracellular deoxyribonucleases. In this regards to intimate the in vivo milieu, in vitro transfection experiments were performed for only 10 min. Surprisingly, in only 10 min transfection, a remarkably higher OVA mRNA expression was detected in IC-21 and L cells treated with FL than by other transfection techniques (Fig. 2, naked plasmid, lane b; liposome, lane c; Lipofectin, lane d; and FL, lane e). For example, OVA expression was lower for a commercially available cationic lipid-based gene delivery method (Lipofectin) than for FL-mediated transfection in L cells. Furthermore, OVA expression was not observed in IC-21 cells in response to the Lipofectin method.

We have demonstrated that FLs deliver their contents via membrane fusion rather than endocytosis and that macrophages preferentially phagocytize positively charged liposomes [29,30]. Together, these studies indicate that plasmid vectors complexed with cationic lipids are taken up through an endocytotic pathway and degraded in endosomes. In contrast, FLs mediate rapid and efficient introduction of the encapsulated plasmid into cells, which leads to antigen processing and presen-

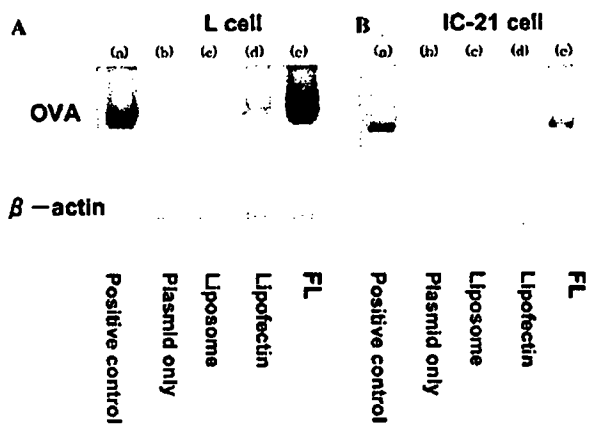

Fig. 2. RT-PCR analysis of OVA in L and IC-21 cells that were transfected by various vectors. L (A) and IC-21 (B) cells were incubated with various vectors at 37 °C for 10 min. after 24 h in culture. Naked 1 μg pCMV-script/OVA plasmid (b), conventional liposome encapsulating 1 μg pCMV-script/OVA (c), 5 μg Lipofectin/pCMV-script/OVA complex (d), and 1 μg pCMV-script/OVA-FL (e) were co-amplified by *Taq* DNA polymerase generating 864 bp PCR fragments. The samples were electrophoresed through a 2.0% agarose gel and stained with ethidium bromide.

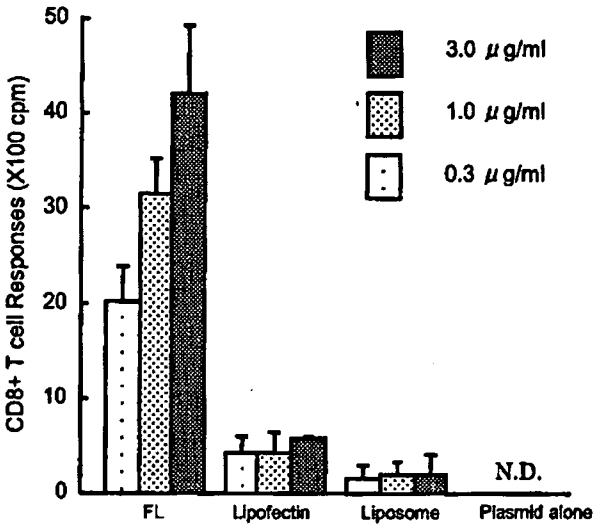

Fig. 3. Presentation of OVA using FL on MHC class I molecules by IC-21 cells. IC-21 cells were incubated with various concentrations of antigen-encoding plasmids entrapped or complexed with various vectors at 37 °C for only 10 min. After 24 h in culture, the IC-21 cells were incubated with CD8OVA1.3 T hybridoma cells for 24 h. The responses of CD8OVA1.3 were determined by their level of IL-2 secretion, using a CTL bioassay. Results are expressed as means ± SD for each group ($n = 3$).

tation to MHC class I molecules in a murine macrophage cell line (IC-21) (Fig. 3).

In the present study, we also investigated anti-OVA IgG responses in mice after subcutaneous immunization with FL. We collected blood samples from mice at the indicated time points after immunization with pCMV-script/OVA-FL and several other plasmid formulations. Both IgG1 and IgG2a antibodies were assessed by ELISA against recombinant OVA proteins (Fig. 4). The OVA-specific IgG1 and IgG2a responses in the serum of mice subcutaneously immunized with pCMV-script/OVA-FLs were remarkably higher than those from mice immunized with naked plasmid, pCMV-script/OVA encapsulated conventional liposome, or Lipofectin/pCMV-script/OVA complexes. These serum Ab responses remained elevated for at least 12 weeks and demonstrated that FL-mediated antigen expression induced efficient and antigen-specific humoral immune responses.

In general, DNA vaccines that do not use an immuno-modulating adjuvant (CpG oligonucleotides, cholera-toxin, and heat-labile toxin) exhibit low immunogenicity [6,13,16,17]. These observations are supported by the results of human trials of DNA vaccination, which are generally ineffective [1]. In this regard, the development of a delivery system for DNA vaccination, which possesses immuno-potentiating adjuvant activity, is desirable. We therefore characterized the Ag-specific splenocytes from mice subcutaneously immunized with pCMV-script/OVA-FLs, and determined Th1 (IFN-γ) and Th2 (IL-4)-specific cytokine production levels (Table 1). Th1- and Th2-type cytokine production in splenocytes increased remarkably in mice immunized with pCMV-script/OVA-FLs. These results indicated that FLs could elicit systemic Th1- and Th2-type CD4+ T cells without co-administration of adjuvants.

As indicated in Fig. 4, Th1-type Ag-specific responses were also induced by FL-mediated DNA vaccination, which suggests that this method could induce efficient and Ag-specific CTL responses. To confirm this theory we performed an in vitro cytotoxicity assay. CTL activity of splenocytes from mice immunized with pCMV-script/OVA-FLs against EG7 cells was stronger than that of splenocytes from mice immunized with other pDNA formulations, such as naked pDNA, Lipofec-

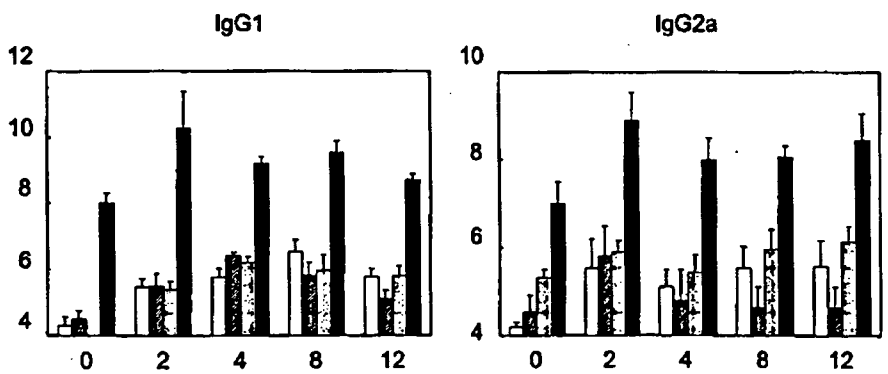

Fig. 4. OVA-specific Ab responses after intradermal immunization with pCMV-script/OVA-FL. Balb/c mice were immunized with 50 μg of naked pCMV-script/OVA (□), 5 μg conventional liposome encapsulating pCMV-script/OVA (▨), 25 μg of a Lipofectin/5 μg pCMV-script/OVA complex (▩), or 5 μg pCMV-script/OVA-FL (■) twice with an interval of two weeks between treatments. Serum samples were collected from each mouse and assayed for OVA-specific IgG1 and IgG2a by ELISA at different time intervals after the primary immunization. Titers were defined as the highest dilution required to reach an OD of 0.1 at 450 nm.

Table 1
Characterization of Th1- and Th2-responses in vivo

Vector	Stimulation	IFN-γ (pg/ml)	IL-4 (pg/ml)
Saline	Medium	157.0 ± 12.7	<7.8
	OVA	161.4 ± 15.3	<7.8
Naked DNA	Medium	170.4 ± 26.4	10.3 ± 2.5
	OVA	443.8 ± 39.5	11.5 ± 1.8
Liposome	Medium	165.7 ± 10.7	9.5 ± 1.5
	OVA	438.1 ± 21.4	9.8 ± 1.2
Cationic liposome	Medium	182.6 ± 14.6	12.8 ± 1.7
	OVA	529.4 ± 25.7	11.4 ± 2.4
Fusogenic liposome	Medium	176.1 ± 18.4	12.4 ± 2.4
	OVA	861.1 ± 58.4	24.2 ± 3.7

Splenocytes (5×10^6 cells/ml) were stimulated in vitro for 48 h with OVA protein (1 mg/ml), and the cytokine levels in the culture supernatant were determined by ELISA. The mean ± SD is shown for each group of three mice. The lower limit of IL-4 detection was 7.8 pg/ml.

tin/pDNA complexes or pDNA encapsulated by conventional liposomes. These findings emphasize that plasmid DNA vaccination combined with FL could induce effective CTL responses (Fig. 5).

Finally, we investigated whether FL-mediated DNA vaccination could induce antigen-specific anti-tumor immunity. In order to determine the effects of FL-mediated prophylactic vaccination, we analyzed tumor growth and survival after vaccination in the EG7 model.

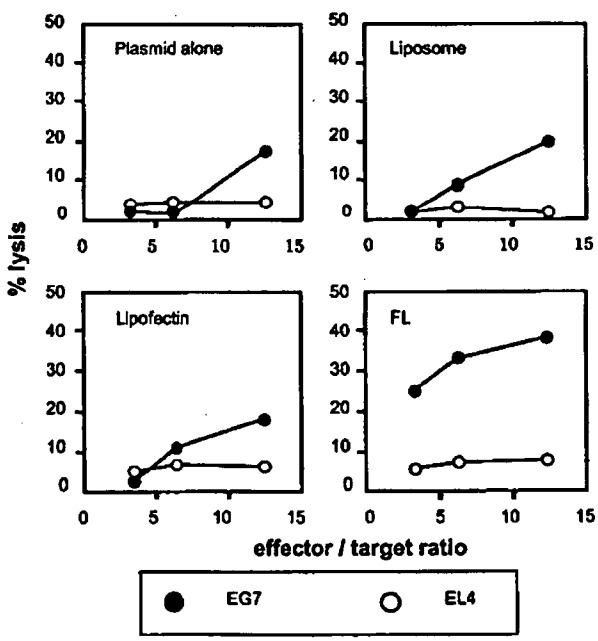

Fig. 5. In vivo immunization with pCMV-script/OVA encapsulated by FL induced antigen-specific CTL responses. Fourteen days after the last immunization, splenocytes from mice immunized with naked pCMV-script/OVA (50 μg), pCMV-script/OVA (5 μg) encapsulated by a conventional liposome, Lipofectin/pCMV-script/OVA (25 μg) complex or FL containing pCMV-script/OVA (5 μg) were isolated and restimulated with MMC-treated EG7 for 5 days to enhance the frequency of Ag-specific CTLs. CTL activity against EG7 or EL4 was measured by a ^{51}Cr release assay. The figure represents the amount of lysis against the ^{51}Cr-labeled EG7 and EL4.

Four weeks after the last immunization, mice were intradermally challenged with 10^6 live EG7 cells in the abdomen. As shown in Fig. 6, high concentrations of naked pCMV-script/OVA, 5 μg pCMV-script/OVA/Lipofectin complexes or pCMV-script/OVA encapsulated in conventional liposomes did not generate protective immunity. In contrast, only 5 μg of pCMV-script/OVA encapsulated in FL exhibited enhanced anti-tumor effects as demonstrated by reduced tumor growth and prolonged survival. Furthermore, Hayashi and Kunisawa et al. reported that FL possesses immuno-stimulating activities derived from Sendai virus accessory proteins displayed on the FL surface, which up-regulate co-stimulatory molecules on cells, including antigen presenting cells such as dendritic cells and splenocytes [27,31]. In the present study, in vitro experiments provide evidence that FLs mediate efficient introduction and expression of antigen genes and enhance MHC class I antigen presentation. Our findings lead us to surmise that these synergistic effects facilitated antigen-specific immune reactions in response to FLs used as genetic vaccine carriers.

Our results demonstrated that FL-mediated genetic immunization can trigger strong and antigen-specific humoral and cell-mediated immunity in mice. FL, which was reconstituted from UV-inactivated Sendai virus and liposomes, is a promising approach for antigen-encoding gene delivery. Sendai virus-derived accessory proteins incorporated into FL not only enhance antigen introduction into the cytoplasm and pDNA expression, but also stimulate inflammatory responses, a major advantage of DNA vaccination. In conclusion, our present study illustrated that FL-mediated genetic vaccination facilitates the antigen presentation by APC in vitro and remarkably enhanced anti-tumor immune responses in vivo. Thus, by overcoming the current problems in genetic immunization, the FL-mediated gene transfer system makes significant progress toward the development of genetic cancer or viral vaccines.

the in vivo pharmacokinetics of synthesized PVP derivatives with various electrically charge or hydrophilic/hydrophobic balance [20]. For instance, the co-polymer between vinylpyrrolidone (VP) and vinyl-lauric acid showed a marked increase in distribution in the spleen compared to that in the liver. Recently, we synthesized a novel polymeric drug carrier, PVD, which was a powerful candidate as a targeting carrier for a renal drug delivery system. In this study, we attempted to optimize the renal drug delivery system using PVD.

To assess the usefulness of PVD as a renal targeting carrier, the relationship between the M_n of PVD and its renal accumulation after intravenous injection was investigated (Fig. 1). Renal accumulation was highest for molecules with an M_n of 6–8 kDa, about 80% of the administered dose accumulate in the kidneys 3 h after intravenous injection. Increase or decrease of an M_n lowered the renal accumulation (Fig. 2). We have previously showed that the safety of PVD was similar to that of PEG and PVP, which are used clinically [17]. Thus, PVD with 6–8 kDa of M_n seems to be an extremely safe polymeric carrier with much higher renal targeting and retention capacity. It has previously been found that PVD was selectively accumulated in renal proximal tubular epithelial cells after intravenous injection [17]. Additionally, the uptake of PVD by renal tubular cells was inhibited by the energy inhibitor (NaN_3) and was not affected by cytochalasin B. Thus, PVD may be taken in by an energy-dependent process except for endocytosis. Several specific molecules play a role in renal transport, and various organic anions transporter exist in the kidney, but these transporters generally carry low-molecular-weight drugs. It is unclear why PVD with a molecular weight of 6–8 kDa was selectively accumulated in the kidneys. To address this question, we are currently in the process of doing some experiments.

We further evaluated the usefulness of PVD as a renal targeting carrier by polymer conjugation to SOD, which has been expected to be a potential drug to renal disease. Many recent studies have reported an association between activated oxygen species such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (·OH), and NO with various pathologic disease processes such as cancer, inflammation, septicemia, and necrosis associated with ischemic reperfusion [21,22]. Several studies have investigated the use of activated oxygen metabolic enzymes and antioxidants as therapeutic agents in diseases where stress oxidation plays a prominent role [23,24]. SOD has shown promise as a therapeutic agent, eliminating O_2^- in the early stages of formation of highly reactive oxygen species such as ·OH. Developments in genetic engineering have now enabled the production of large quantities of human Cu/Zn-SOD, which has attracted attention as a therapeutic agent [25]. With respect to kidney disease, activated oxygen is known to play an indispensable role in the mechanisms of ARF, complications associated with long-term maintenance dialysis, drug toxicity, and various inflammatory conditions [26]. We prepared PVD-SODs with various molecular size (Table 2) and then evaluated their tissue distribution 3 h after intravenous injection (Fig. 3). The renal accumulation of PVD-SODs decreased with increasing their molecular size. About sixfold more L-PVD-SOD with 73 kDa of M_n, which had almost full activity compared with native SOD, was distributed to the kidneys than native SOD. The renal accumulation of PVP-modified SOD with the same molecular size as L-PVD-SOD was similar to that of native SOD (data not shown). This confirms that renal accumulation of L-PVD-SOD is attributable to the properties of PVD. However, extensive PVD modification of SOD did not increase its renal accumulation. These results indicated that L-PVD-SOD was the optimal derivative which had a potential renal targeting capability. Thus, our results revealed that the renal accumulation probably involves not only an optimal introduction rate of electrical charges and/or hydrophobicity to PVD, but also molecular size of PVD-SOD.

Renal disease is one of the most serious problems in the world. Many researchers have attempted to deliver drug to the kidney. For example, Hashida et al. reported that cationized SOD and PEGylated SOD exhibited significant therapeutic effects on ischemic acute renal failure [27,28]. However, there is no report as to delivery of drug to the kidney specifically. On the other hand, PVD accumulated in the kidney about 80% of the administered dose. Therefore, we consider that PVD may be the most superior carrier for delivering drug to the kidney. Thus, the development

of new therapeutic approaches is expected for treating patients with renal disease. As shown in Table 4, L-PVD-SOD showed great potential as a renal antioxidant agent to ARF. These results suggest that L-PVD-SOD may be a candidate as a novel therapeutic agent with high renal targeting capability.

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Augmentation of antigen-specific immune responses using DNA-fusogenic liposome vaccine

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Abstract

In an attempt to enhance the immunological efficacy of genetic immunization, we investigated a new biological means for delivering antigen gene directly to the cytoplasm via membrane fusion. In this context, we investigated fusogenic liposome (FL) encapsulating DNA as a possible genetic immunization vehicle. RT-PCR analysis indicated that a FL could introduce and express encapsulating OVA gene efficiently and rapidly in vitro. Consistent with this observation, an in vitro assay showed that FL-mediated antigen-gene delivery can induce potent presentation of antigen via the MHC class I-dependent pathway. Accordingly, immunization with FL containing the OVA-gene induced potent OVA-specific Th1 and Th2 cytokine production. Additionally, OVA-specific CTL responses and antibody production were also observed in systemic compartments including the spleen, upon immunization with the OVA-gene encapsulating FL. These findings suggest that FL is an effective genetic immunization carrier system for the stimulation of antigen-specific immune responses against its encoding antigen.
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Keywords: DNA vaccine; Tumor vaccine; Liposome; Drug delivery system

Genetic immunization using plasmid DNA (pDNA) encoding antigens from bacteria, viruses, and cancers has often led to protective cell-mediated (Th1) and humoral (Th2) immunity [1]. This system has some practical advantages over conventional vaccines, such as safety, stability, cost-effectiveness for manufacturing, and the ease of modifying and customizing the gene sequence to produce the desired type of recombinant protein for expression in vivo. Although naked DNA vaccines (i.e., pDNA in saline) are effective in small animal models [2–4], recent results from large-animal and non-human primate studies have been disappointing due to sub-optimal immune responses, despite the use of multi-milligram doses of naked pDNA [5]. Therefore, the development of adjuvants and excipients to increase immune responses to DNA vaccines has become an active area of research. The potential of genetic immunization to exert an effective antigen-specific immune response is directly related to both the level of

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expression of the encoded protein and the immunomodulatory activity generated by DNA vaccine formulations [6–8].

Two main causes for the ineffectiveness of genetic immunization have been proposed. First, the pDNAs are generally unstable in vivo. In other words, most injected pDNAs are degraded by extracellular deoxyribonucleases in situ [9–11]. In addition, a limited amount of undegraded pDNA is taken up via endocytosis by neighboring cells around the injection site, including antigen presenting cells (APCs). The endocytosed pDNAs are thus generally degraded in intracellular compartments such as endosomes or lysosomes. Consequently, extremely low levels of pDNAs that escaped from these host factors could express their encoded antigens, resulting in inefficient induction of antigen-specific immunity. Furthermore, in terms of eliciting antibody responses, DNA vaccines are poorly immunogenic relative to other vaccines such as peptide and protein vaccines [12,13]. Because of the extra- and intra-cellular degradation and low immunogenicity of naked pDNAs, genetic immunization exhibits poor performance when administered by routes other than intramuscular [5].

In this context, various approaches, such as combination with adjuvant or cytokines and particulating techniques, have been applied to enhance immune responses to the encoding antigen [9,11]. The carrier-mediated approach is particularly promising because of enhanced pDNA stability and immunogenicity, and also due to the combination with molecular adjuvants. To address these issues, several DNA-particulating approaches have been evaluated [14]. Recently, liposomes and micro- or nano-scale particles have been tested for genetic vaccination [14–20]. However, due to the low immunogenicity and degradation of pDNA by APCs, a novel carrier-mediated approach to improve the potency of DNA vaccines is required.

We previously developed a unique antigen delivery system based on liposomes fused with UV-inactivated Sendai virus, known as the fusogenic liposome, FL [21–25]. The FL efficiently delivered the encapsulated antigens into the cytoplasm of antigen presenting cells via membrane fusion for use in the MHC class I-presentation pathway [26,27]. Furthermore, FL possesses adjuvant activity derived from Sendai virus accessory proteins. It stimulates antigen presenting cells to up-regulate cell surface markers and enhances the expression of inflammatory cytokines by APCs. Additionally, we demonstrated that subcutaneous immunization with antigen-encapsulated FL induced high levels of antigen-specific immune responses at systemic immune compartments such as the spleen [26].

This information and our previous results allowed us to hypothesize that FL could be utilized as genetic vaccine carrier. Thus, the purpose of the present study was to analyze the efficiency of immunization through dermal delivery of model antigen protein-encoding DNA (OVA) delivered by FL.

Materials and methods

Mice and cells. Six- to eight-week-old male C57BL/6 (H-2^b) mice were purchased from SLC (Hamamatsu, Japan). EL4 (Tohoku University, Sendai, Japan) is a C57BL/6 T lymphoma and EG7 is an ovalbumin (OVA)-transfected clone of EL4. IC21 cells are C57BL/6 macrophage clones, H-2Kb. CD8OVA1.3 (provided by Dr. Clifford V. Harding, Case Western Reserve University, Cleveland, OH) is a T-T hybrid cell, which is specific for OVA257-264-Kb.

EL4 and IC21 cells were grown in RPMI1640 medium supplemented with 10% FCS. The CTLL-2 cells were maintained in RPMI1640 medium supplemented with 10% FCS and 1 U/ml human recombinant IL-2. The EG7 cells were maintained in RPMI1640 medium supplemented with 10% FCS and 400 μg/ml G418. CD8OVA1.3 was grown in DMEM supplemented with 10% FCS. All culture media were purchased from Invitrogen (Carlsbad, CA) and supplemented with non-essential amino acids, antibiotics and, 5×10^{-5} M 2-mercaptoethanol (2-ME).

Plasmids. The *Eco*RI fragment of pAc-neo-OVA [28] was cloned into the *Eco*RI site of pBluescriptII KS(−), resulting in pBluescriptII KS(−)/OVA. To construct an OVA gene expression vector, the *Bam*HI/*Sal*I fragment of pBluescript II KS(−)/OVA was ligated into a *Bam*HI/*Sal*I cut pCMV-script (Stratagene), resulting in pCMV-script/OVA (Fig. 1), which is driven by the cytomegalovirus promoter and contains the SV40 poly(A) signal.

Preparation of fusogenic liposome. pCMV-script/OVA containing unilamellar liposomes was prepared by a modified reverse-phase evaporation method using 46 μmol of lipids (egg phosphatidylcholine: L-α-dimyristryl phosphatidic acid: cholesterol = 5:1:4, molar ratio). After three cycles of freezing and thawing, the liposomes were sized by extrusion through a 0.8 μm polycarbonate membrane (Nucleopore; Coaster, Cambridge, USA) and pelleted by ultracentrifugation to remove unencapsulated plasmids. Then, FL encapsulated pCMV-script/OVA was prepared by fusing the liposomes with UV (2000 J/cm^2)-inactivated Sendai virus as described previously [21,26]. The amount of plasmid DNA encapsulated within liposomes was determined by means of fluorometric assay using 3,5-diaminobenzoic acid.

RT-PCR for OVA gene detection. To examine the expression of OVA mRNA, IC-21 cells were incubated in serum free RPMI1640

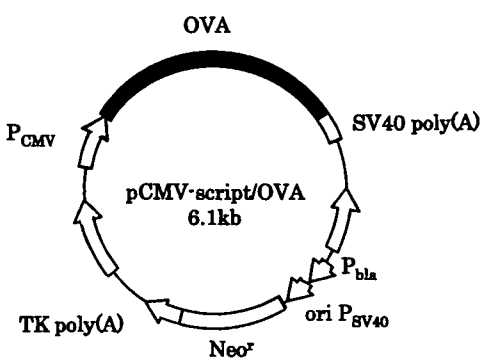


Fig. 1. Structure of OVA expression plasmid pCMV-script/OVA. OVA cDNA was inserted into an expression plasmid (pCMV-script) containing a cytomegalovirus (CMV) promoter and a SV40 polyadenylation site.

medium for 10 min at 37 °C with FL containing pCMV-script/OVA (1 μg), Lipofectin (Invitrogen) complexed with 1 μg plasmid as described in the manufacturer's protocol. After 24 h cultivation, total RNA was extracted from the transfected cells and used as a template for RT-PCR analysis.

Antigen presentation assay. The in vitro assay for antigen processing and presentation was performed as described in other studies [26]. In brief, IC21 cells (10^5 cells/well in 96-well plate) were incubated with various concentrations of antigens-encoding plasmids entrapped or complexes with various vectors at 37 °C for only 10 min. The cells were washed and subsequently incubated at 37 °C for 24 h. Then the cells were fixed with 0.05% glutaraldehyde, washed three times, and cultured with CD8OVA1.3 T hybridoma cells (10^5 cells/well). The response of the CD8OVA1.3 T cells was determined by the level of IL-2 secretion using a CTLL-2 proliferation assay. Results are expressed as means ± SD for triplicate/group.

Detection of OVA-specific antibody production by ELISA. OVA-specific antibody was detected as described previously. ELISA plates were coated with 10 mg/ml OVA in 50 mM bicarbonate buffer. Wells were blocked with 2-fold diluted Block Ace (Dai-Nippon Pharmaceutical) for 16 h at 4 °C. After washing four times with PBS containing 0.05% Tween 20 (PBS-T), each diluted serum was added (50 μl/well) and incubated for 2 h at 37 °C. Serums from non-immunized mice were included as controls. HRP conjugated anti-mouse IgG1 (Southern Biotechnology Associates, Birmingham, AL) was used as the detection Ab. Following 2 h incubation at 37 °C, plates were washed, and the reaction was developed by 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA), and color development was terminated after a 15-min incubation by addition of 2 N H_2SO_4. Endpoint titers were expressed as the reciprocal log2 of the last dilution, which gave an OD at 450 nm of 0.1 greater than non-immunized mice.

Cytokine analysis by ELISA. Cytokine levels in culture supernatants of Ag-stimulated splenocytes were determined by a cytokine-specific ELISA. Briefly, splenocytes from immunized mice were cultured with 1 mg/ml OVA. Culture supernatants were harvested 96 h after incubation, and the levels of Th1 (IFN-γ) and Th2 (IL-4, IL-5, and IL-6)-type cytokines were determined by cytokine-specific ELISA kit (Amersham–Pharmacia Biotech). The concentration of cytokines was calculated by standard curves obtained according to the instruction provided by the manufacturer.

In vitro CTL induction and cytotoxic assay. C57BL/6 mice (7 weeks old, male, H-2b) were immunized twice at two-week intervals with 50 μg of naked DNA, cationic lipid/plasmid complex, and plasmids entrapped in a conventional liposome or FL. Spleen cells from immunized or non-immunized mice were recovered 14 days after the last immunization and were stimulated in vitro with mitomycin C-treated EG7 cells for 5 days. The cytotoxic activity of these effector cells was tested on ^{51}Cr-labeled target cells, OVA-expressed EG7 cells, and EL4 as a control, at various effector/target ratios. The cytotoxicity assay was completed in triplicate. The maximum release was determined by adding 1% Triton X-100 to the target cells. A spontaneous release was obtained in target cells incubated without effector cells. EL4 cells were used as control for specificity. The released radioactivity was measured in the supernatant. The specific lysis was determined as follows: percentage of specific lysis = [(percentage of lysis of positive target) − (percentage of lysis of negative target)].

Tumor rejection assay. C57BL/6 mice were immunized twice s.c. at the tail base using three doses of naked DNA, cationic lipid/plasmid complex, and plasmids entrapped in a conventional liposome or a FL at two-week intervals. Fourteen days after the last immunization (day 0), 1×10^6 OVA-expressing EG7 cells or 1×10^6 EL4 cells were intradermally injected. Six to thirteen mice were used for each experimental group. Tumor growth was monitored by calculating the tumor volume and was individually plotted. The tumor volume was calculated as follows: $V = (\text{length} \times \text{width}^2)/2$. Tumor measurements were determined until they exceeded 1000 mm^3.

Results and discussion

Initially, using RT-PCR analysis, we verified the expression of OVA mRNA transcribed from the plasmid, pCMV-script/OVA (Fig. 1), used for DNA vaccination (Fig. 2, lane a). We also performed in vitro transfection studies to assess the OVA proteins expressed in a transfected mouse macrophage cell line (IC-21) and fibroblasts (L cells). It appears that a majority of the DNA injected in vivo is rapidly degraded by extracellular deoxyribonucleases. In this regards to intimate the in vivo milieu, in vitro transfection experiments were performed for only 10 min. Surprisingly, in only 10 min transfection, a remarkably higher OVA mRNA expression was detected in IC-21 and L cells treated with FL than by other transfection techniques (Fig. 2, naked plasmid, lane b; liposome, lane c; Lipofectin, lane d; and FL, lane e). For example, OVA expression was lower for a commercially available cationic lipid-based gene delivery method (Lipofectin) than for FL-mediated transfection in L cells. Furthermore, OVA expression was not observed in IC-21 cells in response to the Lipofectin method.

We have demonstrated that FLs deliver their contents via membrane fusion rather than endocytosis and that macrophages preferentially phagocytize positively charged liposomes [29,30]. Together, these studies indicate that plasmid vectors complexed with cationic lipids are taken up through an endocytotic pathway and degraded in endosomes. In contrast, FLs mediate rapid and efficient introduction of the encapsulated plasmid into cells, which leads to antigen processing and presen-

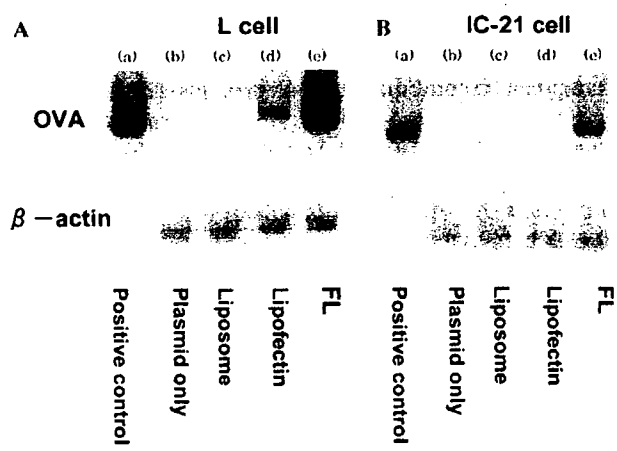
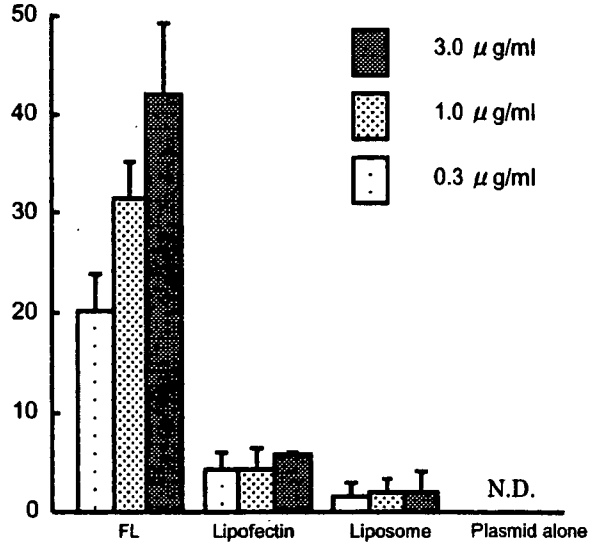


Fig. 2. RT-PCR analysis of OVA in L and IC-21 cells that were transfected by various vectors. L (A) and IC-21 (B) cells were incubated with various vectors at 37 °C for 10 min. after 24 h in culture. Naked 1 μg pCMV-script/OVA plasmid (b), conventional liposome encapsulating 1 μg pCMV-script/OVA (c), 5 μg Lipofectin/pCMV-script/OVA complex (d), and 1 μg pCMV-script/OVA-FL (e) were co-amplified by *Taq* DNA polymerase generating 864 bp PCR fragments. The samples were electrophoresed through a 2.0% agarose gel and stained with ethidium bromide.



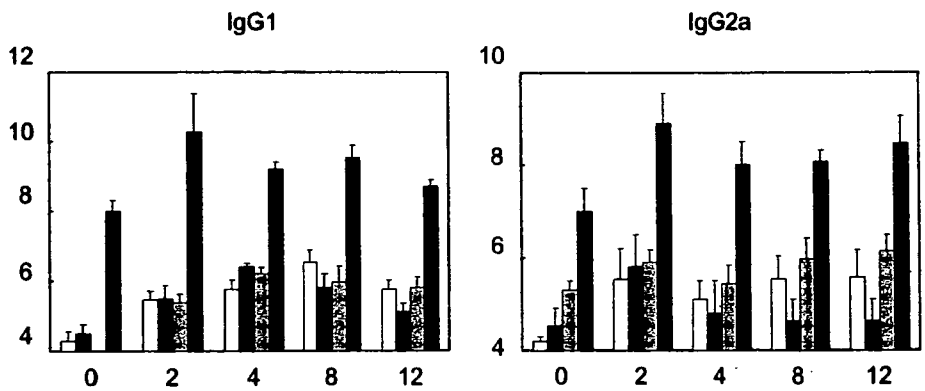
3. Presentation of OVA using FL on MHC class I molecules by
21 cells. IC-21 cells were incubated with various concentrations of
gen-encoding plasmids entrapped or complexed with various
ors at 37 °C for only 10 min. After 24 h in culture, the IC-21 cells
e incubated with CD8OVA1.3 T hybridoma cells for 24 h. The
onses of CD8OVA1.3 were determined by their level of IL-2
etion, using a CTL bioassay. Results are expressed as means ± SD
each group ($n = 3$).

ion to MHC class I molecules in a murine macro-
ıge cell line (IC-21) (Fig. 3).

In the present study, we also investigated anti-OVA
G responses in mice after subcutaneous immunization
h FL. We collected blood samples from mice at the
icated time points after immunization with pCMV-
ipt/OVA-FL and several other plasmid formulations.
th IgG1 and IgG2a antibodies were assessed by ELI-
against recombinant OVA proteins (Fig. 4). The OVA-specific IgG1 and IgG2a responses in the serum of mice subcutaneously immunized with pCMV-script/OVA-FLs were remarkably higher than those from mice immunized with naked plasmid, pCMV-script/OVA encapsulated conventional liposome, or Lipofectin/pCMV-script/OVA complexes. These serum Ab responses remained elevated for at least 12 weeks and demonstrated that FL-mediated antigen expression induced efficient and antigen-specific humoral immune responses.

In general, DNA vaccines that do not use an immuno-modulating adjuvant (CpG oligonucleotides, cholera-toxin, and heat-labile toxin) exhibit low immunogenicity [6,13,16,17]. These observations are supported by the results of human trials of DNA vaccination, which are generally ineffective [1]. In this regard, the development of a delivery system for DNA vaccination, which possesses immuno-potentiating adjuvant activity, is desirable. We therefore characterized the Ag-specific splenocytes from mice subcutaneously immunized with pCMV-script/OVA-FLs, and determined Th1 (IFN-γ) and Th2 (IL-4)-specific cytokine production levels (Table 1). Th1- and Th2-type cytokine production in splenocytes increased remarkably in mice immunized with pCMV-script/OVA-FLs. These results indicated that FLs could elicit systemic Th1- and Th2-type CD4+ T cells without co-administration of adjuvants.

As indicated in Fig. 4, Th1-type Ag-specific responses were also induced by FL-mediated DNA vaccination, which suggests that this method could induce efficient and Ag-specific CTL responses. To confirm this theory we performed an in vitro cytotoxicity assay. CTL activity of splenocytes from mice immunized with pCMV-script/OVA-FLs against EG7 cells was stronger than that of splenocytes from mice immunized with other pDNA formulations, such as naked pDNA, Lipofec-



. 4. OVA-specific Ab responses after intradermal immunization with pCMV-script/OVA-FL. Balb/c mice were immunized with 50 μg of naked
MV-script/OVA (□), 5 μg conventional liposome encapsulating pCMV-script/OVA (▨), 25 μg of a Lipofectin/5 μg pCMV-script/OVA complex
, or 5 μg pCMV-script/OVA-FL (■) twice with an interval of two weeks between treatments. Serum samples were collected from each mouse and
ıyed for OVA-specific IgG1 and IgG2a by ELISA at different time intervals after the primary immunization. Titers were defined as the highest
ıtion required to reach an OD of 0.1 at 450 nm.

Table 1
Characterization of Th1- and Th2-responses in vivo

Vector	Stimulation	IFN-γ (pg/ml)	IL-4 (pg/ml)
Saline	Medium	157.0 ± 12.7	<7.8
	OVA	161.4 ± 15.3	<7.8
Naked DNA	Medium	170.4 ± 26.4	10.3 ± 2.5
	OVA	443.8 ± 39.5	11.5 ± 1.8
Liposome	Medium	165.7 ± 10.7	9.5 ± 1.5
	OVA	438.1 ± 21.4	9.8 ± 1.2
Cationic liposome	Medium	182.6 ± 14.6	12.8 ± 1.7
	OVA	529.4 ± 25.7	11.4 ± 2.4
Fusogenic liposome	Medium	176.1 ± 18.4	12.4 ± 2.4
	OVA	861.1 ± 58.4	24.2 ± 3.7

Splenocytes (5×10^6 cells/ml) were stimulated in vitro for 48 h with OVA protein (1 mg/ml), and the cytokine levels in the culture supernatant were determined by ELISA. The mean ± SD is shown for each group of three mice. The lower limit of IL-4 detection was 7.8 pg/ml.

tin/pDNA complexes or pDNA encapsulated by conventional liposomes. These findings emphasize that plasmid DNA vaccination combined with FL could induce effective CTL responses (Fig. 5).

Finally, we investigated whether FL-mediated DNA vaccination could induce antigen-specific anti-tumor immunity. In order to determine the effects of FL-mediated prophylactic vaccination, we analyzed tumor growth and survival after vaccination in the EG7 model.

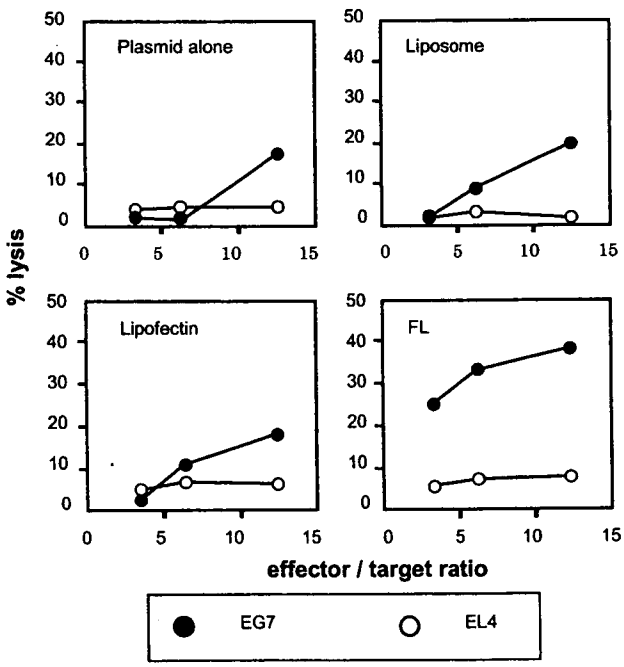


Fig. 5. In vivo immunization with pCMV-script/OVA encapsulated by FL induced antigen-specific CTL responses. Fourteen days after the last immunization, splenocytes from mice immunized with naked pCMV-script/OVA (50 μg), pCMV-script/OVA (5 μg) encapsulated by a conventional liposome, Lipofectin/pCMV-script/OVA (25 μg) complex or FL containing pCMV-script/OVA (5 μg) were isolated and restimulated with MMC-treated EG7 for 5 days to enhance the frequency of Ag-specific CTLs. CTL activity against EG7 or EL4 was measured by a ^{51}Cr release assay. The figure represents the amount of lysis against the ^{51}Cr-labeled EG7 and EL4.

Four weeks after the last immunization, mice were intradermally challenged with 10^6 live EG7 cells in the abdomen. As shown in Fig. 6, high concentrations of naked pCMV-script/OVA, 5 μg pCMV-script/OVA/Lipofectin complexes or pCMV-script/OVA encapsulated in conventional liposomes did not generate protective immunity. In contrast, only 5 μg of pCMV-script/OVA encapsulated in FL exhibited enhanced anti-tumor effects as demonstrated by reduced tumor growth and prolonged survival. Furthermore, Hayashi and Kunisawa et al. reported that FL possesses immuno-stimulating activities derived from Sendai virus accessory proteins displayed on the FL surface, which up-regulate co-stimulatory molecules on cells, including antigen presenting cells such as dendritic cells and splenocytes [27,31]. In the present study, in vitro experiments provide evidence that FLs mediate efficient introduction and expression of antigen genes and enhance MHC class I antigen presentation. Our findings lead us to surmise that these synergistic effects facilitated antigen-specific immune reactions in response to FLs used as genetic vaccine carriers.

Our results demonstrated that FL-mediated genetic immunization can trigger strong and antigen-specific humoral and cell-mediated immunity in mice. FL, which was reconstituted from UV-inactivated Sendai virus and liposomes, is a promising approach for antigen-encoding gene delivery. Sendai virus-derived accessory proteins incorporated into FL not only enhance antigen introduction into the cytoplasm and pDNA expression, but also stimulate inflammatory responses, a major advantage of DNA vaccination. In conclusion, our present study illustrated that FL-mediated genetic vaccination facilitates the antigen presentation by APC in vitro and remarkably enhanced anti-tumor immune responses in vivo. Thus, by overcoming the current problems in genetic immunization, the FL-mediated gene transfer system makes significant progress toward the development of genetic cancer or viral vaccines.

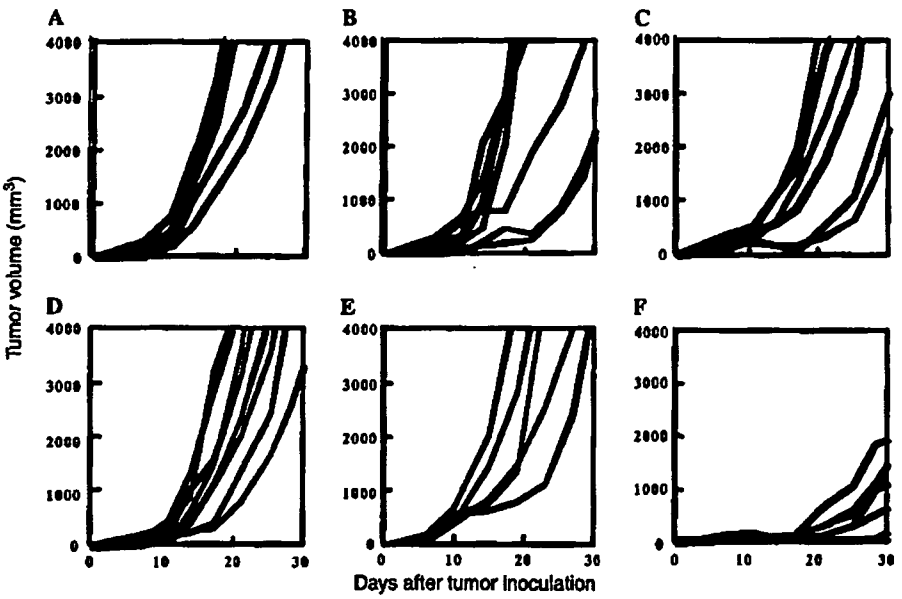


Fig. 6. In vivo immunization with pCMV-script/OVA-FL elicits protective immunity against OVA-expressing tumor. C57/BL6 mice were immunized twice at two-week intervals with saline control (A), 50 μg of naked pCMV-script/OVA (B), Lipofectin/pCMV-script/OVA (5 μg) complex (C), pCMV-script/OVA (5 μg) encapsulated by a conventional liposome (D), pCMV-script/LacZ (5 μg) containing FL (E), or pCMV-script/OVA (5 μg) containing FL (F). All mice were challenged intradermally in the abdomen with 1×10^6 live OVA-expressing EG7 cells four weeks after the last immunization and survival was monitored.

Acknowledgments

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Optimal site-specific PEGylation of mutant TNF-α improves its antitumor potency

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Abstract

Recently, we created a lysine-deficient mutant tumor necrosis factor-α [mTNF-α-Lys(−)] with full bioactivity in vitro compared with wild-type TNF-α (wTNF-α), and site-specific PEGylation of mTNF-α-Lys(−) was found to selectively enhance its in vivo antitumor activity. In this study, we attempted to optimize this PEGylation of mTNF-α-Lys(−) to further improve its therapeutic potency. mTNF-α-Lys(−) was site-specifically modified at its N-terminus with linear polyethylene glycol (LPEG) or branched PEG (BPEG). While randomly mono-PEGylated wTNF-α (ran-$LPEG_{5K}$-wTNF-α) with 5 kDa of LPEG ($LPEG_{5K}$) had about only 4% in vitro bioactivity of wTNF-α, mono-PEGylated mTNF-α-Lys(−) [sp-PEG-mTNF-α-Lys(−)] with $LPEG_{5K}$, $LPEG_{20K}$, $BPEG_{10K}$, and $BPEG_{40K}$ had 82%, 58%, 93%, and 65% bioactivities of mTNF-α-Lys(−), respectively. sp-LPEG-mTNF-α-Lys(−) and sp-$BPEG_{10K}$-mTNF-α-Lys(−) had much superior antitumor activity to those of both unmodified TNF-αs and ran-$LPEG_{5K}$-wTNF-α, though sp-$BPEG_{40K}$-mTNF-α-Lys(−) did not show in vivo antitumor activity. Thus, the molecular shape and weight of PEG may strongly influence the in vivo antitumor activity of sp-PEG-mTNF-α-Lys(−).
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Tumor necrosis factor-α (TNF-α), an antitumor cytokine, has numerous bioactivities such as direct cytotoxicity against tumor cells, activation of immune antitumor response, and selective impairment of tumor-blood vessels [1]. Thus, TNF-α has been considered as a novel antitumor agent [2,3]. However, very high doses of TNF-α as a systemic antitumor agent were required to obtain the sufficient clinical responses, since TNF-α is rapidly cleared from the circulation and is widely distributed to various tissues after its intravenous (i.v.) administration. As a result, TNF-α with pleiotropic in vivo actions exhibited unexpected toxic side-effects, typified by pyrexia and hypertension [4,5]. Its systemic application has been abandoned despite intratumoral administration of TNF-α showing significant antitumor effects in phase I studies [6]. Presently, the clinical application of TNF-α is limited to intratumoral administration, despite its high expectations as a systemic agent. These in vivo drawbacks of TNF-α are also found in the clinical applications of other bioactive proteins [7]. Thus, the development of a drug delivery system (DDS) is necessary for the promotion of protein therapies following recent marked advances in proteomics and structural genomics.

PEGylation has been known as one of the most useful DDS for optimizing protein therapies [8,9]. The PEGylation of proteins increases their molecular size and steric hindrance, both of which are derived from polyethylene glycol (PEG) attached to bioactive proteins, resulting in augmented plasma half-lives and

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in vivo stability. Due to these comprehensive effects of PEGylation, PEGylation of proteins could enhance therapeutic potency and could reduce undesirable effects. We also reported that the PEGylation of proteins such as TNF-α, superoxide dismutase, interleukin-6, and immunotoxin could enhance therapeutic potency and reduce undesirable side effects [10–16]. However, the PEGylation of proteins is mostly non-specific and targeted at all the lysine residues in the protein, some of which may be in or near an active site. As a result, PEGylation of proteins was accompanied by a significant loss of their specific activities in vitro. For instance, randomly mono-PEGylated interferon α2a ($BPEG_{40K}$-IFN-α2a), which is IFN-α2a, conjugated with 40 kDa of branched PEG, has been clinically used for the treatment of hepatitis C. The $BPEG_{40K}$-IFN-α2a, which is a mixture of various positional isomers, had about 10% of its bioactivity compared to unmodified IFN-α2a [17,18]. Thus, clinical application of PEGylated proteins has been limited in most cases except for some bioactive proteins, such as IFN-α.

To overcome this problem of PEGylation, we have developed a novel strategy for site-specific mono-PEGylation using TNF-α for the improvement of its in vivo antitumor potency [16]. We isolated a lysine-deficient mutant TNF-α with full bioactivity [mTNF-α-Lys(−)] from phage libraries expressing mTNF-αs, in which all lysine residues were replaced with other amino acids. In the mTNF-α-Lys(−) molecule, Lys11, Lys65, Lys90, Lys98, Lys112, and Lys128 of wTNF-α were replaced with Met11, Ser65, Pro90, Arg98, Asn112, and Pro128, respectively. This mTNF-α-Lys(−) was site-specifically mono-PEGylated at its N-terminus with 5 kDa of linear PEG ($LPEG_{5K}$). This site-specifically mono-PEGylated mTNF-α showed increased antitumor therapeutic potency, compared to an unmodified wild-type TNF-α (wTNF-α) and a randomly mono-PEGylated wTNF-α. In this study, to optimize the site-specific PEGylation at the N-terminus of mTNF-α-Lys(−) in order to further improve its therapeutic potency, mTNF-α-Lys(−) was modified with LPEG and BPEG, which had different molecular weights (Mw). Additionally, through molecular modeling of the complexes between wTNF-α and TNF receptor-I (TNF-RI), we discussed why mTNF-α-Lys(−) had comparable in vitro bioactivity to wTNF-α, despite reports that some lysine residues were essential for its bioactivity. This study will provide the information necessary to optimally design a PEGylated TNF-α suitable for therapeutic use as a systemic antitumor agent.

Materials and methods

Expression and purification of TNF-αs. Plasmids pYas1-TNF and pYas-mTNF encoding human wTNF-α and mTNF-α-Lys(−), respectively, under the control of a T7 promoter, were prepared. wTNF-α and mTNF-α-Lys(−) proteins were produced in *Escherichia coli* BL21(DE3) harboring the expression plasmid pYas1-TNF and pYas-mTNF as described [14,16]. mTNF-α-Lys(−) had K11M, K65S, K90P, K98R, K112N, and K128P compared with wTNF-α. Endotoxin levels were determined to be <300 pg/mg each in the wTNF-α and mTNF-α-Lys(−).

PEGylation of mTNF-α-Lys(−). As shown in Fig. 1, activated LPEGs with Mw of 5 and 20 kDa ($LPEG_{5K}$, $LPEG_{20K}$) and activated BPEGs with Mw of 10 and 40 kDa ($BPEG_{10K}$, $BPEG_{40K}$) were purchased from Shearwater Polymers (Huntsville, AL). mTNF-α-Lys(−) in PBS was reacted with 50 times molar excess of $LPEG_{5K}$ and $LPEG_{20K}$ as well as 250 times molar excess of $BPEG_{10K}$ and $BPEG_{40K}$ against total primary amine groups of mTNF-α-Lys(−) at 37 °C for 30 min. wTNF-α in PBS was reacted with five times molar excess of $LPEG_{5K}$ against total primary amine groups of wTNF-α at 37 °C for 30 min. Site-specifically mono-PEGylated mTNF-α-Lys(−)s and randomly mono-PEGylated wTNF-α were purified by anion-exchange and gel filtration chromatographies. The specific bioactivities of mono-PEGylated forms were examined by a cytotoxicity assay using LM cells, a cell line derived from L929 cells. Kinetic and equilibrium constants for the interaction between mono-PEGylated TNF-αs and TNF-RI were measured using surface plasmon resonance in a BIAcore 2000, as described.

Antitumor studies. All experimental protocols for animal studies were in accordance with the *Guide for Laboratory Animal Facilities and Care* (NIH publication 85-23, revised 1985). These protocols have been approved by the Committee of the Pharmaceutical School, Osaka University. The antitumor effects of mTNF-α-Lys(−) and sp-PEG-mTNF-α-Lys(−)s were evaluated in mice bearing Meth-A fibrosarcoma. Meth-A cells were implanted intradermally (2×10^5 cells/site) in 5-week-old female BALB/c mice. On day 7, when the tumor diameter reached 7 mm, TNF-α molecules were administered by a single i.v. injection. Antitumor potency was estimated from the tumor volume and tumor hemorrhagic necrosis within 24 h of the injection.

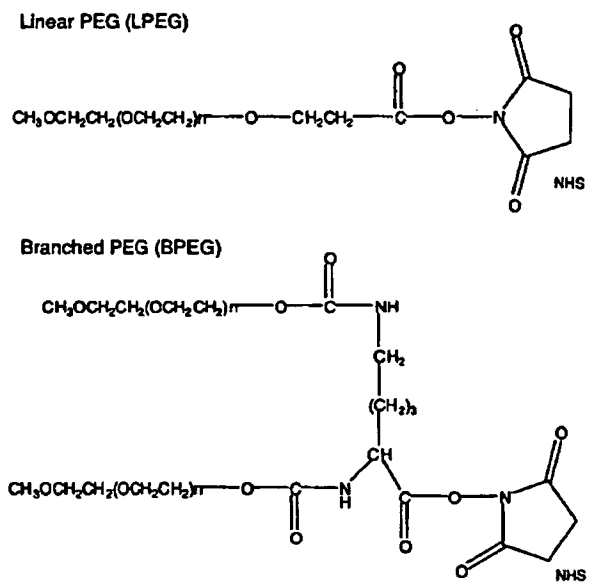

Fig. 1. Chemical structure of activated PEG molecules.

Results

Site-specific PEGylation of mTNF-α-Lys(−)

As shown in Fig. 1A, $BPEG_{10K}$ and $BPEG_{40K}$ consist of two $LPEG_{5K}$ molecules or two $LPEG_{20K}$ molecules, respectively. wTNF-α and mTNF-α-Lys(−) were modified with these activated PEGs. PEGylated TNF-α molecules were detected by SDS–PAGE using a Coomassie blue staining (data not shown). As a result, we found that a single PEG molecule was site-specifically attached to the N-terminus of mTNF-α-Lys(−), whereas a number of $LPEG_{5K}$ molecules were attached to wTNF-α at random. Site-specifically mono-PEGylated mTNF-α-Lys(−)s appeared to have a higher molecular size (Ms) than calculated Ms when compared to the Mw marker proteins, probably due to the high mobility of attached PEG in aqueous solution and its formation of a highly hydrated shield around mTNF-α-Lys(−). To examine the usefulness of site-specifically mono-PEGylated mTNF-α-Lys(−)s, randomly mono-PEGylated wTNF-α, which was composed of positional isomers with PEG at various sites, was separated and purified by anion-exchange and gel filtration chromatographies.

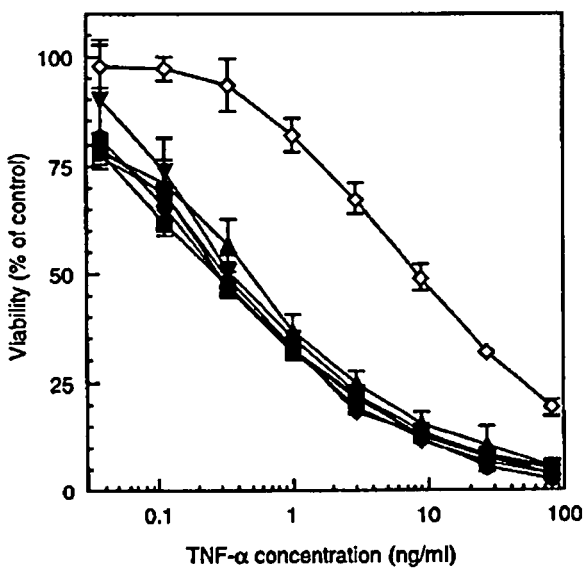

Fig. 2. In vitro bioactivity of mono-PEGylated forms of TNF-αs. The specific activity of mono-PEGylated forms of TNF-α was measured by cytotoxic assay using LM cells in the presence of actinomycin D. Each data value represents means ± SD. □, wTNF-α; ◇, ran-$LPEG_{5K}$-wTNF-α; ■, mTNF-Lys(−); ◆, sp-$LPEG_{5K}$-mTNF-α-Lys(−); ●, sp-$LPEG_{10K}$-mTNF-α-Lys(−); ▲, sp-$LPEG_{20K}$-mTNF-α-Lys(−); and ▼, sp-$LPEG_{40K}$-mTNF-α-Lys(−).

In vitro bioactivity of sp-PEG-mTNF-α-Lys(−)s

To assess the relationship between the shape of PEG with various Mw and in vitro bioactivity of sp-PEG-mTNF-α-Lys(−)s, we assessed the specific bioactivity of sp-PEG-mTNF-α-Lys(−)s using LM cells in the presence of actinomycin D at a concentration of 2 μg/ml (Fig. 2 and Table 1). While the remaining bioactivity (LC50) of ran-$LPEG_{5K}$-wTNF-α was only 4% compared with that of wTNF-α, all sp-PEG-mTNF-α-Lys(−)s had much higher bioactivity than ran-$LPEG_{5K}$-wTNF-α. Additionally, the remaining bioactivities (LC50) of sp-$BPEG_{10K}$-mTNF-α-Lys(−) [93% compared with that of mTNF-α-Lys(−)] or sp-$BPEG_{40K}$-mTNF-α-Lys(−) [65% compared with that of mTNF-α-Lys(−)] were similar to those of $LPEG_{5K}$-mTNF-α-Lys(−) [82% compared with that of mTNF-α-Lys(−)] or sp-$LPEG_{20K}$-mTNF-α-Lys(−) [58% compared with that of mTNF-α-Lys(−)], respectively. These results indicated that site-specific mono-PEGylation of mTNF-α-Lys(−) with BPEG, which had two LPEG molecules, may enable the designing of a sp-PEG-mTNF-α-Lys(−) with higher Ms and stronger in vitro bioactivity.

The affinity of sp-PEG-mTNF-α-Lys(−)s for human TNF-RI was measured using BIAcore (Table 1). The affinity of ran-$LPEG_{5K}$-wTNF-α was markedly reduced (20% of wTNF-α), but all sp-PEG-mTNF-α-Lys(−)s retained sufficient affinity for TNF-RI compared with both wTNF-α and mTNF-α-Lys(−). In brief, the off rates (dissociation rates) of sp-PEG-mTNF-α-Lys(−)s

Table 1
Biological activities and binding parameters of various forms of TNF-α

	LC50[a] (ng/ml)	k_{on}[b] ($\times 10^5\ M^{-1}\ s^{-1}$)	k_{off}[c] ($\times 10^{-4}\ s^{-1}$)	k_d[d] ($\times 10^{-10}$ M)
wTNF-α	0.34	19.0 ± 5.4	2.9 ± 0.5	1.7 ± 0.8
ran-$LPEG_{5K}$-wTNF-α	8.69	2.4 ± 0.4	2.0 ± 0.4	8.5 ± 1.1
mTNF-α-Lys(−)	0.28	17.6 ± 5.7	3.7 ± 0.6	2.3 ± 1.1
sp-$LPEG_{5K}$-mTNF-α-Lys(−)	0.34	15.3 ± 5.7	4.6 ± 0.4	3.4 ± 1.6
sp-$BPEG_{10K}$-mTNF-α-Lys(−)	0.30	12.3 ± 5.2	4.4 ± 0.3	3.9 ± 1.3
sp-$LPEG_{20K}$-mTNF-α-Lys(−)	0.48	10.2 ± 4.4	5.0 ± 0.5	5.5 ± 2.5
sp-$BPEG_{40K}$-mTNF-α-Lys(−)	0.43	9.2 ± 4.6	4.6 ± 1.2	5.6 ± 1.8

Each data value represents means ± SD.
[a] LC50 is the concentration of various PEGylated TNF-αs capable of killing 50% of LM cells.
[b,c] The rate constants k_{on} and k_{off} are from BIAcore optical biosensor assays.
[d] The equilibrium dissociation constant k_d is calculated from the ratio of k_{off}/k_{on}.

Table 2
Antitumor effects of wTNF-α, mTNF-α-Lys(−), and sp-LPEG$_{5K}$-mTNF-α-Lys(−)

	Dose (U/mouse)	Complete regression (%)[a]	IR (%)[b]	Hemorrhagic necrosis (mm^2)[c]
PBS	—	0	—	0
wTNF-α	45	0	88	0
mTNF-α	45	0	79	3.5 ± 2.0
Sp-LPEG$_{5K}$-mTNF-α-Lys(−)	30	70	18	14.4 ± 3.9

Mice were used in groups of five.
[a] Complete regression was defined when tumor was not regrown for more than 30 days.
[b] Inhibition rate (%); tumor volume measured on day 20 after tumor implantation and compared with the mean tumor volume in the saline treated group.
[c] Tumor hemorrhagic necrosis was scored 24 h after the injection. Each data value is the mean ± SD.

were about the same as that of mTNF-α-Lys(−), and the decrease of their k_d (equilibrium dissociation constant) resulted from the decrease of their rates (association rates). There was a good correlation between in vitro bioactivities of PEGylated TNF-αs and their k_d values.

Antitumor effects of sp-PEG-mTNF-α-Lys(−)s

The in vivo antitumor effects of various TNF-αs on Meth-A solid tumors were assessed by a single i.v. injection (Table 2). The tumor hemorrhagic necrotic area on intradermally implanted Meth-A fibrosarcoma was assessed 24 h after injection. In mice treated with both unmodified wTNF-α and mTNF-α-Lys(−), hemorrhagic necrosis was not observed at the dose of 45 U/mouse. In contrast, sp-LPEG$_{5K}$-mTNF-α-Lys(−) at a dose of 30 U/mouse showed significant tumor hemorrhagic necrosis, and complete regression was observed in 70% of mice treated. As shown in Fig. 3, sp-LPEG$_{20K}$-mTNF-α-Lys(−)s and sp-BPEG$_{10K}$-mTNF–Lys(−) effectively induced tumor hemorrhagic necrosis compared with

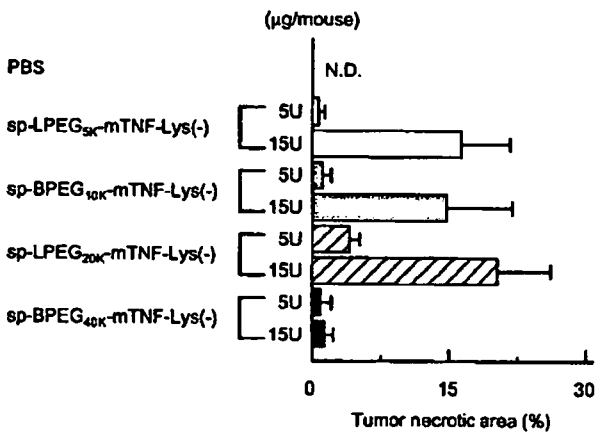

Fig. 3. Antitumor effects of various forms of TNF-α on mice bearing intradermally implanted Meth-A solid tumors. Tumor necrotic effects of i.v.-administered TNF-α and mono-PEGylated TNF-α forms. Tumor hemorrhagic necrosis was scored 24 h after the injection. Mice were used in groups of five. Each data value is the means ± SEM. N.D., not detected.

mTNF-α-Lys(−), but the antitumor effects of sp-BPEG$_{40K}$-mTNF-α-Lys(−) could not be observed.

Discussion

Continuous-infusion or frequent administration of high doses of TNF-α was required to sustain the plasma TNF-α level for observing significant antitumor effects because of its short plasma half-life, and TNF-α as a systemic antitumor agent was found to have unexpected toxic side-effects in phase I studies [6]. This severe toxicity of TNF-α prevented the administration of dosages required for antitumor activity observed in preclinical studies. Thus, TNF-α is administered only into the tumor or the artery controlling cancer in the current cancer chemotherapy [19–22]. The antitumor effects of TNF-α result from not only its direct cytotoxic action against various tumor cells, but also specific damage to the tumor vessels. Additionally, in the process of bleeding necrosis in the tumor vessels, the vascular permeability of the tumor vessels is selectively increased, promoting the transport of macromolecules from blood to the tumor tissue. On the other hand, the increase in blood-residency would lead to a decrease in the distribution of TNF-α in the liver and spleen, which are the major sources of the unfavorable side-effects. Thus, an improvement in blood residency of TNF-α may expand its antitumor therapeutic window. Recently, we prepared phage libraries expressing mTNF-αs, in which all the lysine residues were replaced with other amino acids [16]. A fully bioactive lysine-deficient mutant TNF-α [mTNF-α-Lys(−)] was obtained, despite reports that some lysine residues were essential for its bioactivity. mTNF-α-Lys(−) was site-specifically mono-PEGylated at its N-terminus with LPEG$_{5K}$. This sp-LPEG$_{5K}$-mTNF-α-Lys(−) showed a longer plasma half-life than wTNF-α, resulting in an improvement of in vivo antitumor potency compared to wTNF-α. In this study, to obtain the fundamental information for designing a more useful sp-PEG-mTNF-α-Lys(−) as a systemic antitumor agent, we attempted to predict the structure of mTNF-α-Lys(−) through molecular modeling and assess the various properties of sp-PEG-mTNF-α-Lys(−)s.

A matter of great interest was why mTNF-α-Lys(−) and wTNF-α had comparable in vitro bioactivity after the substitution of all the lysine residues. Site-directed mutagenesis analysis of TNF-α suggested that among six lysine residues, Lys65 and Lys90 were involved in interaction with its receptor, in particular. Fig. 4 showed the model complex between wTNF-α and TNF-receptor I (TNF-RI). In the wTNF-α structure, Lys65 was predicted to repel against Lys78 in TNF-RI. Lys90 forms a hydrogen bond with Glu135 and this interaction is likely to stabilize the loop structure containing residues 84–89, which involved in the receptor binding in the model. In mTNF-α-Lys(−), Lys65 was replaced by Ser65. We considered that the substitutions of Lys65 with a small amino acid, such as Ser65 in mTNF-α-Lys(−), would enable these proteins to bind receptors because of loss of interference between Lys65 in TNF-α and Lys78 in TNF-RI. However, in mTNF-α-Lys(−), Pro90 is likely to be unable to form hydrogen bond with Glu135 and the loop structure became unstable. Therefore, mTNF-Lys(−) had comparable in vitro bioactivity to wTNF-α.

As mentioned above, Lys65, and Lys90 in wTNF-α were reported to play important roles at least for the expression of their bioactivities. Thus, the introduction of PEG to these lysine residues should have caused a significant loss of its bioactivity. In fact, the in vitro bioactivity and receptor-affinity of ran-LPEG$_{5K}$-wTNF-α were markedly lower than those of wTNF-α (Fig. 2 and Table 1). Additionally, we previously found that random PEGylation of wTNF-α with larger Mw of LPEG resulted in much lower bioactivity of ran-LPEG-wTNF-. Previously we showed that ran-LPEG$_{5K}$-wTNF-α was composed of positional isomers with a LPEG$_{5K}$ molecule at various lysine residues, and such isomers could have distinct remaining bioactivity compared with wTNF-α [16]. In most cases, these drawbacks of random PEGylation of bioactive proteins have prevented the clinical application of the PEGylated ones. In contrast, the N-terminus of TNF-α was found to be unimportant for its function, because a deletion mutant lacking eight residues at the N-terminus retained its full bioactivity [16]. As a result, the remaining in vitro bioactivity of each sp-PEG-mTNF-α-Lys(−) was more than 60% compared with both wTNF-α and mTNF-α-Lys(−). In particular, sp-BPEG$_{40K}$-mTNF-α-Lys(−) had a 20-fold higher bioactivity than ran-LPEG$_{5K}$-wTNF-α.

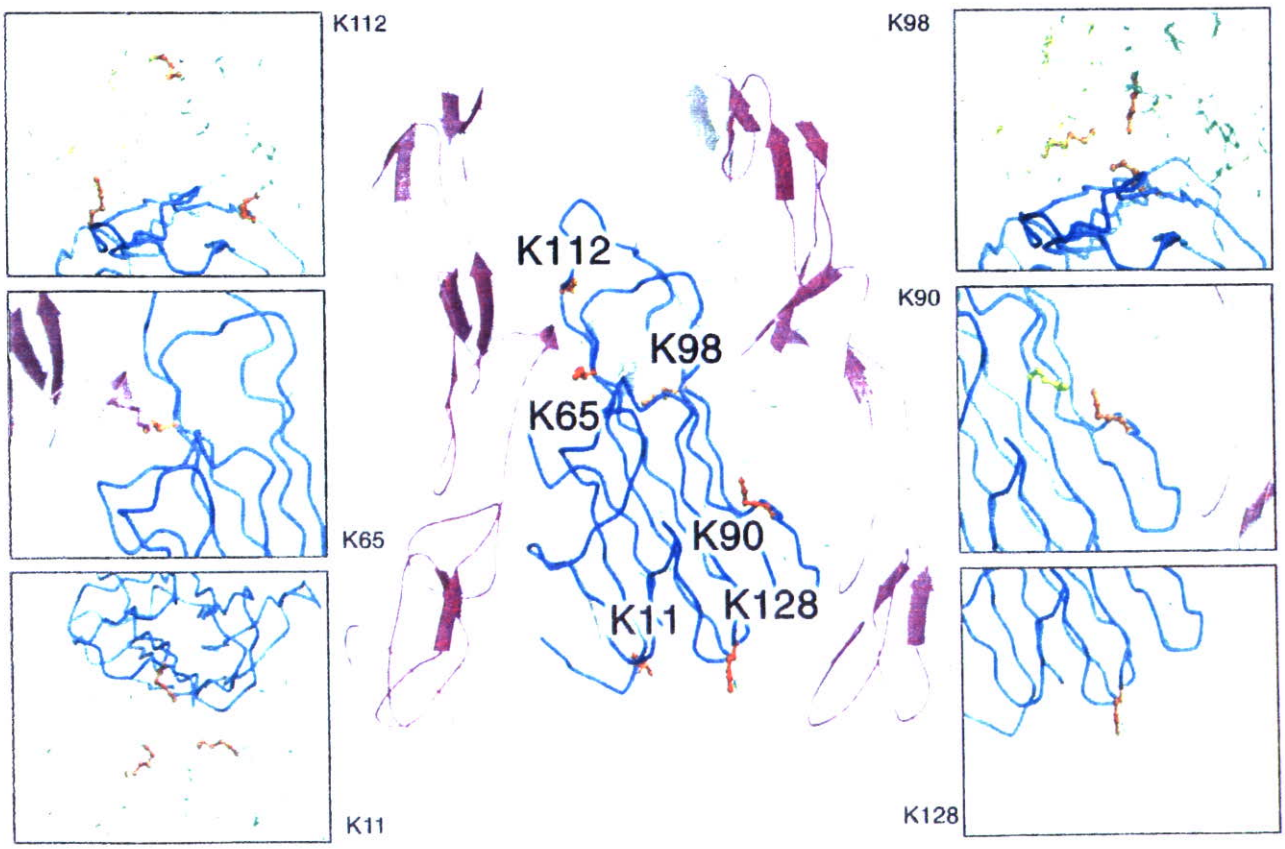

Fig. 4. Ribbon diagrams of the complex model between wTNF-α and TNF-RI. This model is based on crystal structures of wTNF-α trimer (PDB code:1TNF) [23] and TNF-β/TNF-RI complex (PDB code:1TNR) [24]. The images were drawn using Bobscript [25] and Raster3D [26]. Each subunit of the wTNF-α is shown in blue, green, and yellow. TNF-RI is shown in magenta.

Additionally, in the case of mTNF-α-Lys(−), both activated LPEG and BPEG were introduced only into the N-terminal amino group. Thus, the obtained sp-PEG-mTNF-α-Lys(−)s were molecularly uniform. These results strongly indicated the usefulness of our site-specific PEGylation system for lysine-deficient mutant proteins with full bioactivity, which could be created by the phage display technique. The N-terminus of many cytokines, such as interleukin-2, is not important for its function. Thus, at present, the creation of lysine-deficient mutant cytokines and its site-specific PEGylation are being carried out for promotion of cytokine therapies.

$BPEG_{10K}$ and $BPEG_{40K}$ have two LPEG molecules with Mw of 5 and 20 kDa, respectively. Thus, the Ms of PEGylated molecules efficiently increases by PEGylation with BPEG rather than that with LPEG, and BPEG may be more suitable than LPEG. In fact, the remaining in vitro bioactivities and receptor-affinity of sp-$BPEG_{10K}$-mTNF-α-Lys(−) and sp-$BPEG_{40K}$-mTNF-α-Lys(−) were similar to those of $LPEG_{5K}$-mTNF-α-Lys(−) and sp-$LPEG_{20K}$-mTNF-α-Lys(−), respectively (Fig. 2 and Table 1). Additionally, the blood-residency of sp-PEG-mTNF-α-Lys(−) enhanced with an increase in its Ms, irrespective of the molecular shape of PEG (data not shown). In case of PEGylation of TNF-α, sp-$BPEG_{40K}$-mTNF-α-Lys(−), whose remaining in vitro bioactivity was about 65% compared with mTNF-α-Lys(−), did not show any antitumor response (Fig. 3 and Table 1). In vivo antitumor potency of other sp-PEG-mTNF-α-Lys(−)s was higher than that of mTNF-α-Lys(−). A matter of great interest is the difference in in vivo activity of sp-PEG-mTNF-α-Lys(−)s. This difference may be partly accounted for by tissue transport. sp-$BPEG_{40K}$-mTNF-α-Lys(−) might be hard to transport to the tumor tissue than other sp-PEG-mTNF-α-Lys(−)s, because of higher Ms. Thus, we believed that sp-$BPEG_{10K}$-mTNF-α-Lys(−) and sp-$LPEG_{20K}$-mTNF-α-Lys(−) are the most optimal modified products. However, detailed studies on the pharmacokinetics of sp-PEG-mTNF-α-Lys(−)s are necessary to clarify our speculation, and these are currently under investigation.

In a previous study, we showed that the conjugation of TNF-α with polyvinylpyrrolidone (PVP) effectively increased its antitumor effects as compared to that with PEG because PVP has superior character than PEG for the purpose of prolonging plasma half-life and increasing stability [13]. Therefore, we consider that the application of PVP to site-specific bioconjugation of mTNF-α-Lys(−) would be useful as a more potent antitumor therapeutic agent. Our results indicated that it is necessary to optimize the condition of PEGylation to selectively enhance the desirable therapeutic effects, taking into account their mechanism of action. Our results provide the information necessary to design a PEGylated protein, optimally suitable for therapeutic use.

Acknowledgments

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Selective Enhancer of Tumor Vascular Permeability for Optimization of Cancer Chemotherapy

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Clinical approach using tumor necrosis factor-alpha (TNF-α) as selective destruction against tumor endothelial cells and selective enhancer of tumor vascular permeability for effective accumulation of antitumor chemotherapeutic agents has attracted attention. However, the clinical application of TNF-α as a systemic antitumor agent has been limited because of toxic side-effects. To systemically use TNF-α as an antitumor agent and the selective enhancer of tumor vascular permeability, we assessed the usefulness of PEGylated TNF-α (PEG-TNF-α). PEG-TNF-α at a dose of 1000 JRU showed marked hemorrhagic necrosis in S-180 tumors without side-effects due to selective destruction of tumor vasculature, whereas wild-type TNF-α at a dose of 10000 JRU showed a little hemorrhagic necrosis with severe side-effects. PEG-TNF-α induced the enhancement of tumor vascular permeability. The permeability was increased at 1 h, after an i.v. injection of PEG-TNF-α and returned to the basal level at 2 h. In addition, high molecular weight of PEG (molecular weight; 500K) accumulated in tumor tissue as well as low molecular weight of PEG (molecular weight; 12K). On the other hand, PEG-TNF-α didn't affect the permeability of normal tissue and inflammation site. This data suggested that PEG-TNF-α was useful agent as selective enhancer of tumor vascular permeability with safe.

Key words tumor necrosis factor-alpha; permeability; tumor endothelial cell; chemotherapeuctic drug

With the development of tumor biology, molecular biology, and combinatorial chemistry, many therapeutic agents have been developed for tumors. However, the clinical application of antitumor agents such as cytokines, antibodies and chemotherapeutic drugs has failed because of poor accumulation in the tumor tissue.[1,2] Heterogeneous tumor perfusion, vascular permeability, and increased interstitial pressure restricted the penetration of therapeutic agents into tumor tissue from circulation.[1,2]

It has been suggested that tumor necrosis factor-alpha (TNF-α) can overcome these problems. TNF-α was identified as a cytokine that specifically injures tumors and has been highlighted as a potent anti-tumor agent.[3] Furthermore, TNF-α destroyed the tumor vasculature selectively and enhanced the tumor vascular permeability.[4] Regional isolated limb perfusion using TNF-α in combination with melphalan or doxorubicin showed greater therapeutic effects on soft-tissue sarcoma or melanoma than chemotherapeutic drugs alone.[5] It is believed that the synergistic effect between TNF-α and chemotherapeutic drugs results from TNF-α-induced destruction of tumor vasculature and enhancement of tumor vasculature permeability. However, because of its high instability and pleiotropic action *in vivo*, attempts to use TNF-α as a systemic anticancer agent and an enhancer of tumor vascular permeability in humans, failed due to severe systemic side effects such as fever and decreased blood pressure as seen in case of an endotoxin-like shock, before therapeutic doses could be administered.[6]

To overcome these drawbacks and apply TNF-α as a systemic administrator, we have attempted to conjugate TNF-α with polyethylene glycol (PEG) and other water-soluble polymeric modifiers.[7–10] We showed that the antitumor effects of PEG-TNF-α, obtained by PEGylation with a molecular weight of 5000, was enhanced to 100 times that of unmodified TNF-α, without increasing their toxic side effects.[7,8] In this study, we examined the usefulness of PEG-TNF-α as a selective enhancer of tumor vascular permeability. We showed that PEG-TNF-α enhanced the permeability of tumor vasculature without having any effect on the permeability of normal tissue. We considered that PEG-TNF-α would be used with chemotherapeutic drugs for tumor therapy.

MATERIALS AND METHODS

Preparation of ^{125}I-Labeled Polymers Radiolabeled polymeric modifiers were prepared by the chloramine-T method. PEGs (average molecular weight: 12000, 50000, 70000, 500000) (purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan) dissolved in 1,4-dioxan were reacted with *N,N'*-carbonyldiimidazole for 6 h at room temperature. After dialysis in water, the activated polymers were reacted with a 2-fold molar excess of thyramine hydrochloride for 48 h at 4 °C. These reaction mixtures were also dialyzed in water and lyophilized. The polymer–thyramine conjugates, dissolved in a 0.4 M sodium phosphate buffer (2.5 mg/ml) and Na^{125}I (100 mCi/ml), were mixed in a microcentrifuge tube on ice. The labeling reaction was started by the addition of 3.8 mM chloramine T. After iodination, the reaction was stopped by the addition of 2.5 mM sodium pyrosulfate. ^{125}I-Labeled polymer was purified by Gel Filtration Chromatography on the Econo-Pac® 10 DG column.

***In Vivo* Behavior of Polymers** All experimental protocols for animal studies were in accordance with the *Guide for Laboratory Animal Facilities and Care* (NIH publication 85-23, rev 1985). These protocols have been approved by the committee of the Pharmaceutical School, Osaka University, Japan. PEG-TNF-α, in which 56% of the lysine amino groups of natural human TNF-α (Hayashibara Biological Laboratories Inc., Okayama, Japan) were coupled with PEG

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