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:

tumor volume (mm^3) = [major axis (mm)] × [minor axis (mm)]^2 × 0.5236

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.

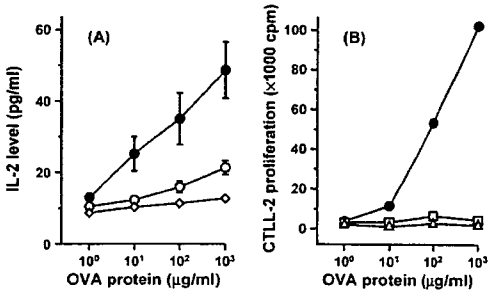

Fig. 1. Antigen Delivery into MHC Class I-Restricted Presentation Pathway on DC2.4 Cells by FLs

(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-

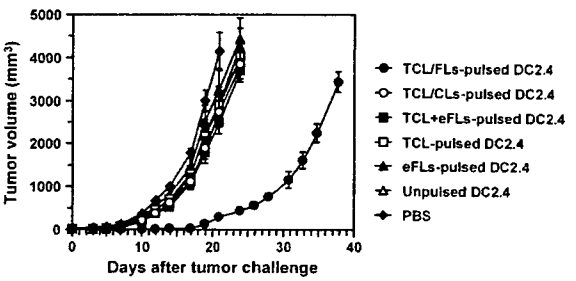

Fig. 2. TCL/FLs-Pulsed DC2.4 Cells-Mediated Prophylactic Effect against B16BL6 Tumor Challenge

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.

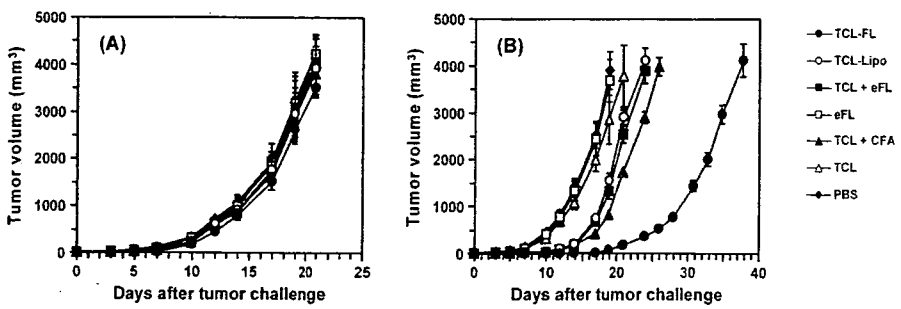

Fig. 3. Inhibitory Effect against B16BL6 Tumor Growth by *in Vivo* Direct CL/FLs-Immunization

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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REFERENCES

1) Cox A. L., Skipper J., Chen Y., Henderson R. A., Darrow T. L., Shabanowitz J., Engelhard V. H., Hunt D. F., Slingluff C. L., Jr., *Science*, **264**, 716—719 (1994).
2) Brichard V., Van Pel A., Wölfel T., Wölfel C., De Plaen E., Lethé B., Coulie P., Boon T., *J. Exp. Med.*, **178**, 489—495 (1993).
3) Ada G., *N. Engl. J. Med.*, **345**, 1042—1053 (2001).
4) de Vries T. J., Fourkour A., Wobbes T., Verkroost G., Ruiter D. J., van Muijen G. N., *Cancer Res.*, **57**, 3223—3229 (1997).
5) Nestle F. O., Alijagic S., Gilliet M., Sun Y., Grabbe S., Dummer R., Burg G., Schadendorf D., *Nat. Med.*, **4**, 328—332 (1998).
6) Guermonprez P., Valladeau J., Zitvogel L., Thery C., Amigorena S., *Annu. Rev. Immunol.*, **20**, 621—667 (2002).
7) Heath W. R., Belz G. T., Behrens G. M., Smith C. M., Forehan S. P., Parish I. A., Davey G. M., Wilson N. S., Carbone F. R., Villadangos J. A., *Immunol. Rev.*, **199**, 9—26 (2004).
8) Mizuguchi H., Nakanishi M., Nakanishi T., Nakagawa T., Nakagawa S., Mayumi T., *Br. J. Cancer*, **73**, 472—476 (1996).
9) Nakanishi T., Hayashi A., Kunisawa J., Tsutsumi Y., Tanaka K., Yashiro-Ohtani Y., Nakanishi M., Fujiwara H., Hamaoka T., Mayumi T., *Eur. J. Immunol.*, **30**, 1740—1747 (2000).
10) Kunisawa J., Nakanishi T., Takahashi I., Okudaira A., Tsutsumi Y., Katayama K., Nakagawa S., Kiyono H., Mayumi T., *J. Immunol.*, **167**, 1406—1412 (2001).
11) Yoshikawa T., Imazu S., Gao J. Q., Hayashi K., Tsuda Y., Shimokawa M., Sugita T., Niwa T., Oda A., Akashi M., Tsutsumi Y., Mayumi T., Nakagawa S., *Biochem. Biophys. Res. Commun.*, **325**, 500—505 (2004).
12) Shen Z., Reznikoff G., Dranoff G., Rock K. L., *J. Immunol.*, **158**, 2723—2730 (1997).
13) Pfeifer J. D., Wick M. J., Roberts R. L., Findlay K., Normark S. J., Harding C. V., *Nature* (London), **361**, 359—362 (1993).

14) Gillis S., Smith K. A., *Nature* (London), **268**, 154—156 (1977).
15) Harding C. V., Song R., *J. Immunol.*, **153**, 4925—4933 (1994).
16) Rosenberg S. A., Yang J. C., Restifo N. P., *Nat. Med.*, **10**, 909—915 (2004).
17) Mitchell M. S., Kan-Mitchell J., Morrow P. R., Darrah D., Jones V. E., Mescher M. F., *Clin. Cancer Res.*, **10**, 76—83 (2004).
18) Chang A. E., Redman B. G., Whitfield J. R., Nickoloff B. J., Braun T. M., Lee P. P., Geiger J. D., Mule J. J., *Clin. Cancer Res.*, **8**, 1021—1032 (2002).
19) Geiger J. D., Hutchinson R. J., Hohenkirk L. F., McKenna E. A., Yanik G. A., Levine J. E., Chang A. E., Braun T. M., Mule J. J., *Cancer Res.*, **61**, 8513—8519 (2001).
20) Shibagaki N., Udey M. C., *J. Immunol.*, **168**, 2393—2401 (2002).
21) Shibagaki N., Udey M. C., *Eur. J. Immunol.*, **33**, 850—860 (2003).
22) Hayashi A., Nakanishi T., Kunisawa J., Kondoh M., Imazu S., Tsutsumi Y., Tanaka K., Fujiwara H., Hamaoka T., Mayumi T., *Biochem. Biophys. Res. Commun.*, **261**, 824—828 (1999).
23) Gilboa E., Nair S. L., Lyerly H. K., *Cancer Immunol. Immunother.*, **46**, 82—87 (1998).
24) Boczkowski D., Nair S. K., Nam J.-H., Lyerly H. K., Gilboa E., *Cancer Res.*, **60**, 1028—1034 (2000).

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.

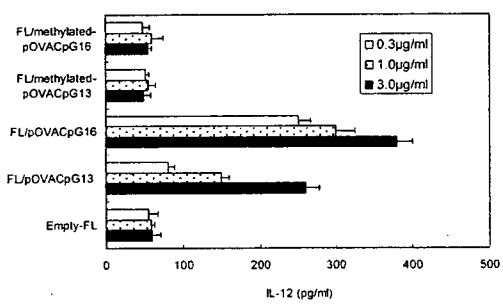

Fig. 1. CpG Enhanced Vector (pOVACpG16) Containing FL Hold Immunostimulatory Effects

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.

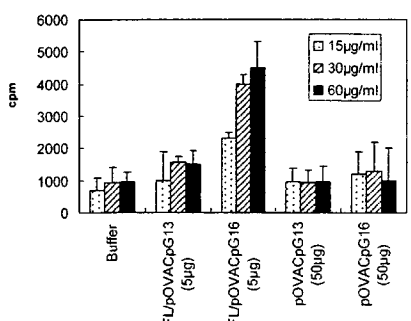

Fig. 2. OVA Specific T Cell Proliferation Derived from Mice Splenocytes Immunized with CpG Enhanced FL-DNA Vaccine

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.

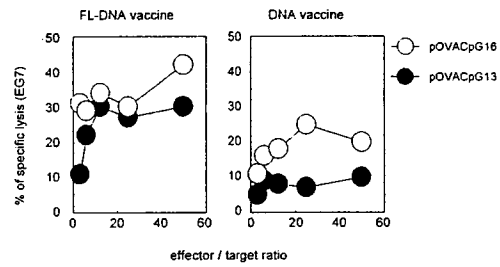

Fig. 3. OVA Specific CTL Response after *in Vivo* Priming with CpG Enhanced FL-DNA Vaccine

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.

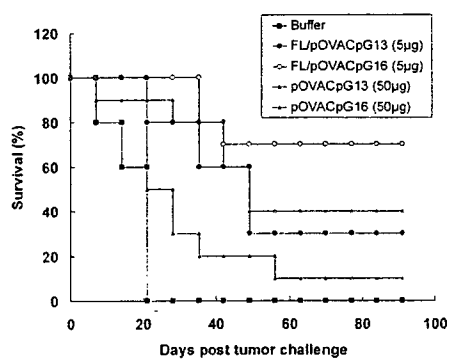

Fig. 4. Survival Analysis of Mice Immunized with DNA-Fusogenic Liposome Vaccine in a Prophylactic Treatment Model

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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REFERENCES

1) Ulmer J. B., Donnelly J. J., Parker S. E., Rhodes G. H., Felgner P. L., Dwarki V. J., Gromkowski S. H., Deck R. R., DeWitt C. M., Friedman A., *et al.*, *Science*, **259**; 1745—1749 (1993).
2) Corr M., von Damm A., Lee D. J., Tighe H., *J. Immunol.*, **163**, 4721—4727 (1999).
3) Donnelly J. J., Ulmer J. B., Shiver J. W., Liu M. A., *Annu. Rev. Immunol.*, **15**, 617—648 (1997).
4) Donnelly J. J., Ulmer J. B., Liu M. A., *Dev. Biol. Stand.*, **95**, 43—53 (1998).
5) Montgomery D. L., Ulmer J. B., Donnelly J. J., Liu M. A., *Pharmacol. Ther.*, **74**, 195—205 (1997).
6) Donnelly J. J., Friedman A., Martinez D., Montgomery D. L., Shiver J. W., Motzel S. L., Ulmer J. B., Liu M. A., *Nat. Med.*, **1**, 583—587 (1995).
7) MacGregor R. R., Boyer J. D., Ugen K. E., Lacy K. E., Gluckman S. J., Bagarazzi M. L., Chattergoon M. A., Baine Y., Higgins T. J., Ciccarelli R. B., Coney L. R., Ginsberg R. S., Weiner D. B., *J. Infect. Dis.*, **178**, 92—100 (1998).
8) Wang R., Epstein J., Baraceros F. M., Gorak E. J., Charoenvit Y., Carucci D. J., Hedstrom R. C., Rahardjo N., Gay T., Hobart P., Stout R., Jones T. R., Richie T. L., Parker S. E., Doolan D. L., Norman J., Hoffman S. L., *Proc. Natl. Acad. Sci. U.S.A.*, **98**, 10817—10822 (2001).
9) Singh M., Kazzaz J., Ugozzoli M., Chesko J., O'Hagan D. T., *Expert. Opin. Biol. Ther.*, **4**, 483—491 (2004).
10) O'Hagan D., Singh J., Ugozzoli M., Wild C., Barnett S., Chen M., Schaefer M., Doe B., Otten G. R., Ulmer J. B., *J. Virol.*, **75**, 9037—9043 (2001).
11) Roy K., Mao H. Q., Huang S. K., Leong K. W., *Nat. Med.*, **5**, 387—391 (1999).
12) Ulmer J. B., DeWitt C. M., Chastain M., Friedman A., Donnelly J. J., McClements W. L., Caulfield M. J., Bohannon K. E., Volkin D. B., Evans R. K., *Vaccine*, **18**, 18—28 (1999).
13) Nakanishi T., Kunisawa J., Hayashi A., Tsutsumi Y., Kubo K., Nakagawa S., Nakanishi M., Tanaka K., Mayumi T., *J. Control. Release*, **61**, 233—240 (1999).
14) Nakanishi T., Kunisawa J., Hayashi A., Tsutsumi Y., Kubo K., Nakagawa S., Fujiwara H., Hamaoka T., Mayumi T., *Biochem. Biophys. Res. Commun.*, **240**, 793—797 (1997).
15) Krieg A. M., Yi A. K., Matson S., Waldschmidt T. J., Bishop G. A., Teasdale R., Koretzky G. A., Klinman D. M., *Nature* (London), **374**, 546—549 (1995).
16) Hemmi H., Kaisho T., Takeda K., Akira S., *J. Immunol.*, **170**, 3059—3064 (2003).
17) Hemmi H., Takeuchi O., Kawai T., Kaisho T., Sato S., Sanjo H., Matsumoto M., Hoshino K., Wagner H., Takeda K., Akira S., *Nature* (London), **408**, 740—745 (2000).
18) Akira S., Hemmi H., *Immunol. Lett.*, **85**, 85—95 (2003).

19) Brazolot Millan C. L., Weeratna R., Krieg A. M., Siegrist C. A., Davis H. L., *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 15553—15558 (1998).
20) Krieg A. M., *Biochim. Biophys. Acta*, **1489**, 107—116 (1999).
21) Kunisawa J., Nakanishi T., Takahashi I., Okudaira A., Tsutsumi Y., Katayama K., Nakagawa S., Kiyono H., Mayumi T., *J. Immunol.*, **167**, 1406—1412 (2001).
22) Mizuguchi H., Nakagawa T., Nakanishi M., Imazu S., Nakagawa S., Mayumi T., *Biochem. Biophys. Res. Commun.*, **218**, 402—407 (1996).
23) Nakanishi T., Hayashi A., Kunisawa J., Tsutsumi Y., Tanaka K., Yashiro-Ohtani Y., Nakanishi M., Fujiwara H., Hamaoka T., Mayumi T., *Eur. J. Immunol.*, **30**, 1740—1747 (2000).
24) Sugita T., Yoshikawa T., Gao J. Q., Shimokawa M., Oda A., Niwa T., Akashi M., Tsutsumi Y., Mayumi T., Nakagawa S., *Biol. Pharm. Bull.*, **28**, 192—193 (2005).
25) Yoshikawa T., Imazu S., Gao J. Q., Hayashi K., Tsuda Y., Shimokawa M., Sugita T., Niwa T., Oda A., Akashi M., Tsutsumi Y., Mayumi T., Nakagawa S., *Biochem. Biophys. Res. Commun.*, **325**, 500—505 (2004).
26) Ahmad-Nejad P., Hacker H., Rutz M., Bauer S., Vabulas R. M., Wagner H., *Eur. J. Immunol.*, **32**, 1958—1968 (2002).
27) Brichard V., Van Pel A., Wolfel T., Wolfel C., De Plaen E., Lethe B., Coulie P., Boon T., *J. Exp. Med.*, **178**, 489—495 (1993).
28) Lethe B., van den Eynde B., van Pel A., Corradin G., Boon T., *Eur. J. Immunol.*, **22**, 2283—2288 (1992).
29) Latz E., *et al.*, *Nat. Immunol.*, **5**, 190—198 (2004).

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．

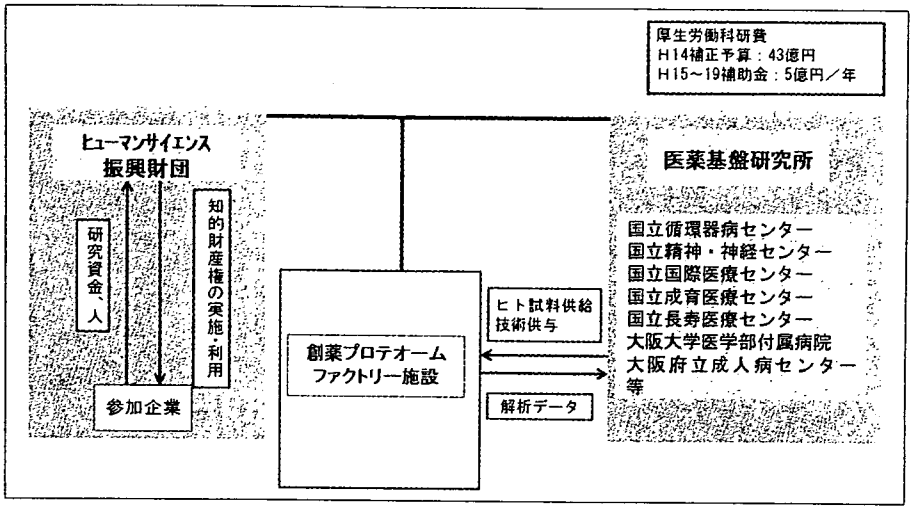

図2 「疾患関連たんぱく質解析研究・創薬プロテオームファクトリープロジェクト」概念図

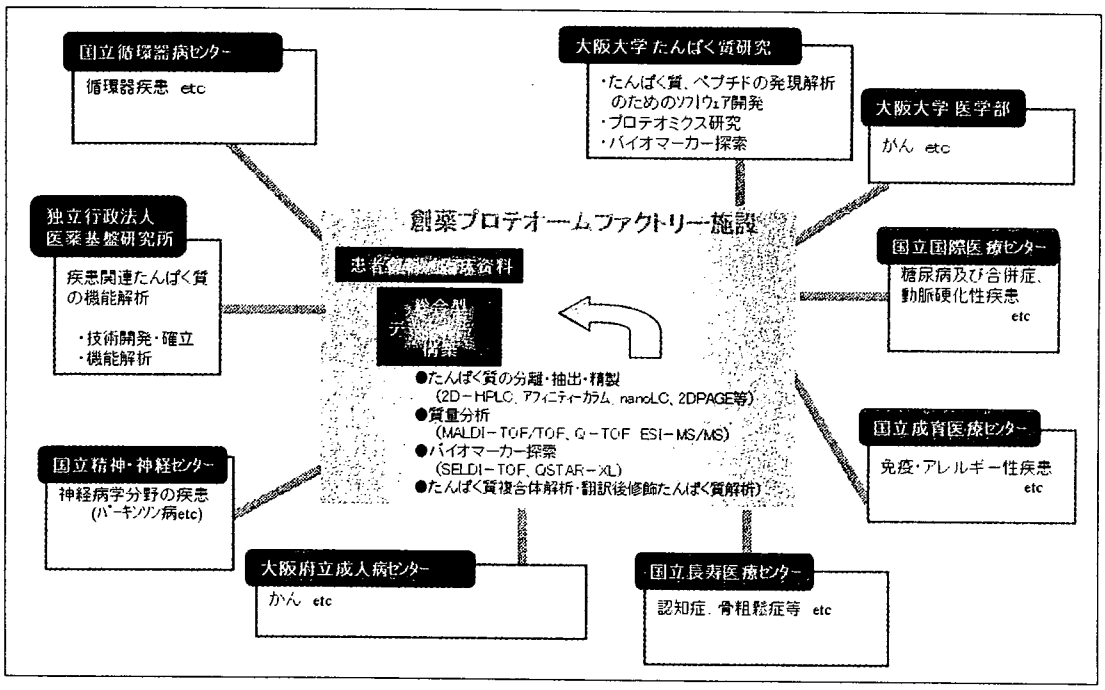

図3 協力機関と対象疾患

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

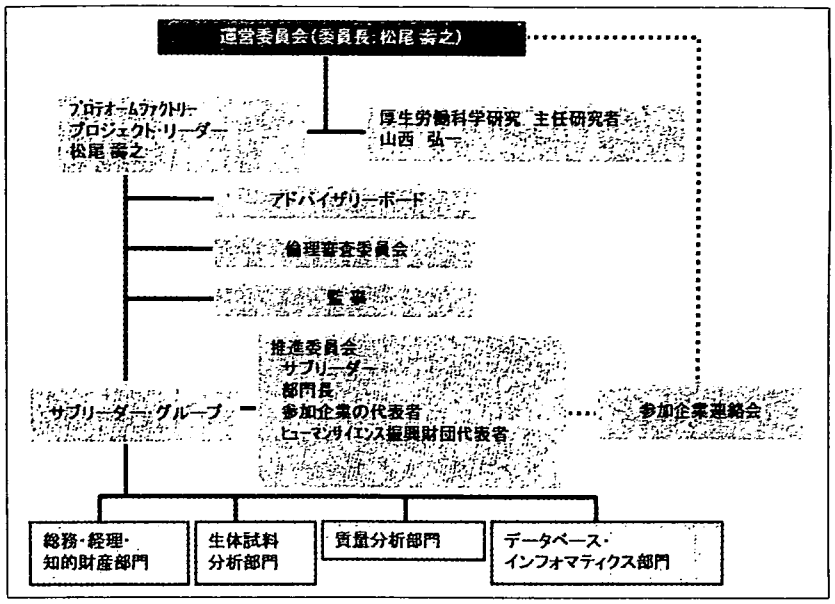

図4 組織体制

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

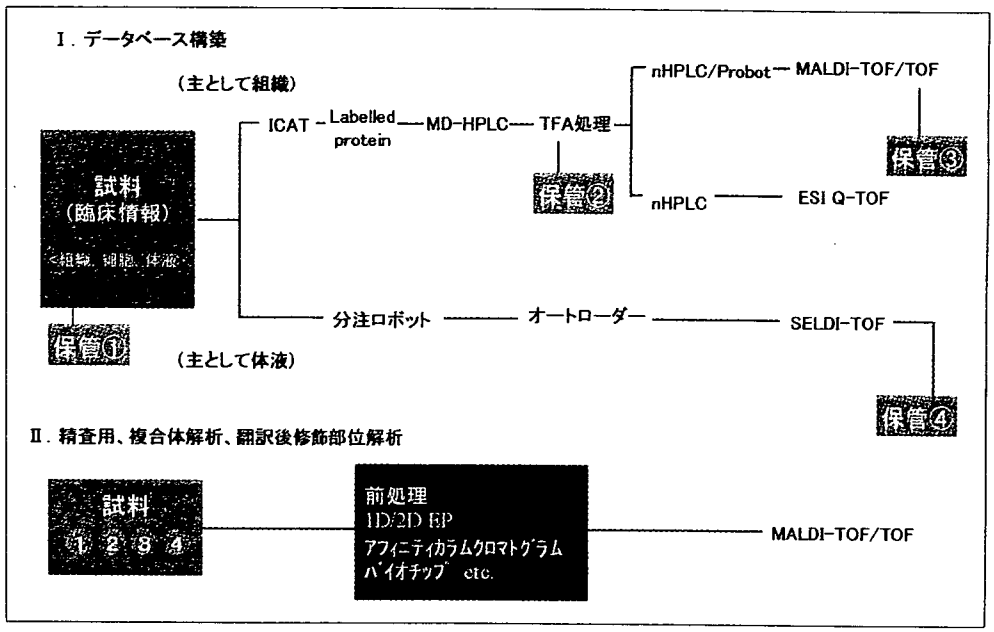

図5　ワークフロー

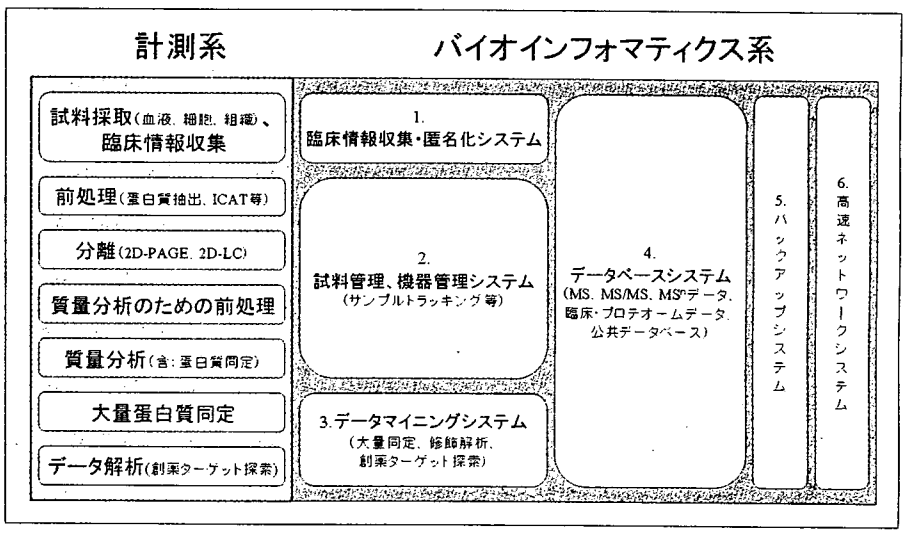

図6　バイオインフォマティクスの構成図

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．

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Deletion of Core Fucosylation on α3β1 Integrin Down-regulates Its Functions*

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The core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues. Recently α1,6-fucosylation has been further reported to be very crucial by the study of α1,6-fucosyltransferase (*Fut8*)-knock-out mice, which shows the phenotype of emphysema-like changes in the lung and severe growth retardation. In this study, we extensively investigated the effect of core fucosylation on α3β1 integrin and found for the first time that *Fut8* makes an important contribution to the functions of this integrin. The role of core fucosylation in α3β1 integrin-mediated events has been studied by using *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ embryonic fibroblasts, respectively. We found that the core fucosylation of α3β1 integrin, the major receptor for laminin 5, was abundant in *Fut8*$^{+/+}$ cells but was totally abolished in *Fut8*$^{-/-}$ cells, which was associated with the deficient migration mediated by α3β1 integrin in *Fut8*$^{-/-}$ cells. Moreover integrin-mediated cell signaling was reduced in *Fut8*$^{-/-}$ cells. The reintroduction of *Fut8* potentially restored laminin 5-induced migration and intracellular signaling. Collectively, these results suggested that core fucosylation is essential for the functions of α3β1 integrin.

α1,6-Fucosyltransferase (*Fut8*) catalyzes the transfer of a fucose residue from GDP-fucose to position 6 of the innermost GlcNAc residue of the hybrid and complex types of *N*-linked oligosaccharides on the glycoproteins (Fig. 1) (1). Core fucosylation (α1,6-fucosylation) of glycoprotein is widely distributed in mammalian tissues and altered under pathological conditions, such as hepatocellular carcinoma and liver cirrhosis (2, 3). A high expression of *Fut8* was observed in 33.3% of papillary carcinoma, and the incidence was directly linked to tumor size and lymph node metastasis, thus *Fut8* expression may be a key factor in the progression of thyroid papillary carcinomas (4). It has also been reported that the deletion of the core fucose from the IgG1 molecule enhances antibody-dependent cellular cytotoxicity activity by up to 50- to 100-fold. This indicates that the core fucose is an important sugar chain in terms of antibody-dependent cellular cytotoxicity activity (5). Recently, the physiological functions of the core fucose have been further investigated by our group using analysis of core fucose-deficient mice (6). The *Fut8*$^{-/-}$ mice showed severe growth retardation, and 70% died within 3 days after birth. The surviving mice suffered from emphysema-like changes in the lung that appear to be due to the lack of core fucosylation of transforming growth factor-β1 receptor, which consequently resulted in a marked dysregulation of transforming growth factor-β1 receptor activation and signaling. We also found that the loss of core fucosylation resulted in the down-regulation of EGF[3] receptor-mediated signaling pathway (7). These results together suggest that core fucose performs the important physiological functions through modification of some important functional proteins.

Cell-extracellular matrix (ECM) interactions play essential roles during the acquisition of migration and invasive behavior of the cells. The integrin family consists of α and β heterodimeric transmembrane receptors for ECM and connects many biological functions, such as development, the control of cell proliferation, protection against apoptosis, and malignant transformation (8). For example, α3β1 integrin, the major receptor for laminin 5 (LN5), is widely distributed in almost all tissues, and α3 knock-out mice have been reported to show the defects in kidney, lung, and skin (9). It has been reported that G-like repeats of LN5 constitute the favored ligand for α3β1 integrin, triggering haptotaxis (10). Especially, the G3 domain is essential for the unique activity of LN5, such as promotion of cell migration (11). Furthermore, α3β1 integrin has been proposed to be involved in tumor invasion (12, 13): the interaction

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[3] The abbreviations used are: EGF, epidermal growth factor; ECM, extracellular matrix; LN5, laminin 5; FN, fibronectin; COL, collagen; mAb, monoclonal antibody; PBS, phosphate-buffered saline; GnT-III, *N*-acetylglucosaminyltransferase III; GnT-V, *N*-acetylglucosaminyltransferase V; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase; AAL, *Aleuria aurantia* lectin; LC, liquid chromatography; MS, mass spectrometry; FT, Fourier transform; GM3, NeuAcα2,3Galβ1,4Glc-ceramide.

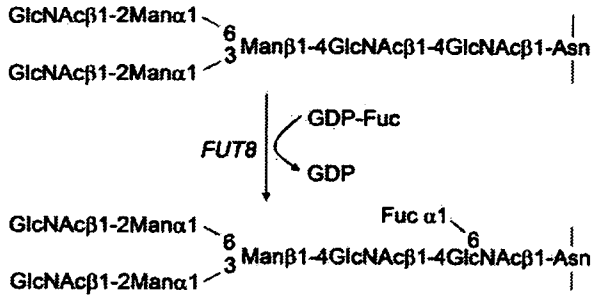

FIGURE 1. **Reaction pathway for the biosynthesis of core fucose by *Fut8*.** *Man*, mannose; *Fuc*, fucose; *GDP-Fuc*, guanosinediphospho-fucopyranoside; *Asn*, asparagine.

of α3β1 integrin with LN5 in exposed basement membrane provides both a molecular and a structural basis for cell arrest during pulmonary metastasis (14). In some malignant tumors, α3β1 integrin is found to be the most predominant integrin expressed (15), and cell invasion on ECM could be inhibited by antibodies against α3 integrin (13) and β1 integrin (14). Thus, α3β1 integrin, which mediates to laminins of basement membrane, preferentially promotes cell migration and metastasis (16–18). Given its various biological functions, α3β1 integrin, as one of most important extracellular adhesive molecules, deserves the more detailed investigation.

It has long been known that various factors can modulate integrin functions, including the status of glycosylation of integrin (19), the partnerships with tetraspanins, growth factor receptors (20–22), and the association with ganglioside GM3 (22), and others. Cell surface integrins are all major carriers of *N*-glycans, therefore *N*-glycosylation of integrins plays an important role in their biological functions (23). For example: the α3 and β1 subunits expressed by the metastasis human melanoma cell lines carry β1,6-branched structures, and these cancer-associated glycan chains may modulate tumor cell adhesion by affecting the ligand properties of α3β1 integrin (23). The linkage and expression levels of the terminal sialic acids of α3β1 integrin play an important role in cell-ECM interactions (24, 25). An increase in β1,6-GlcNAc sugar chains of β1 integrin resulted in the stimulation of cell migration and the organization of F-actin into extended microfilaments in cells plated on FN-coated plates (26). Moreover, a recent study has shown that introduction of bisecting GlcNAc into α5β1 integrin down-regulates cell adhesion and cell migration (27). These previous papers listed above have shown that the functions of integrins were positively or negatively regulated by *N*-glycans catalyzed by GnT-III, GnT-V, sialyltransferases, and others.

However, until now the effect of core fucosylation on integrin functions remains unclear. Here, we described studies comparing embryonic fibroblasts from wild-type and *Fut8*$^{-/-}$ mice to elucidate the role of core fucosylation in α3β1 integrin-stimulated events, and our finding for the first time showed that core fucosylation is required for the functions of α3β1 integrin.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—A polyclonal antibody against mouse α3 integrin and functional blocking monoclonal antibody (mAb) against α2β1 integrin were obtained from Chemicon International, Inc. (Temecula, CA). mAbs against α3 integrin, FAK, FAK (pY397), and functional blocking mAbs against integrin α6 and β1 subunits were from BD Transduction Laboratories (Lexington, KY). A polyclonal antibody against rabbit ERK1/2 and peroxidase-conjugated goat antibody against rabbit IgG were obtained from Cell Signaling (Beverly, MA). A mouse control IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A peroxidase-conjugated goat antibody against mouse IgG was obtained from Promega (Madison, WI), and biotinylated *Aleuria aurantia* lectin (AAL) was from Seikagaku Corp., Japan.

Cell Culture—*Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ mouse embryonic fibroblasts (MEFs) and restored cells were previously established in our laboratory (6). *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ embryonic fibroblasts and restored cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum in the presence of 400 μg/ml Zeocin, and restored cells were maintained in Dulbecco's modified Eagle's medium in the presence of 400 μg/ml Zeocin and 400 μg/ml hygromycin.

Western Blot and Lectin Blot Analysis—Cell cultures were harvested in lysis buffer (20 mM Tris-HCl, pH 7.4, 10 mM EGTA, 10 mM $MgCl_2$, 1 mM benzamidine, 60 mM β-glycerophosphate, 1 mM Na_3VO_4, 20 mM NaF, 2 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton X-100, 0.1 mM phenylmethylsulfonyl fluoride). Cell lysates were centrifuged at 15,000 × *g* for 10 min at 4 °C, the supernatants were collected, and the protein concentrations were determined using a BCA protein assay kit (Pierce). Proteins were then immunoprecipitated from the lysates using a combination of 2 μg of anti-α3 integrin antibody and Protein G-Sepharose beads. Immunoprecipitates were suspended in nonreducing buffer, heated to 100 °C for 3 min, resolved on 7.5% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell). The blots were then probed with anti-α3 integrin antibody and biotinylated AAL, respectively. Immunoreactive bands were visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and an ECL kit (Amersham Biosciences).

Cell Surface Biotinylation—Cell surface biotinylation was performed as described previously (28). Briefly, cells were rinsed twice with ice-cold PBS and then incubated with ice-cold PBS containing 0.2 mg/ml sulfosuccinimidobiotin (Pierce) for 2 h at 4 °C. After incubation, 50 mM Tris-HCl (pH 8.0) was used for the initial wash to quench any unreacted biotinylation reagent, and the cells were washed three times with ice-cold PBS and then solubilized in lysis buffer. The resulting cell lysate was then immunoprecipitated with the anti-α3 integrin antibody as described above. The biotinylated proteins were visualized using a Vectastain ABC kit and an ECL kit.

Migration Assay and Functional Blocking Assay—Transwells (BD Bioscience) were coated with 5 nM of recombinant LN5, as described previously (29), or 15 nM of human plasma FN, 50 μg/ml collagen I (COL, Sigma) in PBS by an overnight treatment at 4 °C followed by an incubation with 1% bovine serum albumin for 1 h at 37 °C. Serum-starved cells (2×10^5) per well in 500 μl of fetal calf serum-free medium were seeded in the upper compartment of the plates. After incubation for 3 h, the cells in the upper chamber of the filter were removed with a wet cotton swab. Cells on the lower side of the filter were fixed and

stained with 0.5% crystal violet. Each experiment was performed in triplicate, and counting was done in three randomly selected microscopic fields within each well. To identify which specific integrin mediates cell migration on LN5, monoclonal antibodies against different types of integrins at concentrations of 10 μg/ml were preincubated individually with fibroblasts for 10 min at 37 °C. Then cells were transferred into Transwells coated with LN5 and then incubated for 2 h 37 °C. The migrated cells were then quantified as described above.

Construction of Small Interference RNA Vector and Retroviral Infection—Small interfering oligonucleotides specific for integrin α3 subunit were designed on the Takara Bio website, and the oligonucleotide sequences used in the construction of the small interference RNA vector were as follows: 5′-GATCCGCTATGGAGAATCACACTGATTCAAGAGATCAGTGTGATTCTCCATAGCTTTTTTG-3′ and 5′-AATTCAAAAAAGCTATGGAGAATCACACTGATCTCTTGAATCAGTGTGATTCTCCATAGCG-3′. The oligonucleotides were annealed and then ligated into BamHI/EcoRI sites of the RNAi-Ready pSIREN-Retro Q vector (Takara Bio). A retroviral supernatant was obtained by transfection of human embryonic kidney 293 cells using a Retrovirus Packaging Kit Eco (Takara Bio) according to the manufacturer's protocol. Embryonic fibroblasts cells were infected with the viral supernatant, and the cells were then selected with 15 μg/ml puromycin for 2–3 weeks. Stable α3 integrin knockdown clones were therefore selected.

Tyrosine Phosphorylation Assay of FAK—Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK (pY397) antibody. Then the equal loading was confirmed by blotting with an antibody against total FAK.

Purification of α3β1 Integrin—The purification of α3β1 integrin was performed as described previously (30). Briefly, confluent cells were detached with TBS(+) (20 mM Tris-HCl, pH 7.5, 130 mM NaCl, 1 mM $CaCl_2$, and 1 mM $MgCl_2$) and washed with TBS(+). The cell pellets were extracted with 50 mM Tris/HCl containing 15 mM NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, pH 7.4, and protease inhibitor mixture (Roche Applied Science), 100 mM octyl-β-D-glucopyranoside at 4 °C. The cell extract was applied to an affinity column prepared by coupling 5 mg of GD6 peptide of laminin α1 chain (30) (KQNCLSSRASFRGCVRNLRLSR residues numbered 3011–3032, Peptide Institute, Inc., Osaka, Japan) to 1 ml of activated CH-Sepharose (Sigma). The bound α3β1 integrin was eluted with 20 mM EDTA in 50 mM Tris/HCl, pH 7.4, containing 100 mM octyl-β-D-glucopyranoside. The elutes containing α3β1 integrin were further purified on 1 ml of a wheat germ agglutinin-agarose column (Seikagaku Corp.) and eluted with 0.2 M *N*-acetyl-D-glucosamine containing 100 mM octyl-β-D-glucopyranoside. The purity of the integrin was verified by SDS-PAGE by means of a silver staining kit (Daichi Pure Chemicals Co., Ltd., Tokyo, Japan).

Analysis of N-Glycan Structure by Liquid Chromatography (LC/Tandem Mass Spectrometry (MS/MS))—Purified α3β1 integrin was applied to SDS-PAGE and excised from the gel then cut into pieces. The gel pieces were destained and dehydrated with 50% acetonitrile. The protein in the gel was reduced and carboxymethylated with dithiothreitol and monoiodoacetic acid according to the reports described by Kikuchi *et al.* (31) with some modifications. *N*-Glycans were released and extracted from the gel pieces as reported by Kustar *et al.* (32). The extracted oligosaccharides were reduced with $NaBH_4$. LC/MS was performed using a quadrupole liner ion trap-Fourier transform (FT) ion cyclotron resonance mass spectrometer (Finnigan LTQ FT™, Thermo Electron Corp., San Jose, CA) connected to a nanoLC system (Paradigm, Michrom BioResource, Inc., Auburn, CA). The eluents were 5 mM ammonium acetate, pH 9.6/2% CH_3CN (pump A), and 5 mM ammonium acetate, pH 9.6/80% CH_3CN (pump B). The borohydride-reduced *N*-linked oligosaccharides were separated on a Hypercarb (0.1 × 150 mm, Thermo Electron Corp.) with a linear gradient of 5–20% of B in 45 min and 20–50% of B in 45 min. FT-full MS scan (*m/z* 450–2000) followed by data-dependent MS/MS for the most abundant ions was performed in both negative and positive ion modes as described in the previous report (33).

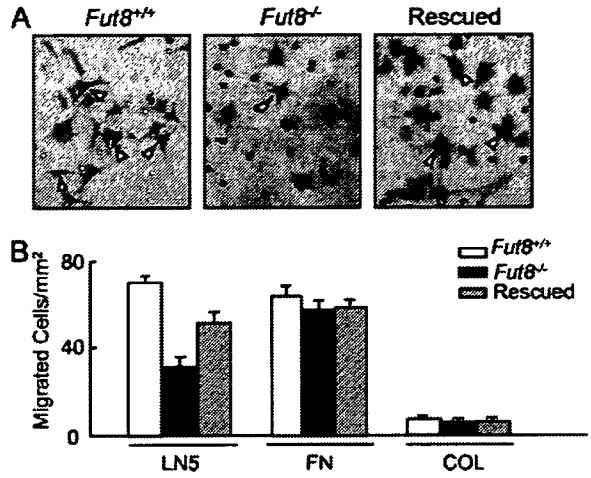

FIGURE 2. **Effects of deficient core fucosylation on cell migration on LN5 but not on FN.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were replated on the upper chamber coated with LN5 (5 nM), FN (15 nM), or 50 μg/ml COL. Cell migration was determined using the Transwell assay described under "Experimental Procedures." *A*, representative fields on LN5 were photographed using a phase-contrast microscope. The *arrowheads* indicate migrated cells. *B*, the numbers of migrated cells on LN5, FN, or COL were quantified and expressed as the means ± S.D. from three independent experiments.

RESULTS

Impaired α3β1 Integrin-mediated Cell Migration Was Found in Fut8$^{-/-}$ *Cells*—One of the major functions of α3β1 integrin is promotion of cell migration. In some malignant tumors, α3β1 integrin was found to be the most predominant integrin expressed (15), and it has made an important contribution to metastasis (14); therefore, cell motility on different ECMs was firstly examined by utilizing a Transwell assay. Cells were applied into the chambers, the bottoms of which had been coated with LN5, FN, or COL. As shown in Fig. 2 (*A* and *B*), *Fut8*$^{-/-}$ cells showed impaired migration on LN5 by a decrease to 44% relative to *Fut8*$^{+/+}$ cells. Consistently, reintroduction of *Fut8* partly restored cell migration by an increase in the percentage of migrating cells from 44% to 74%, indicating that core fucosylation is required for LN5-stimulated cell migration. But in the case of cell migration on FN, a specific ligand for α5β1

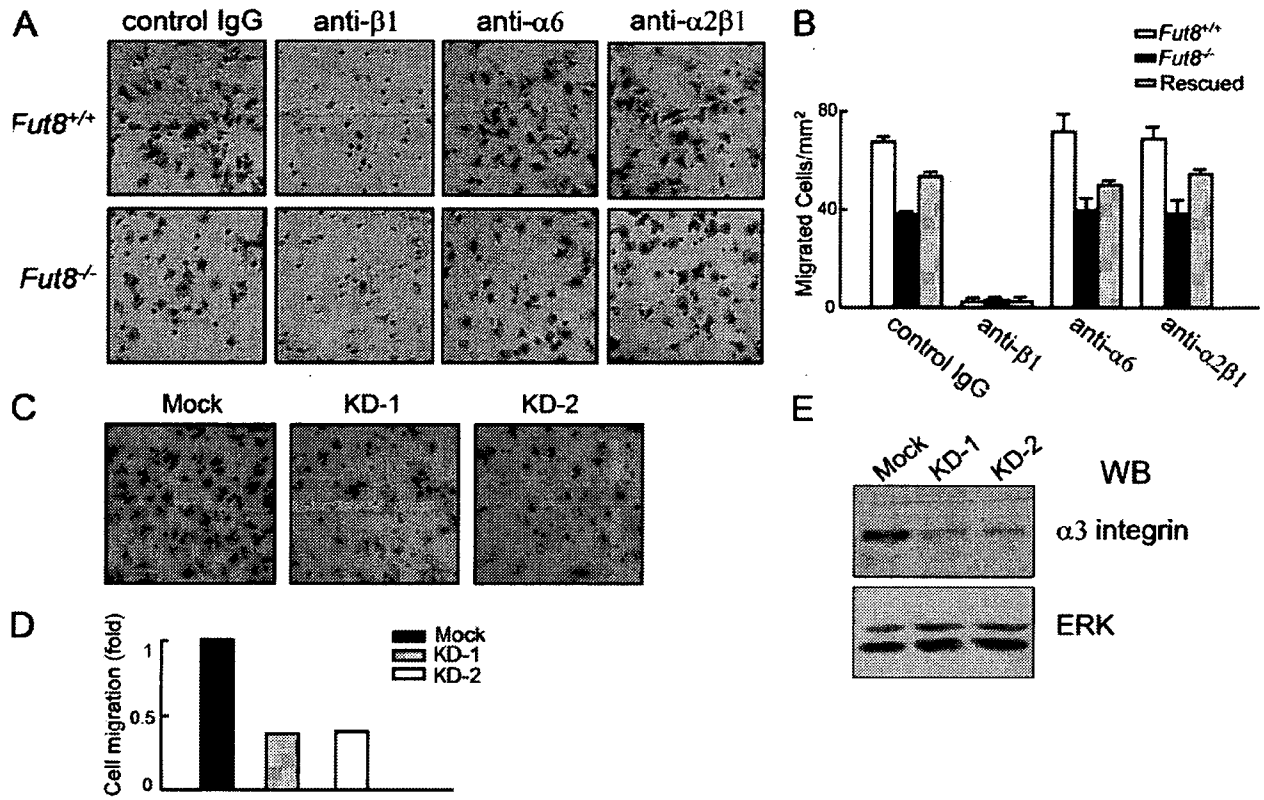

FIGURE 3. **Cell migration on LN5 was mediated by α3β1 integrin.** *A*, $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells were detached, preincubated with mouse control IgG or function-blocking mAbs against β1, α6, or α2β1 integrin for 10 min, replated on the upper chamber coated with LN5 (5 nM), and checked by Transwell assay. Representative fields were photographed using a phase-contract microscope. *B*, the numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *C*, cell migration of α3-knockdown cells on LN5 (5 nM). Representative fields were photographed using a phase-contrast microscope. *Arrowheads* indicate migrated cells. *D*, quantification of migration of mock and α3-knockdown cells. The numbers of migrated cells were quantified and expressed as the means ± S.D. from three independent experiments. *E*, α3-knockdown was confirmed by blotting total cell lysates with anti-α3 antibody (*upper panel*), and equal loading was confirmed by probing with an antibody against total protein ERK1/2 (*lower panel*). *KD1* and *KD2*, α3-knockdown cells.

integrin, the obvious difference among $Fut8^{+/+}$, $Fut8^{-/-}$, and rescued cells was not found. In addition, the motility of these three types of cells on COL, a ligand for α1β1 and α2β1 integrins, was barely detectable (Fig. 2*B*). This suggested that α5β1, α1β1 and α2β1 integrins, unlike receptor of laminin 5, may be not strongly affected by *Fut8*. So MEF cells may favor LN5 as an ECM for cell migration. Furthermore, the cell migration on LN5 was completely blocked by the presence of function-blocking antibodies against β1 but not by α6 or α2β1 integrin antibodies (Fig. 3, *A* and *B*), further excluding the involvement of α6 and α2β1 integrin on LN5-stimulated cell migration. However, so far the function-blocking antibody against mouse α3 is unavailable. To definitely confirm the important function of integrin α3 subunit for the cell migration on LN5, we utilized an RNA interference strategy to silence α3 in MEF cells. After retroviral infection, the cells were selected based on their resistance to puromycin as described under "Experimental Procedures." Expression of α3 but not α5 (data not shown), or other proteins such as ERK, was effectively down-regulated, compared with those in mock cells (Fig. 3*E*). We then tested cell migration on LN5 and found that α3-knockdown resulted in a significant decreased cell migration compared with mock cells (Fig. 3, *C* and *D*). Together with the data in Fig. 3 (*A* and *B*), these results provided the evidence that in this study the cell migration on LN5 was mediated by α3β1 integrin. This result was consistent with the view of previous study that α3β1 integrin is distinct from other integrins and preferentially promotes cell migration (16). The result above was also supported by the previous observation that LN5 as well as LN10/11 promoted cell migration is mainly mediated by α3β1 integrin, but not α6β1 or α6β4 integrins (34). However, we cannot definitely exclude the involvement of syndecan-1 and -4, because it has been reported to have an interaction with LN5 (35, 36); therefore, they might regulate integrin functions in an indirect way. Collectively, these results suggested that α3β1 integrin is a key molecule for cell migration on LN5 in the embryonic fibroblasts and that core fucosylation regulates α3β1 integrin-mediated cell migration.

Integrin-stimulated Phosphorylation of FAK Was Reduced in $Fut8^{-/-}$ Cells—ECM-integrin signaling events are prominently involved in regulating cell migration (16). In particular, the protein-tyrosine kinase FAK plays a prominent role in integrin signaling (37–39). To address the effects of *Fut8* on α3β1 integrin-mediated signaling, we examined FAK phosphorylation in adherent cells on LN5. As shown in Fig. 4, the level of tyrosine phosphorylation was reduced in the $Fut8^{-/-}$ cells compared with $Fut8^{+/+}$ cells, moreover the down-regulation of phosphorylation in $Fut8^{-/-}$ cells was restored in the rescued cells, suggesting that deficient core fucosylation was able to neg-

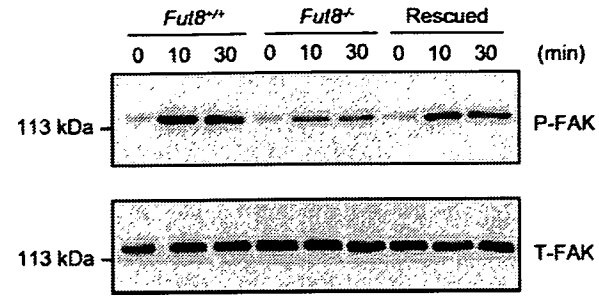

FIGURE 4. **Comparison of tyrosine phosphorylation levels of FAK among *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ and rescued cells on LN5.** Serum-starved cells were detached and held in suspension for 60 min to reduce the detachment-induced activation and then replated on dishes coated with LN5 (5 nM) for the indicated times, and the cell lysates were blotted with anti-phosphotyrosine FAK antibody to detect the amount of phosphorylation. Then the equal loading was confirmed with an antibody against total protein FAK.

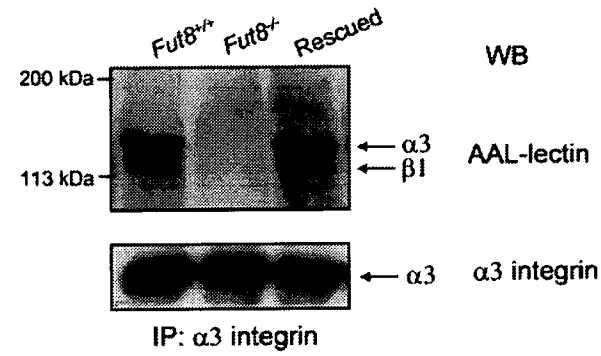

FIGURE 5. **Glycosylation analysis of α3β1 integrin from *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells.** Whole cell lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, and the resulting immunocomplexes were subjected to 7.5% SDS-PAGE under nonreducing condition. After electroblotting, the blots were probed, respectively, by AAL (*upper panel*) and an anti-α3 integrin antibody (*lower panel*).

atively regulate α3β1 integrin-mediated signaling pathway. Considerable evidence implicates FAK in the regulation of cell migration. Most notably, FAK-deficient cells exhibit poor migration ability in response to chemotactic and haptotactic migration (40, 41). Therefore, based on such evidence we suggested that the deficient signal transduction may account for the deficient cell migration on LN5 in *Fut8*$^{-/-}$ cells.

Expression of α3β1 Integrin on the Cell Surface Was Not Influenced by Fut8—Some important glycosyltransferases have been reported to modify and further regulate the functions of integrins by modulating the status of glycosylation on them such as GnT-III and GnT-V; however, there is no such data so far to show the relation of *Fut8* and integrins. Therefore, in Fig. 5, the fucosylation on α3β1 integrin among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells has been examined by using blotting of α3 integrin-immunoprecipitated lysates with AAL lectin (*upper panel*). Equal loadings were verified by blotting with α3 integrin antibodies (*lower panel*). As shown in Fig. 5, the levels of core fucosylation in both α3 and β1 subunits were abolished in *Fut8*$^{-/-}$ cells consistent with no *Fut8* activity in these cells (7), whereas they were rescued by reintroduction of *Fut8*, suggesting α3β1 integrin is the target of *Fut8*. Furthermore, the effect of deficiency of core fucosylation on the expression of α3β1 integrin on the cell surface was also determined, because *N*-glycosylation plays an important role in the quality control of the expression of glycoproteins. The biotinylation of cell surface proteins followed by immunoprecipitation of α3 integrin was examined by blotted. As shown in Fig. 6, the expression levels of α3β1 integrin on the cell surface remained unchanged among *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells, indicating that the expression of α3β1 integrin on cell surface was not influenced by *Fut8*. Collectively, we suggested that the deficiency of core fucosylation resulted in the malfunctions of α3β1 integrin but not its expression level.

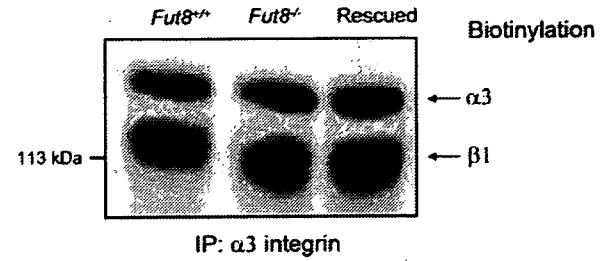

FIGURE 6. **Effects of core fucosylation on expression levels of α3β1 integrin on cell surface.** *Fut8*$^{+/+}$, *Fut8*$^{-/-}$, and rescued cells were biotinylated, whole lysates were immunoprecipitated (*IP*) with anti-α3 integrin antibody, the samples were subjected to 7.5% SDS-PAGE and transferred to a nitrocellulose membrane, and the biotinylated proteins were then detected as described under "Experimental Procedures."

Purified α3β1 Integrin, Rich in Core Fucosylation, Was Shown by LC/MS/MS—The analysis of glycan structural alteration in glycoproteins is becoming increasingly important in the discovery of therapies and diagnostic markers (42). To better understand the detailed modification of *Fut8* for α3β1 integrin, we purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells by using a GD6 peptide affinity column combined with a wheat germ agglutinin affinity column. The purity was evaluated by SDS-PAGE followed by silver staining. Two major bands, migrating at 150 and 110 kDa on SDS-PAGE under nonreducing conditions (Fig. 7*A*, *inset, right panel*), corresponding to the immunoreactivity with the anti-α3 and anti-β1 antibodies, were detected, respectively (data not shown). Then we analyzed *N*-glycan profiles of purified α3β1 integrin by LC/MS and LC/MS/MS. The profiles of the *N*-linked oligosaccharides extracted from purified α3β1 integrin of *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$, respectively, are shown in Fig. 7*A*. They were obtained by full MS scan (*m/z* 450–2000) in the negative ion mode. The FT MS spectra of the *peaks 1–7* (from *Fut8*$^{+/+}$) and *peaks 1′–7′* (from *Fut8*$^{-/-}$) are shown in Fig. 7*B*, respectively. The structures of carbohydrates in these peaks could be deduced from the *m/z* values of protonated ions obtained by FT MS and data-dependent MS/MS spectra. The oligosaccharides released from α3β1 integrin of *Fut8*$^{+/+}$ (*peaks 1–7*) were assigned to fucosylated complex and hybrid type oligosaccharides, whereas those released from α3β1 integrin of *Fut8*$^{-/-}$ (*peaks 1′–7′*) were nonfucosylated forms. The data correspond to that of AAL lectin blot, revealing that α3β1 integrin derived from *Fut8*$^{+/+}$ is highly modified by *Fut8* and suggesting loss of core fucosylation will result in the deficiency of α3β1 integrin function.

DISCUSSION

The physiological importance of fucose modification on proteins has been highlighted by the description of human congen-

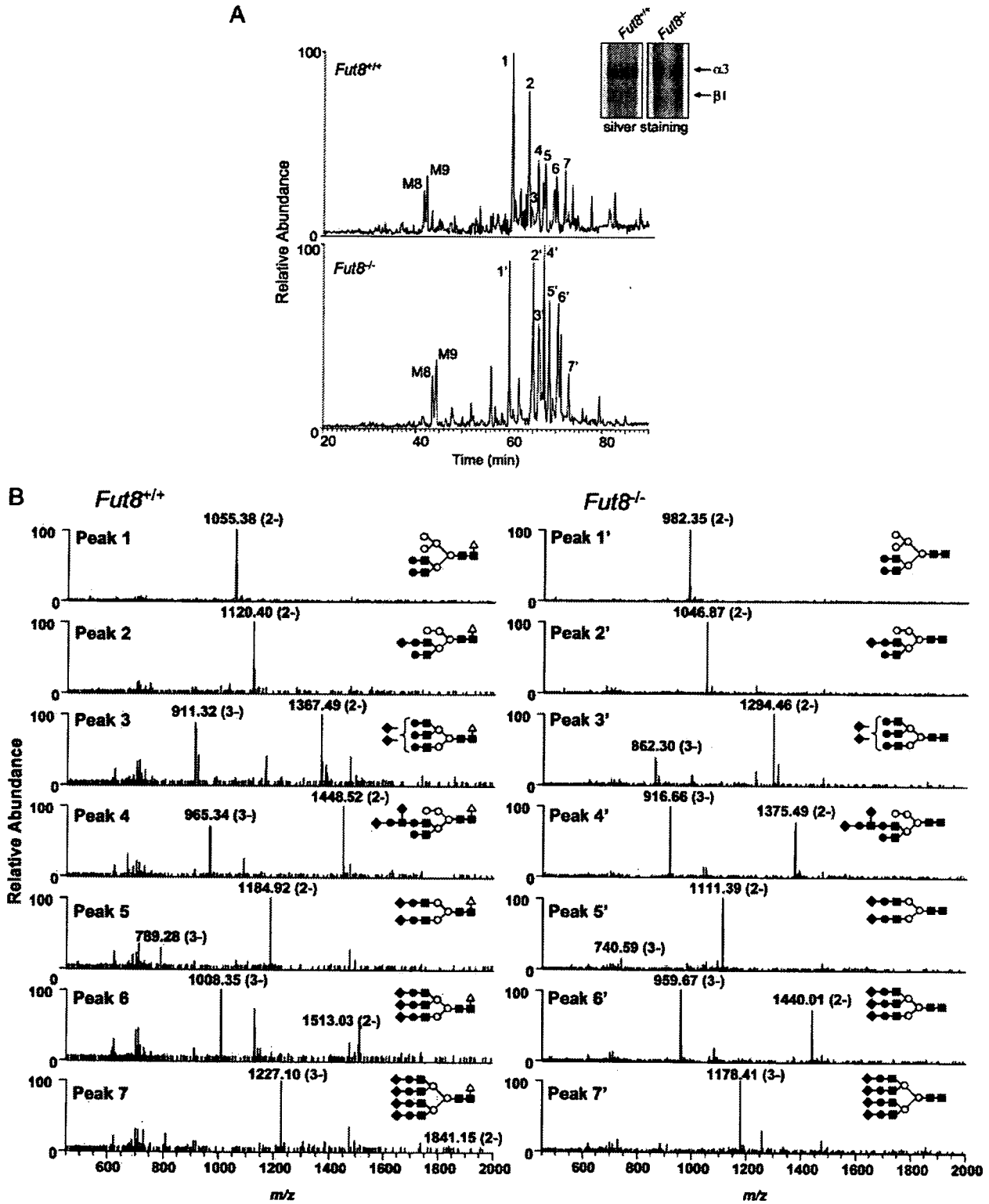

FIGURE 7. **Chromatograms of *N*-linked oligosaccharides extracted from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells.** In *A*: MS, full MS scan (*m/z* 450–2000) in the negative ion mode. LC, pump A, 5 mM ammonium acetate, pH 9.6/2% CH_3CN; Pump B, 5 mM ammonium acetate, pH 9.6/80% CH_3CN; column, hypercarb (0.1 × 150 mm); gradient, 5–20% of B (0–45 min) and 20–50% of B (45–90 min). The purity of α3β1 integrin was verified by silver staining under nonreducing condition as shown in the *right panel* of the *inset*. *B*, FT MS spectra of *N*-glycans from purified α3β1 integrin from *Fut8*$^{+/+}$ and *Fut8*$^{-/-}$ cells. *Peaks 1–7* in *Fut8*$^{+/+}$ cells, *peaks 1′–7′* in *Fut8*$^{-/-}$ cells, and carbohydrate compositions assigned by *m/z* values of protonated ions and MS/MS spectra. △, fucose; ●, galactose; ○, mannose; ■, *N*-acetylglucosamine; ♦, *N*-acetylneuraminic acid.