Anti-tumor Responses Induced by Chemokine CCL19 Transfected into an Ovarian Carcinoma Model *via* Fiber-Mutant Adenovirus Vector

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Considerable attention has recently been paid to the application of chemokines to cancer immunotherapy because of their chemotactic affinity for a variety of immune cells and because several chemokines are strongly angiostatic. In the present study, the recombinant adenovirus vectors encoding chemokine CCL19 or XCL1 in an E1 cassette (AdRGD-mCCL19 and AdRGD-mXCL1) were developed. The constructed fiber-mutant adenovirus vector, which contained the integrin-targeting Arg-Gly-Asp (RGD) sequence in the fiber knob, notably enhanced the transfection efficiency to OV-HM ovarian carcinoma cells compared to that induced by conventional adenovirus vector. The results of an *in vitro* chemotaxis assay for chemokine-encoding vector demonstrated that both AdRGD-mCCL19 and AdRGD-mXCL1 could induce the migration of cells expressing specific chemokine receptors. Of the two chemokine-encoding vectors evaluated *in vivo*, AdRGD-mCCL19 showed significant tumor-suppressive activity in B6C3F1 mice *via* transduction into OV-HM cells, whereas XCL1 did not exhibit any notable anti-tumor effects, suggesting that CCL19 may be a candidate for cancer immunotherapy.

Key words chemokine; CCL19; XCL1; recombinant adenovirus vector; anti-tumor effect; OV-HM cell

Chemokines attract a variety of immune cells and function at inflammatory disease sites as well as lymphoid tissue.[1,2] Considering the eradication of tumor cells as a consequence of interaction with immune cells that have migrated and accumulated in tumor tissue, the usefulness of chemokines for cancer immunotherapy has received considerable attention.[3] By now, more than 40 chemokines have been well characterized, but only a few have been identified as candidates for cancer therapy either independently or with an adjuvant. Tumor-suppressive activity of several chemokines has been observed after transduction into a variety of experimental tumors.[4—7] Tumor cells that were transduced with the CC chemokine gene, CCL3, had reduced tumorgenicity and significantly increased infiltration of macrophages and neutrophils.[8] Another CC chemokine, CCL22, was also strongly chemoattractive to dendritic cells, NK cells and T cells, which resulted in tumor regression in a murine lung carcinoma model due to its efficient induction of anti-tumor immunity.[9] In the present study, we constructed the recombinant viral vector for efficient gene transfection and evaluated the CC family chemokine, EBI1-ligand chemokine (CCL19), and C family chemokine, lymphotactin (XCL1). CCL19 has been shown to chemoattract $CD4^+$, $CD8^+$ T cells and dendritic cells,[11,12] whereas XCL1 is chemotactic for T cells and NK cells but not for monocytes, neutrophils or dendritic cells.[13,14] We anticipated that if tumor cells could be genetically modified by an efficient gene transfer system *in vitro* to produce chemokines *in vivo*, the chemokines could induce accumulation of immune cells in the tumor. The *in vivo* interaction of T cells with the tumor cells should induce anti-tumor immunity, resulting in suppression of tumor growth.

In the present study, we used the adenovirus vector, which exhibits very high gene transduction efficiency.[15] Because a variety of tumor cells contain few Coxsackie adenovirus receptors (CAR),[16] we used a recombinant adenovirus vector with a fiber mutation containing the Arg-Gly-Asp (RGD) sequence in the fiber knob. This fiber-mutant vector possesses higher transduction and anti-tumor activities compared to conventional adenovirus vectors when used in cytokine-gene therapy against melanoma.[17,18] In the present study, ovarian carcinoma OV-HM cells were transfected with a chemokine-encoding recombinant vector, AdRGD-mCCL19 or AdRGD-mXCL1, and both the *in vitro* chemotactive activity and the *in vivo* tumor-suppressive response were investigated.

MATERIALS AND METHODS

Cell Lines and Animals OV-HM ovarian carcinoma cell line[19] were kindly provided by Dr. Hiromi Fujiwara (School of Medicine, Osaka University, Japan) and were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS. A549 human lung carcinoma cells and human embryonic kidney (HEK) 293 cells were cultured in DMEM supplemented with 10% FBS. Murine pre-B lymphoma L1.2 cells and their stable transfectants, L1.2/mCCR7 and L1.2/mXCR cells, which expressing specific receptor for CCL19 and XCL1, respectively, were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS and 2-ME (50 μM, Life Technologies). All the cell lines were cultured at 37 °C in a humidified atmosphere with 5% CO_2. Female B6C3F1 mice were purchased from SLC Inc. (Hamamatsu, Japan) and used at 6—8 weeks of age. All of the experimental pro-

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cedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Construction of Adenovirus Vectors The replication-deficient adenovirus vectors containing a fiber mutation, which were used in this study, were developed based on the adenovirus type 5 backbone with deletions of the E1 and E3 regions.[20] The RGD sequence was inserted into the HI loop of the fiber knob using a two-step method developed by Mizuguchi *et al.*[21] Murine chemokine genes derived from pT7T3D-Pac-mCCL19 and pExCell-mCXL1 were used as sources of cDNA. Recombinant adenovirus vectors with the RGD fiber mutation, AdRGD-mCCL19 and AdRGD-mXCL1, carrying the chemokine cDNA under the control of the cytomegalovirus (CMV) promoter, were constructed by an improved *in vitro* ligation method described previously.[20,22] The luciferase expressing adenovirus vectors with the RGD fiber mutation (AdRGD-luc), serving as a negative control, is identical to the AdRGD-mCCL19 and AdRGD-mXCL1 vectors and contains the luciferase gene in the expression cassette (Fig. 1). Conventional adenovirus vector expressing lusiferase (Ad-Luc) was also developed by Mizuguchi *et al.*[22] The adenovirus vectors were propagated in 293 cells and purified by cesium chloride gradient ultracentrifugation. Virus particle (VP) was accomplished spectrophotometrically.[23] The titer (tissue culture infectious dose$_{50}$; TCID$_{50}$) was determined by plaque-forming assay using 293 cells.[24,25]

Gene Expression by AdRGD-Luc or Conventional Ad-Luc in OV-HM Ovarian Carcinoma Cells 2×10^3 of OV-HM cells in a 96-well plate were treated with Ad-Luc or AdRGD-Luc at 1250, 2500, 5000, and 10000 viral particles/cell for 1.5 h, respectively. Cells were washed with PBS and cultured for an additional 48 h. Subsequently, the cells were washed, collected, and their luciferase activity was measured using the Luciferase Assay System (Promega, U.S.A.) and Microlumat Plus LB96 (Perkin Elmer, U.S.A.) after the cells were lysed with the Luciferase Cell Culture Lysis (Promega, U.S.A.) according to the manufacturer's instruction.

***In Vitro* Chemotaxis Assay** The AdRGD-Luc and indicated AdRGD-chemokine were tranfected into A549 cells for 2 h at a multiplicity of infection (MOI) of 50, and the cells were washed twice with PBS and cultured in media containing 10% FBS. The cells were subsequently washed after 24 h cultivation, and incubated with an assay medium (phenol red-free RPMI 1640 containing 0.5% bovine serum albumin and 20 μM HEPES, pH 7.4) for another 24 h. The resulting conditioned medium was collected, and its chemoattractant activity was measured by an *in vitro* chemotaxis assay across a polycarbonate membrane with 5-μm pores (Chemotaxicell-24; Kurabo, Osaka, Japan) using L1.2 transfectants expressing the specific receptor for chemokines. The culture supernatants of intact A549 cells, AdRGD-Luc-transfected A549 cells, and chemokine gene-transduced A549 cells were prepared. These samples and recombinant chemokines dissolved in the assay medium were added to a 24-well culture plate. Cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1) were suspended in the assay medium (1×10^6 cells) and placed in a Chemotaxicell-24 installed on each well. Likewise, parental L1.2 cells for these transfectants were prepared and added to the Chemotaxicell-24. Cell migration was allowed for 2 h at 37 °C in a 5% CO_2 atmosphere. The cells that migrated to the lower well were lysed and quantitated using a PicoGreen dsDNA quantitation reagent (Invitrogen, Tokyo, Japan). The data are expressed as mean±S.E. of the triplicate results and the migration activity was expressed in terms of the percentage of the input cells. Recombinant chemokines (mouse: mCCL19 and mXCL1) corresponding to each specific receptor (CCR7 and XCR1) were purchased from DakoCytomation (Kyoto, Japan) and used as a positive control.

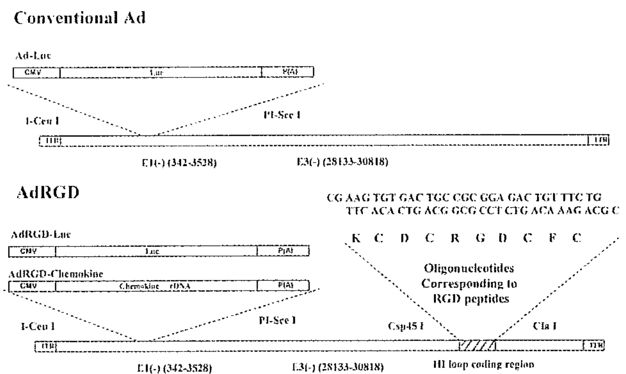

Fig. 1. Schematic Representation of Conventional Ad and AdRGD Used in This Study

Evaluation of Growth of OV-HM Cells Transfected with Chemokine-Encoding Adenovirus Vectors in Immunocompetent Mice OV-HM cells were transfected with AdRGD-mCCL19, AdRGD-mXCL1, or AdRGD-Luc as a control, at a MOI of 10 for 24 h. The cells were then harvested and washed with PBS three times and 1×10^6 cells were inoculated intradermally into the flank of B6C3F1 mice. An aliquot of the OV-HM cells infected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc at a MOI of 10 were cultured for an additional 48 h, and cell viability was examined by MTT assay. For *in vivo* evaluation of OV-HM cell growth, tumor volume was calculated by measuring the length and width of the tumor, twice a week. The mice were euthanized when one of the two measurements was greater than 15 mm.

RESULTS

OV-HM Tumor Cells Transfected with Fiber-Mutant Adenovirus Vector Induced Higher Gene Expression Than That Induced by Conventional Vector To evaluate the gene transfection efficiency of the fiber-mutant adenovirus vector developed for this study, OV-HM cells were transfected with conventional adenovirus vector or fiber-mutant adenovirus vector at indicated particles/cell and luciferase activity was measured. The results shown in Fig. 2 demonstrated that luciferase gene expression induced by fiber-mutant vector was much higher than that induced by conventional adenovirus vector. For example, at 10000 VP/cell transfection, 16-fold greater gene expression was obtained in response to fiber-mutant vector than to Ad-Luc. This demonstrated that the insertion of the RGD peptide into the viral fiber enhanced transfection efficiency to OV-HM cells *via* the adenovirus vector.

Expression of Murine CCL19 and XCL1 by Transfection with Chemokine-Encoding Adenovirus Vector To

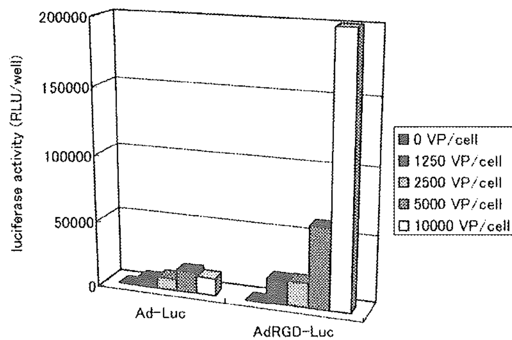

Fig. 2. Luciferase Expression by Ad-Luc or AdRGD-Luc Transfected OV-HM Cells

2×10^3 OV-HM cells were inoculated in a 96-well plate for 12 h, and were transfected with Ad-Luc (left) or AdRGD-Luc (right), respectively, at the indicated viral particles/cell for 1.5 h. The cells were then washed and incubated for another 48 h. After incubation, cells were collected and luciferase activity was measured. Data are presented as mean±S.E. of relative light units (RLU)/well determined from three experiments.

Table 1. Specific Chemoattractant Activity *in Vitro* Induced by Transfection of AdRGD-mCCL19 or AdRGD-mXCL1 into A549 Cells

	L1.2 % of input cells (mean±S.E.)	L1.2/XCR1 % of input cells (mean±S.E.)
Medium	0.2±0.0	0.1±0.0
10 nM mXCL1	0.3±0.0	9.2±0.8
A549	1.2±0.1	1.6±0.1
Luc/A549	1.5±0.0	2.0±0.2
mXCL1/A549	3.7±0.3	11.6±0.7

	L1.2 % of input cells (mean±S.E.)	L1.2/CCR7 % of input cells (mean±S.E.)
Medium	0.2±0.0	0.7±0.1
10 nM mCCL19	0.2±0.0	16.3±1.2
A549	1.2±0.1	2.0±0.1
Luc/A549	1.5±0.0	2.2±0.2
mCCL19/A549	2.5±0.1	8.2±0.5

Chemotaxis assay was performed using L1.2 cells expressing specific receptors for CCL19 (L1.2/CCR7) or XCL1 (L1.2/XCR1).

verify that the CCL19 and XCL1 produced by AdRGD-mCCL19 and AdRGD-mXCL1, respectively, were biologically functional, A549 cells were infected with the vectors for 2 h, and the culture supernatants were harvested after an additional 48 h. In the present study, human lung carcinoma A549 cells were used instead of murine tumor cells because of the very strong background chemotactic activity in the culture supernatant of the latter.[16] Using an *in vitro* chemotaxis assay, we investigated whether A549 cells transfected with each chemokine gene-carrying AdRGD could secrete chemokine protein in its biologically active form into culture supernatants. As shown in Table 1, the culture supernatants of A549 cells transfected with AdRGD-mCCL19 (mCCL19/A549) or AdRGD-mXCL1 (mXCL1/A549) could induce greater migration of cells expressing the corresponding chemokine receptors than those from intact A549 cells or A549 cells transfected with AdRGD-Luc (Luc/A549). The migration of L1.2 cells was not observed in wells containing recombinant chemokines, and only low-level migration was observed in culture supernatants from intact A549, Luc/A549, mXCL1/A549, and mCCL19/A549. These results demonstrated that all AdRGDs could deliver their encoded chemokine gene to target cells, and that transfected cells could secrete the chemokine protein, which maintained its original chemoattractant activity.

Anti-tumor Effect *in Vivo* by Transfection of Chemokine CCL19 into OV-HM Cells *via* Fiber-Mutant Adenovirus Vector OV-HM ovarian carcinoma cells transfected with 10 MOI of AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc as the control vector, were intradermally inoculated into B6C3F1 immunocompetent mice to evaluate their effects on tumor growth *in vivo*. In the present study, 10 MOI of Ad vectors were chosen for transfection because that higher MOI induced the cytotoxicity of OV-HM cells (data not shown). As shown in Figs. 3A and B, the transfection of AdRGD-mCCL19 resulted in significant suppression of tumor growth, while that of AdRGD-mXCL1 did not show any difference from that with AdRGD-Luc. To exclude the possibility that the suppression of tumor cell growth by AdRGD-mCCL19 was due to the cytotoxicity of the adenovirus or chemokine, OV-HM cells transfected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc were cultured for 48 h, and cell viability was measured by the MTT assay. The *in vitro* growth of the cells infected with these vectors was essentially identical to that of control cells (Fig. 3C).

DISCUSSION

Cytokines or chemokines encoded by a viral vector are currently regarded as intriguing options for cancer gene immunotherapy. Adenovirus vector, which shows high gene transduction efficiency and which can infect both dividing and non-dividing cells, is widely used as a carrier for gene therapy. It has been reported that the initial process of adenovirus infection involves at least two sequential steps. The first step is the attachment of the virus to the cell surface, which occurs by binding of the fiber knob to the Coxsackie virus and Adenovirus Receptor (CAR).[26,27] Following this, in the second step, the interaction between the RGD motif of the penton base with αv integrins, the secondary host-cell receptors, facilitates internalization through receptor-mediated endocytosis.[28,29] In other words, if the host cell surface lacks CAR, efficient gene transfer using a conventional adenovirus vector is difficult. Unfortunately, some malignant cells, including ovarian carcinoma, exhibit a resistance to adenovirus-mediated gene transduction due to low CAR expression on their surface. To overcome the low gene expression levels in CAR negative cells by adenovirus vectors, we constructed a fiber-mutant Ad vector with an integrin-targeting RGD peptide by a simple *in vitro* method.[20] The results of gene transfection *in vitro* (Fig. 2) demonstrated that OV-HM transfected using AdRGD-Luc carrying the luciferase gene significantly induced gene expression compared to that induced by the conventional Ad-Luc, suggesting that the recombinant adenovirus vector is a better option for cancer gene therapy.

We also inserted the murine chemokine cDNA of the CC family chemokine, CCL19, and C family chemokine, XCL1, into the E1 cassette of this fiber-mutant adenovirus vector, and AdRGD-mCCL19 and AdRGD-mXCL1 were developed. The expression of chemokine mRNA was reported pre-

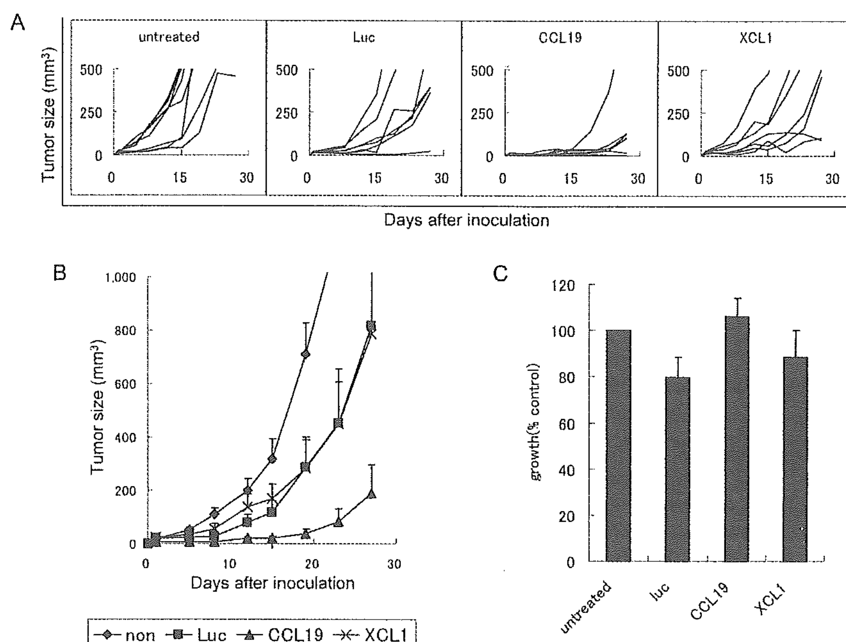

Fig. 3. Growth of OV-HM Tumor Cells in B6C3F1 Mice Transfected with Chemokine-Encoding Adenovirus Vectors

Mice were inoculated intradermally in the flank with 1×10^6 OV-HM cells (100 μl in RPMI 1640) at a MOI of 10 and with AdRGD-mCCL19 or AdRGD-mXCL1 for 24 h. Tumor volume was calculated after measuring the length and width of the tumor at indicated periods of time. Data are expressed as the mean±S.E. Intact OV-HM cells were used as control (untreated), and the OV-HM cells infected with AdRGD-Luc were inoculated into B6C3F1 mice for vector-control. Animals were euthanized when one of the two measured values were greater than 15 mm. At least six mice were used in each group. (A) Individual tumor size in each group and (B) average size in each group. (C) MTT assay results that evaluated the growth of chemokine-gene-transduced OV-HM cells *in vitro*. OV-HM cells were infected with AdRGD-mCCL19, AdRGD-mXCL1 or AdRGD-Luc at a MOI of 10 for 24 h, and then cultured for 48 h. Cell viability was examined by MTT assay. Data are expressed as the means±S.E. of triplicate results. Each of the analyses were performed at least three times.

viously.[30] A chemotaxis assay of chemokine-encoding vectors was conducted *in vitro* to evaluate the biological activity of these vectors. The results demonstrated that the produced protein in the culture supernatants of cells infected with these vectors could efficiently cause migration of the specific receptor-expressing cells (Table 1).

The C family chemokine, XCL1, has been widely used for cancer immunotherapy, but in general, XCL1 by itself did not induce notable anti-tumor effects, even though it is a chemoattractant for both T cells and NK cells.[31] The CC chemokine, CCL19, reportedly induces T cell and dendritic cell migration and exhibits tumor-suppressive effects in several mouse malignant cell models.[32,33] Hillinger *et al.* reported that intratumoral injection of recombinant CCL19 led to significant systemic reduction in tumor volumes. CCL19-treated mice exhibited remarkably increased infiltration of $CD4^+$ and $CD8^+$ T cell subsets as well as dendritic cells at the tumor sites. These cell infiltrates were accompanied by increases in several cytokines and chemokines such as IFN-γ, CXCL9, CXCL10, GM-CSF, and IL-12.[34] We have also shown that CCL19 and XCL1 elicited anti-tumor response, to some extent, through transfection into B16BL6 melanoma cells. But our study, which used eight chemokines to evaluate the anti-tumor effects in three tumor cell types, suggests that the tumor-suppressive activity of chemokine gene immunotherapy is very complicated and is greatly influenced by the type of tumor and activation state of the host's immune system.[30] Moreover, as we previously reported,[10] transfection with the chemokine CCL27 induced tumor-suppressive effects, whereas another chemokine, CX_3CL1, did not show any notable anti-tumor activity. However, both of these chemokines induced the accumulation of T cells as well as NK cells at the tumor site. Our results indicated that the distribution of immune cells that have migrated to the tumor and the angiogenic or angiostatic activity may play an important role in the anti-tumor response.

Several groups have reported much stronger anti-tumor activity when using chemokines as adjuvants with other agents.[35—39] In the present study, CCL19 could not induce complete tumor regression, but merely inhibited its growth. On other hand, remarkable anti-tumor activity could be obtained when XCL1 was combined with cytokines or tranfected into dendritic cells.[40,41] A recent report showed that combination of both XCL1 and CXCL10 can enhance the efficiency of adoptive T cell therapy for EG7 tumor cells *via* accumulation of effector T cells in tumor tissue.[42] Many factors are likely to influence the tumor-suppressive effects of chemokines, but the relatively weak anti-tumor activity and long-term immuno-protective effects of chemokines may be mainly related to the activation level of migrating immune cells. In other words, not only the accumulation but also the activation of immune cells migrating into tumors is important in cancer immunotherapy using chemokines. Therefore, combination therapy using both chemokines and cytokines will increase the anti-tumor effects and improve cancer immunotherapy.

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Vaccine Efficacy of Fusogenic Liposomes Containing Tumor Cell-Lysate against Murine B16BL6 Melanoma

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Recent advances in tumor immunology have facilitated the development of cancer immunotherapy targeting tumor-associated antigens (TAAs). However, because TAAs were identified in only a few types of human cancer, novel vaccine strategies that utilize tumor cell-lysate (TCL), including unidentified TAAs as an antigen source, are needed. Herein, we describe the utility of fusogenic liposomes (FLs) as TCL-delivery carriers for both *ex vivo* dendritic cell-based vaccination and *in vivo* direct immunization in the murine B16BL6 melanoma model. As a result, both *in vivo* direct immunization and *ex vivo* immunization induced anti-B16 melanoma prophylactic effects. *Ex vivo* dendritic cell (DC)-mediated vaccination strategy exert more potent anti-tumor effect than direct immunization. Our results suggest that this flexible system is a promising approach for the development of versatile cancer immunotherapy regimes.

Key words vaccine carrier; tumor cell lysate; liposome; melanoma

Early studies in mice demonstrated that tumor-specific cytotoxic T lymphocytes (CTLs) could control tumor growth and metastasis. The identification of T cell-recognizing tumor-associated antigens (TAAs) in human cancer, particularly in melanoma (*i.e.* MAGE, MART-1, gp100, tyrosinase, and TRP),[1,2] facilitated the development of cancer immunotherapy based on TAA-vaccination with adjuvants to elicit tumor-specific CTLs.[3] However, this immunological approach limits the application of this system only to certain cancer patients because TAAs are not yet identified for most of human cancers. Additionally, the expression levels of known TAAs that may be applicable for immunotherapy vary between tumor cells isolated from patients with cancer.[4] Therefore, it is very difficult to predict which TAA would generate an effective anti-tumor immune response that would make it appropriate for use as a vaccine component for a specific patient.

To overcome this limitation, several researchers have attempted to develop a vaccine strategy using tumor cell-lysate (TCL) as a possible source of TAA.[5] The use of TCL prepared from surgically removed tumors is a promising approach to induce a broader T cell-immune response not only to defined TAAs but also to unknown TAAs. In TCL-based cancer immunotherapy, the development of both an antigen-delivery system and an adjuvant that can efficiently prime and propagate CTLs specific for TAAs included in the TCL is required for achieving sufficient therapeutic effect. CTLs are activated by antigen-presenting cells (APCs), including dendritic cells (DCs), through the major histocompatibility complex (MHC) class I-restricted antigen presentation pathway. Peptides presented on MHC class I molecules are derived in most situations exclusively from endogenous antigens synthesized by cells. Antigens in the extracellular fluids fail to gain access to the MHC class I-pathway in most cells, although class I-presentation of endocytosed antigens also occurs in APCs under certain circumstances.[6,7] Therefore, if we can introduce the TAA-containing TCL directly into the cytoplasm, the TAAs would be definitively delivered to the MHC class I-antigen presentation pathway, much like cytoplasmic proteins.

Fusion active liposomes (fusogenic liposomes; FLs), which are composed of conventional liposomes (CLs) displaying Sendai virus-accessory proteins, retain membrane-fusion activity derived from Sendai-virus and efficiently introduce its contents into cytoplasm.[8] We have previously reported that direct antigen loading into cytoplasm by FLs is an efficient approach for enhancing antigen-specific CTL induction in mice.[9—11] In the present study, in order to evaluate the usefulness of FLs as antigen-delivery carriers for TCL-based cancer immunotherapy, we investigated anti-tumor efficacy of *ex vivo* vaccination using TCL-containing FLs (TCL/FLs)-pulsed DCs and *in vivo* direct TCL/FLs-immunization in the murine B16BL6 melanoma model.

MATERIALS AND METHODS

Cells and Mice B16BL6 cells, a C57BL/6-origin melanoma cell line, were grown in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), and antibiotics. DC2.4 cells, a C57BL/6-derived DC line,[12] were generously provided by Dr. K. L. Rock (Department of Pathology, University of Massachusetts Medical School, Worcester, MA, U.S.A.), and were cultured in RPMI1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 μM non-essential amino acid, 50 μM 2-mercaptoethanol, and antibiotics. CD8-OVA 1.3 cells, a T-T hybridoma against OVA+ H-2Kb,[13] were kindly provided by Dr. C. V. Harding (Department of Pathology, Case Western

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Reserve University, Cleveland, OH, U.S.A.), and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol, and antibiotics. CTLL-2 cells, which proliferate specifically in response to interleukin-2 (IL-2),[14] were maintained in RPMI1640 medium supplemented with 10% FBS, 50 μM 2-mercaptoethanol and 10 U/ml murine recombinant IL-2 (Pepro Tech EC Ltd., London, England). Female C57BL/6 mice (H-2b), aged 7—8 weeks, were purchased from SLC Inc. (Hamamatsu, Japan). All of the experimental procedures were in accordance with the Osaka University guidelines for the welfare of animals in experimental neoplasia.

Preparation of B16BL6 TCL Cultured B16BL6 cells were recovered and washed three times with phosphate-buffered saline (PBS, pH 7.4). Cells were resuspended in a balanced salt solution (10 mM Tris–HCl, 150 mM NaCl, pH 7.6) and lysed by four cycles of freezing and thawing, followed by centrifugation at 13000 ***g*** for 60 min. The soluble fraction was passed through a 0.22-μm membrane filter and the protein concentration was adjusted to 4 mg/ml upon determination with a DC-protein assay kit (Bio-Rad, Tokyo, Japan).

Preparation of CLs and FLs TCL- or ovalbumin (OVA)-containing CLs (TCL/CLs or OVA/CLs) were prepared as follows. Cholesterol, egg phosphatidylcholine, and L-α-dimyristoryl phosphatidic acid were mixed at a molar ratio of 5 : 4 : 1 in chloroform. The lipid mixture was evaporated to obtain a thin-lipid film, and then liposome suspensions were prepared by dispersing the thin-lipid film in 400 μl of TCL or OVA solution. After three cycles of freezing and thawing, the liposomes were sized by two rounds extrusion through 0.8-μm and 0.4-μm polycarbonate membranes and were ultracentrifuged to remove un-encapsulated TCL or OVA. TCL/FLs or OVA/FLs were prepared by fusing the TCL/CLs or OVA/CLs with UV (2000 J/cm^2)-inactivated Sendai virus as described previously.[9] The amount of antigen proteins encapsulated in liposomes and FLs was measured by a DC-protein assay kit and calculated by following formula:

encapsulated antigen protein level
=(total protein level of antigen-containing CLs or FLs)
−(protein level of empty CLs or FLs)

***In Vitro* Antigen Presentation Assay** One hundred microliters of OVA solution, OVA/CLs suspension, or OVA/FLs suspension were added to DC2.4 cells cultured on a 96-well plate at a density of 10^5 cells/well, and the cells were incubated for 5 h at 37 °C. After three washes with PBS, DC2.4 cells were co-cultured for 20 h with 10^5 CD8-OVA 1.3 cells. The response of stimulated CD8-OVA 1.3 cells was assessed by the murine IL-2 ELISA kit (Biosource International, Camarillo, CA, U.S.A.), which determines the amount of IL-2 released into 100 μl of culture supernatants. In another experiment, DC2.4 cells were pre-incubated for 1 h at 37 °C with 10 μM of lactacystin or MG132 (Peptide Institute, Minoh, Japan), and then the cells were incubated for 15 min at 37 °C with OVA/FLs in the presence of inhibitors. After fixation with 0.05% glutaraldehyde and washing three times with PBS, CD8-OVA 1.3 cells were added at 10^5 cells/100 μl/well. After 24 h-cultivation, the response of CD8-OVA 1.3 cells was determined by the level of IL-2 secretion in a CTLL-2 proliferation assay as described previously.[15] Radioactivity derived from [^{3}H]-thymidine uptake by CTLL-2 cells was measured on a liquid scintillation counter, and data were expressed in Δcpm as follows:

Δcpm=(cpm in the presence of OVA/FLs)
−(cpm in the absence of OVA/FLs)

***Ex Vivo* Vaccination Experiment Using TCL-Introduced DC2.4 Cells** DC2.4 cells were pulsed for 5 h at 37 °C with TCL in various formulations (TCL/FLs, TCL/CLs, the mixture of TCL and empty FLs (TCL+eFLs), or TCL alone) at 500 μg TCL/10^7 cells/ml, and then the cells were treated for 30 min at 37 °C with mitomycin C (50 μg/ml) in order to inhibit their proliferation. After three washes with PBS, the cells were intradermally injected into the right flank of C57BL/6 mice at 10^6 cells/50 μl. Likewise, control mice were injected with the unpulsed or eFLs-pulsed DC2.4 cells or PBS. At 1 week after the vaccination, 2×10^5 B16BL6 cells were inoculated into the left flank. The size of tumors was assessed using microcalipers and was expressed as tumor volume calculated by the following formula:

$$\text{tumor volume (mm}^3\text{)}=[\text{major axis (mm)}]\times[\text{minor axis (mm)}]^2\times0.5236$$

Mice containing tumors>20 mm were euthanized.

***In Vivo* Direct Vaccination Experiment** C57BL/6 mice were immunized once or three times at a 1-week interval by intradermal injection of each 100 μg-TCL formulation (TCL/FLs, TCL/CLs, TCL+eFLs, the emulsion of TCL and complete Freund's adjuvant (TCL+CFA), or TCL alone) into the right flank. Likewise, eFLs or PBS was injected into mice as a control. At 1 week after the final vaccination, 2×10^5 B16BL6 cells were inoculated into the mouse left flank, and then tumor volumes were monitored as described above.

RESULTS

MHC Class I-Restricted OVA-Presentation by OVA/FLs-Pulsed DC2.4 Cells

We first compared the levels of MHC class I-restricted antigen presentation between DC2.4 cells treated with various OVA formulations (Fig. 1A). OVA peptide presentation *via* MHC class I on DC2.4 cells was significantly increased by OVA/FLs-treatment in an OVA dose-dependent manner, whereas OVA/CLs-pulsed DC2.4 cells showed slight enhancement of OVA-presentation as compared with the cells pulsed with the soluble form of OVA. This result suggested that OVA delivered directly into the cytoplasm by FLs imitated endogenous antigens in DC2.4 cells. Thus, in order to investigate the antigen presentation pathway in DC2.4 cells treated with OVA/FLs, we used lactacystin and MG132, which inhibit proteasome activity essential for antigen processing and presentation in the classical MHC class I-pathway (Fig. 1B). Both inhibitors could completely suppress MHC class I-restricted presentation under conditions that induced high OVA-presentation levels in OVA/FLs-pulsed DC2.4 cells in the absence of inhibitors. In addition, FLs could sufficiently deliver their encapsulating antigen proteins into the MHC class I pathway while in contact with DC2.4 cells for only 15 min. Collectively, antigen introduction by FLs could greatly enhance antigen presentation *via* MHC class I on APCs, as a result of prompt fusion to the plasma

membrane and direct delivery of their encapsulating antigens into cytoplasm.

Vaccine Efficacy of DC2.4 Cells Pulsed with TCL/FLs DC2.4 cells were pulsed with various B16BL6-TCL formulations at 500 μg-TCL/10^7 cells/ml, and then 10^6 cells were intradermally injected into C57BL/6 mice. One week after vaccination, the mice were challenged with B16BL6 cells (Fig. 2). Mice immunized with eFLs-pulsed or unpulsed DC2.4 cells showed a slight delay in B16BL6 tumor growth as compared with the PBS-injected group. We theorized that this phenomenon was caused by nonspecific immunostimulatory effects that depended on administration of DC2.4 cells. Tumor growth in mice immunized with TCL/CLs- or TCL-pulsed DC2.4 cells was comparable to that in control groups injected with eFLs-pulsed or unpulsed DC2.4 cells. In contrast, vaccination with TCL/FLs-pulsed DC2.4 cells markedly delayed tumor growth and suppressed tumor appearance until day 17 post-challenge, when all groups harbored large (>1000 mm^3) tumors. On the other hand, TCL+eFLs-pulsed DC2.4 cells did not inhibit B16BL6 tumor growth, indicating that the superior vaccine efficacy of TCL/FLs-pulsed DC2.4 cells was the result of efficient TCL-delivery into cytoplasm by FLs. These results clearly revealed that FLs were potential antigen-carriers for the development of DC-based cancer immunotherapy using TCL as antigen source.

Vaccine Efficacy of TCL/FLs by *in Vivo* Direct Immunization In order to evaluate the vaccine efficacy of TCL/FLs in *in vivo* direct immunization, we administered various TCL formulations into mice by one or three intradermal injections. In the single immunization mode, mice injected with any TCL formulation, including TCL/FLs, did not exhibit obvious inhibitory effects against the growth of B16BL6 tumors inoculated at 1 week after immunization (Fig. 3A). On the other hand, triple TCL/FLs-immunization at 1-week intervals dramatically delayed B16BL6 tumor appearance as compared to eFLs- or PBS-administration using the same mode, whereas tumor growth in mice immunized with TCL alone was only slightly suppressed relative to that in the control groups (Fig. 3B). Although mice immunized three times with TCL/CLs or TCL+eFLs exhibited moderate inhibition against B16BL6 tumor growth, as was seen in the TCL+CFA-immunized group, these effects were inferior to those observed in response to TCL/FLs, which prevented the growth of visible tumors in all mice during the 17 d post-challenge. Taken together, these results suggest that FLs are useful antigen-carriers and adjuvants for an *in vivo* direct TCL-vaccination strategy.

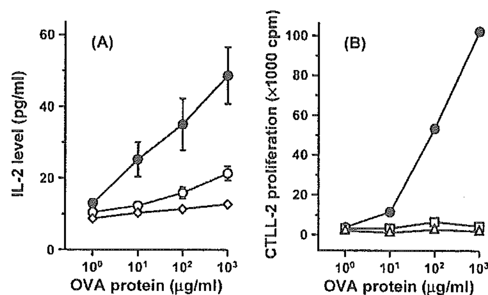

Fig. 1. Antigen Delivery into MHC Class I-Restricted Presentation Pathway on DC2.4 Cells by FLs

(A) DC2.4 cells were incubated for 5 h at 37 °C with OVA/FLs (●), OVA/CLs (○), or OVA solution (◇) at the indicated OVA concentrations. After washing, DC2.4 cells were co-cultured for 20 h with CD8-OVA 1.3 cells. OVA presentation *via* MHC class I molecules on DC2.4 cells was determined by ELISA for IL-2 released from stimulated CD8-OVA 1.3 cells. (B) DC2.4 cells were pre-incubated for 1 h at 37 °C with 10 μM lactacystin (□) or 10 μM MG132 (△) or without any additives (●). The cells were incubated for 15 min at 37 °C with OVA/FLs at the indicated OVA concentrations in the presence of inhibitors. After washing and glutaraldehyde fixation, CD8-OVA 1.3 cells were added and cultured for 24 h. The IL-2 released from CD8-OVA 1.3 cells was measured by CTLL-2 proliferation assay. Results are expressed in Δcpm as described in Materials and Methods. All data are presented as mean±S.D. of three independent cultures in the presence of inhibitors.

DISCUSSION

Recent advances in tumor immunology have identified various TAAs presented on MHC molecules, which has facil-

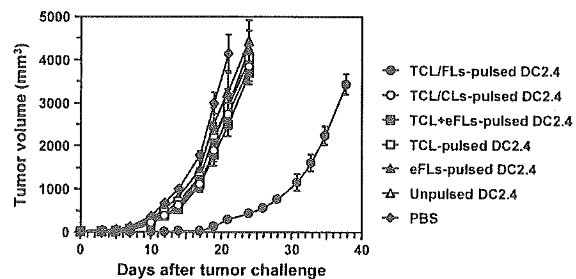

Fig. 2. TCL/FLs-Pulsed DC2.4 Cells-Mediated Prophylactic Effect against B16BL6 Tumor Challenge

C57BL/6 mice were immunized by intradermal injection of DC2.4 cells pulsed with various TCL formulations into the right flank at 10^6 cells, and then 2×10^5 B16BL6 cells were inoculated into the mouse left flank 1 week post-vaccination. Control mice were immunized with eFLs-pulsed DC2.4 cells, unpulsed DC2.4 cells, or PBS. The size of tumors was assessed using microcalipers three times per week. Each point represents the mean±S.E. from 6—12 mice.

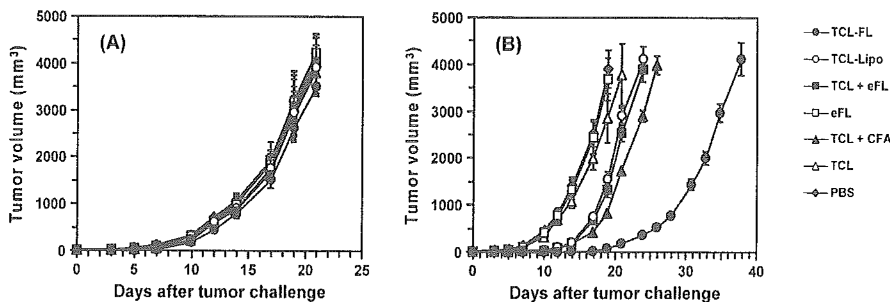

Fig. 3. Inhibitory Effect against B16BL6 Tumor Growth by *in Vivo* Direct CL/FLs-Immunization

Various TCL (100 mg) formulations were intradermally injected once (A) or three times at 1-week intervals (B) into the right flank of C57BL/6 mice. Likewise, control mice were injected with eFLs or PBS. At 1 week after the final immunization, the mice received a 2×10^5 B16BL6 cells-challenge in the left flank and tumor volumes were monitored. Each point represents the mean±S.E. from 6—12 mice.

itated the development of vaccine strategies for cancer.[16] However, immunotherapeutic application using TAAs as a vaccine component is limited to patients with a particular cancer because TAAs have been identified for only a few human cancers. TCL, which probably includes both known and unknown TAAs, is a very attractive antigen source for the development of versatile cancer immunotherapy. In fact, several studies demonstrated that TCL-pulsed DCs could offer the potential advantage of augmenting a broader T cell-immune response against both defined and undefined TAAs.[17—19] To improve the CTL response against TCL, we need excellent TCL-delivery carriers and adjuvants that can increase the immunogenicity of weak and rare TAAs. Thus, we evaluated the potential of FLs as a TCL vaccine vehicle in both *ex vivo* DC-based immunotherapy and *in vivo* direct vaccination.

We previously reported that FLs, composed of CLs fused with inactivated Sendai virus, could directly introduce their contents into cytoplasm by fusion with the cell membrane.[9,10] The *in vitro* antigen presentation assay showed that FLs delivered their encapsulating antigens into the classical MHC class I-restricted pathway for antigen processing and presentation in DC2.4 cells more efficiently than CLs (Fig. 1). Other approaches for developing an effective vaccine strategy have also been tested. Shibagaki *et al.* reported that an HIV-1-derived TAT protein transduction domain (PTD) conjugation technique could directly introduce antigens into cytosol of DCs.[20] Immunization of mice with DCs containing PTD-antigen fusion proteins induced anti-tumor effects through potent antigen-specific CTL activity.[21] However, the application of this technique to DC-based immunotherapy is limited to the treatment of cancer for which TAAs have been identified. In contrast, our antigen delivery system using FLs does not rely on a specific antigen source. Therefore, TCL/FLs-pulsed DC2.4 cells could demonstrate effective vaccine efficacy against B16BL6 tumor challenge (Fig. 2). This antigen delivery system using FLs against DCs would greatly contribute to the development of DC-based immunotherapy applicable to a wide variety of cancer types.

Furthermore, a triple *in vivo* direct immunization with TCL/FLs was more effective against B16BL6 tumor growth than the same immunization mode with TCL+CFA. This result suggested that FLs might efficiently deliver their encapsulating antigen into APCs at the administration site, although it is necessary to examine the biodistribution of antigens and the ratios of APCs containing the antigens after administration of antigen-encapsulating FLs. Additionally, we found that Sendai-virus accessory proteins displayed on FLs possessed mitogenic activity[22] and that FLs could enhance the expression of MHC class I/II molecules and co-stimulatory molecules (CD40 and CD80) and the secretion of IL-6, IL-12 and TNF-α in DCs (unpublished data). Therefore, FLs are not only efficient antigen-delivery carriers but also potential adjuvants in an *in vivo* direct immunization protocol.

With a view of potential therapeutic use for TCL/FL vaccines, then we tested whether this vaccine would facilitate eradication against established B16 melanoma. However, TCL/FL-immunized mice did not show inhibitory effect against growth of tumors (data not shown). From these results, we hypothesized that the concentration of TAA proteins involved in TCL/FL is too small to induce anti-melanoma therapeutic effect. As a potential solution to this problem, tumor cell derived total RNA is useful to induce multiple TAA specific immunity. It has been shown that vaccination with tumor derived RNA transfected DC can be remarkably effective in stimulating CTL and tumor immunity in *in vitro* and *in vivo* models.[23,24] Since multiple TAAs encoded by tumor derived RNA can be amplified from few tumor cells by PCR, FLs might be applicable to transfect it to dendritic cells and *in vivo* direct immunization strategy.

In conclusion, we demonstrated the usefulness of FLs as TCL-delivery carriers for *ex vivo* DC-based immunotherapy and *in vivo* direct immunization in the murine B16BL6 melanoma model. Because FLs can encapsulate various antigen candidates, such as crude tumor lysate or tumor extract, purified whole or partially processed TAA, and TAA-coding DNA or RNA, this simple and flexible system is a promising approach for the development of versatile cancer immunotherapy.

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Highlighted paper selected by Editor-in-chief

Non-Methylated CpG Motif Packaged into Fusogenic Liposomes Enhance Antigen-Specific Immunity in Mice

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DNA rich in non-methylated CG motifs (CpGs) enhances induction of immune responses against co-administered antigen encoding genes. CpGs are therefore among the promising adjuvants known to date. However, naked plasmid DNA, even which contains CpG motifs, are taken up by antigen presenting cells *via* the endocytosis pathway. Endocytosed DNAs are thus degraded and their gene expression levels are inefficient. In this context, an effective plasmid delivery carrier is required for DNA vaccine development. We show in the present study that packaging plasmids containing CpGs into fusogenic liposomes (FL) derived from conventional liposomes and Sendai virus-derived active accessory proteins is an attractive method for enhancing the efficacy of a DNA vaccine. These CpG-enhanced plasmids (possessing 16 CpG repeats) that were packaged into FL, enhanced ovalbumin (OVA)-specific T cell proliferation and cytotoxic T cell activity after immunization. In fact, vaccination with CpG enhanced plasmid-loaded FL induced effective prophylactic effects compared with 13 repeats CpG containing plasmid in a tumor challenge experiment. Thus, the development of a CpG-enhanced DNA-FL genetic immunization system represents a promising tool for developing candidate vaccines against some of the more difficult infectious, parasitic, and oncologic disease targets.

Key words DNA vaccine; CpG motif; fusogenic liposome

DNA vaccines have been widely used in laboratory animals and human primates over the last decade to induce humoral and cellular immune responses.[1—5] This approach to immunization has generated sustained interest because of its speed, simplicity, and ability to induce immune responses against naïve protein antigens expressed from plasmid DNA. There has been substantial work on DNA immunization in many species, including humans and large animals.[6—8]

In striking contrast, vaccination with antigen expressing genes usually fails to induce significant immune responses. Various methods are under evaluation to augment the potency of DNA vaccines, such as combination with gene delivery devices to increase the transfection of cells or to target the DNA or with the adjuvants which enhance inflammatory cytokine expression.[9—14] The extent of DNA degradation by extracellular deoxyribonucleases is unknown, but degradation could be considerable. It follows that approaches to protect DNA from the extracellular biological milieu and thereby introduce it into cells more efficiently, should contribute to optimal DNA vaccine design. In this context, not only efficient gene delivery devices but also immunostimulatory adjuvants are essential for augmentation of DNA vaccination.

Interestingly, the sequence composition of plasmid DNA itself also has been shown to increase the potency of the DNA vaccine.[12] This is because the bacterial DNA sequences result in the plasmid which possesses different methylation pattern from mammalian DNA. Bacterial oligonucleotides having the sequence purine–purine–cytosine–guanosine–pyrimidine–pyrimidine, in which the CpG sequence is unmethylated, can activate innate immune system, resulting in an augmentation of the antigen-specific immunity.[15] Recently, it was established that the innate immune system of vertebrates recognizes non methylated CpG motifs flanked by specific bases in bacterial DNA as a danger signal through toll-like receptor 9 (TLR9) expressed on the antigen presenting cells.[16—18] The cytokine profile induced by CpG motifs *in vitro* is consistent with their ability to induce a Th1-biased immune response when used as an adjuvant in vaccine formulations.[19] Therefore, CpG motifs may have potential as adjuvants in protein- and DNA-based vaccine formulations.[20]

CpG DNA is internalized *via* a clathrin dependent endocytic pathway and rapidly moves into a lysosomal compartment.[29] Since it has been known that TLR9 is localized in lysosomal compartment, CpG containing plasmids should be delivered to endosome–lysosome pathway even if plasmids were degraded in endosomes. Recently, several reports are suggested that TLR9 is expressed in ER prior to stimulation and translocate to a CpG containing lysosomal compartment for ligand binding and signal transduction.[29] In this context, with a view of plasmid based DNA vaccine development, CpG DNA targeting to translocating TLR9 is more useful to avoid endosomal DNA degradation.

Previously, we developed a highly unique antigen delivery carrier, fusogenic liposomes (FL), which consist of conventional liposomes and ultra-violet inactivated Sendai virus-derived accessory proteins.[21—24] This carrier could introduce

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its contents into various types of mammalian cells *via* membrane fusion but was not subject to endocytosis. FL introduced encapsulating genes into mammalian cells *in vitro* and *in vivo*. Furthermore, FL mediated DNA immunization induce efficient antigen specific immunity.[25] However, improvement of the efficacy of the FL-mediated gene delivery system is important for the development of a DNA vaccine.

In this study, we, therefore, created a novel genetic immunization system combined with a CpG-containing plasmid backbone and FL. The principal aim of this study was to induce potent antigen-specific immunity to the antigens encoded in the plasmid encapsulated in FL and combined with the CpG motif and a model antigen, chicken egg ovalbumin (OVA), thereby formulating a DNA vaccine.

MATERIALS AND METHODS

Animals and Cells Male C57BL/6 (H-2^b) mice, 7 weeks old, were purchased from SLC Inc. (Hamamatsu, Shizuoka, Japan). EL4 (Tohoku University, Sendai, Japan) is a C57BL/6 T lymphoma and EG7 is an ovalbumin (OVA)-transfected clone of EL4. IC21 cell is a C57BL/6 macrophage clone, H-2Kb. CD8OVA1.3 (provided by Dr. Clifford V. Harding, Case Western Reserve University, Cleveland, OH, U.S.A.) 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 a DMEM medium supplemented with 10% FCS. All culture media were purchased from Invitrogen (Carlsbad, CA, U.S.A.) and supplemented with non-essential amino acids, antibiotics, and 5×10^5 μM 2-mercaptoethanol (2-ME).

Plasmids The EcoRI fragment of pAc-neo-OVA was cloned into the EcoRI site of pBluescriptII KS(−), resulting in pBluescriptII KS(−)/OVA. To construct an OVA gene expression vector, the BamHI/SalI fragment of pBluescript II KS(−)/OVA was ligated into BamHI/SalI cut pCMV-script (Stratagene), resulting in pCMV-script/OVA (Fig. 1), which is driven by cytomegalovirus promoter and contains a SV40 poly(A) signal. This pCMV-script/OVA containing 13 repeats of the CpG motif, was named pOVACpG13. Furthermore, the plasmid containing 16 CpG motif repeats, pOVACpG16, was constructed as follows. SspI and AlwNI fragments of the pGL3-control vector (Promega) were ligated into pCMV-script digested with AlwNI and blunt ended, resulting in the CpG-enhanced vector, pCMV-script/CpG(+). Then, the BamHI/SalI fragment of pBluescript II KS(−)/OVA was introduced into the BamHI/SalI digested pCMV-script/CpG (+). This plasmid contained 16 CpG motif repeats. Methylated plasmids were prepared by SssI treatment for 4 h at 37 °C. These methylated plasmids were used for experiments after purification by phenol/chloroform precipitation.

Preparation of fusogenic liposome plasmid vector 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, MA, U.S.A.) and pelleted by ultracentrifugation to remove un-encapsulated plasmids. Then, FLs encapsulating pCMV-script/OVA were prepared by fusing the liposomes with UV (2000 J/cm^2)-inactivated Sendai virus as described.[21—24] The amount of plasmid DNA encapsulated within the liposomes was determined by means of fluorometric assay using 3,5-diaminobenzoic acid.

Proliferative Responses of Antigen-Specific T Cells from Immunized Mice Fourteen days after final immunization, lymphocytes were obtained from spleen. B cells were then depleted by using goat anti-mouse IgG (H&L)-coupled micro beads and a MACS column (Miltenyi Biotec, Sunnyvale, CA, U.S.A.). Purified T cells were cultured at a density of 2×10^5 cells/ml with 1 mg/ml OVA for 3 d. To measure cell proliferation, 1 μCi of [^{3}H] thymidine was added to individual culture wells 8 h before termination, and the uptake of [^{3}H] thymidine by dividing cells was determined by scintillation counting.

IL-12 Expression Analysis by ELISA IL-12 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 (or various indicated concentrations). Culture supernatants were harvested 48 h after incubation, and the levels of IL-12 were determined by an IL-12-specific ELISA kit (Biosource). The concentration of cytokines was calculated by standard curves obtained according to the instructions provided by the manufacturer.

***In Vitro* CTL Induction and Cytotoxic Assay** C57BL/6 mice (7 weeks old, male, H-2^b) were immunized twice at 2 week intervals with 50 μg of naked or 5 μg of Fusogenic liposome encapsulated pOVACpG13 or pOVACpG16, respectively. Spleen cells from immunized or non-immunized mice were recovered 14 d after the last immunization and were stimulated *in vitro* with mitomycin C treated EG7 cells for 5 d. 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 different effector/target ratios. A cytotoxicity assay was conducted in triplicate. The maximum release was determined by adding 1% Triton X-100 to the target cells. A spontaneous release was obtained in the case of 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:

$$\text{percentage of specific lysis} = 100\times[(\text{release of CTLs})-(\text{spontaneous release})]/[(\text{maximal release})-(\text{spontaneous release})]$$

Tumor Challenge Experiments C57BL/6 mice (7 weeks old, male, H-2b) were immunized s.c. at the tail base twice at 2 week intervals with 50 μg of naked or 5 μg of fusogenic liposome encapsulated pOVACpG13 or pOVACpG16. Fourteen days after the last immunization (day 0), 1×10^6 OVA expressing EG7 cells were intradermally injected. Six to 13 mice were used for each experimental group. Tumor survival in tumor bearing mice was monitored weekly. Mice that developed tumors larger than 4000 mm^3 were considered to have developed lethal tumors.

RESULTS

***In Vitro* Enhancement of IL-12 Expression by CpG-Enhanced Vectors Combined with FL** Initially we evaluated the immunostimulatory effect of CpG-enhanced vector encapsulated in FL by IL-12 production (Fig. 1). ELISA analysis showed that IL-expression of FL/pOVACpG16-stimulated splenocytes tended to enhance IL-12 production compared with non-CpG enhanced vector (pOVACpG13) containing FL. In addition, methylated plasmid vector encapsulated in FL or empty FL did not enhance IL-12 expression. These results clearly showed that CpG-enhanced vectors retained their immunostimulatory effect even when encapsulated in FL, and IL-12 expression increased depending on the number of CpG motifs.

Vaccination with CpG-Enhanced Vector Combined with FL Significantly Enhances Antigen Specific T Cell Mediated Immune Responses in Vaccinated Mice Examination of antigen-specific proliferation of lymphocytes in immunized mice (Fig. 2) indicated that FL/pOVACpG16 vaccination dramatically enhanced proliferation. On the other hand, FL/pOVACpG13- or naked CpG-enhanced or non-enhanced vector immunization did not induce antigen-specific proliferation. These results indicated that the combination of CpG immuno stimulatory sequences and FL significantly enhanced antigen specific T cell proliferation under a very low dose (5 μg). Next, the immunogenicity of FL/pOVACpG16 was tested by CTL assay (Fig. 3). The best response was obtained for pOVACpG16 combined with FL, which exhibited *ex vivo* killing of *ca.* 40% at an E : T ratio of 50. The corresponding killing obtained by pOVACpG13 combined with FL was in the range of 30%.

Protection against the Growth of OVA-Expressing Tumors in Mice Vaccinated with CpG-Enhanced Vectors by FL To determine whether the observed enhancement in antigen-specific T cell mediated immunity translated to a significant anti-tumor immunity and prolonged survival, we performed an *in vivo* tumor protection experiment using an OVA expressing tumor-model, EG7. As shown in Fig. 4, 70% of mice receiving the pOVACpG16 vaccine combined with FL survived 90 d after the EG7 challenge. In contrast, the survival rate of unvaccinated mice and mice receiving pOVACpG13 or pOVACpG16 alone or a combination vaccine of pOVACpG13 and FL was less than 40%. A two-fold improvement was observed in the response of mice treated with a prophylactic vaccine treatment consisting of pOVACpG16 combined with FL. These results indicated that the combination of CpG enhanced vectors and FL was a more effective genetic immunization system for prophylactic tumor vaccine.

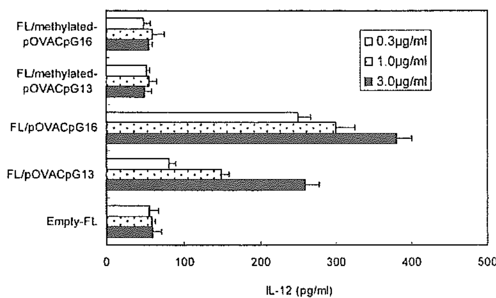

Fig. 1. CpG Enhanced Vector (pOVACpG16) Containing FL Hold Immunostimulatory Effects

Splenocytes from naïve mice were cultured for 2 d in the presence of FL-pOVACpG13, FL-pOVACpG16, FL-methylated pOVACpG13 and FL-methylated pOVACpG16 at indicated concentrations. Then IL-12 levels in the culture supernatants were determined by ELISA.

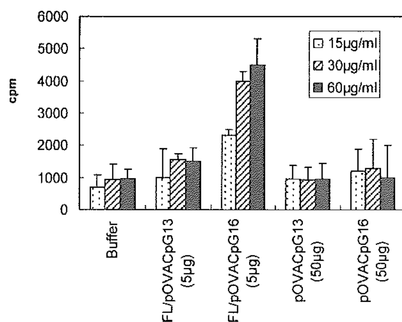

Fig. 2. OVA Specific T Cell Proliferation Derived from Mice Splenocytes Immunized with CpG Enhanced FL-DNA Vaccine

Spleen cells from C57/Bl6 mice immunized with balanced salt solution (Buffer), 5 μg FL-pOVACpG13, 5 μg FL-pOVACpG16, 50 μg pOVACpG13 and 50 μg pOVACpG16 were assayed for proliferation assay. Then the splenocytes were incubated with 15 (□), 30(▨), 60(■) μg/ml OVA in culture medium for 3 d. OVA specific proliferative responses were determined by [^{3}H]-thymidine uptake.

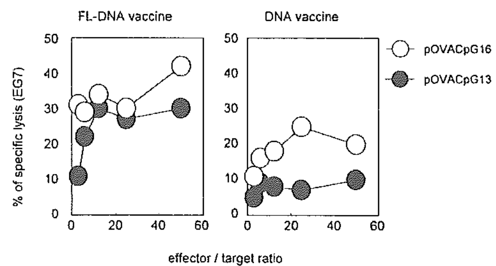

Fig. 3. OVA Specific CTL Response after *in Vivo* Priming with CpG Enhanced FL-DNA Vaccine

Spleen cells from C57/Bl6 mice that had been immunized with 50 μg FL-pOVACpG13, 50 μg FL-pOVACpG16, 5 μg pOVACpG13, 5 μg pOVACpG16 were assayed for cytotoxic activity, after *in vitro* stimulation with EG7 tumor cells for 5 d. The figure represents the amount of specific lysis against the 51Cr labeled EG7 cells.

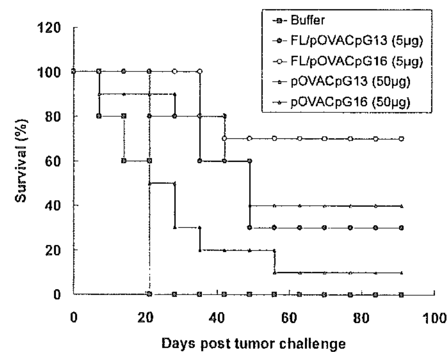

Fig. 4. Survival Analysis of Mice Immunized with DNA-Fusogenic Liposome Vaccine in a Prophylactic Treatment Model

C57/Bl6 mice were immunized with Buffer, 50 μg pOVACpG13 or 50 μg pOVACpG16 as control vaccine, 5 μg FL-pOVACpG13 or 5 μg FL-pOVACpG16 twice with an interval of two weeks between treatments. Four weeks after last immunization, immunized mice were challenged i.d. in the abdomen with 1×10^6 cells. Comparison of survival curves of two groups were significantly different ($p<0.01$).

DISCUSSION

In the present study, we demonstrated that a combination of CpG-enhanced vectors and FL strengthened IL-12 expression by splenocytes from naïve mice, and this approach enhanced the potency of DNA vaccines using OVA as a model antigen, leading to effective OVA specific T cell proliferation, CTL responses, and prophylactic anti-tumor effects. Our previous study showed that immunization of mice with conventional OVA expression vector, pOVACpG13 using FL, induced antigen-specific antibodies and strong CTL responses.[25] In the present study, we utilized CpG immunostimulatory sequences to enhance FL-mediated DNA vaccination therapy. The results demonstrated that CpG introduction was effective for *in vitro* inflammatory cytokine production by APCs and this leads to dramatically enhanced proliferation of antigen-specific T cell proliferation, because IL-12 production and OVA specific T cell proliferation was significantly weaker in conventional CpG containing plasmid vector (pOVACpG13) or even in combination with FL in immunized mice.

Generally, the CpG motif, even in a plasmid backbone, stimulates APCs *via* TLR9 receptor signaling.[16,17] Although these activation mechanisms are available to the endocytosis pathway,[26] previous studies have not reported any investigations of immunostimulatory ability of directly introduced CpG motifs *via* membrane fusion. Recent report suggested that TLR is expressed in ER prior to stimulation, and translocate to lysosomal compartment through cytosolic compartment by inflammatory stimuli.[29] So we hypothesized that CpG enhanced plasmid in cytosol could bind to TLR9, which is translocating from ER to lysosome through cytosol. Another hypothesis is that DNAs adsorbed on FL or released from FL may interact with TLR9. Overall, although our data indicate that the direct introduction of CpG-enhanced vectors *via* membrane fusion retained their stimulatory effects, detailed studies are needed to clarify activation mechanisms. Our data indicated that antigen specific T cell proliferation and CTL responses were more effective than the combination of FL and conventional pOVACpG13 in vaccinated mice. When challenged with OVA-expressing EG7 tumors, mice immunized with the CpG-enhanced vector combined with FL exhibited prolonged survival compared with conventional vector immunized groups, even when combined with FL.

Although the anti-tumor effects presented in Fig. 4 are somewhat striking, they hold little relevance to immunological therapy against tumors. We should have tested their vaccines in a therapeutic mode (tumor first and vaccine after) and not solely in a prophylactic fashion. Moreover, these experiments do not address the issue of potential immunological tolerance to real tumor antigens, which in many cases are also expressed to some extent by normal cells, since OVA is a totally foreign antigen. Studies conducted using a real tumor antigen in murine models, such as TRP2 for B16 melanoma,[27] P1A for P815 mastocytoma,[28] or anything equivalent, could potentially provide additional information that better simulates actual conditions.

In summary, our findings indicate that the introduction of three CpG immunostimulatory sequences and FL is able to enhance inflammatory cytokines and elicit more effective antigen-specific T cell activity and prophylactic anti-tumor effects *in vivo* than a previously developed conventional plasmid backbone (pOVACpG13 and FL combination vaccine). This approach may be promising for future vaccine development to control cancer, which expresses self antigens, or infectious diseases, and may be particularly useful in patients with reduced immune responses, particularly human immunodeficiency virus (HIV) or human T cell leukemia virus (HTLV)-infected patients. Studies are in progress to clarify the efficacy of FL mediated genetic immunization systems on tumor-associated antigens and virus-related antigen expression vectors.

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話題

WADAI

疾患関連たんぱく質解析研究・創薬プロテオームファクトリープロジェクト

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1. はじめに

疾患の原因解明や診断，有効な治療法や予防方法の確立，さらには画期的な新薬創製等を目指して，疾患関連タンパク質の探索研究が世界中で進められている．ヒト試料中に存在する多種多様なタンパク質から疾患に関連する微量タンパク質を効率良く探索・同定するためには，高性能の分析機器や大量情報処理システムの整備とともに高精度の解析技術の開発が必要である．さらに，倫理面への対応も含めた適切な研究協力体制の構築と適切かつ慎重な運営が必須である．既に欧米では国家規模で産官学が大規模な協力体制を組んで研究を進めており，新規タンパク質の発見と知的所有権確保の競争が激しさを増している．我が国においても2002年10月の総合科学技術会議評価を受けて，日本製薬工業協会(製薬協)の協力のもとに厚生労働省(厚労省)，ヒューマンサイエンス振興財団(HS財団)等が中心となって産官学の協力により立ち上げた「疾患関連たんぱく質解析研究・創薬プロテオームファクトリープロジェクト」が進行中であるので，その概要を紹介する．

2. 目的

本プロジェクトでは，疾患患者と健常者との間の生体タンパク質の種類・質・量の相違について解明する「疾患からのアプローチ」により，疾患関連タンパク質のデータベースを作成するとともに，新しい解析技術を開発したり疾患関連タンパク質を発見した場合その知的財産権を確保する．この成果を速やかに研究協力機関の研究者やコンソーシアム参加企業にフィードバックすることにより，適切な診断方法や有効な治療方法の開発等に貢献するとともに，画期的な医薬品の研究開発に繋がるシーズを提供し医薬品開発に貢献することを目的としている．

1)国家プロジェクトとして、わが国の主要疾患である糖尿病、がん、高血圧、認知症等を対象とした疾患関連たんぱく質の探索・同定の研究を推進する。

2)これらの疾患の診断、治療、予防に関心を持つわが国の医療関係者、研究者等が共に利用できる新規技術の開発、バイオマーカーや創薬ターゲット候補タンパク質を見つけて、創薬のための基盤的データベースを構築する。

3)新規技術、新規タンパク質やデータベースに関する知的財産権を確保・活用して、これらの疾患に対する画期的な医薬品等の創出に役立てる。

図1 「本プロジェクト」の目的

3. 設立の経緯

欧米を中心とした疾患関連タンパク質解析研究の動向を踏まえ，2002年に製薬協・研究開発委員会による「医薬品産業の立場から期待する科学技術政策研究」のなかで，創薬プロテオームファクトリーの早期創設が提言された．また厚労省が策定した「医薬品産業ビジョン」のアクションプランとして「疾患関連たんぱく質解析と創薬基盤研究所の推進」が示された．同年10月の総合科学技術会議において「疾患関連たんぱく質解析プロジェクト」はS評価を受けた．12月に発表されたバイオテクノロジー戦略大綱の「バイオ行動計画2002」において，“たんぱく質構造・機能解析，遺伝子発現解析等のポストゲノム研究を進め，その研究成果を基に創薬基盤研究を確立する”ことが提言された．このような経緯を経て2003年度から5年間の国の研究事業として，長尾　拓氏(国立医薬品食品衛生研究所(国立衛研))を主任研究者とする厚生労働科学研究「疾患関連たんぱく質解析研究」の開始が決定された．なお，2005年度から主任研究者は山西弘一氏(医薬基盤研究所(基盤研))が引き継いでいる．

一方，HS財団は独自に欧米のプロテオーム解析施設等を視察しその動向に関する情報収集を行い，産官学共同でプロテオーム解析に取り組む国家プロジェクトを立ち上げるべく，2003年6月に創薬プロテオームファクトリーに興味を持つ企業を募り，「PF設立準備委員会」を設置した．この委員会において財団賛助会員企業21社によるワーキンググループを作り本プロジェクトの目的・目標案設定，5年間の事業計画案作成及び知的所有権確保等に関する諸問題を検討し，8月には報告書をまとめた．またこの委員会では，国立衛研，大学，国立高度専門医療センター，製薬企業等の研究者並びに機器メーカー専門家等からの情報及び意見も含めて「機器選定案」を作成し，HS財団を通して国立衛研に提出した．これに基づき厚労省，国立衛研で検討を重ねた後，公開入札により，本プロジェクト遂行に必要な各種分析機器類等及びインフォマティクスシステムを整備した．また，HS財団は2003年9月に公募を実施し，民間企業22社によるコンソーシアムを立ち上げ，国立衛研と共同で「疾患関連たんぱく質解析研究・創薬プロテオームファクトリープロジェクト」を開始した．機器購入にあたり，国は2002年度補正予算として43億円を交付し，さらに2003～2007年度まで厚生労働科学研究費補助金として5億円/年規模の交付を予定し，コンソーシアム参加企業からの参加費年5.5億円/年とあわせて本プロジェクトを運営実施している．

4. 全体構成

本プロジェクトは2003年度から5年間で実施される国家プロジェクトである．前項で述べた通り，厚生労働科学研究「疾患関連たんぱく質解析研究」事業とHS財団が主宰するコンソーシアムによる創薬プロテオームファクトリー事業で構成されている．核となる基盤研とHS財団は共同研究契約を締結し，微量タンパク質解析技術の確立，分離技術の確立，高度分析法の開発，疾患関連タンパク質の探索・同定，解析データベースの構築・提供等について協力して研究を進めている．一方，国立循環器病センター(循環器病センター)，国立精神・神経センター，国立国際医療センター，国立生育医療センター，国立長寿医療センター，大阪府立成人病センター，大阪大学医学部附属病院及び大阪大学蛋白質研究所は研究協力機関として，HS財団及び基盤研と研究協力契約を締結し，別紙対象疾患について倫理指針に基づきインフォームドコンセントのもとに，患者さんの十分な理解と承諾を得た良質なヒト試料の収集に協力するとともに，プロテオーム解析結果について詳細研究を進めている．

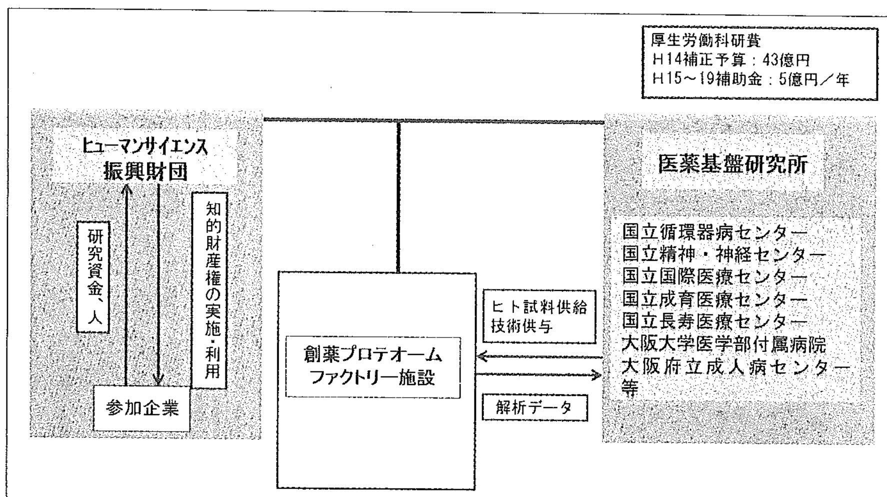

図2 「疾患関連たんぱく質解析研究・創薬プロテオームファクトリープロジェクト」概念図

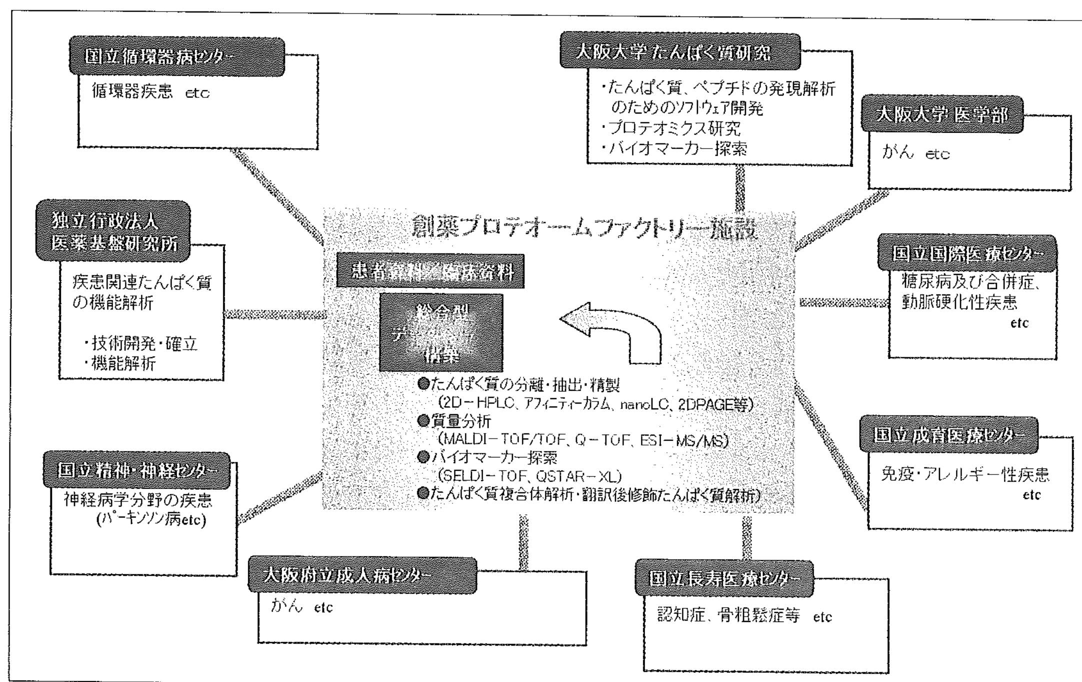

図3 協力機関と対象疾患

5. 組織体制

本プロジェクトは松尾壽之氏(循環器病センター)をリーダーとして，寒川賢治氏(循環器病センター)，南野直人氏(循環器病センター)，高尾敏文氏(阪大蛋白質研究所)，瀬谷　司氏(大阪府立成人病センター)，金子　勲氏(創薬プロテオームファクトリー)の各氏がサブリーダーで協力する．また，山西弘一氏(基盤研)は厚生労働科学研究の主任研究者として共同研究を進めている．本プロジェクトはその基本計画策定，運営，管理その他を審議，決定する運営委員会のもとに運営され，リーダーの諮問機関として倫理審査委員会が設置されている．さらにリーダーに対する助言等サポートとしてアドバイザリーボードも設置されている．

主体となる研究施設は創薬プロテオームファクトリー施設という名称で，大阪市西淀川区に

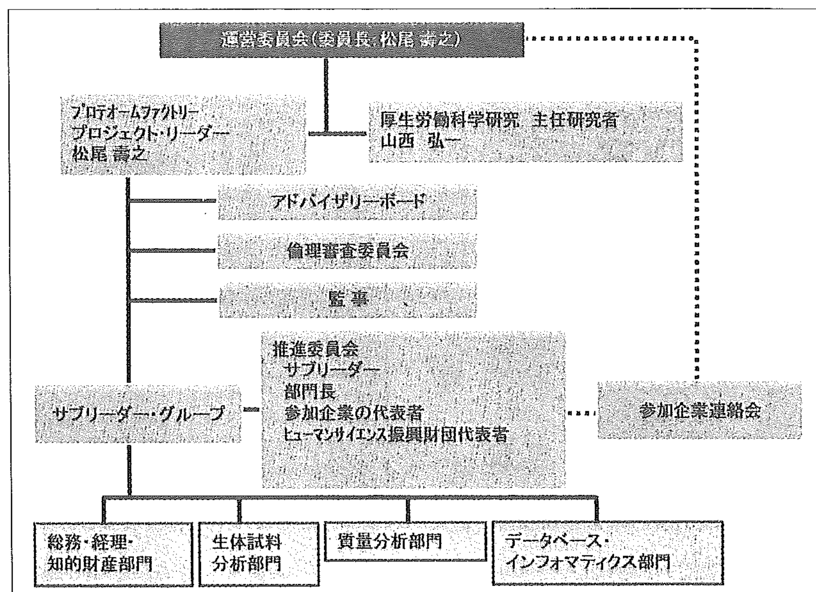

図4 組織体制

設置した．この施設では，ヒト試料からの生体微量タンパク質分離・精製等を行う生体試料分析部門，測定に関わる質量分析部門，データ解析とデータベース構築・提供に関わるデータベース・インフォマティクス部門に産官学の研究者を配備して効率的で質の高い業務を進めている．さらに本プロジェクトの成果に関する知的財産権を取得・管理することも重要であり，総務・経理・知的財産部門も設置し，HS財団に設立されたヒューマンサイエンス技術移転センター(厚労省認定TLO)と協力して対応している．

なお提供されるヒト試料は，各協力機関及び当施設において二重の匿名化を行うとともに，付随する個人情報については当施設のプロジェクトリーダーが任命した個人情報管理者により厳重に管理され，情報の漏洩が起こらないよう慎重に配慮している．

6. 解析の基本方針と進捗状況

従来，プロテオーム解析において汎用され多大な実績を挙げてきた2次元電気泳動法と質量分析計を組み合わせる方法での問題点として，微量タンパク質の分離能，high throughput性，多量に存在するhouse keeping gene productの処理等が懸念される．本プロジェクトでは多量のヒト試料解析を最優先課題として，夾雑タンパク質除去方法の開発，nanoHPLCの活用，最新鋭の質量分析計の導入，大容量の情報処理に対応するバイオインフォマティクスシステム構築が必要である．

機器等の設置に合わせて研究者の配備も進め，これまでにヒト試料の受け入れ，臨床情報匿名化，試料・機器管理(LIMS；laboratory information management system)，前処理法，タンパク質分離・抽出法，大量タンパク質同定解析システム及び創薬ターゲット探索データベース構築用インフォマティクスまでを一括管理できる体制を整えた．低発現タンパク質解析システムを構築するとともに，ヒト血清タンパク質解析フローを確立し，患者血清の解析も本格的に進められている．また組織試料についても解析検討を進めている．この成果の1つとして，微量タンパク質解析方法に関する特許を1件申請中である．

今後，タンパク質同定・比較定量の一層の精度向上を目指すとともに，対象疾患を中心として各種患者試料の解析を進める予定である．さらに，ターゲット候補となるタンパク質につい

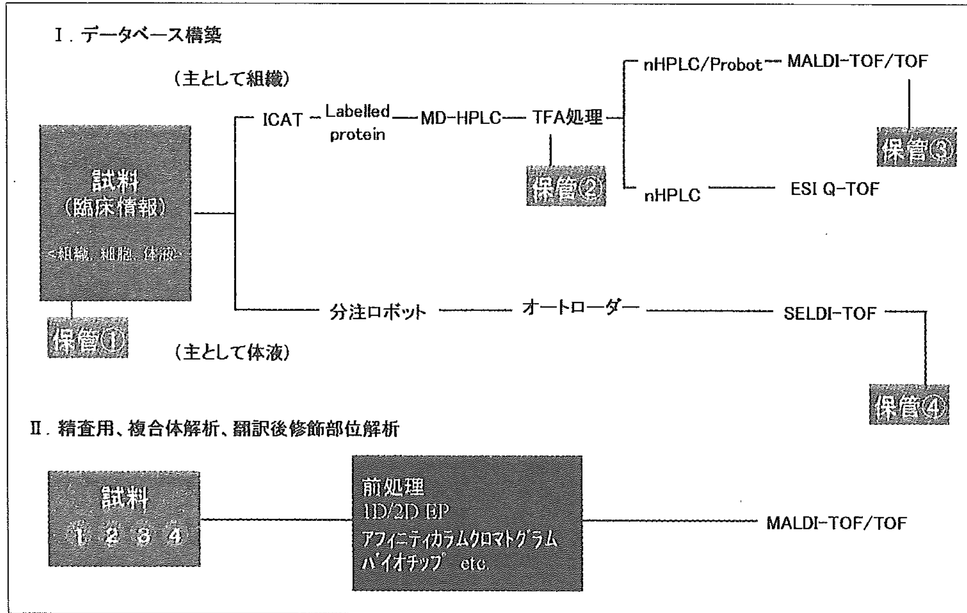

図5　ワークフロー

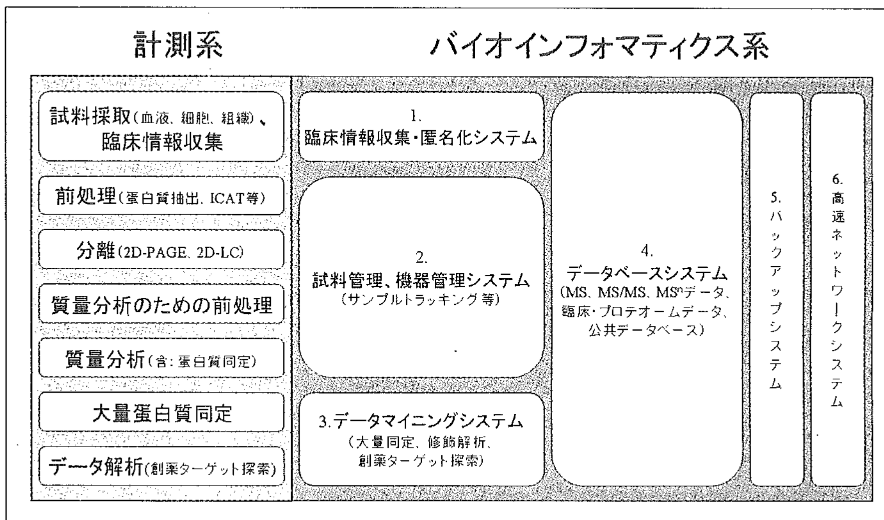

図6　バイオインフォマティクスの構成図

ては，アフィニティーカラム，プロテインチップの開発利用，標識方法の改良等により高性能質量分析計の特性を生かした機能解析を予定している．なお，プロジェクトの成果については，特許取得後に論文公表も進める．

7. おわりに

本プロジェクトの成果として，疾患関連タンパク質に関する創薬基盤データベースの構築，新規プロテオーム解析技術の開発，新規な創薬標的タンパク質や疾患マーカーとなる新規タンパク質の発見等により，疾病の原因解明や治療法，画期的な新薬創製等に貢献することが期待されている．そのためには，今後も国の支援はもちろんのこと，産官学の研究者が一体となった関係各位の一層の協力が必須である．