Expression of Toll-Like Receptor 4 on Dendritic Cells Is Significant for Anticancer Effect of Dendritic Cell-Based Immunotherapy in Combination with an Active Component of OK-432, a Streptococcal Preparation

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INTRODUCTION

OK-432, a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) that was developed by Okamoto et al. (1), is successfully used as an immunotherapeutic agent in malignancies (2, 3). We have also reported that OK-432-based immunotherapy exhibits a marked antitumor effect in patients with oral squamous cell carcinomas (4, 5). It has been reported that OK-432 induces interleukin (IL)-12 and polarizes the T-cell response to a helper T-cell 1 (Th1)-dominant state in mice (6), that local injection of OK-432 augments the Th1-type T-cell response of tumor-draining lymph node cells (7), and that OK-432 induces lymphokine-activated killer cells that exhibit higher cytotoxic activity than the original OK-432 (7). We have reported that OK-PSA is a far more potent inducer of Th1-type cytokines as well as killer cell activities in human peripheral blood mononuclear cells (PBMCs) than the original OK-432, and that it engages in marked antitumor activity in tumor-bearing mice (9, 11–16). It was clearly demonstrated that OK-PSA is an active component of OK-432. Furthermore, we have reported that Toll-like receptor (TLR) 4 signaling is involved in regulating OK-PSA-induced antitumor immunity in tumor-bearing mice (17), and that oral cancer patients who do not express or faintly express TLR4 or MD-2 gene, did not secrete IFN-γ and did not obtain a satisfactory therapeutic effect in response to OK-432 (5). TLRs are transmembrane proteins and represent a newly recognized family of vertebrate pattern recognition receptors in the innate immune system (18). Among the identified family of TLRs, TLR4 recognizes bacterial cell wall components, namely lipopolysaccharide (LPS; Ref. 19). MD-2 acts as a significant coreceptor in the TLR4 signaling. It is physically associated with TLR4 on the cell surface, and the TLR4/MD-2 complex confers responsiveness on bacterial components (20).

Dendritic cells (DCs) are potent antigen-presenting cells that play a central role in initiating adaptive and innate immune responses. Since their original identification by Steinman, much attention has been focused on the role of DCs in eliciting the antitumor effect and in potential therapeutic applications, and the recent insights may provide the basis for generating more effective antitumor immune responses (21–23). In most tissues, including tumor tissues, DCs are present in an immature state. The immature DCs (iDCs) are unable to stimulate T cells and are extremely well equipped to capture antigens. The iDCs are matured by the stimulation associated with capturing antigens such as bacteria, viruses, and apoptotic cancer cells, and by other stimulating agents including LPS, tumor necrosis factor (TNF)-α, IL-1β and CD40 ligand. In the primary tumor sites, the antigen-bearing DCs that are followed by appropriate maturation and that strongly express CD80, CD83, CD86, MHC class I and MHC class II molecules, migrate to the paracortical T-cell-rich area of the draining lymph nodes, present antigens to T cells, and induce tumor-specific CTLs as well as Th cells (23). The immunomodulator that can induce the maturation of human DCs appropriately in vivo and in vitro may be a useful adjuvant for DC-based immunotherapy in patients with malignant diseases.

Recently, it has been demonstrated that OK-432 stimulates mature DCs, and that DCs stimulated with OK-432 can induce antigen-specific CTLs (24–26). In the present study, we first examined the effects of OK-PSA in the maturation of human DCs by in vitro...
experiments using healthy donor-derived as well as head and neck-cancer patient-derived DCs with or without the expression of TLR4 or MD-2 mRNA, and then tested the in vivo anticancer effect of an intratumoral administration of DCs followed by OK-PSA in wild-type and TLR4-deficient mice bearing syngeneic tumor.

MATERIALS AND METHODS

Preparation of OK-PSA. The antigen recognized by TS-2 monoclonal antibody that is an active component of OK-432 (Chugai Pharmaceutical Co., Ltd, Tokyo, Japan), was isolated by affinity chromatography on cyogen bromide-activated Sepharose 4B-bound TS-2 and was designated OK-PSA, as described previously (9). An endospecy test revealed no LPS contamination in the OK-PSA (data not shown). The endospecy test was performed by using the Endospecy ES-30M set (Seikagaku Kogyo, Tokyo, Japan) according to the manufacturer’s recommendation. Furthermore, in the in vitro experiments with human PBMCs, OK-PSA-induced cytokine production was not inhibited by the addition of 25 µg/ml polymixin B (Sigma-Aldrich Fine Chemicals), an LPS inhibitor, whereas cytokine-production induced by LPS derived from Escherichia coli 055:B5 (Sigma-Aldrich) was almost completely neutralized by polymixin B, as described elsewhere (5).

Study Subjects. This study was carried out in accordance with the standards of the Institutional Committee for the Protection of Human Subjects, Tokushima University. Written informed consent was obtained from 10 patients with head and neck cancer and 5 healthy donors derived from our laboratory staff (4 males and 1 female; ranging in age from 28 to 40 years old), and the collection of the samples was approved by the Institutional Review Board. The peripheral blood (200 ml per person) of healthy donors and patients (drawn before the treatment) was submitted for the preparation of DCs. After peripheral blood was collected, all 10 patients had chemotherapy [UFT (tegafur/uracil, 1:4); 400 mg/day for 6 days, p.o.; Taiho Pharmaceutical Co., Tokyo, Japan] and immunotherapy (OK-432 for 6 weeks) simultaneously in combination with radiotherapy (40 Co; a total irradiation dose of 60 Gy; Refs. 4 and 5). We administered OK-432 to these patients both intradermally and peritumorally in a week. Two Klinische Einheit (KE; i.e., 0.2 mg) of OK-432 was injected intradermally, and 3 KE were injected peritumorally. Total 5 KE/week was administered, except in patient 1 with the tumor in maxillary sinus, we administered 5 KE of OK-432 only intradermally once a week, because it was technically difficult to inject OK-432 peritumorally.

Cells and Media. LL2, a Lewis lung carcinoma cell line (27), was grown in Eagle’s MEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS, Bio-Whittaker, Walkersville, MD). Human erythroleukemic cell line K-562 (28), mouse Molony lymphoma cell line MD-2 (29), and mouse fibrosarcoma cell line Meth-A (30) were cultured in RPMI-1640 (Life Technologies, Inc.) supplemented with 10% heat-inactivated FBS. All of the cell lines were grown in a humidified atmosphere of 95% air and 5% CO2 at 37°C.

Experimental Protocol of the in Vitro Experiments. PBMCs were isolated from heparinized venous blood by Ficoll-Hypaque gradient density centrifugation according to standard procedures (31). These PBMCs (9 × 10⁸ cells per 3 ml/well) were placed into 6-well plastic tissue-culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) in RPMI 1640 containing 10% heat-inactivated FBS (Bio-Whittaker). After 2 h of incubation at 37°C, nonadherent cells were removed, and the adherent cells were cultured in RPMI 1640 containing 10% heat-inactivated FBS, granulocyte-macrophage colony-stimulating factor (GM-CSF, 500 units/ml; PropepTech, London, England), and IL-4 (250 units/ml; Genzyme, Boston, MA) to generate iDCs (32). The population of the adherent cells remaining in the wells was composed of 95.6 ± 3.3% CD14⁺ monocytes. After 6 days of the cultivation, DC-maturing agents [OK-PSA (10 µg/ml), OK-432 (10 µg/ml), LPS (1 µg/ml; derived from E. coli 055:B5; Sigma-Aldrich), or TNF-α (10 ng/ml; Endogen, Boston, MA)] were added into the culture. After 2 days of additional stimulation, cells and supernatants were collected and were supplied for the following analysis. The concentrations of the DC-maturing agents were determined by the preliminary experiments (data not shown).

Flow-Cytometric Analysis of Cell Surface Antigens. Cell surface staining was performed using the following antihuman monoclonal antibodies. FITC-labeled anti-HLA-A, -B, -C (MHC class I), anti-HLA-DR, -DP, -DQ (MHC class II), anti-CD80, anti-CD83 and anti-CD86 were purchased from PharMingen (San Diego, CA). Phycocyanin-conjugated anti-CD1a and anti-CD1c were obtained from Immunotech (Marseille, France). Isotype-matched control mouse IgG, conjugated with FITC or phycoerythrin, were also purchased from PharMingen. The cells were resuspended in PBS containing 0.1% sodium azide and 0.2% BSA and then were incubated for 30 min at 4°C with a saturating concentration of each monoclonal antibody. After the cells were washed twice, their fluorescence intensity was determined using a flow cytometer (EPICS XL-MCL; Beckman Coulter, Fullerton, CA).

Assay for Cytokines and Chemokines. Cytokines and chemokines in the supernatants of the DC cultures were measured by commercial ELISA kits. The ELISA kits for TNF-α, IL-1β, IL-12, macrophage inflammatory protein (MIP)-1α, regulated-on-activation, normal-T-cell-expressed-and-secreted (RANTES; BioSource International, Inc., Camarillo, CA), IL-18 (MBL, Nagoya, Japan), MIP-1β, and IFN-γ-inducible protein-10 (IP-10; R&D System, Minneapolis, MN) were used for the present study.

Allogeneic Mixed Lymphocyte Reaction. To evaluate the antigen-presenting ability of DCs, an allogeneic mixed lymphocyte reaction (MLR) was performed. Different numbers of irradiated (30 Gy) DCs were cultured with 2 × 10⁶ allogeneic T cells (95% CD3⁺) purified from PBMCs by anti-CD3-coated microbeads and a magnetic cell-sorting system (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). After 5 days of cultivation, [3H]thymidine (1 µCi/well) was added to the cultures. Eighteen h later, [3H]thymidine uptake was measured using a liquid scintillation counter. Cell-free supernatants were harvested and stored at −80°C until the assay for IFN-γ.

51Cr Release Assay for Allo-Specific CTL Activity. The cytotoxic activities of the T cells harvested from allogeneic MLR culture (DC:T ratio = 1:20) were assayed against allo-specific target cells, namely iDCs derived from the same donor as the DCs used in the MLR, and against nonspecific target cells, K-562, which are sensitive target cells for human natural killer-cell activity, in a specific 51Cr-release test (33). For cell-mediated cytotoxicity assays, 1.0, 2.0, or 5.0 × 10⁴ effector cells were mixed in the wells of 96-well microtiter plates (Falcon; Becton Dickinson Labware, Lincoln Park, NJ) with 1.0 × 10⁴ 51Cr-labeled target cells (E:T ratio = 10:1, 20:1, or 40:1) in a total volume of 200 µl of medium and were incubated at 37°C for 4 h. The percentage of specific 51Cr-release was calculated according to the formula: [(E - S) / (M - S)] × 100, where E was experimental 51Cr-release, S was spontaneous 51Cr-release, and M was maximum 51Cr-release.

Isolation of Total RNA and Semiquantitative Reverse Transcription-PCR. Human monocyte-derived iDCs, generated by cultivation with GM-CSF and IL-4 for 6 days, were harvested, and total RNA was then extracted by a modified acid-guanidinium-thiocyanate-phenol-chloroform method (34) using ISOGEN RNA extracting mixture (Nippon Gene, Toyama, Japan), according to the manufacturer’s recommendations. Expression of mRNAs for TLR4, MD-2, and MD-2 receptor for Lipopolysaccharide (LPS) was studied using semiquantitative reverse transcription-PCR (RT-PCR) method. The nucleic acid primer was used: 5'-TGTTAGCATGGTCTCCTTATTAAG-3' as an upstream primer and 5'-GAATATGGGACCGACCCCTTC-3' as a downstream primer for human TLR4 (506 bp; Ref. 35); 5'-GAATTCGTTACCATCCTGTTTCTGT-3' as an upstream primer and 5'-GAATTCATTGATTTAGGTGG-3' as a downstream primer for human MD-2 (493 bp; Ref. 35); and 5'-GAATATCCGACCGACATCCTACCAAG-3' as an upstream primer and 5'-GTGTTGGACCTCATGCCCACCATG-3' as a downstream primer for human GAPDH (781 bp; Ref. 36). One µg of total RNA was reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) at 42°C for 60 min in a 20-µl mixture with random primer (Life Technologies, Inc.). Two µl of reverse transcription mixture was subjected to PCR in a 20-µl mixture [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 20 mM each dNTP (A,G,T,C), 0.5 units Taq polymerase (Takara Bio Inc., Otsu, Japan), and 0.25 pmol of primer]. Twenty-eight cycles of reaction at 94°C, 55°C, and 72°C for 60, 90, and 150 s, respectively, were carried out in a DNA Thermal Cycler (Takara Bio). Amplified cDNA was subjected to electrophoresis in 1.5% agarose gels containing 100 ng/ml ethidium bromide. At the completion of electrophoresis, gels were viewed and photographed under UV light illumination (Polaroid type 667 film; Polaroid Corp., Cambridge, MA). Densitometric analysis for the band patterns of RT-PCR was performed by using NIH Image 1.59 software (NIH, Bethesda, MD). A relative density of each specific

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Table 1 Effect of OK-PSA on maturation of human dendritic cells (DCs) derived from healthy donors: DC surface markers

<table>
<thead>
<tr>
<th>Class</th>
<th>Class II</th>
<th>CD1a</th>
<th>CD1c</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
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<tr>
<td>Control</td>
<td>752</td>
<td>1504</td>
<td>871</td>
<td>975</td>
<td>107</td>
<td>52</td>
</tr>
<tr>
<td>OK-PSA</td>
<td>1486</td>
<td>5280</td>
<td>1214</td>
<td>984</td>
<td>234</td>
<td>126</td>
</tr>
<tr>
<td>OK-432</td>
<td>1523</td>
<td>3442</td>
<td>806</td>
<td>718</td>
<td>131</td>
<td>59</td>
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<tr>
<td>LPS</td>
<td>1259</td>
<td>2188</td>
<td>821</td>
<td>1153</td>
<td>142</td>
<td>63</td>
</tr>
<tr>
<td>TNF-α</td>
<td>972</td>
<td>4285</td>
<td>753</td>
<td>1007</td>
<td>175</td>
<td>126</td>
</tr>
</tbody>
</table>

* LPS, lipopolysaccharide; TNF, tumor necrosis factor; iDC, immature DC.

RT-PCR band was expressed as a ratio to the density of GAPDH; relative density < 0.1 was defined as −, relative density ≥ 0.5 to 0.5 was defined as ±, and relative density ≥ 0.5 was defined as +. The identification of each amplified product was confirmed by automated DNA sequencing.

**Transient Transfection.** iDCs derived from patient 10, in whom TLR4 gene was not detected, were transiently transfected with 2 μg of pEFS plasmid containing TLR4 gene (provided by Drs. Miyake and Akashi) using SuperFect Transfection Reagent (Qiagen, Hilden, Germany). Twenty-four h later, the iDCs were treated with OK-PSA (10 μg/ml) for 48 h at 37°C; then cell surface phenotype, IL-12 production, and allogeneic T-cell stimulation to produce IFN-γ were analyzed as described above. Our preliminary study revealed that the efficiency of transfection of DCs with the expression plasmid by using SuperFect Transfection Reagent is 50 to 60%. We concluded that this efficiency is sufficient to evaluate the role of TLR4 in OK-PSA effect.

**Animals.** The mutant mice deficient in TLR4 (TLR4 in DC THERAPY

| Table 2 Effect of OK-PSA on maturation of human DCs derived from healthy donors: cytokines and chemokines (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>TNF-α</th>
<th>IL-1 b</th>
<th>IL-12</th>
<th>IL-18</th>
<th>IP-10</th>
<th>MIP-1 α</th>
<th>MIP-1 β</th>
<th>RANTES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25</td>
<td>51</td>
<td>28</td>
<td>16</td>
<td>101</td>
<td>103</td>
<td>252</td>
<td>44</td>
</tr>
<tr>
<td>OK-PSA</td>
<td>4440</td>
<td>318</td>
<td>3976</td>
<td>83</td>
<td>772</td>
<td>5045</td>
<td>4377</td>
<td>498</td>
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<tr>
<td>OK-432</td>
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<td>109</td>
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<td>107</td>
<td>366</td>
<td>3387</td>
<td>3469</td>
<td>82</td>
</tr>
<tr>
<td>LPS</td>
<td>2885</td>
<td>86</td>
<td>2151</td>
<td>89</td>
<td>773</td>
<td>3672</td>
<td>3281</td>
<td>242</td>
</tr>
<tr>
<td>TNF-α</td>
<td>N.E.</td>
<td>75</td>
<td>481</td>
<td>65</td>
<td>194</td>
<td>425</td>
<td>837</td>
<td>69</td>
</tr>
</tbody>
</table>

* DC, dendritic cell; TNF, tumor necrosis factor; IL, interleukin; IP, (IFN-γ-)inducible protein; MIP, macrophage inflammatory protein; RANTES, regulated-on-activation, normal-T-cell-expressed-and-secreted (protein); LPS, lipopolysaccharide; N.E., not examined.

Results with allogenic MLR assay

<table>
<thead>
<tr>
<th>T-cell proliferation (cpm)</th>
<th>IFN-γ (pg/ml)</th>
<th>Allo-specific CTL (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10533 ± 2810</td>
<td>254 ± 35</td>
</tr>
<tr>
<td>OK-PSA</td>
<td>24248 ± 804**</td>
<td>573 ± 37*</td>
</tr>
<tr>
<td>OK-432</td>
<td>17348 ± 1431*</td>
<td>509 ± 35*</td>
</tr>
<tr>
<td>LPS</td>
<td>16088 ± 1408*</td>
<td>395 ± 29*</td>
</tr>
<tr>
<td>TNF-α</td>
<td>18485 ± 1534*</td>
<td>305 ± 43</td>
</tr>
</tbody>
</table>

* a, P < 0.01; b, P < 0.05 as compared with controls.
* a, DC, dendritic cell; MLR, mixed lymphocyte reaction; LPS, lipopolysaccharide; TNF, tumor necrosis factor.
molecules. The levels of the surface markers on the DCs after the OK-PSA treatment were compared with those on DCs treated with other DC-maturing stimuli such as OK-432, LPS, and TNF-α. OK-PSA increased the expression of MHC class II, CD80, CD83, and CD86 far better than the other stimuli tested (Table 1).

The amounts of TNF-α and IL-1β (DC-maturing cytokines), IL-12 and IL-18 (Th1-inducing cytokines), and IP-10, MIP-1α, MIP-1β, and RANTES (chemokines for Th1 cells) in the supernatants of the DC cultures were significantly increased by the stimulation with OK-PSA, OK-432, LPS, or TNF-α, and OK-PSA was most effective among all of the stimuli tested except in IL-18 production. In IL-18 production, OK-432 was most potent. TNF-α

Fig. 1. Effect of OK-PSA on maturation of head and neck cancer patient-derived dendritic cells (DCs). A, monocytes derived from head and neck cancer patients were cultured for 6 days in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4, and immature DCs (iDCs) were induced. Total RNAs isolated from the iDCs were assayed for TLR4, MD-2, and GAPDH by using a semiquantitative reverse transcription-PCR (RT-PCR) method. B, cell surface phenotype of patient-derived DCs stimulated by OK-PSA (10 μg/ml) for 2 days was analyzed by flow cytometry. □, untreated; □, OK-PSA. C, IL-12 production by patient-derived DCs stimulated by OK-PSA (10 μg/ml) for 2 days was measured by ELISA. Bars, SD of triplicate samples. *, P < 0.01 as compared with untreated controls. Open box, untreated; hatched box, OK-PSA. D, patient-derived DCs stimulated by OK-PSA (10 μg/ml) for 2 days were cocultured with allogeneic T cells derived from another patient (DC:T = 1:20) for 5 days, and then the supernatants of the cocultivation were analyzed for IFN-γ by ELISA. Bars, SD of triplicate samples. *, P < 0.01 as compared with controls when untreated DCs were cocultured with allogeneic T cells. E, the cytotoxic activities of the T cells harvested from the above allogeneic mixed lymphocyte reaction (MLR) culture were assayed against antigen-specific target cells, iDCs derived from the same donor to the DCs used in MLR, as well as against nonspecific target cells, K-562, in a 51Cr-release test. Bars, SD of triplicate samples. *, P < 0.01 as compared with untreated controls. O, untreated; ●, OK-PSA.
had a weak effect on the induction of cytokines and chemokines in the DCs (Table 2).

The antigen-presenting ability of the DCs was assessed by allogeneic MLR assay. Immature DCs were treated with OK-PSA, OK-432, LPS, or TNF-α for 2 days, and then the DCs were cocultured with allogeneic T cells. In comparison with the DCs treated with OK-432, LPS, or TNF-α, OK-PSA-treated DCs showed a higher ability to stimulate the proliferation of allogeneic T cells (data from the allogeneic MLR culture at DC:T ratio = 1:20 are shown in Table 3). The cell-free supernatants derived from the allogeneic MLR culture (DC:T ratio = 1:20) were analyzed for IFN-γ. IFN-γ was markedly induced when T cells were stimulated by allogeneic DCs treated with OK-PSA as well as with OK-432. IFN-γ production by T cells that were stimulated by DCs treated with LPS or TNF-α was relatively weak (Table 3). IFN-γ was not detected in the supernatants that were derived from the cultivation of only DCs treated with any of the stimulators, including OK-PSA (data not shown).

Next, these OK-PSA-treated or untreated DCs were irradiated with 30 Gy of X-ray and then were cultured with allogeneic T cells (DC:T ratio = 1:20) derived from another patient. After 5 days of the cocultivation, the supernatants were analyzed for IFN-γ. IFN-γ was markedly induced when T cells were cocultured with patient 1-derived DCs treated with OK-PSA in comparison with patient 6- or patient 9-derived DCs (Fig. 1).

Table 4 summarizes the clinical backgrounds and the results of cell surface markers, IL-12 production, and allogeneic MLR in the DCs with or without OK-PSA treatment, derived from 10 patients. Patient 1, 3, 4, and 5, in whom both TLR4 and MD-2 were expressed, had complete remission in response to OK-432 and UFT (tegafur:uracil, 1:4) in combination with radiotherapy. No complete remission was observed in the patients who showed TLR4(+)MD-2(−), and that patients 9 and 10 showed TLR4(−)MD-2(+) (Fig. 1; Table 4). These patient-derived iDCs induced by 6 days of cultivation with GM-CSF and IL-4 were stimulated with OK-PSA for 2 days. Data from patients 1, 6, and 9, are shown in Fig. 1. OK-PSA stimulation increased the expression of MHC class II, CD80, CD83, and CD86 in iDCs derived from patient 1. The expression of these cell surface antigens was also increased by OK-PSA stimulation on patient 6-derived DCs but not on patient 9-derived DCs (Fig. 1B).

Although DCs derived from both patient 1 and patient 6 produced IL-12 as a result of OK-PSA treatment, IL-12 secretion by patient 1-derived DCs was greater than that by patient 6-derived DCs. No IL-12 secretion by patient 9-derived DCs was observed (Fig. 1C).

Effect of OK-PSA on Maturation of DCs Derived from Head and Neck-Cancer Patients in Whom Expression of TLR4 and/or MD-2 mRNA Was Detected or Not Detected. Semiquantitative RT-PCR analysis of the patient-derived iDCs, revealed that patients 1 to 5 showed TLR4(+)/MD-2(−), that patients 6, 7, and 8 showed TLR4(−)/MD-2(−), and that patients 9 and 10 showed TLR4(−)/MD-2(+) (Fig. 1; Table 4). These patient-derived iDCs induced by 6 days of cultivation with GM-CSF and IL-4 were stimulated with OK-PSA for 2 days. Data from patients 1, 6, and 9, are shown in Fig. 1. OK-PSA stimulation increased the expression of MHC class II, CD80, CD83, and CD86 in iDCs derived from patient 1. The expression of these cell surface antigens was also increased by OK-PSA stimulation on patient 6-derived DCs but not on patient 9-derived DCs (Fig. 1B).

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Next, these OK-PSA-treated or untreated DCs were irradiated with 30 Gy of X-ray and then were cultured with allogeneic T cells (DC:T ratio = 1:20) derived from another patient. After 5 days of the cocultivation, the supernatants were analyzed for IFN-γ. IFN-γ was markedly induced when T cells were cocultured with patient 1-derived DCs treated with OK-PSA in comparison with patient 6- or patient 9-derived DCs (Fig. 1D).

Furthermore, the allospecific CTL activity of the T cells harvested from the above culture was also examined. CTL activity was significantly increased only when the T cells were cocultured with patient 1-derived DCs activated by OK-PSA but not when cocultured with DCs from patient 6 or patient 9 (Fig. 1E).

Table 4 summarizes the clinical backgrounds and the results of cell surface markers, IL-12 production, and allogeneic MLR in the DCs with or without OK-PSA treatment, derived from 10 patients. Patient 1, 3, 4, and 5, in whom both TLR4 and MD-2 were expressed, had complete remission in response to OK-432 and UFT (tegafur:uracil, 1:4) in combination with radiotherapy. No complete remission was observed in the patients who showed TLR4(−)/MD-2(−). We reported that OK-432 administration induced IFN-γ protein in the sera...
Intratumoral injection of DCs followed by OK-PSA resulted in cells, the antitumor effect of DC administration alone was not significantly. Results are shown in Fig. 3. In wild-type mice bearing LL/2 mice were given bone marrow-derived DCs and/or OK-PSA intratumorally. We next examined the antitumor effect of DCs and OK-PSA in wild-type and TLR4 mice.

**Table 4: Summary of patients’ profile and OK-PSA effect on maturation of dendritic cells (DCs) derived from the patients with head and neck cancer**

<table>
<thead>
<tr>
<th>Donors, patient no.</th>
<th>Sex/Age</th>
<th>Primary site</th>
<th>TNM classification</th>
<th>Response</th>
<th>TLR4</th>
<th>MD-2</th>
<th>In vitro treatment</th>
<th>Surface markers</th>
<th>IL-12 (pg/ml)</th>
<th>IFN-γ (pg/ml)</th>
<th>allo-specific CTLs (% cytotoxicity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M/69</td>
<td>Maxillary sinus</td>
<td>T2N0M0</td>
<td>CR</td>
<td>+</td>
<td>+</td>
<td>Untreated</td>
<td>Class II</td>
<td>28.3 ± 107</td>
<td>57.5 ± 11.3</td>
<td>17.7 ± 2.5</td>
</tr>
<tr>
<td>2</td>
<td>M/63</td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>PR</td>
<td>+</td>
<td>+</td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>71.1 ± 347</td>
<td>623 ± 52</td>
<td>42.5 ± 3.3</td>
</tr>
<tr>
<td>3</td>
<td>F /70</td>
<td>Upper alveolus and gingival</td>
<td>T2N0M0</td>
<td>CR</td>
<td>+</td>
<td>+</td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>69.7 ± 289</td>
<td>1291 ± 73</td>
<td>35.9 ± 6.1</td>
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<td>4</td>
<td>F /55</td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>CR</td>
<td>+</td>
<td>+</td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>728.5 ± 95</td>
<td>527.8 ± 4.7</td>
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<td>5</td>
<td>F /68</td>
<td>Upper alveolus and gingival</td>
<td>T2N0M0</td>
<td>CR</td>
<td>+</td>
<td>+</td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>20.6 ± 810</td>
<td>104.5 ± 12.5</td>
<td>5.2 ± 1.0</td>
</tr>
<tr>
<td>6</td>
<td>M/72</td>
<td>Upper alveolus and gingival</td>
<td>T2N0M0</td>
<td>PR</td>
<td>+</td>
<td></td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>767.0 ± 73</td>
<td>829 ± 79</td>
<td>26.4 ± 3.5</td>
</tr>
<tr>
<td>7</td>
<td>M/66</td>
<td>Buccal mucosa</td>
<td>T2N0M0</td>
<td>PR</td>
<td>+</td>
<td></td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>726.9 ± 377</td>
<td>289.3 ± 31</td>
<td>18.2 ± 1.5</td>
</tr>
<tr>
<td>8</td>
<td>M/64</td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>PR</td>
<td>+</td>
<td></td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>2945.1 ± 173</td>
<td>51.2 ± 3.2</td>
<td>6.2 ± 0.7</td>
</tr>
<tr>
<td>9</td>
<td>F /64</td>
<td>Tongue</td>
<td>T2N0M0</td>
<td>NC</td>
<td></td>
<td></td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>1951 ± 232</td>
<td>116.2 ± 12</td>
<td>7.7 ± 1.1</td>
</tr>
<tr>
<td>10</td>
<td>M/52</td>
<td>Upper alveolus and gingival</td>
<td>T2N0M0</td>
<td>PR</td>
<td>+</td>
<td></td>
<td>Untreated</td>
<td>OK-PSA</td>
<td>1173 ± 77</td>
<td>129 ± 53</td>
<td>16.6 ± 2.2</td>
</tr>
</tbody>
</table>

**Table 5: Effect of transfection of patient 10-derived dendritic cells (DCs) with TLR4-expressing plasmid in OK-PSA-induced expression of DC surface markers**

<table>
<thead>
<tr>
<th>TLR4 transfection</th>
<th>Treatment</th>
<th>MHC class II</th>
<th>CD80</th>
<th>CD83</th>
<th>CD86</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)</td>
<td>Untreated</td>
<td>972 ± 126</td>
<td>27.3 ± 5.4</td>
<td>96.7 ± 14.2</td>
<td></td>
</tr>
<tr>
<td>(+)</td>
<td>OK-PSA</td>
<td>1263 ± 84</td>
<td>25.1 ± 2.7</td>
<td>82.3 ± 5.7</td>
<td></td>
</tr>
</tbody>
</table>

*P < 0.01 as compared with untreated controls.

derived from patients 1, 2, 3, 4, and 5 but not from patients 6, 7, 8, 9, and 10 (5).

**Acquisition of Responsiveness of TLR4(−) Patient-Derived DCs to OK-PSA by Transfection with Expression Plasmid Containing TLR4 Gene**

We transiently transfected the DCs derived from patient 10, in whom the TLR4 gene was not expressed and who did not respond to OK-PSA (Table 4), with the expression vector including TLR4 cDNA. The treatment with OK-PSA, of the DCs transfected with the TLR4 gene significantly, increased the expression of surface molecules (MHC class II, CD80, and CD86; Table 5), as well as the production of IL-12 (Fig. 2A). Furthermore, the OK-PSA-treated DCs stimulated allologenic T cells to produce IFN-γ (Fig. 2B).

**Antitumor Effect of Intratumoral Administration of DCs in Combination with OK-PSA in Wild-Type and TLR4(−) Mice.**

We next examined the in vivo antitumor effect of intratumoral administration of DCs in combination with OK-PSA. Tumor-bearing mice were given bone marrow-derived DCs and/or OK-PSA intratunorally. Results are shown in Fig. 3. In wild-type mice bearing LL/2 cells, the antitumor effect of DC administration alone was not significant. OK-PSA inhibited the tumor growth slightly but significantly. Intratumoral injection of DCs followed by OK-PSA resulted in marked inhibition of the tumor growth (Fig. 3A). In TLR4(−) mice, the antitumor effect of OK-PSA as well as of the combination therapy with DCs and OK-PSA was not significant (Fig. 3B). Interestingly, in TLR4(−) mice bearing LL/2, the administration of wild-type-mouse-derived DCs and OK-PSA exhibited a marked antitumor effect (Fig. 3C). Both in wild-type and in TLR4(−) mice bearing LL/2, after completion of the treatment with wild-type DCs and OK-PSA on day 25, rapid regrowth of the tumors was not observed. Untreated and TLR4(−)–DC-injected animals were dead in 8 to 11 weeks after tumor inoculation. All of the animals treated both with wild-type DCs and with OK-PSA survived over 12 weeks after tumor inoculation (data not shown).

Cytotoxic Activities of TILs and Draining Lymph Node Cells Derived from LL/2-Bearing Mice Given DCs and/or OK-PSA.

The cytotoxic activities of TILs and draining lymph node cells derived from tumor-bearing mice that were given DCs and/or OK-PSA against the inoculated tumor cells LL/2, and against nonspecific target cells YAC-1 and Meth-A, were examined by a 51Cr-release test (Fig. 4). In wild-type mice, the OK-PSA treatment increased the killer-cell activities of TILs against LL/2 significantly, and further enhancement of the cytotoxic activities of TILs was induced by combination therapy with DCs and OK-PSA. Slight enhancement in the killer-cell activities of the TILs against YAC-1 and Meth-A was observed in the mice that received the therapy with OK-PSA or with OK-PSA plus DCs. There were no significant differences between mice given OK-PSA alone and those given DCs and OK-PSA. In TLR4(−) mice, the therapy with DCs and/or OK-PSA did not increase any cytotoxic activities tested. Even in TLR4(−) mice bearing LL/2 tumors, the cytotoxic activity of TILs against LL/2 was significantly augmented by the administration of wild-type-mouse-derived DCs in combination with OK-PSA. The cytotoxicity of TILs against YAC-1 was also
enhanced slightly but significantly by wild type-DCs and OK-PSA (Fig. 4). Similar results were obtained for the cytotoxicities of draining lymph node cells (data not shown).

**IFN-γ Secretion in LL/2-Bearing Mice Given DCs and/or OK-PSA.** IFN-γ in sera from mice that received therapy with DCs and/or OK-PSA was assayed by ELISA. IFN-γ secretion was significantly increased by DCs followed by OK-PSA in wild-type mice but not in TLR4−/− mice. By means of the intratumoral injection of wild-type DCs in combination with OK-PSA, IFN-γ was significantly increased even in the sera from TLR4−/− mice (Fig. 5).

**DISCUSSION**

We have reported that TLR4 signaling is heavily involved in regulating antitumor immunity induced by OK-PSA as well as by OK-432 (5, 17). It was recently reported by several investigators that
OK-432 is able to stimulate DCs into maturity in vitro (24–26). In the present study, we investigated by using human samples, including those from patients with head and neck cancer, as well as by using animal models, whether OK-PSA, which is an active component of OK-432, may stimulate DCs in vitro to mature or not; whether OK-PSA may augment the in vivo anticancer effect of intratumoral administration of DCs or not; and whether the signaling via TLR4/MD-2 may be involved in the anticancer effect of local DC therapy followed by OK-PSA or not.

We demonstrated in the present in vitro experiments that OK-PSA...
is able to mature human iDCs derived from healthy donors, as well as those from patients with head and neck cancer. OK-PSA was more effective than LPS and TNF-α, known DC-maturing agents, as well as the original OK-432, in the maturation of the DCs. Furthermore, OK-PSA-matured DCs markedly produced IL-12 and IL-18, as well as chemokines against Th1 cells, and induced IFN-γ production and antigen-specific cytotoxic activity in allogeneic T cells most effectively among all of the agents tested. It was strongly suggested that OK-PSA-matured DCs might induce antigen-specific CTLs and Th1 cells strongly, by presenting antigens to T cells with promoting Th1-leading situation via induction of IL-12 and IL-18 in the local environment, especially in primary tumor sites and in draining lymph nodes. It was reported that in the local tumor sites, DC maturation and activation as well as DC migration into the tumor tissues are inhibited by Th2 cytokines such as IL-6 and IL-10, as well as by TGF-β, released by the tumor cells (22). OK-PSA, which is a DC-maturing agent as well as a strong Th1 inducer, may be a most potent adjuvant for DC-based antitumor immunotherapy. Actually, OK-PSA significantly augments the in vivo anticancer effect of an intratumoral administration of DCs in the present animal model. Furthermore, the fact that a rapid regrowth of the tumors was not observed after completion of the treatment with wild-type DCs and OK-PSA on day 25, strongly suggests that CTLs recognizing tumor antigen(s) expressing in inoculated LL/2 cells have been induced by the present therapy. In addition, it was elucidated that these effects of OK-PSA depend on the expression of TLR4 on the DCs injected intratumorally, at least in part. We have already reported that there are oral cancer patients who do not express or faintly express the TLR4 or the MD-2 gene, and that these patients’ antitumor immunity was not enhanced in response to OK-432 or OK-PSA (5). Thus, we considered that OK-432/OK-PSA might be useful for cancer therapy in TLR4(+)/MD-2(+) patients. For the patients who shows TLR4(−)/ or MD-2(−), it may be better to change OK-432 or OK-PSA to the other immunotherapeutic agents that may be more effective for the patients who show TLR4(−)/ or MD-2(−), e.g., CpG-DNA (TLR9 ligand), at this point in time. In addition, it was suggested that the transfer of TLR4 and/or MD-2 gene(s) may be useful to lead OK-432 or OK-PSA to elicit an antitumor effect. However, a strategy of gene transfer that can be used in clinics has not been established. The present findings indicate that the expression of the TLR4 gene in all of the immune cells in the hosts may not be essential to augment the antitumor effect of local DC therapy followed by OK-PSA, and that the effect may be obtained if TLR4 signaling is activated only in DCs injected into the tumor tissues, even in TLR4-deficient hosts. In the present study, we did not investigate the antitumor effect of TLR4-deficient DCs and OK-PSA in wild-type mice because, in the treatment of human cancer, we do not carry out the therapy using DCs deficient in TLR4 signaling against a TLR4-expressing host. We considered that first, the intratumorally injected DCs might phagocytize tumor cells; then the OK-PSA might mature the DCs that captured the tumor cells (tumor antigens) via TLR4 signaling to induce tumor-antigen-specific CTLs, and the antitumor activity of OK-PSA might be elicited. In the future, when DC local therapy followed by OK-432 and/or OK-PSA will be in use for cancer patients in whom TLR4 signaling is deficient, the strategy by which the DCs transfected ex vivo with TLR4 and/or MD-2 gene(s) are administered intratumorally may be useful. The ex vivo gene transfer is relatively convenient as compared with the in vivo gene transfer, and a high efficiency of gene transfection may be obtained. The finding that TLR4-deficient DCs acquired the responsiveness to OK-PSA so as to mature by transfection of the TLR4 expression vector (Fig. 2; Table 5), strongly supports the hypothesis described above. For an in vivo study as well as a clinical trial, we are now reconstructing an adenovirus vector containing TLR4 cDNA that should make the transfection of DCs highly efficient.

Clinical study of DC-based cancer immunotherapy has been performed in many types of malignancies. Several strategies to enhance the host responses to tumors have been developed in which syngeneic DCs are armed with tumor-specific antigens as cancer vaccines. Examples of loading DCs to initiate therapeutic antitumor responses include the following: (a) DCs pulsed with defined peptides, proteins, or tumor cell lysate; (b) DCs genetically modified to express tumor antigens; and (c) DCs fused with tumor cells (39-41). However, there are several problems with the above strategies, namely, (a) that the homing efficiency of CTLs induced by antigen-loaded DCs to the tumor sites may be relatively low; (b) that the tumor-specific antigen(s) have never been established in most malignancies; and (c) that it is difficult to induce antigen-specific CTLs suitable for the variety of tumor antigens in a tumor tissue. The effect of an alternative strategy, namely, intratumoral administration of iDCs, was reported in humans as well as in mice (42-44). In this strategy, for the purpose of enhancing antigen-specific antitumor immunity, it is important to bring to maturity the DCs bearing tumor antigens in the local tumor sites. We look forward to the establishment of the adjuvant(s) appropriate for maturing DCs in vivo. OK-432, which has already been used as an immunotherapeutic agent for patients with malignancies, may be a useful adjuvant for DC-based immunotherapy; and OK-PSA, an active component of OK-432, may be more potent as an adjuvant for local DC therapy than the original OK-432.

In clinical outcome of the head and neck cancer patients described in the present report, patient 2 did not have a complete remission in response to the therapy, even though he expressed TLR4 and MD-2, and patient 10 showed partial response, even when not expressing the TLR4. Although TLR4 signaling is essential for eliciting the anticancer effect by immunotherapy with OK-432, it may be difficult for patients with head and neck cancer to attain complete remission with only OK-432 therapy. Actually, all 10 patients received therapy with OK-432 and UFT simultaneously in combination with radiotherapy. The cancer cells in patient 2 may be relatively low in the sensitivity to radiation and/or UFT, even though OK-432 is effective, and the therapy using radiation and UFT may be effective for the tumor of patient 10. We have previously reported that among 28 patients, 10 (50%) of 20 TLR4(+)/MD-2(+) patients became histopathologically tumor-free after the therapy, and without surgical resection.

A clinical Phase I-Phase II study of intratumoral administration of DCs in combination with OK-432 has already been started in our clinic, and a satisfactory therapeutic effect was obtained, at least in oral cancer patients in whom both TLR4 and MD-2 genes are expressed. Furthermore, we are now planning a clinical study of OK-PSA. We believe that OK-PSA may be an effective immunotherapeutic agent for malignant diseases, especially as a DC-maturing agent as well as a Th1 inducer.

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Expression of Toll-Like Receptor 4 on Dendritic Cells Is Significant for Anticancer Effect of Dendritic Cell-Based Immunotherapy in Combination with an Active Component of OK-432, a Streptococcal Preparation


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