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Facilitating the presentation of antigen peptides on dendritic cells for cancer immunotherapy using a polymer-based synthetic receptor†‡

Cuicui Li,^a Masafumi Takeo,^a Masayoshi Matsuda,^a Hiroko Nagai,^a Sun Xizheng,^a Wataru Hatanaka,^b Akihiro Kishimura,^c ^{abcd} Hiroyuki Inoue,^e Kenzaburo Tani,^e Takeshi Mori ^{abc} and Yoshiaki Katayama^{*abcdfg}

The introduction of proteins into dendritic cells (DCs) *ex vivo* is a critical step for the DC-based immunotherapy of cancer. Here, we developed a biotin-modified polymer with multiple hydrophobic membrane anchors for cells that functions as a synthetic receptor for an antigen protein, ovalbumin (OVA), to introduce it efficiently into DCs compared with the conventional pulsing method. Our method showed significant advantages, including the rapid incorporation of OVA and the activation of antigen-specific T cells in a MHC-restricted manner. When mice were immunized by DCs treated with our method, tumor growth was completely suppressed, indicating that our method can be used to prepare adjuvant DCs.

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1. Introduction

Cancer immunotherapy using dendritic cells (DCs) as cellular adjuvants is effective because DCs are activated by loading antigens through an *ex vivo* process.^{1,2} DCs are professional antigen-presenting cells that present antigen peptides derived from exogenous proteins *via* MHC class I through cross-presentation.³ For therapeutic applications, autologous DCs are treated with antigen proteins or peptides that form an antigen peptide/MHC complex on the cell surface. Then, these DCs are administered into patients for immunization against cancer. In this therapeutic protocol, the introduction of antigen proteins into DCs is a critical step for effective immunother-

apy. Conventional methods simply mix antigen proteins or peptides with DCs, termed antigen pulsing.⁴

To improve the efficacy of the protein introduction process, electroporation^{5,6} and sonoporation^{7,8} have been utilized, in which proteins are introduced into DCs through transient pores formed on the cell membrane. However, because this transient pore formation might cause cellular toxicity, careful tuning of the experimental conditions to minimize toxicity is required. Another method to improve the efficacy of protein introduction is the utilization of cationic reagents that form a complex with proteins followed by adhesion of the complex on the negatively charged cell membrane for endocytotic uptake. Many reagents including polymers, peptides, and lipids have been developed to date.^{9–15} However, such cationic reagents are limited by the small number of applicable proteins with an anionic charge.

To overcome this issue, we previously reported a method to introduce proteins irrespective of their charge into cells by modifying polymer-based synthetic receptors on the cell membrane.¹⁶ A schematic representation of this method and the chemical structure of a receptor polymer are shown in Fig. 1 and Chart 1, respectively. In this method, a target protein is modified with biotin followed by complex formation with streptavidin (SA). Then, the complex is bound to the receptor polymer on the cell membrane and rapidly taken up by endocytosis. Here, we applied this method to introduce an antigen protein, ovalbumin (OVA), into DCs as a cellular adjuvant for cancer therapy. Interestingly, our method activated the MHC class I-mediated presentation of antigen peptides, indicating that the polymer activates DCs probably *via* Toll-

^a Graduate School of Systems Life Sciences, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan. E-mail: ykatatcm@mail.cstm.kyushu-u.ac.jp; Tel: +81 092 802 2850

^b Department of Applied Chemistry, Faculty of Engineering, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^c Center for Advanced Medical Innovation, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^d International Research Center for Molecular Systems, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^e Division of Molecular and Clinical Genetics, Medical Institute of Bioregulation, Kyushu University, Fukuoka, Japan

^f Center for Advanced Medical Innovation, Kyushu University, 744 Motoooka, Nishi-ku, Fukuoka 819-0395, Japan

^g Department of Biomedical Engineering, Chung Yuan Christian University, 200 Chung Pei Road, Chung Li District, Taoyuan 32023, Taiwan

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Fig. 1 (A) Preparation of a complex formed from a biotin-modified antigen protein and SA. (B) Scheme of endocytotic uptake of the complex into dendritic cells mediated by the receptor polymer. OVA is processed by dendritic cells and the resultant antigen peptides are presented on the cell surface *via* MHC class I and class II.

Chart 1 Chemical structure of the receptor polymer.

like receptors. The obtained DCs are highly effective for cancer immunotherapy.

2. Materials and methods

2.1. Materials & reagents

Dextran (Dex, $M_w = 40\,000$), 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride (DMT-MM), and triethylamine were purchased from Tokyo Kasei Industry (Tokyo, Japan). Ethylenediamine, biotin, N,N' -carbonyldiimidazole (CDI), and dimethylsulfoxide (DMSO) were purchased from

Wako Pure Chemicals (Osaka, Japan). Palmitoyl chloride was purchased from Kanto Chemical (Tokyo, Japan). Fluoro-Link™ Cy™3-labelled streptavidin was purchased from Amersham Biosciences. TruStain Fc γ ™ (anti-mouse CD16/32) antibody was purchased from Sony Biotechnology Inc. (Japan). FITC anti-mouse H-2K^b/H-2D^b and FITC anti-mouse I-A^b were purchased from BioLegend Inc. (San Diego, CA, USA). Recombinant IL-2 was purchased from Peprotech. Mitomycin C was purchased from Focus Biomolecules. The lactate dehydrogenase (LDH) assay kit was purchased from Takara Biomedicals.

2.2. Cell culture

DC2.4, an immortalized murine bone marrow-derived dendritic cell line previously characterized as an immature C57BL/6DC line (H-2K^b),¹⁷ was kindly provided by Dr. K. L. Rock (University of Massachusetts Medical School, Worcester, MA) and was grown in RPMI 1640 medium containing 10% FBS (Gibco Invitrogen Co.), 2 mM L-glutamine, 55 μ M 2-mercaptoethanol (2-ME), 1% MEM non-essential amino acid, 100 U mL⁻¹ penicillin, 100 μ g mL⁻¹ streptomycin, and 0.25 μ g mL⁻¹ amphotericin B (Invitrogen, Grand Island, NY, USA). CD8-OVA1.3 cells, a T-T hybridoma against an OVA_{257–264}/H-K^b complex, were kindly provided by Dr. Clifford V. Harding (Case Western Reserve University, Cleveland, OH) and were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, 55 μ M 2-ME and antibiotics.^{18,19} OT4H.1D5 cells, a T-T hybridoma against an OVA_{265–277}/I-A^b complex, were kindly provided by Dr. Judith A. Kapp (Emory University, Atlanta, GA) and were cultured in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 55 μ M 2-ME, and antibiotics.²⁰ E.G7-OVA, a chicken egg OVA gene-transfected clone of EL4 that presents OVA *via* MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA). E.G7-OVA cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 50 μ M 2-ME and 400 μ g mL⁻¹ G418.

2.3. Animals

Female C57BL/6 mice (6 weeks old) were purchased from Kyudo Ltd. (Saga, Japan) and were maintained at 22 ± 1 °C with a 12 h light/dark cycle. Animal studies were performed in accordance with the Guidelines for Animal Care and Use Committee at Kyushu University (Fukuoka, Japan).

2.4. Generation of DCs from mouse bone marrow cells

DCs were generated from bone marrow cells isolated from C57BL/6 mice. The bone marrow cells were cultured in RPMI 1640 supplemented with 10% FBS, 55 μ M 2-ME and antibiotics, 50 U mL⁻¹ penicillin, 50 μ g mL⁻¹ streptomycin, 10 ng mL⁻¹ mouse IL-4 (Miltenyi Biotec), and 10 ng mL⁻¹ mouse granulocyte macrophage colony-stimulating factor (Sigma-Aldrich). After 7 days of culture, non-adherent cells were collected and used as DCs.

2.5. Polymer synthesis

Polymers were synthesized as described previously.^{16,21} Briefly, 10 mg mL⁻¹ dextran (M.W. = 40 000, 1.24 mmol glucose units) in anhydrous DMSO was mixed with an equal mole of CDI. After 4 hours of stirring at RT, a 10-fold molar excess of ethylenediamine (EDA) was added dropwise and reacted for 24 hours. After dialysis with Spectra/Por6 dialysis bag (MWCO = 10 000) against water for 3 days and lyophilization, the resulting polymer, Dex-EDA, was dissolved in DMSO. Biotin, palmitoyl and DMT-MM were added to the Dex-EDA solution at molar ratios of 0.1, 0.02, and 0.12 fold of Dex-EDA. The dialysis and lyophilization were repeated to yield solid products. The degree of substitution of each component was determined to be 6 mol% for palmitoyl and 8 mol% for biotin by ¹H-NMR. The obtained polymer was dissolved in a 1:1 (v/v) mixture of DMSO/H₂O and filtered for sterilization. The polymer solution was further dissolved in cold DPBS to 100 µg mL⁻¹ before use.

2.6. Preparation of rhodamine- and biotin-modified OVA

One mg mL⁻¹ NHS-rhodamine in anhydrous DMSO was added dropwise to 10 mg mL⁻¹ OVA in a 50 mM sodium bicarbonate solution (pH 8.5) at a 1:2 molar ratio, and the resulting mixture was stirred for 5 hours to form rhodamine-modified OVA. The unreacted rhodamine was removed with a VIVASPIN 20 tube (MWCO = 30 000). Biotin modification to the resulting rhodamine-modified OVA was conducted similarly with NHS-biotin. The content of rhodamine in one OVA molecule was 0.6.

2.7. OVA uptake

DC2.4 cells were pelleted by centrifugation, resuspended in DPBS, and washed with DPBS 3 times. Then, a 50 µL polymer solution (150 µg mL⁻¹ polymer, containing 2.7 nmol of biotin) was added to the cell pellet (1 × 10⁶ cells) and the resulting suspension was gently mixed for 30 min at 4 °C. Next, a 1:1 complex (0.07 nmol) between streptavidin (SA) and rhodamine- and biotin-modified OVA was added to cells (1 × 10⁵) in DPBS and the resulting suspension was gently mixed for 10 min at 4 °C. DPBS was replaced with 10% FBS containing culture medium which was then incubated for 1 hour at 37 °C.

2.8. MHC expression of OVA-introduced DCs

DC2.4 cells were introduced with the OVA/SA complex following the method described in section 2.7. Then, the cells were incubated for 24 hours at 37 °C. After washing with DPBS, the cells (5 × 10⁵) were incubated on ice for 30 min with 100 µL stain buffer (DPBS containing 0.1% BSA and 0.01% NaN₃) containing TruStain FcTM (CD16/32) antibodies to block Fc receptors. Surface antigens were detected using FITC-labeled monoclonal antibodies (mAbs) against anti-H-2K^b/D^b (28-8-6) and anti-I-A^b (AF6-120.1) for MHC class I and MHC class II, respectively. The cells were suspended in 100 µL of stain

buffer containing H-2K^b/D^b or I-A^b antibodies on ice, using the amount recommended by the manufacturer. After 30 min of incubation, the cells were washed three times with staining buffer and analyzed using Guava easyCyteTM flow cytometry and InCyte software.

2.9. *In vitro* antigen presentation assay

DC2.4 cells were introduced with the OVA/SA complex following the method described in section 2.7. Then, the cells were transferred to a 96-well plate at a density of 5 × 10⁴ per well. The same number of CD8-OVA1.3 or OT4H.1D5 T-hybridoma cells was then mixed with DCs in 10% FBS-containing medium at 37 °C. After 24 hours of incubation, supernatants were collected and IL-2 levels were measured using a murine IL-2 ELISA kit (PeproTech USA Ltd.).

2.10. Tumor rejection assay

Bone marrow-derived DCs (BMDCs) were introduced with the OVA/SA complex following the method described in section 2.7. The cells were incubated for 24 hours at 37 °C. C57BL/6 mice were immunized twice on days 0 and 7 with BMDCs treated by each method. Then, 1 × 10⁶ cells per 100 µL were subcutaneously (s.c.) injected into the left side of the back of the mice. One week after the final vaccination, 1 × 10⁶ E.G7-OVA tumor cells were s.c. injected into the right side of the back of the mice. Tumor size was assessed using microcalipers and was expressed as tumor volume, calculated by using the following formula: tumor volume = (major axis) × (minor axis)² × 0.5236. Mice with a tumor size larger than 2.5 cm or a tumor volume larger than 2500 mm³ were euthanized. On day 40 after tumor challenge, all survivors were euthanized.

3. Results and discussion

3.1. Uptake of OVA *via* polymer-mediated endocytosis into DCs

The receptor polymer was synthesized following our previous procedure.^{16,21} Ethylenediamine was modified onto biocompatible dextran, and then other groups were modified through the ethylenediamine. The substitution degree of palmitoyl and biotin measured by ¹H-NMR was 6 and 8 mol%, respectively (Fig. S1†). An antigen protein, OVA, was modified with biotin and then labeled with rhodamine. The obtained biotin-modified OVA was mixed with an equimolar quantity of streptavidin (SA) to prepare an OVA/SA complex (schematic shown in Fig. 1A).

We utilized the DC2.4 cell line, an immortalized murine bone marrow-derived DC, for these experiments. The receptor polymer was modified on suspended DC2.4 cells by simply mixing cells with the receptor polymer in DPBS for 30 min at 4 °C. This low temperature was chosen to suppress the endocytosis of the modified receptor polymer. Then, the OVA/SA complex was added to the cell suspension. To examine the effect of the amount of the modified polymer, cell modification

was conducted at various receptor polymer concentrations up to $150 \mu\text{g mL}^{-1}$. Fig. 2A shows the fluorescence images of DC2.4 cells at 1 and 4 hours after the addition of the OVA/SA complex at 37°C . Red fluorescence resulting from rhodamine-labeled OVA was observed inside the cells and the fluorescence intensity increased with the polymer concentration. The internalization of OVA indicates endocytotic uptake of the receptor/OVA/SA complex formed on the cell surface. After 4 hours of incubation, the cells adhered to the plate surface irrespective of the amount of modified polymer indicating that polymer modification does not affect cell adhesion. Fig. 2B shows the polymer concentration dependence of rhodamine fluorescence inside the cells after 1 hour of incubation.

Fig. 2 Polymer concentration-dependent uptake of the OVA/SA complex by DC2.4 cells. Cells were pretreated with polymer for 30 min at 4°C and then incubated with $0.1 \mu\text{g mL}^{-1}$ OVA/SA complex for 1 or 4 hours at 37°C . (A) Merged images between fluorescence and bright view images of cells treated with various concentrations of polymer. Red color indicates rhodamine-labeled OVA. (B) Polymer concentration dependence of fluorescence intensity observed from cells after 1 hour of incubation. Results are expressed as the mean of 50 cells with standard deviation.

Fig. 3 Immunofluorescence analysis of the presentation of antigen peptides via MHC class I and MHC class II on DC2.4 cells. Polymer-modified cells were incubated with the OVA/SA complex for 4 hours ("receptor polymer"). Unmodified cells pulsed with OVA for 4 hours ("pulse") and unmodified cells not treated with OVA ("non-treat") were used as controls. Values in each panel indicate the mean fluorescence intensity. Filled histogram indicates the non-mAb-treated DC2.4 cells with a mean fluorescence intensity of 13.8.

tion. Rhodamine fluorescence intensity proportionally increased with the polymer concentration used for cell modification. This result clearly shows that cellular uptake of the OVA/SA complex is mediated by the receptor polymer. The amount of introduced OVA/SA complex increased more than 6-fold by polymer modification at $150 \mu\text{g mL}^{-1}$.

Fig. 4 IL-2 production from CD8-OVA1.3 and OT4H.1D5 co-cultured with DC2.4 cells. DC2.4 cells modified by the receptor polymer at various concentrations were mixed with the OVA/SA complex for 4 hours. The resulting DC2.4 cells were used for co-culture with two T cell hybridomas. Concentrations of IL-2 were determined after co-culture of DC2.4 cells with CD8-OVA1.3 (filled bar) as MHC class I-specific (OVA₂₅₇₋₂₆₄/H-2K^b) cells or OT4H.1D5 (open bar) as MHC class II-specific (OVA₂₆₅₋₂₇₇/I-A^b) cells for 24 hours. CD8-OVA1.3 and OT4H.1D5 cells without DC2.4 were used as controls (Ctr.), respectively. Each bar represents the mean $\pm$ SD of three independent cultures. Statistical results were compared with controls. ***: $P < 0.001$, **: $P < 0.01$, N.S.: not significant.

Fig. 5 Antitumor effect mediated by immunization with BMDCs. (A) Experimental procedures for BMDC treatment and animal experiments. (B) Change of tumor volume of mice after tumor cell inoculation into the right back of mice. BMDCs treated by each method were subcutaneously administered into the left back of mice: naked BMDCs (closed circle), naked BMDCs pulsed with OVA (open triangle), and BMDCs treated by our method (closed square). Mice without immunization (open circle) were used as control. Each point indicates the mean $\pm$ SD of 5 mice.

compared with conventional pulsing (*i.e.*, cells without polymer modification). Hereafter, we used the polymer concentration of $150 \mu\text{g mL}^{-1}$ for cell modifications.

3.2. Activation of peptide presentation on DCs by polymer-mediated endocytosis

Next, we examined the extent of peptide presentation on DC2.4 modified by our method. Peptide/MHC class I and class II complexes were detected by specific mAb labeled with FITC. The mAbs used here are specific for the kind of MHC (H-2K^b/D^b or I-A^b) but are not specific for the kind of peptide presented on these MHCs. As shown in Fig. 3, cells treated by our method (“receptor polymer”) showed a higher presentation of both peptide/MHC I and peptide/MHC II complexes compared with OVA-pulsed cells (“pulse”). This increased presentation of the peptide/MHC complexes is probably a result of the modified receptor polymer on DC2.4. Molecules including multiple fatty acid esters were reported to activate the presentation of peptide/MHC complexes in antigen-presenting cells through Toll-like receptors.^{22,23} Thus, the modified multiple palmitoyl group on the receptor polymer may activate DC2.4 through a similar mechanism. The presentation of the peptide/MHC complexes by our method especially activated the MHC class I presentation, indicating that this method facilitates CTL activation.

3.3. IL-2 secretion by activated T cells

Polymer-modified DCs treated with the OVA/SA complex should activate specific T cells in a MHC-restricted manner. T cells activated by DCs produce IL-2. Thus, we estimated the extent of T cell activation by detecting the IL-2 concentration in supernatants. We utilized two C57BL/6-derived OVA-specific T cell hybridomas, CD8-OVA1.3 and OT4H.1D5, which recognize peptide/MHC class I complexes or peptide/MHC class II complexes, respectively. DC2.4 cells modified

with various concentrations of the receptor polymer were treated with the OVA/SA complex, and the resulting DC2.4 cells were co-cultured with each T cell hybridoma for 24 hours. The secreted IL-2 concentration was determined by ELISA. As shown in Fig. 4, the amount of IL-2 secreted from both hybridomas increased with the concentration of the receptor polymer used for the modification of DC2.4 cells. This indicated that our method enhanced the presentation of antigen peptides on MHC class I and MHC class II on DCs to stimulate the hybridoma T cells. The increment of IL-2 secretion from pulse (*i.e.*, no polymer modification in Fig. 4) is smaller in MHC class I-mediated stimulation of hybridomas than that in the MHC class II-mediated one. This may indicate the lack of endosome escaping nature of the receptor polymer. Yuba *et al.* reported that the MHC class I-mediated stimulation of hybridomas is more efficient than the MHC class II-mediated one when they use antigen carriers with endosome escaping nature.²⁴ Of note, the surface modification of DCs with the receptor polymer did not inhibit the presentation of peptide/MHC complexes on the DC surface.

3.4. *In vivo* tumor rejection assay

We examined the superiority of our treatment method of DCs for antigen-specific tumor rejection *in vivo* by comparing it with the conventional pulse method. The experimental procedures are summarized in Fig. 5A. BMDCs treated by our method were subcutaneously injected into the left back of mice on days 0 and 7. Then, E.G7-OVA tumor cells expressing MHC class I/OVA-derived peptide complexes on the cell surface were subcutaneously inoculated to the right back of mice one week after the second injection of BMDCs. As shown in Fig. 5B, immunization with BMDCs pulsed with OVA for 4 hours (open triangles) had a negligible effect on tumor suppression compared with non-treated BMDCs (closed circles). In contrast, immunization with polymer-modified BMDCs

treated with OVA/SA (closed squares) resulted in complete rejection of the inoculated tumor cells. These results indicate that BMDCs treated by our method efficiently induced T cell activation *in vivo* despite the short antigen treatment time.

4. Conclusions

In this study, we developed the receptor polymer-mediated endocytotic introduction of an antigen protein into DCs for efficient cancer immunotherapy. Modification of the receptor polymer on the cell surface was completed in 30 min, and then the cellular uptake of the OVA/SA complex was completed in 4 hours. Interestingly, our method activated the MHC-mediated presentation of antigen peptides, especially *via* MHC class I. Because of the efficient presentation of the antigen peptides on DCs, complete rejection of cancer growth was observed in mice. Compared with other representative methods, such as cationic reagent-mediated endocytosis, our method can be used with many antigen proteins irrespective of their surface charge.

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