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ABSTRACT

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer globally. For the treatment of HCC, although many different kinds of treatment are performed according to the practical guidelines, the prognosis of patients is not still satisfactory because the effects of treatments are limited for advanced tumor and the recurrence rate of HCC, even in early stages, is very high. Therefore, immunotherapy is strongly anticipated as a new treatment method for HCC. For the development of new HCC therapy, we attempted to establish immunotherapy using dendritic cells (DC) and peptide vaccine. In several clinical trials that we performed, we confirmed that the immunotherapy was safe and well tolerated by HCC patients. We observed that DC therapy prolonged the recurrence-free survival of patients compared with that of patients without DC infusion, as well as the radiological anti-tumor effect in HCC patients with peptide vaccine. In this chapter, we summarize the results of previous studies using DC and peptide vaccine, including our own data, and describe the prospects of immunotherapy for HCC.

1.1 Introduction

Hepatocellular carcinoma (HCC) is the sixth most common type of cancer globally. Annually, more than 550,000 people die due to HCC (El-Serag and Rudolph 2007; Parkin 2001), about 35,000 of whom are from Japan. The main risk factor of HCC is chronic liver disease, such as chronic hepatitis or liver cirrhosis caused by hepatitis B (HBV) or C virus (HCV). Recently, metabolic abnormalities, such as diabetes, obesity, and fatty liver, have been determined to be risk factors of HCC; in association with this, HCC has been increasing in Western and Asian countries and has become a serious health issue.

For the treatment of HCC, many different kinds of treatment, such as surgical resection, liver transplantation, radiofrequency ablation (RFA), transarterial chemoembolization (TACE), chemotherapy and sorafenib, are performed according to the practical guidelines (El-Serag et al. 2008). Although these treatments have been performed for HCC, their effects are limited and the recurrence rate of HCC is very high. Since HCC arises from an injured hepatocyte, recurrence occurs at a high rate, even when the existing tumor is removed, unless the background liver disease is completely

cured. Moreover, patients who have already developed HCC are mostly in an advanced stage at the time of diagnosis, so it is difficult for them to receive curative treatment.

Therefore, new treatments are needed to improve the outcome of HCC patients; these include treatment to prevent recurrence after curative treatment and treatment for advanced HCC exhibiting an anti-tumor effect through a mechanism different from those of existing treatments. In this regard, immunotherapy is strongly anticipated as a new treatment method for HCC. For the development of new HCC therapy, we have tried to establish immunotherapy using dendritic cells (DCs) and peptide vaccine.

1.2 Dendritic cell therapy

For the development of immunotherapy for HCC, we first undertook an analysis of the host anti-tumor immunological status in patients with HCC. In this basic study, we identified some tumor-associated antigens (TAAs) and their cytotoxic T-cell (CTL) epitope to which T cells in peripheral blood mononuclear cells (PBMCs) of HCC patient responded (Mizukoshi et al. 2006; Mizukoshi et al. 2006; Mizukoshi et al. 2008; Mizukoshi et al. 2011; Mizukoshi et al. 2012). Next, we developed methods to prepare the DCs from the PBMCs of an HCC patient and to deliver the DCs to a local tumor site

(Nakamoto et al. 2007). Using these methods and HCC-associated TAA-derived epitope, we investigated the safety and immunological effect of DC immunotherapy in patients with HCC.

In the first clinical trial, DCs were injected to a local tumor site using an arterial catheter in patients who had HCC and had been treated with transarterial embolization (TAE) (Nakamoto et al. 2007). DCs were prepared as follows. PBMCs were plated in six-well tissue culture dishes and allowed to adhere to the plastic for 2 h. Adherent cells were then cultured with 1000 U/ml recombinant human interleukin (IL)-4 and 100 ng/ml recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF) for 7 days. On day 7, the cells were harvested. Then, 5 X 10^6 cells were reconstituted in 5 ml of normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge and infused through an arterial catheter following Lipiodol injection during selective TAE therapy.

In this clinical trial, we identified the following phenomena: 1) DC infusion is associated with no clinical or serological evidence of adverse events, including hepatic failure or autoimmune responses, in addition to those due to TAE alone. 2) Following the infusion of ^{111}indium-labeled DCs, DCs were detectable inside and around the HCC

nodules for up to 17 days. 3) T-lymphocyte responses were induced against peptides derived from some tumor antigens, human epidermal growth factor receptor 2 (Her-2/neu), multiple drug resistance-associated protein (MRP) 3, human telomerase reverse transcriptase (hTERT) and alpha-fetoprotein (AFP) in some patients. 4) The cumulative survival rates were not significantly changed by this strategy. On the basis of these results, we conclude that transcatheter arterial DC infusion into tumor tissues following TAE treatment is feasible and safe for patients with cirrhosis and HCC. Furthermore, the antigen-non-specific, immature DC infusion may induce immune responses to unprimed tumor antigens, providing a plausible strategy to enhance tumor immunity.

In the next step, we designed a clinical trial to examine the protective effect of DC therapy for HCC recurrence after local treatment (Nakamoto et al. 2011). In this study, we used DCs pulsed with OK-432, which is a streptococcus-derived anti-cancer immunotherapeutic agent, and identified the following phenomena: 1) OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity. 2)

Administration of OK432-stimulated DCs to patients was feasible and safe. 3) Administration of OK432-stimulated DCs prolonged recurrence-free survival of patients compared with that of patients without DC infusion. 4) The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumor necrosis factor-α and the chemokines CCL4 and CCL11. On the basis of these results, we conclude that a DC-based, active immunotherapeutic strategy in combination with loco regional treatments exerts beneficial anti-tumor effects against HCC.

In addition to our studies, several immunotherapies using DCs have been reported in patients with HCC (Table 1) (Ladhams et al. 2002; Iwashita et al. 2003; Stift et al. 2003; Lee et al. 2005; Chi et al. 2005; Butterfield et al. 2006; Palmer et al. 2009; Zhou et al. 2011; Qiu et al. 2011; Tada et al. 2012). Most of these used DCs generated ex vivo from the peripheral blood of the patient, pulsed with tumor lysate or TAA-derived peptides. These studies have shown that DC therapy is safe and well tolerated in HCC patients. They have also indicated that tumor-specific immune response is induced by DC infusion, and an anti-tumor effect consisting of prolonged recurrence-free survival after treatment and overall survival is observed in some cases.

In several studies, partial radiological response is also observed. However, the strength of the anti-tumor effect and the frequency of patients who have a clinical benefit are still not satisfactory. To establish a treatment using DCs as an immunotherapy for HCC, further clinical trials are necessary to prove the clinical efficacy in more patients with HCC.

1.3 Peptide vaccine

In addition to DC therapy, we also attempted to develop peptide vaccine as an immunotherapy for HCC. The first step to establish the treatment is identification of TAA expressed in HCC. Since HCC is complicated by background liver damage in many cases, the establishment of a safe and effective peptide vaccine for HCC depends on whether an immune response killing only tumor cells without injuring normal hepatocytes can be induced, for which it is necessary to discover HCC-specific TAA with high-level immunogenicity. Generally, HCC had not been considered as an “immunogenic” tumor, but previous studies reported that the risk of recurrence after treatment was low and the outcome was favorable in HCC patients with many infiltrating lymphocytes in the tumor, suggesting that anti-tumor immunity is also

present and tumor progression is inhibited through an immunological mechanism in HCC patients (Wada et al. 1998). Actually, including our data, many TAAs and their CTL epitope expressed in HCC have been discovered over the past 10-15 years (Butterfield et al. 2001; Zerbini et al. 2004; Korangy et al. 2004; Komori et al. 2006; Mizukoshi et al. 2006; Mizukoshi et al. 2006).

However, to date, there have been no reported studies of T-cell responses to previously identified TAAs or their epitopes being measured simultaneously and comparatively in many HCC patients. Therefore, to identify suitable epitopes for peptide vaccine, we performed a simultaneous and comparative analysis of immune responses to 27 different CTL epitopes derived from 14 previously reported TAAs in the PBMCs of 31 HCC patients (Mizukoshi et al. 2011). In this study, we made the findings and selected suitable epitopes: 1) The TAAs consisting of cyclophilin B, squamous cell carcinoma antigen recognized by T cells (SART) 2, SART3, p53, MRP3, AFP and hTERT were frequently recognized by T cells and these TAA-derived peptides were capable of generating peptide-specific CTLs in HCC patients, which suggested that these TAAs are immunogenic. 2) HCC treatments enhanced TAA-specific immune responses with an increased number of memory T cells and induced de novo T-cell

responses.

On the basis of the above data, we are now conducting several clinical trials on HCC patients using hTERT-, AFP-, MRP3-, SART2- and SART3-derived peptides. In the clinical trial of hTERT-derived peptide vaccine, HCC patients treated by RFA were enrolled. The peptide was administered emulsified with incomplete Freund's adjuvant by subcutaneous immunization 3 times biweekly. The maximum toxicity observed was grade 2 according to the common terminology criteria and mainly consisted of skin reactions at the vaccination sites. Several immunological assays revealed that the vaccination induced hTERT-specific immunity. Although the HCC recurrence-free survival time after RFA did not differ between patients without vaccination and those with the hTERT-specific immune response after vaccination, the recurrence rate in patients with vaccination was reduced beyond one year after RFA.

In the clinical trial of AFP-derived peptide vaccine, advanced HCC patients, who had been treated by standard therapy including surgical resection, RFA, TACE, chemotherapy and sorafenib but did not show a clinical benefit, were enrolled. The method of vaccination was almost the same as that for hTERT-derived peptide vaccine, but it was continued until the confirmation of tumor progression. The observed toxicity

mainly consisted of skin reactions at the vaccination sites, and severe adverse events were not observed. To date, we have observed one patient with complete response (CR) and one patient with long-term stable disease (SD). AFP-specific immune responses were observed in these 2 patients by several immunological assays. These results are encouraging for the possibility of using peptide vaccines for HCC.

Regarding an immunotherapy using peptide vaccines for HCC, several studies have been reported (Table 2) (Butterfield et al. 2003; Greten et al. 2010; Sawada et al. 2012). A clinical trial of HCC immunotherapy using AFP-derived peptides has been performed, in which AFP-specific CTL increased after treatment in 6 of 6 HCC patients. In addition to AFP, clinical trials of vaccines comprised of the CTL epitopes of hTERT and GPC3 involving HCC patients have been performed (Greten et al. 2010; Sawada et al. 2012). hTERT-derived peptide vaccine was administered in combination with cyclophosphamide to 40 patients with advanced HCC, but no potentiation of immune reactions to the peptide was observed, and, regarding the anti-tumor effect, no patient showed a CR or partial response (PR) (Greten et al. 2010). In the clinical trial of GPC3-derived peptide, vaccine was administered to 33 advanced HCC patients (Sawada et al. 2012). A partial response was noted in one patient, and an effect maintaining an

SD condition for more than 2 months was noted in 19 patients. In addition, tumor necrosis and size reduction were noted in 4 of the 19 patients in whom the disease condition was stabilized, supporting the possibility of using TAA-targeting immunotherapy for HCC. No serious adverse events occurred in AFP-, hTERT-, or GPC3-targeted immunotherapy, confirming that immunotherapy by peptide vaccine can be safely applied in advanced HCC patients with reduced hepatic reserve capacity.

1.4 Prospects of immunotherapy for hepatocellular carcinoma

In previous studies, including our own, the anti-tumor effect induced by immunotherapy for HCC was not so strong and frequent. Several mechanisms have been considered as reasons for the insufficient anti-tumor effect of cancer immunotherapy. Similarly to other cancer types, HCC has a mechanism to escape from host immune responses. In particular, the presence and mechanism of cells leading immune responses in the negative direction, such as regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), in HCC have recently been clarified. Tregs are the most strongly characterized suppressor cells shown to inhibit anti-tumor immunity in many studies. Increased Tregs in PBMCs and tumor-infiltrating

lymphocytes (TILs) in HCC patients have been reported (Unitt et al. 2005). In a study in which low-dose cyclophosphamide was administered to eliminate Tregs in advanced HCC patients, the frequency and function of peripheral blood Tregs were reduced and AFP-specific T-cell reactions resumed (Greten et al. 2010).

MDSCs have been reported to induce Foxp3 and IL-10 through the arginase activity in $CD4^+$ T cells and to inhibit T-cell function through the induction of Tregs (Hoechst et al. 2008). A recent study reported that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were increased in the peripheral blood of HCC patients and negatively correlated with TAA-specific T-cell responses (Mizukoshi et al. 2012). Unfortunately, no drug or antibody directly inhibiting this function of MDSCs is available, but elucidation of the detailed T-cell-inhibitory mechanism of MDSCs and elimination of this inhibition may lead to the potentiation of anti-tumor immunity.

It is known that the inhibition of T-cell-induced anti-tumor immunity is also exhibited through inhibitory receptors. The typical molecules are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death-1 (PD-1). These molecules transmit inhibitory signals to T cells by binding to specific ligands present on the surface of antigen-presenting or cancer cells and inhibit T-cell functions, such as

proliferation, cytokine secretion, and cytolysis. Many study results support the assertion that potent anti-tumor immunity can be achieved by inhibiting this T-cell-inhibitory system using an antibody. Currently, ipilimumab and tremelimumab are available as CTLA-4 antibodies. Ipilimumab has been approved as the first-line treatment for melanoma patients with metastasis (Robert et al. 2011; Ribas 2010). Regarding PD-1, in immunotherapy using an antibody against PD-1 and a ligand of PD-1 (PD-L1), objective responses (CR or PR) were observed in patients with treatment-refractory metastatic solid tumors (melanoma, renal-cell cancer, non-small-cell lung cancer, and ovarian cancer) (Topalian et al. 2012; Brahmer et al. 2012). At present, no clinical data on these antibodies from a large number of HCC cases have been reported, but they will appear in the future.

As described above, recent studies on anti-tumor immunity have contributed to a marked advancement, and further development is expected in the field of HCC immunotherapy. In particular, there are high expectations for the development of a combination treatment method using DCs, peptide vaccines and immune-modulating antibodies to treat advanced HCC. To develop a new effective immunotherapy for HCC, it is necessary to establish the method of DC therapy, identify a highly immunogenic

HCC-specific TAA-derived T-cell epitope and elucidate the anti-tumor immunity-inhibitory mechanism of HCC.

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Table 1 Clinical trials of DC therapies for HCC

Year	Author	No. of Patients	Setting for DCs	Responses
2002	Ladhams et al.	2	GM/IL-4 DC + tumor	1/2 inhibition of tumor growth
2003	Iwashita et al.	10	GM/IL-4 DC + tumor lysate + TNF-α + KLH	1/10 MR
2003	Stift et al.	2	GM/IL-4 DC + tumor lysate + TNF- α + IL-2	no PR or CR
2005	Mazzolini et al.	8	GM/IL-4 DC + tumor + adenovirus IL-12	2/8 SD
2005	Lee et al.	31	DC + tumor lysate	improved survival
2005	Chi et al.	14	Radiotherapy + DC	immune response
2006	Butterfield et al.	10	DC with AFP peptides	no PR or CR
2007	Nakamoto et al.	10	TAE + GM/IL-4 DC	immune response
2009	Palmer et al.	35	GM/IL-4 DC + HepG2 cells lysate + TNF- α	25 patients were assessed, 1/25 PR and 6/25 SD
2010	Nakamoto et al.	13	TAE + GM/IL-4 DC + OK-432	prolonged recurrence-free survival
2011	Zhou et al.	10	PMWA + GM/IL-4 DC + CIK + CTL	immune response
2011	Qiu et al.	9	GM/IL-4 DC + TNF- α + tumor lysate contained α-Gal epitope	3/9 PR and 3/9 SD
2012	Tada et al.	5	GM/IL-4 DC + recombinant protein (AFP, GPC3, MAGE-1) + cytokine cocktail	1/5 SD
2012	Ansary et al.	15	GM/IL-4 DC + HepG2 cells lysate	2/15 PR and 9/15 SD

GM-CSF, granulocyte-macrophage colony stimulating factor; DC, dendritic cell; HCC, hepatocellular carcinoma; IL, interleukin; TNF, tumor-necrosis factor; KLH, keyhole limpet hemocyanin; TAE, transarterial embolization; PMWA, percutaneous microwave ablation; CIK, cytokine-induced killer cell; CTL, cytotoxic T lymphocyte; AFP, alpha-fetoprotein; GPC3, glypican-3; MAGE, melanoma-associated antigen; MR, mixed response; CR, complete response; PR, partial response; SD, stable disease

Table 2 Clinical trials of peptide vaccines for HCC

Year	Author	No. of Patients	Setting for peptides	Responses
2003	Butterfield et al.	6	AFP-derived peptides + Montanide adjuvant, HLA-A2	no PR or CR
2010	Greten et al.	40	hTERT-derived peptides + cyclophosphamide + GM-CSF	no PR or CR
2012	Sawada et al.	33	GPC3-derived peptides + Montanide adjuvant, HLA-A24 and A2	1/33 PR and 19/33 SD
2012	Mizukoshi et al.	12	SART2-derived peptides + Montanide adjuvant, HLA-A24	immune response
2012	Mizukoshi et al.*	14	hTERT-derived peptides + Montanide adjuvant, HLA-A24	prolonged recurrence-free survival and immune response
2012	Mizukoshi et al.*	20	AFP-derived peptides + Montanide adjuvant, HLA-A24	15 patients were assessed, 1/15 CR and 8/15 SD

GM-CSF, granulocyte-macrophage colony stimulating factor; HCC, hepatocellular carcinoma; AFP, alpha-fetoprotein; hTERT, human telomerase reverse transcriptase; GPC3, glypican-3; SART2, squamous cell carcinoma antigen recognized by T cells 2; CR, complete response; PR, partial response; SD, stable disease; *, present study