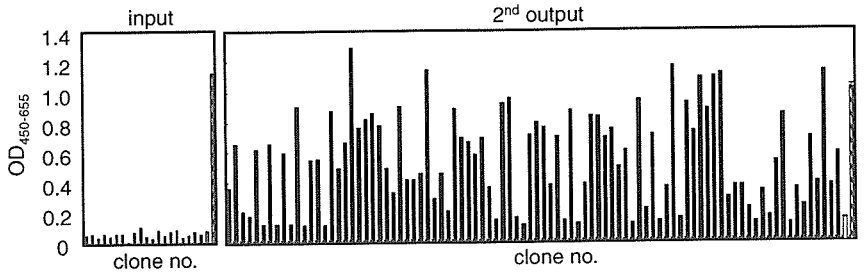

Fig. 4. Affinities of monoclonal phages for anti-TNF-α antibody. Ninety clones were selected randomly from input or second output phage clones and their affinities for the antibody were estimated by phage ELISA. Phage clone expressing TNF-α was used as a positive control (striped column), and IFN-α was used as a negative control (open column).

	1 10 20 30 40
TNF-α:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRRANALLANG
Clone 16/19/26/72:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRP
Clone 20:	LVVANPQAEGQLQWLNRQ
Clone 21:	YVVANPQAEGQLQWLNRRD
Clone 22/47/51:	YVVANPQAEGQLQWLNRP
Clone 30/36/70:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNQ
Clone 34:	HVVANPQAEGQLQWLNRRE
Clone 35:	NVVANPQAEGQLQWLNRRE
Clone 38:	YVVANPQAEGQLQWLNRH
Clone 42:	VRSSSRTPSDKPVAHVVANPQAEGQLQWLNRP
Clone 46:	VHVVANPQAEGQLQWLNRRE
Clone 49:	LVVANPQAEGQLQWLNRRD
Clone 57:	TAHVVANPQAEGQLQWLNRRG
Clone 61:	HFVANPQAEGQLQWLNRQR
Clone 66:	LVVANPQAEGQLQWLNRR
Clone 68/82:	HVVANPQAEGQLQWLNRRE
Clone 71:	HVVANPQAEGQLQWLNHQ
Clone 86:	FRSSSRTPSDKPVAHVVANPQAEGQLQWLNRL
Clone 88:	FVVANPQAEGQLQWLNRK

Fig. 5. Amino acid alignment of peptides presented by phage clones bound to anti-TNF-α antibody. The amino sequences of fragments which strongly bound to the anti-TNF-α antibody in Fig. 4 and their sequence alignment with TNF-α are shown.

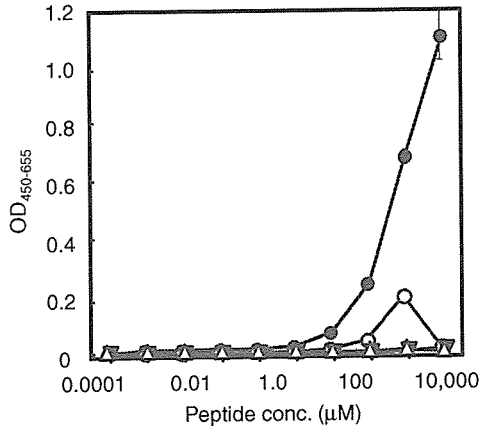

Fig. 6. Binding analysis of synthetic peptide to anti-TNF-α antibody by ELISA. Biotinylated epitope peptide (HVVANPQAEGQLQWLNRRA :●) and biotinylated control peptides (Mab1-peptide; EKGDRLSAEIN:▼), Mab4-peptide (NALLANGVELRD:△), and 3D6-peptide (AEGQLQWL NRRA:○) were applied to solid-phase anti-TNF-α antibody. Binding peptides were detected by avidin-HRP.

antibody by ELISA. Although control peptides did not bind to anti-TNF-α antibody, this synthetic peptide containing TNF-α fragment peptide dose-dependently bound to the antibody. These results indicated that the displayed peptides on the phage surface behaved similarly to free peptides and amino acids 15–33 were actually epitope of the antibody (Fig. 6).

Discussion

In this study, we improved the method for construction of gene fragment phage library and applied this library to epitope mapping. Although gene fragment libraries have been expected to be superior in availability [18,19], they are constructed from cDNA fragments generated by digestion with a non-specific endonuclease, resulting in blunt-end ligation (very low efficiency) and contamination of reversely oriented fragments [12–14]. Thus it is inefficient to identify functional peptides and epitope peptides from gene fragment library constructed by this conventional method. Therefore, focusing on unidirectionality of reverse transcription reaction, we created gene fragments using reverse transcription following transcription of mRNA by T7 RNA polymerase (Fig. 1). This process made it possible to insert gene fragments retaining proper orientation into phagemid vector and ligate each protruding ends.

Additionally, using FLAG tag selection which removed phage clones causing frame shifts and expressing nonspecific peptides, we successfully ameliorated the method of gene fragment library to be superior in quality and diversity (Fig. 2). However, gene fragments from domain 2 and 3 tended to be deflected to the 5′ end of template. We think this problem will be resolved by appropriately changing a temperature of annealing in RT-PCR for each template. In consideration of this point, we are now constructing gene fragment library of some virus envelope proteins for searching functional peptides.

To assess the availability of our strategy, we tried epitope mapping of anti-TNF-α antibody from TNF-α fragment library. After selection of anti-TNF-α antibody, all amino acid sequences of peptides which strongly bound to the antibody contained amino acids 15–33 sequence of TNF-α (Fig. 5). There are very few reports to confirm that selected phage clones almost encode convergent sequence like this. We predicted there are two reasons: rabbit anti-human TNF-α antibody is easy to recognize the epitope containing an amino acid sequence that differs between human and rabbit TNF-α; and TNF-α fragment library constructed in this study is of dramatically higher quality and diversity than conventional phage libraries. In fact, residues 20–32 of TNF-α have low homology among species and the peptides obtained after the panning contained residues 20, 22, 30, and 31, residues which differ between human and rabbit TNF-α [20]; thus, the peptide was recognized as an epitopic region. Additionally, compared to random peptide library, phage clones bound to the antibody were quite efficiently concentrated from our TNF-α fragment library (Fig. 3). Our system provided a useful strategy for comprehensively searching and identifying functional peptides from various proteins, such as cytokines, extracellular matrix, and coat proteins of viruses. This novel method is likely to be useful for the development of pharmaceuticals, targeting peptides, molecular biological tools, and vaccines.

Acknowledgments

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References

[1] E. Ruoslahti, M.D. Pierschbacher, New perspectives in cell adhesion: RGD and integrins, Science 238 (1987) 491–497.
[2] S.R. Schwarze, A. Ho, A. Vocero-Akbani, S.F. Dowdy, In vivo protein transduction: delivery of a biologically active protein into the mouse, Science 285 (1999) 1569–1572.
[3] M.S. O'Reilly, L. Holmgren, Y. Shing, C. Chen, R.A. Rosenthal, M. Moses, W.S. Lane, Y. Cao, E.H. Sage, J. Folkman, Angiostat: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma, Cell 79 (1994) 315–328.
[4] M.S. O'Reilly, T. Boehm, Y. Shing, N. Fukai, G. Vasios, W.S. Lane, E. Flynn, J.R. Birkhead, B.R. Olsen, J. Folkman, Endostat: an endogenous inhibitor of angiogenesis and tumor growth, Cell 88 (1997) 277–285.
[5] D.R. Stanworth, V.M. Jones, I.V. Lewin, S. Nayyar, Allergy treatment with a peptide vaccine, Lancet 336 (1990) 1279–1281.
[6] S. Matsueda, H. Takedatsu, A. Yao, M. Tanaka, M. Noguchi, K. Itoh, M. Harada, Identification of peptide vaccine candidates for prostate cancer patients with HLA-A3 supertype alleles, Clin. Cancer Res. 11 (2005) 6933–6943.
[7] Y. Oka, A. Tsuboi, T. Taguchi, T. Osaki, T. Kyo, H. Nakajima, O.A. Elisseeva, Y. Oji, M. Kawakami, K. Ikegame, N. Hosen, S. Yoshihara, F. Wu, F. Fujiki, M. Murakami, T. Masuda, S. Nishida, T. Shirakata, S. Nakatsuka, A. Sasaki, K. Udaka, H. Dohy, K. Aozasa, S. Noguchi, I. Kawase, H. Sugiyama, Induction of WT1 (Wilms' tumor gene)-specific cytotoxic T lymphocytes by WT1 peptide vaccine and the resultant cancer regression, Proc. Natl. Acad. Sci. USA 101 (2004) 13885–13890.
[8] I. Haro, S. Perez, M. Garcia, W.C. Chan, G. Ercilla, Liposome entrapment and immunogenic studies of a synthetic lipophilic multiple antigenic peptide bearing VP1 and VP3 domains of the hepatitis A virus: a robust method for vaccine design, FEBS Lett. 540 (2003) 133–140.
[9] T. Matsubara, Y. Hiura, O. Kawahito, M. Yasuzawa, K. Kawashiro, Selection of novel structural zinc sites from a random peptide library, FEBS Lett. 555 (2003) 317–321.
[10] T. Matsubara, D. Ishikawa, T. Taki, Y. Okahata, T. Sato, Selection of ganglioside GM1-binding peptides by using a phage library, FEBS Lett. 456 (1999) 253–256.
[11] J.J. Devlin, L.C. Panganiban, P.E. Devlin, Random peptide libraries: a source of specific protein binding molecules, Science 249 (1990) 404–406.
[12] L. Bentley, J. Fehrsen, F. Jordaan, H. Huismans, D.H. du Plessis, Identification of antigenic regions on VP2 of African horsesickness virus serotype 3 by using phage-displayed epitope libraries, J. Gen. Virol. 81 (2000) 993–1000.
[13] A. Holzem, J.M. Nahring, R. Fischer, Rapid identification of a tobacco mosaic virus epitope by using a coat protein gene-fragment-pVIII fusion library, J. Gen. Virol. 82 (2001) 9–15.
[14] L.F. Wang, D.H. Du Plessis, J.R. White, A.D. Hyatt, B.T. Eaton, Use of a gene-targeted phage display random epitope library to map an antigenic determinant on the bluetongue virus outer capsid protein VP5, J. Immunol. Methods 178 (1995) 1–12.
[15] T. Okamoto, Y. Mukai, Y. Yoshioka, H. Shibata, M. Kawamura, Y. Yamamoto, S. Nakagawa, H. Kamada, T. Hayakawa, T. Mayumi, Y. Tsutsumi, Optimal construction of non-immune scFv phage display libraries from mouse bone marrow and spleen established to select specific scFvs efficiently binding to antigen, Biochem. Biophys. Res. Commun. 323 (2004) 583–591.
[16] T. Nishi, R.J. Budde, J.S. McMurray, N.U. Obeyesekere, N. Safdar, V.A. Levin, H. Saya, Tight-binding inhibitory sequences against pp60(c-src) identified using a random 15-amino-acid peptide library, FEBS Lett. 399 (1996) 237–240.
[17] C. Schaffitzel, J. Hanes, L. Jermutus, A. Pluckthun, Ribosome display: an in vitro method for selection and evolution of antibodies from libraries, J. Immunol. Methods 231 (1999) 119–135.
[18] M.B. Irving, O. Pan, J.K. Scott, Random-peptide libraries and antigen-fragment libraries for epitope mapping and the development of vaccines and diagnostics, Curr. Opin. Chem. Biol. 5 (2001) 314–324.

[19] F. Fack, B. Hugle-Dorr, D. Song, I. Queitsch, G. Petersen, E.K. Bautz, Epitope mapping by phage display: random versus gene-fragment libraries, J. Immunol. Methods 206 (1997) 43–52.

[20] J. Yamagishi, H. Kawashima, N. Matsuo, M. Ohue, M. Yamayoshi, T. Fukui, H. Kotani, R. Furuta, K. Nakano, M. Yamada, Mutational analysis of structure–activity relationships in human tumor necrosis factor-alpha, Protein Eng. 3 (1990) 713–719.

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Optimization of anti-tumor necrosis factor-α single chain Fv displayed on phages for creation of functional antibodies

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In this study, we converted the immunoglobulin-type anti-human tumor necrosis factor-α (TNF-α) monoclonal antibody (Mab) to a scFv-type antibody in order to assess its basic properties. The immunoglobulin VH and VL genes were isolated from the hybridoma that produced an anti-TNF-α neutralizing Mab, and they were then linked together to create scFvs of the VL-VH or VH-VL-form. The binding affinity to TNF-α was retained in both scFvs. Interestingly, the VL-VH-type scFv effectively inhibited the TNF-α-mediated cytotoxicity, while this neutralization activity was dramatically decreased in the VH-VL-type scFv. These results suggest that the VL-VH-type scFv is a suitable template to create improved versions of the anti-TNF-α antibody using a phage display system, and they also show that the structural format must be taken into account in manufacturing scFvs.

Recently, targeting therapy and cytokine neutralization therapy using Mabs such as trastuzumab and infliximab have been applied to treat cancer and various inflammatory diseases. However, there are several issues that should be resolved in order to establish effective Mab-based therapies for various diseases. The first issue is that the molecular weight of the immunoglobulin (Ig)-form of a Mab (approximately 150 kDa) is too high to allow it to diffuse and reach target antigens that exist deep in the tissue (Batra et al. 2002). The second issue is that it is difficult to carry out large-scale preparations of antibodies using mammalian cell culture. A single chain Fv (scFv) is a genetically engineered antibody manufactured by conjugating the VH and VL domains of immunoglobulin with a flexible linker sequence. Due to their small molecular size (approximately 25 kDa), scFvs can diffuse into tissues more efficiently than immunoglobulin-form antibodies (Batra et al. 2002). In addition, the generation and large scale production of genetically modified scFvs such as humanized antibodies and immunotoxins are relatively easy, so clinical applications of scFvs and their modified versions are highly anticipated (Chen et al. 2005; Onda et al. 2004). However, because the binding affinity and *in vivo* stability of scFvs are generally lower than those of immunoglobulin-form Mabs, frequent administrations and high dosages are necessary for clinical efficacy. Therefore, improvements in the binding affinity and stability of scFvs are important challenges for research.

Recently, attention has been focused on the phage display system as a method to construct protein libraries having huge diversity on the phage surface (Clackson et al. 1991; Kolonin et al. 2004; Smith 1985). Using this method, peptides or protein libraries can be intensively explored to identify high-affinity targets. In particular, phage display scFv antibody libraries allow researchers to isolate antibodies to various antigens *in vitro* to produce antibodies modified by genetic engineering (Ho et al. 2005).

We report here the conversion of an anti-human TNF-α neutralizing antibody to the scFv-form using a phage display system. The VL and VH genes were isolated from a hybridoma producing an anti-TNF-α neutralizing antibody. The scFv-form antibodies were prepared as both VL-VH and VH-VL types connected by a flexible linker peptide. It is necessary to determine whether or not the binding and neutralizing activities of scFvs are maintained similar to the parent immunoglobulin type. We compared the properties of the VL-VH and VH-VL types of anti-TNF-α scFvs and found unexpected differences in their binding properties.

The scFv-type antibodies have been utilized in many research fields to date. Two types of scFvs, VL-VH and VH-VL, may be prepared, but there is not any kind of standard formula to decide which type of construct is more suitable for a given application. Because we could not determine in advance which type would be more suitable for our application, we investigated the binding and neutralizing activities of both VL-VH and VH-VL type scFvs.

In the M13 phage display system, scFv molecules are expressed as fusions with the N-terminal region of the gene III protein (g3p), which is a phage minor coat protein. The C-terminus of the VH or VL is connected with g3p in the VL-VH-type or VH-VL-type scFv, respectively. It is generally assumed that connecting of scFvs to g3p will not affect the scFv function, because X-ray crystallography has revealed that the C terminal region of the V domain is localized far from the antigen-binding site (Kaufmann et al. 2002). In fact, we were able to confirm that both types of phage-displayed anti-TNF-α scFvs bound to TNF-α in a dose dependent manner, with only small differences in their binding properties (Fig. 1).

Effective anti-TNF-α neutralization therapy requires that the antibody has both high binding affinity to TNF-α and TNF-α neutralizing activity. We constructed anti-TNF-α scFvs from the hybridoma producing the neutralizing antibody, and examined whether the scFvs maintained their TNF-α neutralizing activity. We prepared the soluble forms of the C-terminal FLAG-tagged anti-TNF-α scFvs from the supernatants of *E. coli* HB2151 and estimated their inhibitory effect on TNF-α-mediated cytotoxicity towards L-M cells (Fig. 2). Interestingly, neutralizing activity against TNF-α-mediated cytotoxicity was observed only for VL-VH-type scFvs, while VH-VL-type scFvs showed no inhibitory effect. The concentrations of anti-TNF-α scFvs in the culture supernatants were shown to be comparable by ELISA using TNF-α for the solid phase

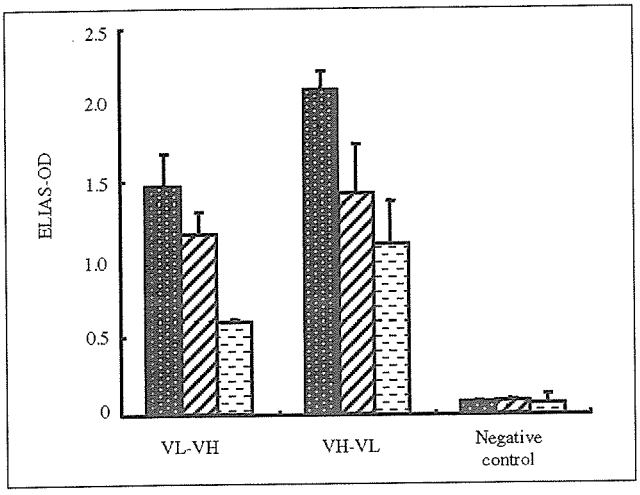

Fig. 1: Binding activities of anti-TNF-α scFvs. Phages displaying anti-TNF-α scFvs were added to immobilized TNF-α, and their binding activities were detected by an anti-M13 HRP conjugate. ■; 3×10^{10} CFU, ▨; 6×10^{9} CFU, ▩; 1.2×10^{9} CFU. The negative control phage displayed an anti-CD25 scFv. This experiment was performed three times and each value is given as the Mean ± SD

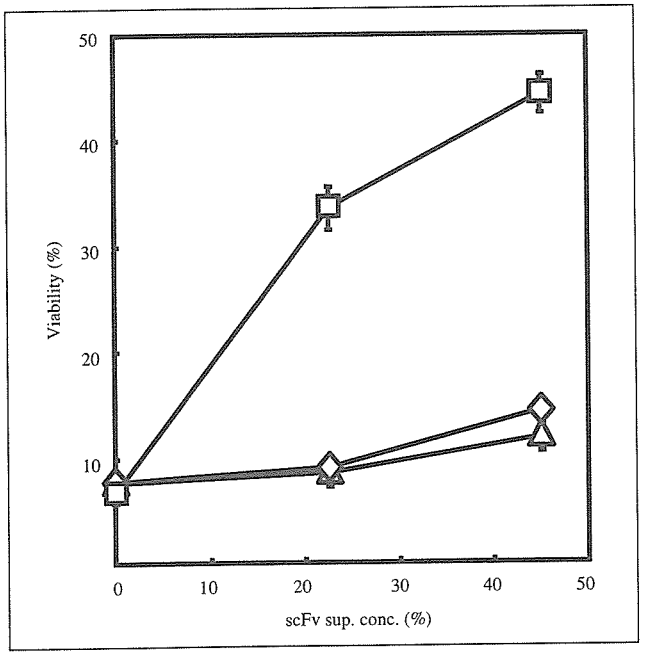

Fig. 2: Neutralizing activities of anti-TNF scFv constructs. Neutralization activities were measured using an inhibition assay against the L-M cell line in the presence of TNF-α and actinomycin D. L-M cells were seeded in a 96-well tissue culture plate at 5×10^{4} cells/well. Anti-TNF-α scFvs were produced in *E. coli* HB21 51 supernatant following the protocol of Recombinant Phage Antibody System (GE Healthcare Bio-Sciences Corp.). They were incubated with 0.05ng of TNF-α for 30min at 37 °C and then added to the L-M cells. After 24 h incubation, cellular viability was measured by a methylene blue assay. □; VL-VH scFv, ◇; VH-VL scFv, △; anti-CD25 scFv. This experiment was performed three times and each value is given as the Mean ± SD

and an anti-FLAG antibody for detection (data not shown). Therefore, the loss of neutralizing activity in the VH-VL-type scFvs suggested that some conformational changes occurred upon conversion of the immunoglobulin-form antibody to the VH-VL-type scFv. Antibodies bind to antigens at complementarity determining regions (CDRs) formed by both the VL and VH domains. Recently, it was reported that some antibody clones were able to recognize their antigens through either the VL or VH alone (Tanaka et al. 2003). Therefore, it is possible that in this case, although both scFv types were able to bind TNF-α, the VH-VL type scFv may have undergone a slight conformational change so that it no longer blocked binding of the scFv-TNF-α complex to the TNF-α receptor, and therefore it lost its neutralizing activity. These results indicate that even if the antibodies used are Mabs from hybridomas whose binding properties have been confirmed, it is necessary to determine the properties again after converting from the immunoglobulin-type to the scFv type.

We concluded that the VL-VH type TNF-α scFv was a suitable antibody for functional modification using the phage display system because both the binding affinity and neutralizing activity of the original immunoglobulin were maintained. Though scFvs are generally thought to have lower binding affinities, a current report (Ho et al. 2005) indicates that affinities may be improved using the phage display system. We have previously established that this methodology that can be used to create functional mutant proteins such as cytokines with modified functions (Yamamoto et al. 2003). Furthermore, the *in vivo* stabilities of cytokines were improved using this technique (Shibata et al. 2004). We expect that various antibody therapies will be developed by applying this type of methodology to production of scFv antibodies in the future.

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References

Batra SK, Jain M, Wittel UA, Chauhan SC, Colcher D (2002) Pharmacokinetics and biodistribution of genetically engineered antibodies. Curr Opin Biotechnol 13: 603–608.

Chen LH, Huang Q, Wan L, Zeng LY, Li SF, Li YP, Lu XF, Cheng JQ (2005) Expression, purification, and in vitro refolding of a humanized single-chain Fv antibody against human CTLA4 (CD1 52). Protein Expr Purif 46: 495–502.

Clackson T, Hoogenboom HR, Griffiths AD, Winter G (1991) Making antibody fragments using phage display libraries. Nature 352: 624–628.

Ho M, Kreitman RJ, Onda M, Pastan I (2005) *In vitro* antibody evolution targeting germline hot spots to increase activity of an anti-CD22 immunotoxin. J Biol Chem 280: 607–617.

Kaufmann M, Lindner P, Honegger A, Blank K, Tschopp M, Capitani G, Pluckthun A, Grutter MG (2002) Crystal structure of the anti-His tag antibody 3D5 single-chain fragment complexed to its antigen. J Mol Biol 318: 135–147.

Kolonin MG, Saha PK, Chan L, Pasqualini R, Arap W (2004) Reversal of obesity by targeted ablation of adipose tissue. Nat Med 10: 625–632.

Onda M, Wang QC, Guo HF, Cheung NK, Pastan I (2004) *In vitro* and *in vivo* cytotoxic activities of recombinant immunotoxin 8H9(Fv)-PE38 against breast cancer, osteosarcoma, and neuroblastoma. Cancer Res 64: 1419–1424.

Shibata H, Yoshioka Y, Ikemizu S, Kobayashi K, Yamamoto Y, Mukai Y, Okamoto T, Taniai M, Kawamura M, Abe Y, Nakagawa S, Hayakawa T, Nagata S, Yamagata Y, Mayumi T, Kamada H, Tsutsumi Y (2004) Functionalization of tumor necrosis factor-alpha using phage display technique and PEGylation improves its antitumor therapeutic window. Clin Cancer Res 10: 8293–8300.

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. Science 228: 1315–1317.

Tanaka T, Lobato MN, Rabbitts TH (2003) Single domain intracellular antibodies: a minimal fragment for direct *in vivo* selection of antigen-specific intrabodies. J Mol Biol 331: 1109–1120.

Yamamoto Y, Tsutsumi Y, Yoshioka Y, Nishibata T, Kobayashi K, Okamoto T, Mukai Y, Shimizu T, Nakagawa S, Nagata S, Mayumi T (2003) Site-specific PEGylation of a lysine-deficient TNF-alpha with full bioactivity. Nat Biotechnol 21: 546–552.

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.

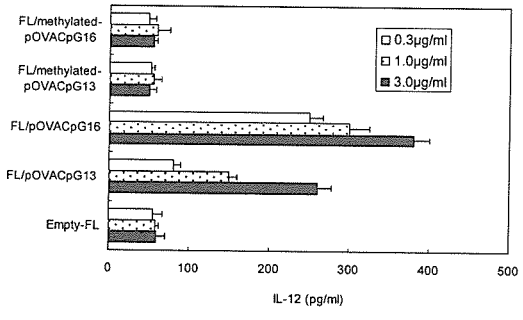

Fig. 1. CpG Enhanced Vector (pOVACpG16) Containing FL Hold Immunostimulatory Effects

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.

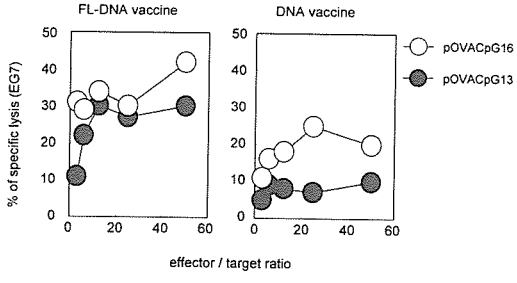

Fig. 3. OVA Specific CTL Response after *in Vivo* Priming with CpG Enhanced FL-DNA Vaccine

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.

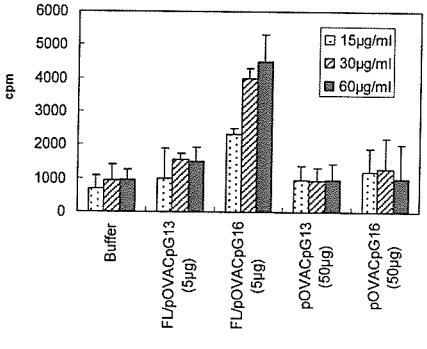

Fig. 2. OVA Specific T Cell Proliferation Derived from Mice Splenocytes Immunized with CpG Enhanced FL-DNA Vaccine

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.

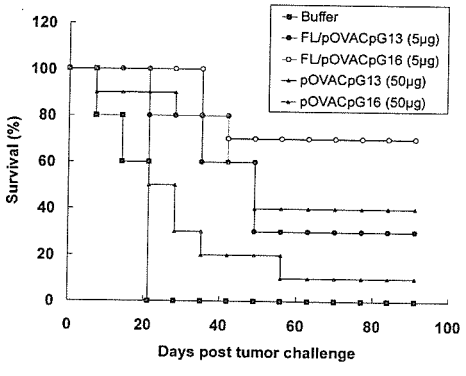

Fig. 4. Survival Analysis of Mice Immunized with DNA-Fusogenic Liposome Vaccine in a Prophylactic Treatment Model

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).

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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:

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.

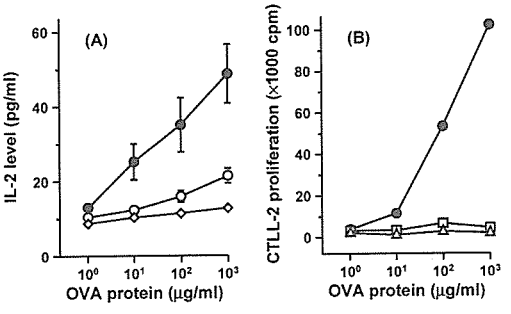

Fig. 1. Antigen Delivery into MHC Class I-Restricted Presentation Pathway on DC2.4 Cells by FLs

(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-

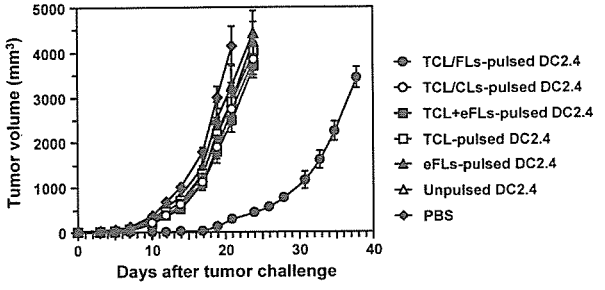

Fig. 2. TCL/FLs-Pulsed DC2.4 Cells-Mediated Prophylactic Effect against B16BL6 Tumor Challenge

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.

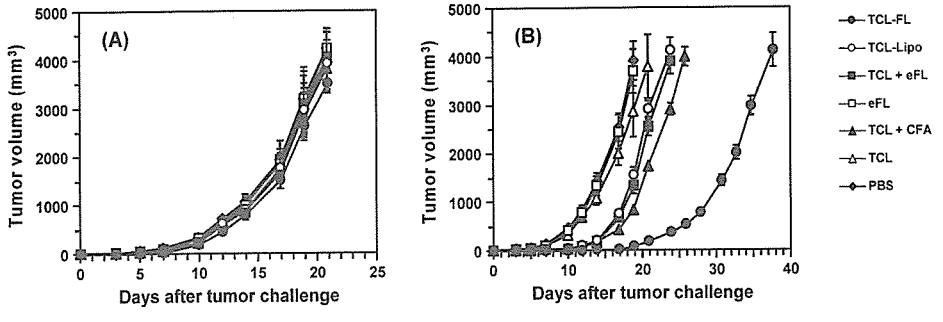

Fig. 3. Inhibitory Effect against B16BL6 Tumor Growth by *in Vivo* Direct CL/FLs-Immunization

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).

Promotion of Optimized Protein Therapy by Bioconjugation as a Polymeric DDS

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Abstract: In recent years, clinical applications of recombinantly produced bioactive proteins such as cytokines have attracted attention. However, since these recombinant proteins are rather unstable *in vivo*, their clinical use as therapeutic agents requires frequent administration at a high dosage. This regimen disrupts homeostasis and results in severe side effects. To overcome these problems, bioactive proteins have been conjugated with water-soluble synthetic (WSS) polymeric carriers. Chemical modification of a protein with a WSS polymeric carrier (bioconjugation) regulates tissue distribution, resulting in a selective increase in its desirable therapeutic effects and a decrease in undesirable side effects. Among several drug delivery system (DDS) technologies, bioconjugation has been recognized as one of the most efficient methods for improving therapeutic potency of proteins. However, for further enhancement of the therapeutic potency and safety of conjugated bioactive proteins, more precise regulation of the *in vivo* behavior of each protein is necessary for selective expression of its therapeutic effect. Therefore, alternative WSS polymeric modifiers in which new functions such as targeting and controlled release of drugs can be added are required for further development of bioconjugated drugs. Recently, we have synthesized a novel polymeric drug carrier, poly(vinylpyrrolidone-co-dimethyl maleic anhydride) [PVD], which was a powerful candidate drug carrier for cancer therapy. In this review, we introduce useful information that enabled us to design polymeric drug carriers and their application for protein therapy.

Key Words: Polyethylene glycol (PEG), bioconjugation, drug delivery system (DDS), polyvinylpyrrolidone (PVP), poly (vinylpyrrolidone-co-dimethyl maleic anhydride) [PVD], cancer therapy.

INTRODUCTION

In the postgenomic era, life sciences research has shifted focus from genome analyses to the functional analyses of genes and their products (proteins), and recently, there have been dramatic advances in pharmacoproteomics. The functions of numerous proteins will be clarified due to the recent advances in structural genomics. Thus, the therapeutic application of bioactive proteins such as newly identified proteins and cytokines has been highly anticipated [1-6]. However, the clinical application of most of these proteins is limited because of their unexpectedly low therapeutic effects. The reason behind this limitation is that these proteins are immediately degraded by various proteases *in vivo* and are rapidly excreted from blood circulation [1,6-9]. Therefore, frequent administration at an excessively high dose is required for manifestation of their therapeutic effects *in vivo*. This however disrupts homeostasis and leads to unexpected side effects.

Recently, to overcome the problems associated with the clinical application of bioactive proteins, attachment of water-soluble synthetic (WSS) polymers such as polyethylene glycol (PEG) to the surface of these proteins has been developed (Fig. **1**). The covalent conjugation of proteins with PEG is specifically referred to as PEGylation. Bioconjugation of proteins decreases their renal excretion rate due to the increased molecular size. In addition, since the WSS polymers cover the protein surface, attack by proteases is blocked by steric hindrance, resulting in prolongation of the *in vivo* half-life. A similar steric effect results in a decrease in the antigenicity and immunogenicity in an immune response, resulting in prolongation of *in vivo* clearance and stabilization. All these advantages lead to an increase in *in vivo* stability, and this in turn, enables a decrease in the dose as well as time of administration.

In fact, PEGylated granulocyte-colony stimulating factor (PEG-G-CSF; PEG filgrastim) , PEGylated interferon-alpha (PEG-IFN-α; PEGASYS, PEG-Intron), PEGylated asparaginase (PEG-Asp; ONCASPER), PEGylated adenosine deaminase (PEG-ADA; ADAGEN) and Poly(stylene-co-maleic acid)-conjugated neocarzinostatin (SMANCS) have demonstrated a marked improvement in therapeutic efficacy in comparison with the native forms, and their clinical applications have already been reported [10-17]. SMANCS dissolved in lipiodol exhibits a marked antitumor effect when used in chemotherapy for targeting hepatomas [13,14]. Furthermore, studies have demonstrated the efficacy of PEGylated Interleukin-2 (PEG-IL-2) in the treatment of patients with metastatic melanoma and carcinoma [18-20]. These results suggested that bioconjugation with a WSS polymeric carrier is a very pragmatic approach for successful therapies with various drugs such as enzymes and antitumor agents. In

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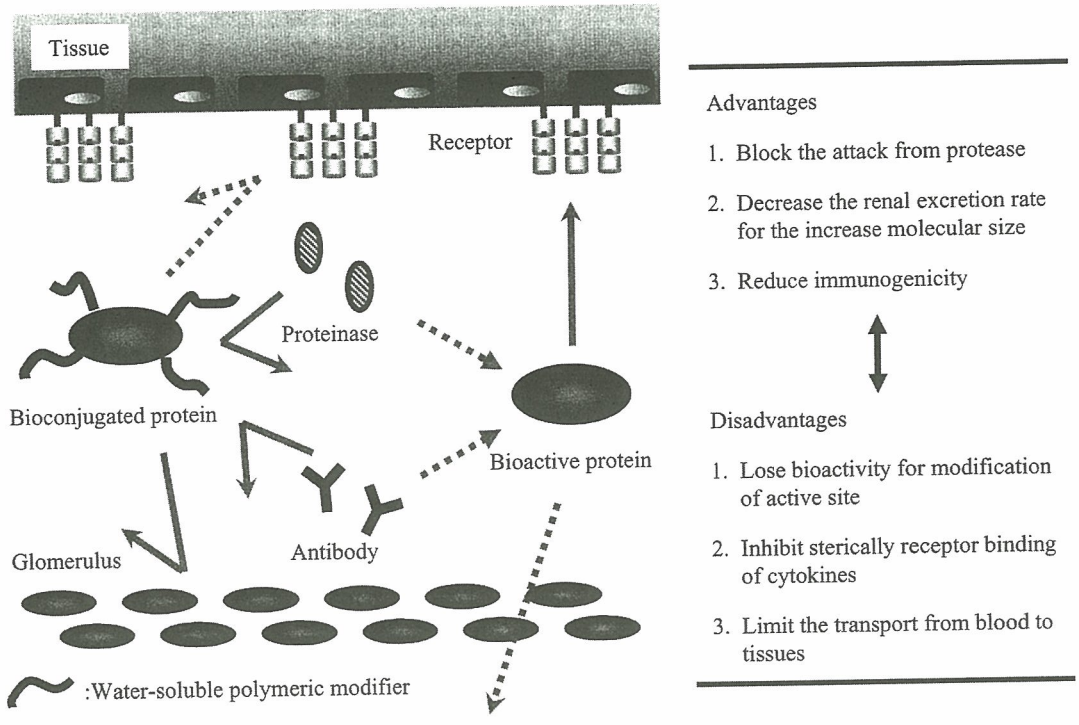

Fig. (1). Characteristics of bioconjugation.

the future, bioconjugated drugs with a WSS polymeric carrier should be more extensive clinical application.

However, with the exception of a few examples, the clinical use of bioconjugated proteins remains limited. This is due to the conflicting effects of polymer conjugation of bioactive proteins; conjugation with a polymeric modifier inhibits the transport from blood to tissues and the binding to their receptors. In addition, specific activities of proteins are decreased by the attachment of polymeric modifiers to active sites. Therefore, it is important to select an optimal modifying molecule based on its suitability for bioconjugation and the properties of individual bioactive proteins and accumulate basic data such as the details of the relationship among molecular weight, modification rate, and activity of the modified protein. Additionally, we must consider the optimal polymer-conjugation conditions to (*a*) increase plasma half-life and stability, (*b*) control behavior in the body (well-balanced tissue transport), and (*c*) selectively enhance desirable therapeutic activities of bioactive proteins without increasing their side effects.

In this review, we first show the fundamental information enabling us to design the bioconjugated bioactive proteins applicable to therapeutic use. Next we discussed the novel polymeric carries with desired DDS functions such as targeting capability and controlled release of drugs for cancer therapy.

NONIONIC WSS POLYMERIC CARRIERS THAT ARE SUITABLE FOR IMPROVEMENT IN BLOOD RESIDENCY OF DRUGS

The fate and distribution of the conjugates between WSS polymeric carriers and drugs (including proteins as drugs) can be attributed to the physicochemical properties of polymeric modifiers, such as molecular weight, electric charge, and hydrophilic balance [21-23]. Therefore, selecting a polymeric modifier by considering the influence of physicochemical characteristics on its pharmacokinetics is extremely important. PEG is a WSS polymeric modifier with low toxicity and antigenicity, and it has been frequently used for bioconjugation. However, PEG also has some disadvantages as a drug carrier—primarily the fact that PEG has a functional group only at the end of the chain, which limits the possibilities of adding new functions to the drugs for a more precise control of their pharmacokinetics and tissue distribution. From this viewpoint, modifiable polymeric modifiers are required to control the biopharmaceutical characteristics of conjugated drugs. In view of this, in a study on mice bearing solid tumors, we focused on nonionic WSS polymers and assessed the pharmacokinetic properties of various polymeric modifiers that could be modified by changes in their physicochemical properties.

The polymer formulations that were used were PEG, polyvinylpyrrolidone (PVP), polyacrylamide (PAAm), polydimethylacrylamide (PDAAm), polyvinyl alcohol (PVA), and dextran. PVP, PAAm, and PDAAm were functionalized by the introduction of various co-monomers during radical polymerization. PVA and dextran have many primary OH groups that can be used for conjugation on the side chain. Mice were intravenously injected with various ^{125}I-labeled WSS polymers of the same molecular size (MW: 5000) for studying the elimination profiles of these polymers (Fig. **2**). All polymers showed biphasic elimination patterns. PEG and dextran, which are used frequently as drug carriers, were rapidly eliminated from blood circulation. On the other hand, PVA and PVP circulated for a longer period than the other

polymers, while these nonionic polymers had the same molecular size as PEG. PVP exhibited the longest plasma half-life among all the nonionic WSS polymers studied, and 25% of the injected dose remained in the body after 3 h. Pharmacokinetic analysis revealed definite differences among the polymers with respect to plasma clearance and tissue distribution. PVP showed the longest mean residence time (MRT) among all polymers examined. The total clearance of PVP was approximately 10-fold lower than that of PEG. The distribution volume of dextran was the highest among all these polymers; its volume was twice that of PVP. In this study, although all the polymers had the same molecular weight dispersity and were nonionic and water soluble, each polymer showed a characteristic distribution. PVP had the longest circulation time, and its tissue distribution was extremely restricted. In addition, PVP could be easily mixed with various functional groups by radical polymerization in order to control its physicochemical properties and to add functions such as targeting or sustained release. These results suggest that PVP is one of the most feasible polymeric modifiers for localizing conjugated drugs in blood. In fact, PVP-conjugated tumor necrosis factor-alpha (TNF-α) showed a higher half-life than PEG-conjugated TNF-α despite having the same molecular size [24].

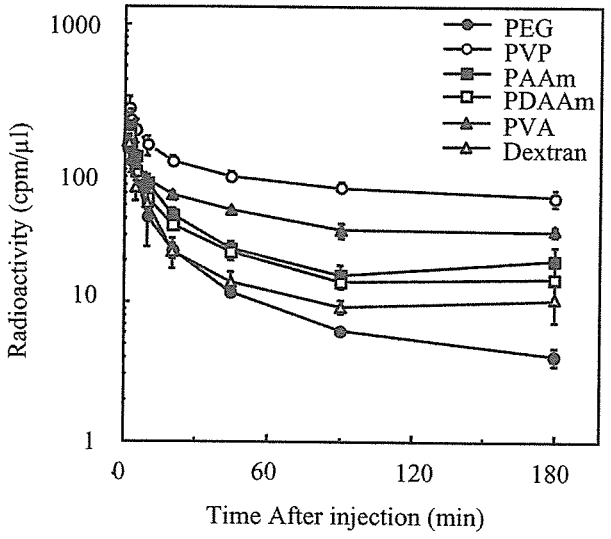

Fig. (2). Plasma clearace of various WSS polymers in mice after intravenous injection [24].

Here we show, using TNF-α as examples, the possibility that PVP-conjugated proteins overcomes their drawbacks, allowing their clinical application. The antitumor effects of TNF-α result not only from its direct cytotoxic action against various tumor cells, but also from activation of antitumor effector immune cells in the blood and specific damage to the tumor vessels. In addition, in the process of bleeding necrosis in the tumor vessels, the vascular permeability of the tumor vessels is selectively increased, promoting transport from blood to the tumor tissue. On the other hand, the increase in blood-residency would lead to a decrease in the distribution of TNF-α in the liver and spleen, which are the major sources of unfavorable side-effects. Therefore, improvement in blood stasis enhances all antitumor action mechanisms of TNF-α increasing its bioavailablity. As a result, PVP-conjugated TNF-α had a more potent antitumor effect than PEG-conjugated TNF-α, without any toxic side effects [25,26]. This phenomenon has also been observed in PVP-conjugated interleukin-6 (IL-6) and leukemia inhibitory factor [27].

DEVELOPMENT OF TISSUE-TARGETING POLYMERIC CARRIERS

It has been reported that bioconjugation of TNF-α and IL-6 with PEG and PVP improved their resistance to proteinase and their plasma half-lives, thereby resulting in greater therapeutic potency [24-31]. However, for further enhancement of therapeutic potency and safety of conjugated bioactive proteins, a more precise control of the *in vivo* behavior of each protein is necessary. Thus, the development of novel WSS polymeric carriers with targeting capability to specific tissue is expected; PEG and PVP are useful and powerful polymeric carriers for improving plasma half-lives. Using PVP as a backbone polymer, we have evaluated the *in vivo* pharmacokinetics of synthesized PVP derivatives with various electric charges or hydrophilic-hydrophobic balance. For example, hydrophobic PVP derivatives that contain vinyl laurate and stylene accumulated in the spleen and liver, respectively, after intravenous injection [32]. In contrast, anionized PVP derivatives were retained in the blood or accumulated in the kidneys (Fig. **3**). Carboxyl PVP, which contained an optimal amount of acrylate, distributed to the kidneys but over-carboxylation resulted in excretion through urine [33]; the same pattern was observed with sulfonated PVP. The *in vitro* cytotoxicity of carboxylated PVP against renal tubular cells was low, and its renal targeting capacity was better than that of other carriers. Anionic polyaspartamides are transiently distributed in the kidneys and are rapidly excreted in the urine [34]. However, we found that these anionic polymers were unsuitable as renal targeting carriers because conjugates composed of these anionic polymers and the drug did not accumulate in sufficient quantities to produce therapeutic effects.

Based on a series of research studies, we have synthesized a novel polymeric drug carrier, namely, poly(vinylpyrrolidone-co-dimethyl maleic anhydride) [PVD], by radical copolymerization and mixed the reactive comonomers [dimethyl maleic anhydride (DMMAn) and vinylpyrrolidone (VP)] in a ratio of 1:5 (Fig. **4**). We found that approximately 80% of the dose of PVD (1:5) selectively accumulated in the kidneys 24 h after intravenous injection. Although the PVD (1:5) that was accumulated in the kidneys was gradually excreted in the urine, approximately 40% was retained 96 h after the treatment was commenced [35]. The high renal accumulation and retention of PVD (1:5) makes it a more useful targeting carrier than other agents. Although most anionized polymers are safer than cationized polymers, they exhibit cytotoxicity at high doses. Poly(VP-co-MAn), which has the same molecular size, polydispersity, and carboxyl group content as PVD (1:5), produced cytotoxicity in LLC-MK2 cells at higher concentrations (Fig. **5**). In contrast, PVD (1:5) produced no evidence of pathological effects in mice at a dose of 10 mg/day for 28 days. A subcutaneous dose of 50 mg PVD (1:5) was well tolerated by mice. The safety of PVD (1:5) appears to be similar to that of PEG and PVP,

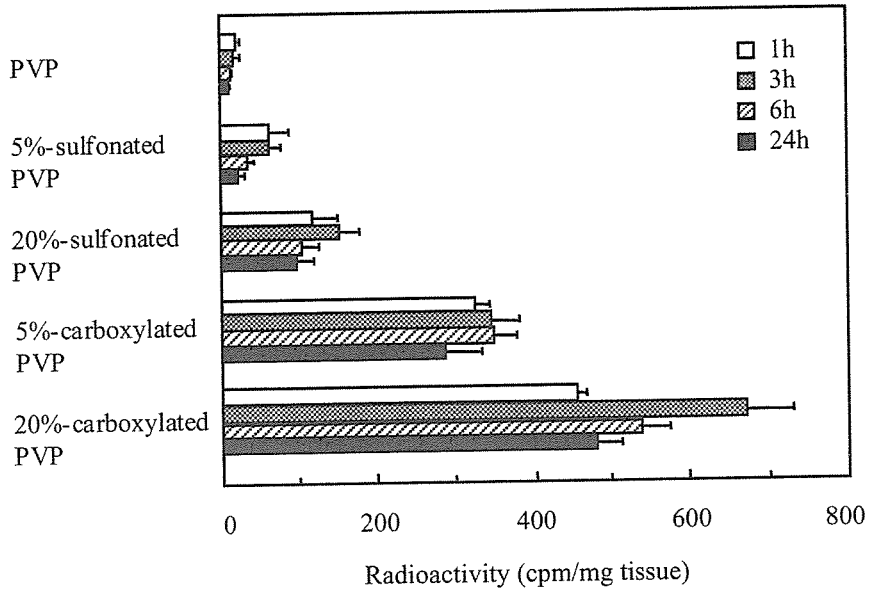

Fig. (3). Kidney accumulation of PVP and anionized PVP derivatives after intravenous injection in mice [33].

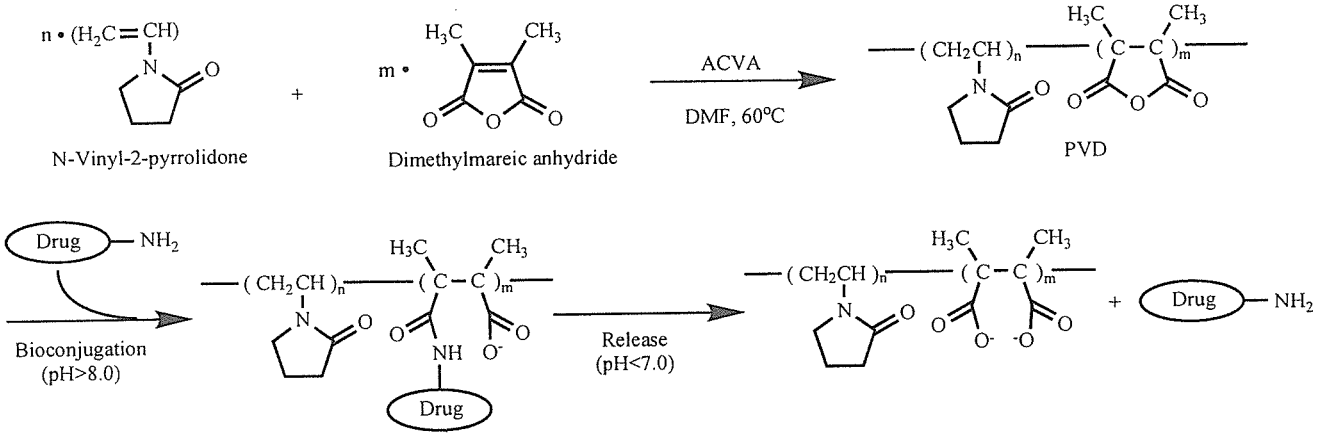

Fig. (4). Chemical structures and characteristics of poly(vinylpyrrolidone-co-dimethyl maleic anhydride)[PVD].

which are used clinically. Thus, PVD (1:5) appears to be a safe polymeric carrier with a considerably higher renal targeting and retention capacity than any other renal targeting carrier.

Renal disease is a serious health problem on the increase in the world. There is no cure for renal disease, and few strategies are available for prevention [36,37]. Bioactive proteins, such as superoxide dismutase (SOD) and interleukin-10, have been expected to prevent the progression of renal disease, but their therapeutic potency were too low because they were poorly distribution to the kidney. The development of a renal delivery system that selectively carries drugs to the kidneys is a promising approach for limiting tissue distribution and controlling toxicity. Several renal DDS have been previously described [38,39]. One approach involves prodrugs that are cleaved by kidney-associated enzymes to release the drugs in the kidney [40]. However, these prodrugs generally do not accumulate in the kidneys as a result of plasma protein binding and limited transport to the kidney. Low-molecular weight proteins, such as lysozyme, have been used as carriers because they are reabsorbed by the kidneys. Unfortunately, they also produced strong renal toxicity and cardiovascular side effects [41]. Streptavidine carriers bind to biotin in the kidney, but they are immunogenic and have limited renal accumulation due to their large molecular size [42,43]. Thus, it is important to develop an effective renal DDS that not only targets the kidney but also has excellent safety. In this regard, we attempted to design novel targeting polymeric modifier to renal system. To cite a case, PVD (1:5)-modified SOD accumulated in the kidneys after intravenous injection and accelerated recovery from $HgCl_2$-induced acute renal failure [35,44]. In contrast, PVP-modified SOD and native SOD were not as effective, because of its poor renal accumulation. Thus, PVD (1:5) represents a promising candidate as a renal targeting carrier.

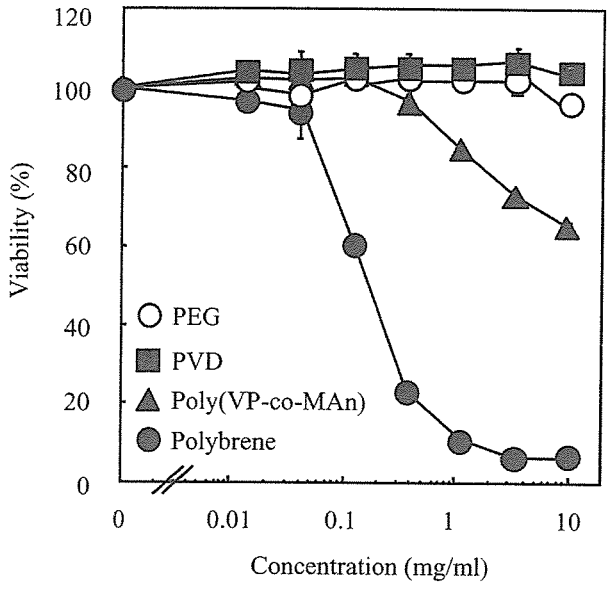

Fig. (5). *In vitro* cytotoxicity of PVD [35].

POLYMERIC DDS FOR OPTIMIZED CANCER CHEMOTHERAPY

The major limitation of antitumor agents, typified by Adriamycin (ADR, doxorubicin), used in clinical applications, is its severe toxicity, such as bone marrow suppression and cardiotoxicity [45-48]. This is caused by the high and frequent dose of antitumor agents, which have a very short half-life and a wide tissue distribution. The chemical conjugation of antitumor agents with WSS polymeric carriers has been found recently to overcome these defects. The conjugation of low molecular weight antitumor agents to WSS polymeric carriers, such as *N*-(2-hydroxypropyl)methylacrylamide, divinylether-co-maleic anhydride, styrene-co-maleic anhydride, dextran, and PEG, offers a potential mechanism to improve cancer chemotherapy [14,49-53]. Distribution of the conjugates, which have a higher molecular weight, is usually restricted to the intravascular space after intravenous injection due to the low permeability in most organs with a continuous capillary bed. It is known that vascular permeability of macromolecules into solid tumors and its retention in tumor tissues are enhanced compared with normal tissues. This is generally called the enhanced permeability and retention (EPR) effect [54-56]. As a result, the polymeric DDS may selectively expand the therapeutic windows of antitumor agents.

However, there is a restriction on the clinical application of this polymeric DDS for cancer chemotherapy. For instance, after the ADR that is taken up into the tumor cells intercalates between double strands of DNA, its antitumor activity is induced by inhibition of DNA replication and topoisomerase activity in the tumor cells [57]. However, the intercalation of polymer-conjugated ADR between double strands of DNA is based on macromolecular interactions, which are sterically hindered by the attached polymeric carrier. Thus, for obtaining *in vivo* antitumor effects, a sufficient amount of antitumor agents is required to be released from the conjugates, because polymer-conjugated anticancer drugs themselves seldom show antitumor activity. However, in most cases, the conjugate between an antitumor agent and a polymeric carrier is formed through stable covalent bonding. As a result, the antitumor therapeutic effects of these conjugates have often not been observed in their clinical trials. To overcome these problems, a relatively unstable linker was used for the conjugation between an antitumor agent and a polymeric carrier. Most of the antitumor agents released from the conjugates have a linker fragment. Furthermore, these modified antitumor agents show much lower specific activities than original antitumor agents in their native form, because the linker fragment is attached to an active functional group of the antitumor agents [58]. Thus, it is necessary to develop a novel polymeric DDS for optimization of cancer chemotherapy.

There are certain characteristics needed by the polymeric drug carrier to (*a*) be excellent in blood residency for effectively obtaining the EPR effect in tumors, (*b*) gradually release the fully active form (native form) of antitumor agents, and (*c*) efficiently release the native antitumor agents under the slightly acidic conditions, if possible, because it is known that the pH of tumor tissues is slightly lower than that of normal tissues [59,60]. From such a viewpoint, some polymeric carriers, typified by divinylether-co-maleic anhydride and styrene-co-maleic anhydride, were developed [50,55]. Some maleic anhydride, that is one of the acid anhydride, were contained in the structure of these polymeric carriers, and the antitumor agents were conjugated with these polymeric carriers *via* the formation of amide bonds between the amino group of antitumor agents and the acid anhydride groups. However, the amide bonds formed through maleic anhydride are very stable near neutral pH, and the antitumor agents are released from the conjugates under strong acidic conditions (<pH 3.0). As a result, the antitumor therapeutic effects of these conjugates have often not been observed in their clinical trials.

DMMAn with a double bound in its structure is used as a pH-reversible protective agent of amino groups in proteins and chemical compounds [61,62]. DMMAn binds to an amino group by forming an amide bond through its acid anhydride group at a pH higher than 8.0, and then reversibly dissociates from the amino group, thereby changing into a slightly acidic from neutral. Therefore, if a polymeric carrier with this function of DMMAn is synthesized, it meets the above conditions and will release a native drug in response to changes in pH.

APPLICATION OF PVD FOR CANCER THERAPY

As described above, PVD contains DMMAn that reacts with an amino group of a drug by forming an amide bond through its acid anhydride group in response to changes in pH. Reflecting the property of DMMAn, PVD could release fully active drugs in the native form in response to the change in pH near neutrality, and gradually released drugs at neutral pH 7.0 and slightly acidic pH 6.0 (Fig. **6**). Further, since the pH of inflammatory tissues and tumor tissues is well-known to be lower than that of normal tissues [59,60], the PVD conjugates would possibly release free drugs more efficiently in inflammatory tissues and tumor tissues.

In view of this, to clarify the usefulness of PVD as a polymeric drug carrier for optimization of cancer therapy, we