Review Articles

Toll-Like Receptor-Targeting Immunotherapy for Oral Cancer

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Abstract: It is important to augment the anti-cancer host response in cancer treatment. Recent studies have suggested that the signaling that occurs via the Toll-like receptors (TLRs), which are newly identified receptor molecules recognizing many pathogens, are involved in the induction of anti-cancer immunity. OK-432, a penicillin-killed and lyophilized preparation of Streptococcus pyogenes, is being successfully used as an immunotherapeutic agent in many types of malignancies. However, the molecular mechanisms of OK-432, a whole bacterial preparation, and the active components which make it effective against cancer, remain uncertain. We have succeeded in isolating the active component of OK-432 (lipoteichoic acid-related molecule, OK-PSA) by affinity chromatography of a butanol extract of OK-432 on the CNBr-activated sepharose 4B bound TS-2 monoclonal antibody that neutralizes the interferon (IFN)-α-inducing activity of OK-432. OK-PSA induced Th1-type cytokines both in human and in mice, and elicited an anti-cancer effect in tumor-bearing mice via TLR4. Furthermore, our clinical study revealed that TLR4 signaling is intimately involved in the anti-cancer effect achieved by OK-432 in patients with oral cancer. We elucidated that OK-432 is first captured and digested by phagocytes, such as dendritic cells and macrophages, and then its active component, OK-PSA, which is released from the phagocytes, stimulates TLR4 signaling. It is strongly suggested that OK-PSA is the molecule most responsible for the anti-cancer effect of OK-432, and that TLR4 may be a definite molecular target for cancer immunotherapy with OK-432/OK-PSA.

Key words: OK-432, Toll-like receptor, Anti-cancer immunotherapy, Innate immunity, Dendritic cells

Introduction

OK-432, which is a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of Streptococcus pyogenes (group A) that was developed by Okamoto et al. in 1967, is being successfully used as an immunotherapeutic agent in many types of malignancies, including oral cancer. We have also reported that OK-432-based immunotherapy exhibits a marked therapeutic effect in patients with oral squamous cell carcinoma. It has been reported that OK-432 elicits anti-tumor effects by stimulating immunocompetent cells such as macrophages, T cells, and natural killer (NK) cells, and by inducing multiple cytokines, especially interferon (IFN)-α. Further studies have suggested that OK-432 induces interleukin (IL)-12 and polarizes the T-cell response to a helper T-cell 1 (Th1) dominant state in mice, and that local injection of OK-432 augments the Th1-type T-cell response of tumor-draining lymph node cells. We have previously reported that OK-432 induced IFN-α as well as...
augmentation of the NK cell activity in 16 of 18 oral cancer patients (88.9%). Most recently, both we and other investigators demonstrated that OK-432 induces the maturation of dendritic cells (DCs), which are professional antigen-presenting cells, and that OK-432-stimulated DCs can induce tumor antigen-specific cytotoxic T-lymphocytes (CTLs). It has been elucidated that OK-432, which is generally called a “non-specific immunotherapeutic agent” or “old BRM”, induces tumor-antigen specific immunity, and therefore OK-432 is being watched with great interest. While previous studies have helped to clarify the cellular mechanism of the OK-432-induced anti-cancer immunity which was described above, there has been limited progress in elucidating the molecular mechanism, i.e., the identification of effective molecule(s) for inducing anti-cancer immunity in the whole bacterial preparation OK-432 and their molecular target(s), such as receptors and signal transducers in immunocompetent cells. We recently succeeded in isolating an active component, which we designated OK-PSA, and which is most responsible for the anti-cancer effect of OK-432. In addition, we clarified, at least in part, the molecular mechanism of its enhancing effect on anti-cancer immunity.

Toll-like receptors (TLRs), which are expressed mainly on macrophages and DCs, are recently identified receptor molecules that recognize many types of pathogens. Macrophages and DCs are not only primarily involved in innate immunity, but are also essential for the establishment of adaptive immunity as antigen-presenting cells (APCs). Thus, TLR signaling promotes activation of an innate immune response, and then triggers antigen-specific adaptive immunity. In immunotherapy against malignant diseases, it has been suggested that the induction of tumor antigen-specific CTLs is crucial for eliminating tumor cells, and most immuno-oncologists have discussed how to induce adaptive immunity against cancer. However, the precedent activation of the innate immune system is essential for the subsequent induction of antigen-specific immunity. The TLR-mediated activation of innate immunity should be important for establishing an effective anti-cancer immune response. Recently, several investigators have proposed the significance of the TLR signaling in the induction of anti-cancer immunity. We have also reported that TLR4 signaling is intimately involved in the anti-cancer immune response induced by OK-432, as well as by OK-PSA.

In this review, we discuss the progress we have made to date in our studies, which are focused on the establishment of the most effective immunotherapy for oral cancer based on OK-432/OK-PSA and TLR4 signaling.

1. OK-PSA, an active component of OK432

In an attempt to clarify the molecular mechanism of OK-432-induced anti-cancer immunity as well as to prepare the more effective immuno-adjuvant for cancer therapy, we isolated an active component of OK-432, and designated it OK-PSA.

1) Isolation of OK-PSA

Our strategy to isolate the component that induces anti-cancer immunity in OK-432 was to use monoclonal antibody (mAb), which recognizes the IFN-α-inducing molecule, as described in Fig. 1A. We generated an IgM mouse mAb TS-2 that neutralizes IFN-α as well as the
killer cell-inducing activities of OK-432. OK-PSA was isolated as an active component of OK-432 by affinity chromatography of a butanol extract of OK-432 on CNBr-activated Sepharose 4B bound TS-2 mAb. OK-PSA is a glycolipid that has a certain chemical structure included in lipoteichoic acids (LTAs) in common. LTAs are membrane-associated amphiphilic macromolecules present in Gram-positive bacteria, and usually consist of a poly (1,3) glycerophosphate backbone covalently linked to a glycolipid or phosphatidyl glycolipid. LTA-1 and LTA-2 contain 2 and 4 acyl lipid anchors, respectively (Fig. 1B), and it has been reported that LTA-2 but not LTA-1 is a potent inducer of tumor necrosis factor (TNF) and IFN-2. In addition, other molecules of the LTA family have also been reported. However, OK-PSA is not always consistent with several known molecules in the LTA family in terms of some of its biological activities and in its chemical and physical characteristics. Our preliminary data demonstrated that OK-PSA contains poly (1,3) glycerophosphate, a major structure of LTAs, along with glycerol and only 2 types of sugar, glucose and mannose. It is possible that OK-PSA is a novel structure in LTA family. A determination of the chemical structure of OK-PSA is now in progress in our laboratories.

2) Effect of OK-PSA on augmentation of anti-tumor immunity
Accumulating evidence suggests that the Th1-type cytokines such as IFN-2, TNF-2, TNF-2, IL-2, IL-12 and IL-18 are greatly involved in killer cell-induction and in eliminating cancer cells, while the Th2 cytokines inhibit the Th1-mediated anti-cancer effect. It has also been reported that the cytokine balance shifts to Th2-dominant in patients with malignant diseases, and that this shift leads to inhibition of anti-tumor immunity as well as tumor cachexia. Thus, it is important in anti-cancer immunotherapy to selectively induce Th1 type-cytokines, and to shift the cytokine balance to Th1-dominant in cancer patients.

Our in vitro experiments revealed that OK-PSA is a more potent inducer of Th1-type cytokines and killer cell activities on human peripheral blood mononuclear cells (PBMCs) than the original OK-432. We next tested the in vivo anti-cancer effect of OK-PSA in syngeneic Meth-A tumor-bearing BALB/c mice. The intraperitoneal injection of OK-PSA significantly extended the survival time of Meth-A-bearing mice in a dose-dependent manner (Fig. 2A). OK-PSA administration resulted in a marked induction of Th1-type cytokines but not of Th2 cytokines (Fig. 2B), and killer cell activity against antigen-specific target Meth-A but not against non-specific target YAC-1 (Fig. 2C). The accelerated cytotoxic activity against Meth-A was neutralized by anti-MHC class I Ab as well as by anti-CD8 Ab (data not shown). It was clearly indicated that the major effector cells in the OK-PSA-induced cytotoxic activity were MHC class I-restricted CD8+ CTLs. In addition, OK-PSA elicited a significant anti-tumor effect in athymic nude mice bearing human salivary gland cancer, but the effect was relatively weaker than that in syngeneic tumor-bearing normal BALB/c mice. This indicates that NK cells also play, at least in part, a significant role in OK-PSA-induced anti-cancer immunity, because T-cell function is lost in nude mice. In addition, we recently demonstrated that OK-PSA matures DCs, professional APCs, and that OK-PSA-stimulated DCs induce antigen-specific CTLs in vitro.

2. TLRs

1) TLRs and their ligands
The toll gene controls dorsoventral pattern formation during the early embryonic development of Drosophila melanogaster. Interestingly, toll participates in the anti-microbial immune responses upon infection in adult Drosophila. Recently, several mammalian homologues of the Drosophila Toll receptor protein (TLRs) were identified. TLRs are transmembrane proteins and represent a newly recognized family of vertebrate pattern recognition receptors in the innate immune system. A prerequisite for the development of an effective host defense is the recognition of pathogens. TLRs are involved in this first step. Of the 10 TLRs described to date (TLR1 to TLR10), the ligands for 9 of the receptors have been identified. The major exogenous ligands recognized by individual TLRs are summarized in Table 1.

2) Downstream signaling of TLRs
Downstream signaling of TLRs is summarized in Fig. 3. Subsequent to pathogen-associated molecular pattern engagement, TLRs initiate the signaling via sequential recruitment of myeloid differentiation
**Table 1** TLRs and their ligands (cited from references 17, 35, 36)

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Fig. 2 Augmentation of anti-cancer immunity by OK-PSA. (A) Prolongation of the survival time of tumor-bearing mice by OK-PSA. BALB/c mice which were inoculated i.p. syngeneic Meth-A cells, were treated with i.p. administration of 100 μg OK-PSA every other day from day 1 through day 9 after the inoculation of Meth-A. *, p<0.01; #, p<0.05 as compared with control group given saline, analyzed by Kaplan-Meier methods. n=8. (B) Cytokines in the sera derived from Meth-A tumor-bearing BALB/c mice administered 100 μg of OK-PSA i.p. Bars denote SD of triplicate samples. *, p<0.01 as compared with respective controls treated with saline. (C) Cytotoxic activities against YAC-1, a nonspecific target, and against Meth-A, an antigen-specific target, of the OK-PSA (1 μg/ml)-treated spleen cells derived from Meth-A-bearing BALB/c mice. Bars denote SD of triplicate samples. *, p<0.01 as compared with respective controls treated with saline.
protein (MyD) 88, IL-1R-associated kinase (IRAK), and TNFR-associated factor (TRAF) 6, which in turn activate downstream mediators such as the nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs). It was reported that a newly identified molecule, Toll-interleukin 1 receptor (TIR) domain-containing adapter protein (TIRAP), acts as an adapter in the MyD88-dependent signaling pathways initiated via TLR2 and TLR4. In addition, experiments using MyD88-deficient (MyD88−/−) mice revealed that TLR4 mediates the signaling in a MyD88-independent fashion in addition to a MyD88-dependent fashion. Kawai et al. reported that a transcription factor, interferon (IFN)-regulatory factor (IRF) 3, translocated into the nucleus in response to LPS in MyD88−/− mice. It was suggested that the IRF3 activation contributes to the MyD88-independent pathway. Recent reports suggested that TIR domain-containing adapter including IFNs (TRIF), a novel adapter molecule, is involved in the MyD88-independent signaling pathway mediated by TLR3 and TLR4.45, 37.

3) TLRs in cancer therapy

The activation of TLR signaling triggers the innate and adaptive immune response, and frequently induces the Th1-type T-cell response. Therefore, it is possible that the ligands of TLRs are able to be effective immunoadjuvants for cancer therapy. In this section, we review the recent progress made by several investigators who have been focusing on the effect of TLR ligands in cancer.

3-1) **Bacillus Calmette-Guerin** cell wall skeleton (BCG-CWS): a ligand of both TLR2 and TLR4

Heat-killed mycobacterial cells suspended in mineral oil are potent immunoadjuvants to induce both cell-mediated and humoral immunity, and the CWS fraction of the BCG is the active immunoadjuvant component. It was reported that BCG-CWS enhances the cytotoxic activity of T cells and macrophages against cancer cells, and elicits an anti-tumor effect in mice and rats bearing transplantable and autochthonous tumors. In addition, clinical trials with BCG-CWS were performed in patients with several types of malignancies, and it was demonstrated that BCG-CWS was effective in prolonging the survival of patients, especially those with gastric cancer or lung cancer.40–41

Seya and his co-investigators have reported findings which strongly suggest that BCG-CWS augments the anti-cancer host response through TLR2 and TLR4. They demonstrated that BCG-CWS induces TNF-α secretion from DCs via both TLR2 and TLR4, and that
the secreted TNF-α induces the maturation of DCs. The distribution profile of TLR2 and TLR4 matches the response profile of cells for BCG-CWS, and further investigation by the Seya group showed that the peptidoglycan portion of BCG-CWS is an active center for cytokine induction and DC maturation via Toll signaling. These findings strongly suggest that signaling via TLRs is closely involved in BCG-CWS-induced anti-tumor immunity.

3-2) Unmethylated CpG-DNA: a ligand of TLR9

The specific immunostimulatory effect of bacterial genomic DNA was first reported by Tokunaga et al., who demonstrated that bacterial DNA activates NK cells and induces IFN production in addition to tumor regression in some mouse models, but vertebrate DNA does not. In 1995, Krieg et al. demonstrated that CpG motifs in bacterial DNA trigger direct B-cell activation. They also reported that CpG content and methylation distinguish vertebrate and bacterial DNAs. Genomic DNA from vertebrates, but not from bacteria, contains very few CpG dinucleotide motifs. Furthermore, CpGs are commonly methylated in vertebrates, while CpGs are not methylated in bacteria and viruses. This suggests the possibility that the immune system may have evolved a defense mechanism based on the recognition of unmethylated CpG-DNAs, which could be a sign of foreign DNA.

The immune response of synthesized oligodeoxynucleotides (ODN) with CpG motifs was examined. Many studies demonstrated that unmethylated CpG-ODN strongly activates immunocompetent cells such as DCs, macrophages, NK cells, T cells, and B cells, and induces the Th1-like T-cell response including IFN-γ production and CTL induction, both in in vitro and in vivo models. The potential of the CpG-DNA as an adjuvant for cancer vaccines was also examined. The CpG-DNA-induced activation of DCs creates a Th1-like cytokine and chemokine environment in the secondary lymphoid organs that promotes cross-priming with strong IFN-γ-secreting CTLs to the antigens derived from tumors, and elicits marked anti-tumor activity. Despite its promising clinical use, the molecular mechanism by which CpG-DNA activates immune cells has remained unclear.

In 2000, the Akira group discovered the receptor molecule recognizing bacterial DNA. The identified protein, TLR9, recognizes the unmethylated CpG motif in bacterial DNA, and mediates an innate immune response. The researchers generated TLR9−/− mice and examined the immune effect of CpG-ODN using those mice. TLR9−/− mice did not show any response to CpG-DNA, including the proliferation of splenocytes, inflammatory cytokine production from macrophages, and maturation of DCs. TLR9−/− mice showed resistance to the lethal effect of CpG DNA without any elevation of serum pro-inflammatory cytokine levels. The in vivo CpG-DNA-mediated Th1 response was also abolished in TLR9−/− mice. Thus, it was clarified that signaling via TLR9 plays an important role in the CpG-DNA-induced host response. Clinical trials of CpG-DNA as an immunotherapeutic agent for cancers and as an anti-allergic agent are currently taking place.

3. TLR4 signaling in OK-432/OK-PSA-induced anti-cancer immunity

We hypothesized that signaling via TLRs may be involved in the anti-cancer immunity induced by OK-432 or OK-PSA, because OK-432 and OK-PSA are bacterial preparations derived from Streptococcus pyogenes.

1) OK-PSA as a specific ligand of TLR4/MD-2 complex

We first investigated the role of TLRs in the anti-cancer host response induced by OK-PSA, an active component of OK-432. Splenocytes from wild type, TLR2−/− or TLR4−/− mice were cultured in the presence of OK-PSA or OK-432 for 48h, and the supernatants were analyzed for IFN-γ. Although OK-PSA induced IFN-γ on the splenocytes derived from wild type and from TLR2−/− mice, it did not induce IFN-γ on the splenocytes derived from TLR4−/− mice. Unexpectedly, OK-432 induced IFN-γ on the splenocytes from all of the types of mice examined. The role of TLR4 in the in vivo anti-cancer effect of OK-PSA was examined. OK-PSA significantly inhibited the tumor growth in wild type mice, while the anti-cancer effect of OK-PSA was not observed in TLR4-mutant mice (C3H/HeJ) or TLR4−/− mice. Furthermore, we have also observed evidence that TLR4 is involved in the DC maturation induced by OK-PSA in mice as well as in patients with oral cancer.
2) Involvement of TLR4 signaling in OK-432-induced anti-cancer immunity

The above findings indicate that OK-PSA is a ligand of TLR4/MD-2, and that TLR4 signaling is intimately involved in OK-PSA-induced anti-cancer immunity, whereas the role of TLR4 signaling in the OK-432 effect remains uncertain. We next examined the involvement of TLR4 in the anti-cancer effect of OK-432.

2-1) Basic study using TLR4<sup>-/-</sup> mice

The effect of an in vitro long-term OK-432 treatment on changes in IFN-γ production from mouse splenocytes was tested. At 48-168h after OK-432 stimulation, the amounts of IFN-γ secreted by the OK-432-stimulated splenocytes derived from the TLR4<sup>-/-</sup> mice was significantly lower than those from the wild-type mice (Fig. 4B). In the in vivo model, the OK-432 administration into the mice significantly increased the serum IFN-γ levels in the wild-type and TLR2<sup>-/-</sup> mice, but not in the TLR4<sup>-/-</sup> mice (Fig. 4C). Furthermore, in syngeneic-tumor bearing mice, the peritumoral injection of OK-432 resulted in a marked inhibition of tumor growth in the wild-type mice, while in the TLR4<sup>-/-</sup> mice, anti-tumor effect of OK-432 was not observed (Fig. 4)<sup>5</sup>.

2-2) Clinical study in oral cancer patients

We examined whether the expression of TLR4 and MD-2 genes may be associated with OK-432-induced anti-cancer immunity. PBMCs from 28 patients with oral cancer were analyzed for TLR4 and MD-2 mRNA expression by reverse transcription-polymerase chain reaction (RT-PCR) analysis. The patients’ sera were collected 24h after OK-432 administration either peritumorally or intradermally, and were then examined for IFN-γ protein by ELISA. The results are shown in Table 2. A statistically significant relationship between the expression of the TLR4 and MD-2 genes and the OK-432-induced IFN-γ production was observed (p = 0.0005 in Fisher’s exact test)<sup>5</sup>.

All 28 patients examined in the current study received therapy with OK-432 and UFT, an oral fluoropyrimidine formulation combining tegafur and uracil in a 1:4 ratio (Taiho Pharmaceutical Co., Tokyo, Japan), in combination with radiotherapy. Among these patients, 10 of 20 TLR4<sup>+</sup> MD-2<sup>+</sup> patients (50%) became histopathologically tumor-free after the therapy, and without surgical resection. By contrast, all 8 patients who were TLR4<sup>-</sup> or MD-2<sup>-</sup>, have surgically resected their tumors, because the tumors still remained after the therapy was completed (Table 2)<sup>5</sup>.

3) Mechanism of OK-432 effect via TLR4: Role of phagocytes

The findings described above indicate that OK-PSA augments anti-cancer immunity via TLR4 signaling, that the in vitro IFN-γ-inducing effect of OK-432 not via TLR4 is a passing phenomenon, and that TLR4 is greatly involved in the in vitro continuous production of IFN-γ, and in the in vivo anti-cancer effect induced by OK-432. We previously reported that OK-432 injected into tumor-bearing mice is first captured by phagocytes<sup>48</sup>, such as macrophages and DCs. Thus, we hypothesized that OK-PSA, an active component of OK-432, which is captured, digested, and released by phagocytes, may elicit its effect through TLR4 signaling, and the in vitro experiments were conducted to investigate this working hypothesis.

We first tested the effect of cytochalasin B (Sigma), a phagocytosis inhibitor, in OK-432-induced cytokine production. The blocking of the phagocytosis by cytochalasin B significantly inhibited the production of cytokines (IFN-γ and IL-12) by mouse peritoneal macrophages and human monocyte-derived DCs. Similar findings were recently reported by other investigators<sup>14</sup>. The immunofluorescence staining which was performed by using TS-2 mAb recognizing OK-PSA revealed that OK-432 was captured and digested by macrophages and DCs in vitro (Fig. 5A). OK-PSA was detected in the supernatants from OK-432-treated DC culture by the ELISA system using TS-2 mAb as a primary Ab. In addition, the supernatants derived from OK-432-treated DCs increased NF-κB activity in the TLR4/MD-2-expressing cells by a luciferase assay system using an NF-κB-dependent reporter construct, and the increased NF-κB activity was significantly neutralized by TS-2 mAb, as well as by anti-TLR4 mAb (Fig. 5B) (manuscript in preparation). Stimulation of the TLR4/MD-2-expressing cells with OK-432 did not increase the NF-κB activity<sup>5</sup>. The mechanism presumed from the results of this study is presented in Fig. 5C.
4. TLR4 ligand, OK-432/OK-PSA, as an adjuvant for DC therapy for cancer

Since their original identification by Steinman, much attention has been focused on the role of DCs in eliciting the anti-tumor effect and in potential therapeutic applications, and recent insights may...
Table 2  Relationship between expression of TLR4/MD-2 genes and IFN-α induction by OK-432 administration in oral cancer patients. aExpression of TLR4 and MD-2 mRNAs in patient-derived PBMCs were analyzed by semiquantitative RT-PCR analysis. Densitometric analysis for the RT-PCR band patterns was done by using NIH Image 1.59 software. The relative density (RD) of each specific RT-PCR band was expressed as a ratio to the density of GAPDH and RD < 0.1 was defined as −, 0.1 ≤ RD < 0.5 as ±, and RD ≥ 0.5 as +. bIFN-α levels in sera collected from the patients 5h before and 24h after OK-432 administration were measured. Serum IFN-α protein was not detectable (i.e., was less than 7.8 pg/ml) in any of the patients before OK-432 treatment (data not shown). Therefore, the cases in that IFN-α was detected in the sera collected after OK-432 administration, were expressed as −++. cAll 28 patients received therapy with OK-432 and UFT in combination with radiotherapy. −++ means that the tumors have surgically resected, because the tumors still remained after the therapy was completed. The patients who became histopathologically tumor-free after the therapy, without surgical resection, expressed −−++.  

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Fig. 5  Mechanism of OK-432-induced host response: phagocytosis, digestion, release of the active components, and TLR4 signaling. (A) One μg/ml of OK-432 was added into the culture of human monocyte-derived DCs or mice peritoneal macrophages. After the cultivation for 6, 12, 24 or 48 h, 100 μg of the culture media including the cells were collected, dropped on glass plates, dried, and fixed with 3 ̀ paraformaldehyde. Immunofluorescence staining using biotinylated TS-2 mAb, an anti-OK-PSA mouse IgM, was done, then microscopic analysis was conducted under the conditions of fluorescent light. (B) Luciferase assay by using an NF-κB reporter construct was performed. Mouse pro-B cell line Ba/F3 stably expressing human TLR4 and MD-2, which have been also transfected with p55Igκ Luc, an NF-κB reporter construct, were treated with LPS (100 ng/ml), OK-PSA (1 μg/ml) or the filtrated supernatants derived from OK-432-treated DC culture (OK-432 DC Sup.). After 5 h-treatment, cells were harvested and lysed, and luciferase activity was measured. Bars denote SD of triplicate samples. *, p<0.01 as compared with controls pretreated with control Ab. (C) Schema of the mechanism of OK-432-induced host response.
provide the basis for generating more effective anti-tumor immune responses\(^{49,50}\). 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. In the primary tumor sites, the antigen-bearing iDCs that are followed by appropriate maturation, and that strongly express CD80, CD83, CD86, MHC class I and MHC class II, 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 helper T lymphocytes\(^{50}\). The immunomodulator that can induce the maturation of human DCs, e.g., TLR ligands, may be a useful adjuvant for DC-based immunotherapy in patients with malignant diseases. In this section, we demonstrate the effect of OK-432/OK-PSA, which can mature DCs in vitro as described above, as an adjuvant for local DC therapy.

We conducted a mouse model to examine the in vivo anti-cancer effect of an intratumoral administration of iDCs in combination with OK-432. Apoptotic tumor cells are relatively easy to be captured and processed, and present their antigens to T cells by DCs present in tumor tissues. Thus, we used TS-1, an oral fluoropyrimidine anti-cancer drug combining tegafur, Gimeracil, and Oteracil Calcium in a 1 : 0.4 : 1 ratio (Taiho), to induce the apoptosis of cancer cells before an injection of iDCs and OK-432. We have already reported that a relatively low dose of 5-fluorouracil (5-FU) causes the augmentation rather than the inhibition of anti-cancer immunity mediated by the induction of cytokines and killer cell activities in patients with oral cancer\(^{51,52}\). In syngeneic tumor-bearing BALB/c mice, after the apoptosis of tumor cells was induced by 1-week oral administration of TS-1, syngeneic bone-marrow-derived iDCs were injected intratumorally, and then the intratumoral administration of OK-432 was done at 6h after the DC injection to induce their maturation. This local DC therapy in combination with TS-1 and OK-432 significantly regressed the growth of tumors in both the injection and distant sites. However, tumors grew rapidly in the control and iDC+OK-432 groups. TS-1 alone initially suppressed tumor growth, but could not sustain this suppression, and a significant difference was observed from that of the TS-1 + DC + OK-432 group in the degree of suppression of tumor growth (Fig. 6A). Consistent with the significant regression of tumor growth in the mice treated with TS-1 + DC + OK-432, the same group also showed the highest survival rate (Fig. 6B). Tumor infiltrating lymphocytes and draining lymph node cells derived from the mice in the TS-1 + DC + OK-432-treated group, but not in the other groups, elicited a marked cytotoxic activity against the inoculated tumor cells, but not against the non-specific target cells, and the major effector cells of the cytotoxicity were MHC class 1-restricted CD8\(^{+}\) T cells. Furthermore, we also observed that the therapy was not effective in TLR4-deficient mice bearing syngeneic tumors\(^{53}\).

An early-stage clinical trial of local DC therapy against oral cancer, in combination with chemotherapeutic agents and OK-432, is currently taking place, and the preliminary findings from these trials are encouraging.

**Discussion and future perspectives**

Although the major therapies for patients with oral cancers are surgical resection, radiotherapy, and chemotherapy, the accumulated evidence demonstrates that stimulating the anti-cancer host response is essential to completely eliminating the remaining cancer cells, e.g., the micro-invaded or micro-metastatic cancer cells, and the radio- and/or chemo-resistant cancer cells. In addition, the induction of memory T lymphocytes, which recognize tumor antigen(s), is an important component in preventing the recurrence of tumors. Augmenting anti-cancer immunity in patients is important both for curing the diseases, and for increasing the quality of life of cancer patients.

Recent studies strongly suggest that TLR ligands can be usefully applied for immunotherapy for cancer patients. The schema of TLR ligand-induced anti-cancer immunity is shown in Fig. 7. In cancer tissues, DCs bearing tumor antigen(s) may be stimulated by bacterial components such as OK-432/OK-PSA, BCG-CWS, or CpG-DNA via TLRs. TLR-mediated signaling stimulates the maturation of DCs. The matured DCs, in which the expression of MHC and costimulatory molecules has been increased by TLR stimulation, migrate to the regional lymph nodes, and then present antigen(s) to the T cells. TLR-stimulated DCs also enhance the producing ability of cytokines such as IL-12 and IL-18, which are potential Th1-inducing cytokines.
Fig. 6 Anti-tumor effect of an intratumoral administration of DCs in combination with TS-1 and OK-432. BALB/c mice were inoculated s.c. with syngeneic fibrosarcoma cells Meth-A (1 × 10^6/mouse) in both right and left flanks. Four days after tumor inoculation, some groups of mice received oral dose (200 µg/mouse/day) of TS-1 and continued for 7 days. On day 12, some groups of mice received intratumoral DC injection (2 × 10^6/mouse) and the same mouse received intratumorally OK-432 (1 KE: 100 µg/mouse) 6 h after DC therapy. (A) Tumor volume. Bars denote SD of 8 determinants. *, p<0.01; #, p<0.05 as compared with control given saline. (B) Survival rate. *, p<0.01; #, p<0.05 as compared with control given saline. N = 8.

Fig. 7 Schema of the mechanism for establishment of anti-cancer immunity induced by TLR ligands.
Therefore, TLR-stimulated DCs may effectively induce tumor-antigen specific Th1 and CTL by presenting antigens to the CD4⁺ and CD8⁺ T cells while promoting a Th1-leading situation. In addition, some TLR ligands activate NK cells which express several types of TLRs, including TLR2, TLR4, and TLR9⁵⁴.⁵⁵ Then, the activated NK cells elicit non-specific killing against cancer cells, as well as induce IFN-α. It is possible that certain ligands of TLRs are able to be effective immunotherapeutic agents for patients with cancer.

Because of the discovery of TLRs as immunoadjuvant receptors, great progress has been made in the use of immunoadjuvants to treat human cancer. The expression of TLRs in patients may prove a useful marker to discriminate between responders and non-responders to TLR ligands used therapeutically. Strong support is provided for this opinion by the clinical study we conducted in oral cancer patients, which is described in section 3.2-2, and in Table 2⁵. For example, if a patient does not express TLR4/MD-2, BCG-CWS or CpG-DNA but not OK-432/OK-PSA should be selected as a therapeutic application. In addition, the transfer of the genes encoding TLR4/MD-2 may be effective to augment the anti-cancer effect of OK-432/OK-PSA in these patients. This possibility is now under investigation in our laboratory.

The residual problem is that all types of signaling mediated by TLRs do not induce the Th1-type T-cell response. Although Agrawal et al. recently demonstrated that signaling via TLR4 and TLR5 but not via TLR2 stimulates the Th1-type host response⁵⁶, the molecular mechanism for this difference between TLR4, TLR5, and TLR2 remains uncertain. The downstream molecular events of TLRs which induce the Th1 response need to be clarified, because the establishment of a Th1-dominant state in cancer patients is of great importance in curing malignant diseases.

The findings of our studies strongly suggest that TLR4 signaling is involved in regulating anti-cancer immunity induced by OK-PSA which is an LTA-related molecule. Several investigators reported that Gram-positive bacteria-derived LTA is recognized by TLR2⁵⁷,⁵⁸ whereas it has been also reported that TLR2 recognizes LTA⁵⁹, as shown in Table 1. Recent studies by Hartung et al. have demonstrated that the butanol-extracted LTA in addition to synthetic LTA from Staphylococcus aureus induce cytokines through TLR2 but not through TLR4⁶⁰,⁶¹. Further, it was also reported that LTA from Bacillus subtilis and from Staphylococcus aureus induced the maturation of murine DCs via TLR2⁶². Since TS-2 mAb recognizes LTAs⁶³, and neutralizes LTA-induced cytokine production⁶⁴, it is suggested that OK-PSA has a certain chemical structure that LTAs share in common, whereas OK-PSA is not always consistent with several known molecules in LTA family in cytokine-inducing activity and in chemical and physical characteristics as described in the section “Isolation of OK-PSA”. In addition, recent evidence in LPS recognition suggests that there are structural and functional differences among LPS molecules from different bacteria. An LPS with a conical shape (e.g. from Escherichia coli) induces cytokines via TLR4, while a more cylindrical LPS (e.g. from Porphyromonas gingivalis) induces a different set of cytokines via TLR2⁶⁴. It is possible that OK-PSA, a ligand for TLR4, may be a novel member of the LTA family with a different structure. Our laboratories are currently working to determine the chemical structure of OK-PSA.

The original OK-432, a whole bacterial preparation, contains many components which may induce Th2-type cytokines and cause serious side effects¹.³⁰. Furthermore, although OK-432 should be captured and digested by phagocytes before eliciting its anti-cancer effect, OK-PSA directly activates TLR4 signaling. We believe that OK-PSA, which augments the anti-cancer immune response by acting as a potent Th1 inducer far better than the original OK-432, could possibly be applied clinically in therapies used for treating human cancers, including oral cancer. Before attempting to apply these agents to the treatment of human cancers, the chemical structure of OK-PSA needs to be fully determined. It is now under investigation in our laboratories as described above, and we expect to be able to conduct clinical trials in the future.

References


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