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Cancer immunotherapy by fusions of dendritic cells and tumor cells

Dendritic cells (DCs) are potent professional antigen-presenting cells and play a critical role in the induction of primary immune responses. DC-based vaccination represents a potentially powerful strategy for cancer immunotherapy. Thus, the use of cancer vaccines to eliminate residual tumor cells is a promising area of investigation. The immunotherapy of tumor antigen-loaded DCs has now been demonstrated in cancer patients and some clinical responses without any significant toxicity. Fusions of DCs and tumor cells represent an alternative but promising approach to overcome the inability of tumor antigens to induce a sustainable T-cell response. This review deals with recent progress in the immunotherapy of cancer with fusions of DCs and tumor cells.

KEYWORDS: cancer vaccine ▪ CD4⁺ T cell ▪ CD8⁺ T cell ▪ dendritic cell ▪ dendritic/tumor fusion cell ▪ tumor-associated antigen

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Dendritic cell-based vaccine

The field of cancer vaccines is currently in an active state of preclinical and clinical investigations. A major area of investigation in cancer immunotherapy involves the design of dendritic cell (DC)-based cancer vaccines. The antigen-presenting cells (APCs) most suitable for cancer immunotherapy are DCs, which can be distinguished from B lymphocytes and macrophages by their abundant expression of costimulatory molecules and ability to efficiently prime both CD4⁺ helper and CD8⁺ cytotoxic immunity [1,2]. DCs are efficient stimulators for both B and T cells. B cells can directly recognize native antigens through their receptors. However, T cells need the tumor-associated antigens to be processed and presented to them by APCs. Effective antigen processing and presentation are crucial to antitumor immunity. There are two different pathways for antigen presentation by DCs. Endogenously synthesized proteins, such as those in viral infections and certain exogenous antigens, are processed and presented through the MHC class I-restricted pathway to CD8 T cells [1–3]. By contrast, exogenous antigens are processed and displayed in association with MHC class II molecules and recognized by CD4 T cells [1,2]. DCs take up exogenous antigens and migrate to draining lymph nodes, where the antigens are presented to CD4⁺ T cells through MHC class II pathways. In addition, DCs are capable of initiating a CD8⁺ T-cell response through a cross-presentation pathway. Exogenous antigens from tumor cells can be translocated to the cytoplasm, processed and presented through an endogenous pathway [3]. The induction of antitumor immunity also requires homing of effector lymphocytes to the tumor site where recognition of restriction elements leads to tumor elimination. DCs are specialized to capture and process antigens into peptides that are presented on MHC molecules and recognized by T and B cells [1,2]. Recent insights into the role of DCs as the pivotal APCs that initiate immune responses may provide the basis for generation of effective antitumor immune responses.

Loading MHC class I and II molecules on the cell surface of DCs with peptides derived from defined antigens is the most commonly used strategy for DC-based cancer vaccines. This strategy has some limitations: a limited number of known tumor peptides available in many HLA contexts whose immunogenicity is uncertain; the relatively rapid turnover of exogenous peptide–MHC complexes, which results in comparatively low antigen presentation by DCs; and the induction of restrictive repertories of T-cell clones resulting in eradication of small subsets of tumor through the down-regulation of antigens. Although DCs pulsed with antigen-specific peptides have been used in clinical trials for patients with cancer, the results show that clinical responses have been found in a small number of patients [4,5]. Moreover, several DC-based cancer vaccines have been developed to date, including various tumor-derived peptides [6,7], tumor cell lysates [8], and vaccines transfected with tumor cell-derived DNA [9] or RNA [9,10]. An alternative approach for the

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induction of antitumor immunity is the use of fusions of DCs and tumor cells [11,12]. In this approach, a broad spectrum of tumor-associated antigens, including both known and unidentified, are endogenously processed and presented by MHC class I and II pathways in the context of the potent immune-stimulatory machinery of the DCs, resulting in induction of polyclonal cytotoxic T lymphocyte (CTL) responses against tumors [13].

DC/tumor fusion strategy

The DC/tumor fusion strategy has been generated and successfully used as a vaccine in murine models [11–17]. The strategy for DC/tumor fusion-cell vaccine is based on the fact that DCs are the most potent APCs, whereas tumor cells express abundant tumor-associated antigens [1,2]. The fusion of syngeneic DCs and tumor cells creates a heterokaryon with both tumor-derived antigens and DC-derived MHC class II costimulatory molecules (B7.1 and B7.2), intracellular adhesion molecule (ICAM)-1, lymphocyte function-associated antigen (LFA)-1 and -3, and CD40, all of which are efficient antigen-processing and -presentation machinery [18–20]. In our initial report on fusion-cell vaccine, murine MC38 adnocarcinoma cells stably transfected with the human *MUC1* gene were fused to syngeneic bone marrow-derived DCs (DCs/MUC1) in the presence of polyethylene glycol (PEG). In this model, the fusion cells have been proven to be effective in causing host rejection of established local and metastatic tumors [11]. DC/tumor fusion cells can possess the elements essential for processing and presenting tumor antigens to host immune cells and for inducing an effective immune response that is able to break T-cell tolerance to tumor-associated antigens. Many studies have demonstrated that the fusion-cell vaccination not only provides protection against challenge with tumor cells, but also regresses established tumors, including melanoma [21–27], colorectal [13,28–30], hepatocellular carcinoma (HCC) [31,32], myeloma [14,33], lung cancer [34], mastocytoma [35], mammary [17,36], renal cell carcinoma [37] and sarcoma [38].

We have used *MUC1*-transgenic (MUC1.Tg) mice as a preclinical model. The human MUC1 glycoprotein is overexpressed and aberrantly glycosylated in human breast, pancreatic, colon and other carcinomas [13,39–44]. MUC1.Tg mice that express MUC1 in a pattern and at a level similar to that found in humans are unresponsive to MUC1 antigen [45]. Thus, MUC1.Tg mice provide a potential model to assess the induction of anti-MUC1 immune responses [45]. Wild-type mice with established MC38/MUC1 tumors can be eliminated after receiving vaccination with DCs transfected with MUC1 RNA. By contrast, there is little, if any, anti-MUC1 induced when with the transfected DCs vaccinated in MUC1.Tg mice [10]. However, vaccination with the DC/tumor fusion cells expressing MUC1 is highly effective in inducing cellular and humoral immunity against MUC1 antigen in MUC1.Tg mice [13]. Vaccination with the fusion cells can reverse clonal anergy against MUC1 and induce new anti-MUC1 CTLs that had been removed from the repertoire by clonal deletion [13,45]. Unresponsiveness to the MUC1 antigen can be reversible by vaccination with DC/tumor fusions that express MUC1. Thus, the fusion-cell vaccine may represent an effective strategy for the treatment of human tumors, including MUC1-positive targets.

The injected tumors usually grow quickly and the host immune system, while potentially competent, does not have sufficient time to generate an effective antitumor responses. These transplantable tumor mice models are not appropriate for cancer vaccine studies because the tumors lack the multiple stages of cancer development found in human cancers. Mice with spontaneous tumor development provide a powerful tool to study the efficacy of tumor vaccines, since they mimic tumor development in humans. Thus, we have produced a transgenic murine model that expresses MUC1 antigen and the polyomavirus middle-T oncogene under control of the mouse mammary tumor virus promoter long terminal repeat [46], and develops spontaneous mammary carcinomas that express the MUC1 antigen. Using this model, prophylactic vaccination of the transgenic mice with DC/tumor fusion cells can induce polyclonal CTL activity against spontaneous mammary carcinoma cells and render 57–61% of the mice free of the disease at the end of experiment (180 days) [17].

Antigen processing & presentation

Immature DCs from patients with cancer can be generated in granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 medium for 6–7 days and successfully fused to freshly isolated tumor cells by 50% PEG solution. The DCs display a characteristic phenotype with expression of MHC class I and II and costimulatory molecules (CD80 and CD86), but not tumor-associated antigens. By contrast, tumor cells express high levels of tumor-associated antigens (WT1, CEA or MUC1), MHC

class I, but not MHC class II and costimulatory molecules. We have previously demonstrated that fusions of immature DCs and autologous tumor cells, including leukemia [47], breast [48,49], ovarian [42,43,49], gastric [50], colorectal cancers [44,51] and HCC [52], result in the formation of heterokaryons that express the tumor-associated antigens and DC-derived costimulatory and adhesion molecules. The fusion cells possess the properties of both parent cells. After fusion, the cytoplasm of the two cells has been integrated, whereas their nuclei remain separate entities. We have demonstrated that tumor-associated antigens (MUC1) are colocalized with HLA-DR molecules on the fused cells under the immunoelectron microscope [49]. Such a structure makes it possible to maintain the functions of both original live cells, at least in part, including synthesis of antigens and costimulatory molecules. Moreover, the fusion approach can also deliver mRNA encoding the tumor-associated antigens. The integration of cytoplasm from DCs and tumor cells facilitates the entry of tumor-associated antigens that are synthesized *de novo* in the heterokaryons into the DC endogenous pathway of antigen-processing and -presentation machinery. The advantage of DC/tumor fusion cell vaccine over pulsing DCs with tumor lysates is that endogenously synthesized antigens have better access to MHC class I pathway [53]. DCs pulsed with apoptotic tumor cell fragments or tumor lysates rely on antigen being cross-presented, which is usually not very efficient. Indeed, it has been demonstrated that fusion vaccine is superior to those involving other methods of DC loading with antigenic protein or peptide, tumor cell lysates or irradiated tumor cells [25]. The ability of human DC/tumor fusion cells to process and present intracellular proteins derived from tumor cells has been demonstrated [54]. In this report, allogeneic DCs were fused with 888mel cells that do not express any of these MHC molecules, but do express multiple melanoma-associated antigens. The fusion cells can efficiently present MHC class I-restricted epitopes from the melanoma-associated antigens, strongly suggesting that tumor-associated antigens are processed along the endogenous pathway, through the antigen-processing machinery of DCs. Moreover, we have also fused autologous DCs and allogeneic tumor cell lines that do not express same MHC class I molecules as autologous DCs [51,55]. These fusion cells can induce antigen-specific CTL responses against autologous tumor cells through the cross-priming.

Stimulation of CD4$^+$ & CD8$^+$ T cells

Tumor antigens are taken up by DCs, mature into IL-12-producing cells, and stimulate Th1-type CD4$^+$ T cells in the draining lymph node, resulting in IFN-γ production. These cells help during the priming of CD8$^+$ T cells to increase their capacity for optimal secondary expansion upon re-encounter with antigens. Memory CD8$^+$ T cells rapidly expand following exposure to a secondary antigen, even in the absence of CD4$^+$ T cells. Expanded CD8$^+$ CTLs can destroy tumor cells through effector molecules granzyme B and perforin [56]. Therefore, efficient CTL induction required the stimulation of both CD4$^+$ and CD8$^+$ T cells [15]. Moreover, the fusion cells, similar to DCs, can also migrate into regional lymph nodes in MUC1.Tg mice as early as 18 h after injection [15]. The fusion cells localize to the T-cell area and form clusters with CD4$^+$ and CD8$^+$ T cells in the lymph node. The fusion cells, unlike DCs, do not have to take up exogenous tumor-associated antigens. The fusion cells also act as a carrier of tumor-associated antigens, and host DCs or Langerhans cells intake degraded fusion cells *in vivo*. Therefore, both direct antigen presentation by fusion cells and cross-presentation by host DCs participate in the T-cell activation and overcome the unresponsiveness of the immune system to MUC1 antigen in MUC1.Tg mice [15]. CD4$^+$ T cells activated by fusion cells are multifunctional effectors that can produce IL-2, IFN-γ, IL-4 and IL-10. Moreover, both CD4$^+$ and CD8$^+$ T cells specific for MUC1 antigen can be primed *in vivo* by fusion-cell vaccine [15].

To dissect the role of antigen presentation through MHC class I and/or II pathways by DC/tumor fusion cells, several types of fusion-cell preparation have been created in a murine model: wild-type fusion cells; MHC class I negative fusion cells; class II negative fusion cells; and class I and II negative fusion cells. In this study, DC/tumor fusion cell vaccine deficient in MHC class II antigen presentation severely affects the downstream involvement of CD4$^+$ and CD8$^+$ T cells. Although maximal antitumor immune responses require both MHC class I and II antigen presentation, presentation through MHC class II plays a more important role in the antitumor immunity by fusion cells [57]. This is due to lack of help from MHC class II-restricted CD4$^+$ T cells in the priming phase, whereas the induction of CTL and antitumor immunity by MHC class I-negative fusion cells is elicited through cross-priming by the host DCs. Theoretically, cross-priming by host DCs

may be more effective in activation of $CD4^+$ T cells through an exogenous pathway. There is increasing evidence that CD4 T cells play a broader role in antitumor immunity [58]. For the design of efficient antitumor vaccine, activation of tumor-specific $CD4^+$ T cells is particularly important [59]. Both $CD4^+$ and $CD8^+$ T cells stimulated by human DC/tumor fusion cells *in vitro* can also produce IFN-γ and be blocked by anti-MHC class I and/or class II antibodies, indicating antigen presentation by the fusion cells through MHC class I and class II pathways [44]. IFN-γ production in both $CD4^+$ and $CD8^+$ T cells is abolished by MHC class II antibody, further suggesting the importance of activation of $CD4^+$ T cells [44]. A few T cells stimulated by the fusion cells produce IL-10. But the low level of IL-10 production does not influence the induction of CTL responses.

Induction of antigen-specific polyclonal CTL response

Cancer vaccine approaches that rely on induction of immunity against a particular antigen are potentially subject to tumor-cell resistance mediated by the downregulation of the single antigen. Although DCs pulsed with a single peptide derived from the known tumor antigen have been used in clinical trials for patients with cancer, the results demonstrate that clinical responses have been found in a small number of patients [4,5]. A major drawback of this strategy using single antigen for DC-based vaccine comes from the limited number of known tumor peptides available in many HLA contexts. Importantly, various subsets of tumor cells downregulate certain tumor antigens, which often appear during the course of tumor progression [60]. In addition, tumor cells express a variety of specific antigens that have not yet been identified. Therefore, it is important to induce antigen-specific polyclonal CTLs. The induction of single antigen-specific CTLs may not result in eradication of various subsets of tumor cells through the downregulation of single antigens. Preclinical human studies have demonstrated that DC/tumor fusion cells induce antigen-specific polyclonal CTL responses *in vitro* [44,49]. Fusion cells can induce carcinoembryonic antigen (CEA)- and MUC1-specific CTL responses simultaneously in HLA-A2- and/or -A24-restrictive manners *in vitro*. The CTLs also lyse semiallogeneic tumor cells positive for CEA, MUC1, and HLA-A2 and/or -A24. In addition, administration of the T cells regressed 7-day-old human tumors in SCID mice and rendered mice free of disease up to the end of experiment (90 days) [55]. Therefore, fusion cells can be used as a vaccine for active immunotherapy or as stimulators to activate and expand T cells for adoptive immunotherapy.

Generation of regulatory T cells

$CD4^+$ $CD25^{high}$ $Foxp3^+$ T regulatory cells (Tregs) are a minor but functionally unique population of T cells, which maintain peripheral tolerance and the control of autoimmunity [61]. Tumor-specific Tregs require ligand-specific activation and cell-to-cell contact to exert their suppressive activity [61], resulting in impairment of effector function of $CD8^+$ CTL and promotion of tumor progression [62,63]. Tregs exist in markedly higher proportions within tumor-infiltrating lymphocytes, peripheral blood lymphocytes and/or regional lymph node lymphocytes in patients with cancer, and are related to tumor progression and inversely correlated with the efficacy of treatment [56,61–64]. The balance between the stimulatory and suppressive forces is in favor of tumor-induced suppression in patients with advanced cancer [65,66]. The immunosuppressive environment in patients with cancer may stifle the effect of therapeutic vaccines, both the induction and effector phase of the immune response [56]. Depletion or blockade of Tregs can enhance immune protection from tumor-associated antigens that are expressed as self-antigens [67]. The exposure of DCs with TGF-β is crucial for the development of Tregs [68]. In fusion-cell vaccine, tumor-derived TGF-β reduces the ability of DC/tumor fusion cell vaccine to stimulate T cells and inhibits the induction of antitumor immunity through Treg generation in a murine model [29,65]. However, the blockade of TGF-β reduces Treg generation by DC/tumor fusion cell vaccine and enhances antitumor immunity [65]. Moreover, vaccination of cancer patients with DC/tumor fusion cells induces immune responses in the majority of patients, but only a subset of patients demonstrate evidence of tumor regression. Stimulation of T cells with human fusion cells also results in induction of not only CTL but also Treg *in vitro* [69]. We have generated fusions of DCs and HCC cells in the presence of supernatants derived from the HCC cells. DCs generated in the presence of the soluble factors fail to undergo full maturation upon stimulation with the Toll-like receptor (TLR)4 agonist. Moreover, fusions of immature DCs generated in the presence of the soluble factors and the HCC cells promote the generation of $CD4^+$ $CD25^{high}$ $Foxp3^+$ Treg and inhibit CTL induction [52]. Tumor cells take advantage

of Treg to protect them by suppressing immune responses elicited by vaccines. The lack of therapeutic efficacy with fusion-cell vaccine in cancer patients may be correlated, at least in part, with generation of Treg. A combination of control of Treg and concomitant induction of CTLs may be a more effective immunotherapy to reduce recurrence and prolong survival after surgery.

From autologous to allogeneic fusion-cell vaccine

The field of tumor vaccination is currently undergoing a shift in focus, from individualized tailor-made vaccines to more generally applicable vaccine formulations [70]. In the clinical setting of patients with cancer, a major difficulty for the fusion-cell vaccine is the preparation of sufficient amounts of autologous tumor cells. The specimen of tumor from primary lesion may not provide sufficient numbers of viable tumor cells owing to the length of culture time and potential contamination of bacteria and fungus. We have fused autologous DCs with allogeneic cancer cell lines with shared antigens instead of autologous cancer cells. DC/allogeneic tumor fusion cells as well as DC/autologous tumor fusion cells stimulate both $CD4^+$ and $CD8^+$ T cells and induce CTL responses that lyse autologous tumor cells [51,55]. This demonstration of cross-priming against shared tumor-associated antigens opens the possibility of using allogeneic tumor cell lines to deliver tumor-associated antigens to autologous DCs for fusion-cell vaccination protocols. This strategy has numerous advantages:

- Allogeneic tumor cell lines are well characterized as tumor-associated antigen source;
- Allogeneic tumor cell lines shared with tumor-associated antigen can grow well *in vitro*; thus, there is no limiting factor for preparation of tumor cells;
- It is not necessary to determine HLA typing of patients and allogeneic tumor cells as a partner for fusion cells;
- HLA typing of autologous DCs and allogeneic tumor cells does not need to match.

The tumor cells provide tumor antigens and the fusion process simply facilitates the delivery of tumor antigens to the efficient antigen-processing and -presentation machinery of the DCs in fusion cells. The tumor antigens are then presented in the context of MHC class I and II molecules derived from DCs. Moreover, a patient's response to allogeneic MHC molecules may promote an effective T-cell response to self-MHC-restricted tumor peptides [71]. Thus, these allogeneic responses by fusions of autologous DCs and allogeneic tumor cells may successfully be harnessed to promote the immune eradication of cancer (FIGURE 1). In Phase I clinical study, autologous DCs loaded with allogeneic apoptotic/necrotic tumor cells or allogeneic tumor lysates have been used to treat melanoma patients and induced objective clinical responses and antigen-specific $CD8^+$ T-cell immunity [72–75].

Although DCs from cancer patients are defective as APCs because of tumor effects or as a result of cancer therapy [52,76], there are numerous studies that *ex vivo*-generated DCs from cancer patients retain their functions (uptake, maturation, trafficking and presentation) [43,44,47,49–53]. A potential benefit of using allogeneic DCs is that DCs from healthy donors with functional APCs are readily available in unlimited amounts. The fusions of allogeneic DCs and autologous tumor cells express DC-derived allogeneic HLA class II molecules for direct stimulation of alloreactive $CD4^+$ T cells. This allogeneic response might fortuitously involve a cross-reaction with the self-MHC-restricted response to tumor peptides. Indeed, fusions of ovarian carcinoma cells and DCs are functional when generated with either autologous or allogeneic DCs *in vitro* [42]. However, T cells activated by the fusion cells are less effective in lysis of autologous tumor cells. In murine models, it has been reported that allogeneic DC/autologous tumor fusion cells lack therapeutic effects [38]. If the allogeneic DCs and the patient do not share any HLA antigens, self-MHC-restricted presentation of tumor peptides by the DCs is not possible. If the HLA typing of allogeneic DCs share with that of patient, the situation changes dramatically. Where there is sharing of HLA class I molecules, the direct $CD4^+$ T-cell response to the allogeneic HLA class II antigens on the DCs will provide potent T-cell help for the generation of $CD8^+$ CTL responses to tumor peptides presented by the shared HLA class I molecules [71]. In Phase I/II studies, 20 patients with metastatic renal cell carcinoma were partially HLA matched with one or more donors used to generate allogeneic DC/tumor fusion cell vaccine. The vaccination resulted in antitumor immune responses in approximately 50% of patients. Two patients demonstrated a partial clinical response and eight patients had stabilization of their disease [77]. Strategies to enhance the immunogenicity of DC/tumor fusion cells to treat cancer patients must be investigated.

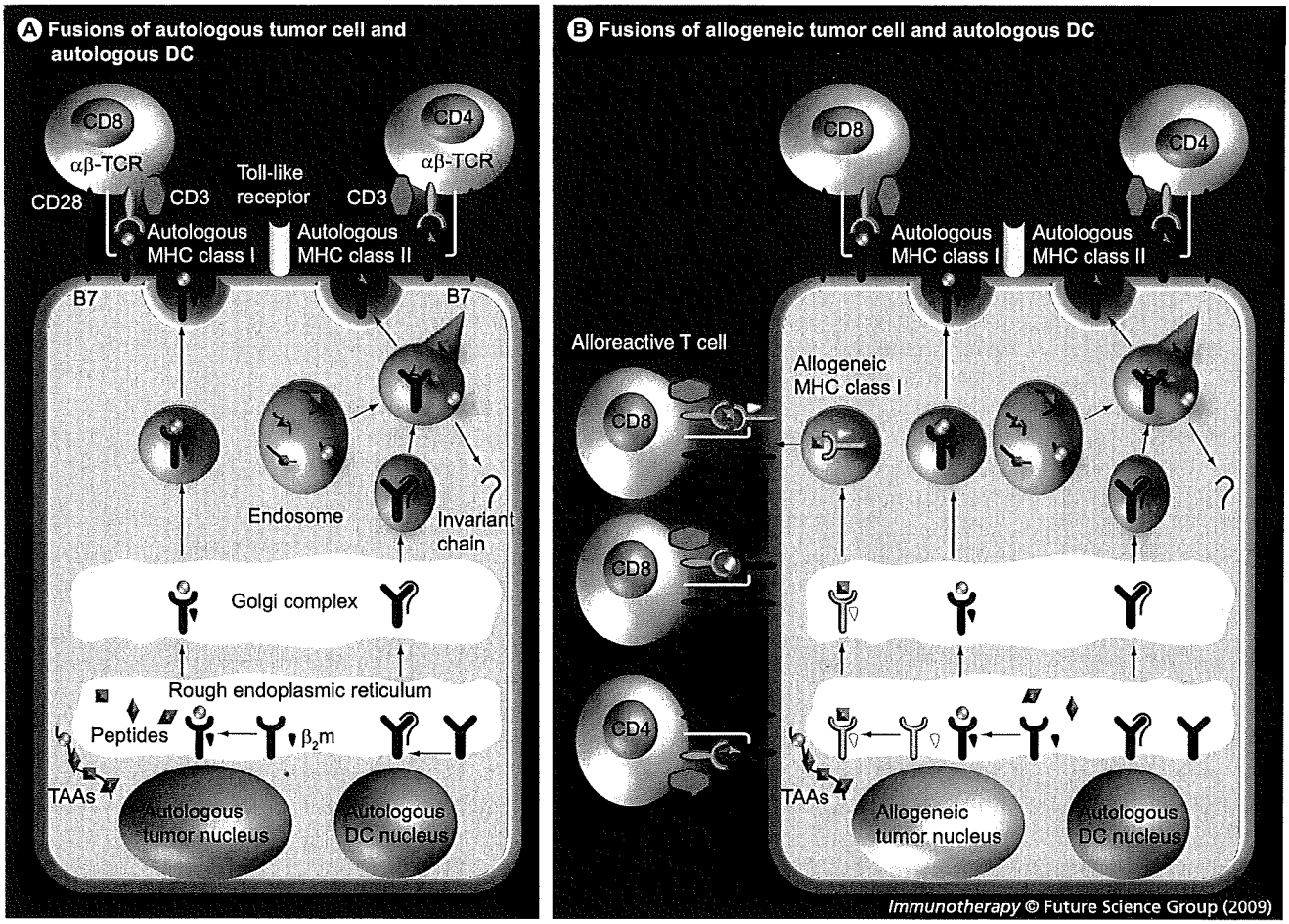

Figure 1. Antigen processing and presentation by dendritic cell/tumor fusion cells. DC/tumor fusion cells express MHC class I and II, costimulatory molecules and Toll-like receptor as well as tumor antigens. **(A)** Fusions of autologous DCs and autologous tumor cells are able to process tumor-derived peptides and MHC class I peptides derived from DCs. They form MHC class I–peptide complexes in the endoplasmic reticulum, which are transported to the surface and presented to $CD8^+$ T cells through the MHC class I-restricted pathway. Similarly, fusion cells can also synthesize MHC class II peptides derived from DCs in the endoplasmic reticulum, which are transported to the cytoplasm where MHC class II–peptide complexes are assembled with tumor-derived peptides. These complexes are presented to $CD4^+$ T cells, which are involved in cytotoxic T lymphocyte induction, through the MHC class II pathway. **(B)** Fusions of autologous DCs and tumor cells are also able to stimulate $CD4^+$ and $CD8^+$ T cells as same as fusions of autologous DCs and autologous DCs. Moreover, fusions of autologous DCs and allogeneic tumor cells are also able to stimulate alloreactive T cells owing to the presence of allogeneic HLA class I molecules from allogeneic tumor cells as a fusion partner. Self-MHC molecules present foreign peptide derived from allogeneic tumor cells to T cells selected to recognize self-MHC–foreign peptide complexes. In addition, T cells also recognize an allogeneic MHC molecule whose structure resembles the self-MHC–foreign peptide complexes and structure formed by both the allogeneic MHC molecules and the bound peptide.
DC: Dendritic cell; TAA: Tumor-associated antigen.

Modified DC/tumor fusion cells

There are still certain obstacles for DC-based antitumor vaccine strategy to achieve therapeutic efficacy in the clinical setting. Appropriate protocol for the clinical trial requires the points below:

- Appropriate subject
- Generation of the best DCs
- Preparation of appropriate tumor-associated antigens
- Method for antigen loading to DCs
- Administration route of the vaccine cells (control behavior of the vaccine cells after administration)
- Overcoming the tumor-induced immune suppression

The DC/tumor fusion strategy would have some advantage to overcome these obstacles. Indeed, vaccination with DC/tumor fusion cells in patients with cancer has been associated with immunological responses in Phase I/II

clinical trials, early clinical trials have shown only limited success [50,77–82]. Many adjuvants, including IL-2 [83], IL-12 [14,84], IL-18 [85], synthetic oligodeoxynucleotides (ODNs) containing specific bacterial unmethylated CpG motifs (CpG-ODNs; TLR9 agonist) [26], and polyinosinic:polycytidylic acid (poly[I:C]; TLR3 agonist) [86], have been used to enhance the ability of DC/tumor fusion cell vaccines to evoke antitumor immune responses in murine models. These results suggest that adjuvants are essential to enhance antitumor immunity when DC/tumor fusion cell vaccines are used to treat patients with cancer. A key question in DC/tumor fusion cell vaccine is how to eliminate or reverse the suppressive function of Tregs by potent adjuvant effect. Recent studies indicate that TLRs directly regulate cancer immunity and tolerance through innate immune responses mediated by Tregs, DCs and other immune cells [87]. Linking TLR signaling to the functional control of Treg may open intriguing opportunities to shift the balance between Th1-type $CD4^+$ T cells and Treg, in ways that may improve the outcome of DC-based cancer vaccine [88,89]. Coadministration of TLR ligands with cancer vaccine regulates the function of Tregs and DCs through numerous mechanisms:

- Stimulation of DCs by TLR signaling results in increased expression of peptide/MHC class I and II complexes, costimulatory molecules (CD80 and CD86) and cytokines (IL-12);
- TLR signaling activation on DCs can render naive T cells refractory to suppression mediated by Treg;
- TLR ligands activate DCs at the tumor site and enhance antigen crosspresentation, migration into regional lymph node and induction of antigen-specific CTL responses;
- TLR ligands, such as CpG, prevent activation-induced cell death in CTLs by increasing the expression of antiapoptotic mediators (Bcl-xL and c-FLIP), allowing these cells to survive and migrate into the tumor site [87,89].

Recently, we have demonstrated that using an immunologic adjuvant composed of a TLR4 ligand OK-432 can increase the production of antitumor CTLs produced by human DC/tumor fusion cell vaccine in a preclinical model. OK-432, penicillin-inactivated and lyophilized preparation of the low-virulence strain (Su) of *Streptococcus pyogenes* (group A), is one of the biological response modifiers and a good manufacturing practice grade agent [90,91]. OK-432 has been widely used and is safe for patients with cancer. OK-432 has been demonstrated to activate neutrophils, macrophages, lymphocytes and NK cells by inducing multiple cytokines, such as IL-12 and IFN-γ, and to polarize the T-cell response to a Th1-dominant state [92–96]. Moreover, OK-432 promotes functional maturation of DCs through the TLR4 and β_2-integrin system to enhance antigen-specific CTL responses to a greater extent than does a previously reported mixture (consisting of TNF-α, IL-1β, IL-6 and PGE_2) or LPS [96–99]. Recently, we have fused OK-432-stimulated DCs and autologous colorectal carcinoma cells [93]. The OK-432-stimulated fusion cells (OK-FCs) coexpress significantly higher levels of CD86, CD83 and IL-12 than those obtained with unstimulated fusion cells. Interestingly, OK-FCs are more efficient in stimulating $CD4^+$ and $CD8^+$ T cells capable of high levels of IFN-γ production and cytolysis of autologous tumor. By increasing the cross-talk between the innate and adaptive immune systems, TLR ligands may drive expansion and memory of CTLs that destroy cancer cells.

Other effective adjuvants for enhancing the induction of antigen-specific CTL are heat-shock proteins (HSPs), to which the ability of heat-treated tumor cells to enhance immunogenicity has been attributed [100–105]. The HSP family includes both constitutively expressed proteins and proteins environmentally induced under stress, including heat treatment. The HSPs are chaperone proteins that can carry peptides, including antigenic peptides. Cancer vaccines based on chaperone proteins appear promising as these proteins naturally exist as complexes with various protein fragments, including those derived from tumor-associated antigens. Moreover, the HSP–peptide complexes can be carried by DCs through receptors and presented in MHC class I and II molecules on DCs [100,106,107]. Recent studies have demonstrated that crosspriming is based on the transfer of proteasome substrates that are transcriptionally upregulated by heat treatment in tumor cells [108]. This is potentially important for the rational design of vaccines that elicit $CD8^+$ T-cell responses. The concept offers additional effects by which heat treatment of tumor cells might enhance antigen processing and presentation in MHC class I and II molecules on the surfaces of fusion cells. Moreover, extracellular HSPs act as chaperone peptides and interact with DCs in a receptor-mediated manner, leading to maturation as well

as proinflammatory responses, all of which are likely to be key danger signals to the immune system. Therefore, we have generated fusion cells by fusing OK-432-stimulated DCs and heat-treated tumor cells (OK/HS-FCs) [109]. OK/HS-FCs are more active than OK-FCs, as demonstrated by: upregulation of multiple HSPs, MHC class I and II, CEA, CD80, CD86, CD83 and IL-12; activation of $CD4^+$ and $CD8^+$ T cells able to produce IFN-γ at higher levels; efficient induction of CTL activity specific for CEA or MUC1 or both against autologous tumor; and superior abilities to induce $CD107^+$ IFN-γ^+ $CD8^+$ T cells and $CD154^+$ IFN-γ^+ $CD4^+$ T cells. The intervention strategy in the combination of OK-432 and upregulated HSPs may act synergistically through the fusion process and result in efficient antigen processing and presentation in the context of upregulated MHC and costimulatory molecules. Factors that may play important synergistic roles in generating augmented CTL activity by OK/HS-FCs include: enhanced expression of CD86, increased production of IL-12p70 and HSP70, and enrichment of intracellular HSPs in OK/HS-FCs (Figure 2). Synergism between OK-DCs and heat-treated tumor cells enhances the immunogenicity of fusion cells and may provide a promising means of inducing therapeutic antitumor immunity. The membranes of fused cells are integrated into a single cell, whereas the nuclei remain separate in the primary hybrid cells [49]. Therefore, the biggest advantage in DC/tumor fusion strategy is that modifications of DCs and tumor cells are independently possible while their characters persist after the fusion. This is an important difference between the DC/tumor fusion strategy and whole-tumor loading strategy.

Clinical trials

In clinical trials, vaccination of cancer patients with autologous DC/tumor fusion cells is associated with immunological and clinical responses in a subset of patients (Table 1). The fusion vaccine

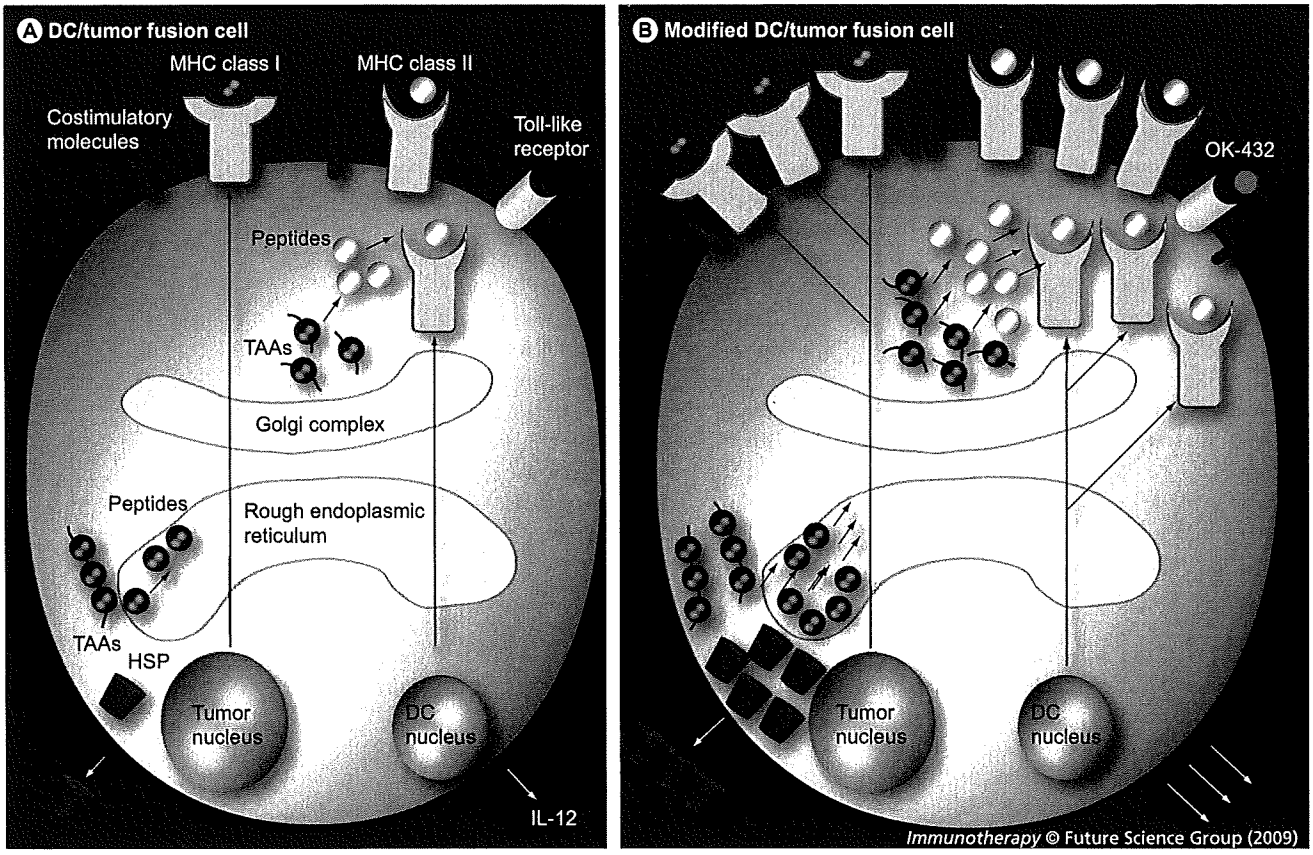

Figure 2. Modified dendritic cell/tumor fusion cells. Fusion cells generated by fusing Toll-like-receptor stimulated DCs (OK-432-stimulated DCs) and heat-stressed tumor cells (modified fusion cells) have a characteristic phenotype with upregulation of multiple HSPs, MHC class I and II, costimulatory molecules (CD80 and CD86), maturation marker CD83, tumor antigens and IL-12. Compared with initial fusion cells **(A)**, synergism between OK-432-stimulated DCs and heat-stressed tumor cells enhances the immunogenicity of fusion cells **(B)**. DC: Dendritic cell; HSP: Heat-shock protein.

Table 1. Asessment of the fusion-cell vaccine.

Tumor	Fusion cell vaccine	Patients (n)	Clinical response	Ref.
Melanoma	Autologous DC/allogeneic tumor	16	1 (CR), 1 (PR), 5 (SD) and 9 (SD)	[110]
Melanoma	Autologous DC/autologous tumor	17	1 (PR),1 (SD) and 15 (PD)	[112]
Glioma	Autologous DC/autologous tumor	8	2 (PR), 1 (SD) and 5 (PD)	[78]
Glioma	Autologous DC/autologous tumor with rhIL-12	12	3 (PR), 2 (MR), 4 (SD) and 3 (PD)	[79,81]
Breast cancer	Autologous DC/autologous tumor with rhIL-12	2	1 (SD) and 1 (PD)	[79,81]
Gastric cancer/ colorectal carcinoma	Autologous DC/autologous tumor with rhIL-12	3	1 (SD) and 2 (PD)	[79,81]
Ovarian carcinoma	Autologous DC/autologous tumor with rhIL-12	3	2 (SD) and 1 (PD)	[79,81]
Melanoma	Autologous DC/autologous tumor with rhIL-12	4	4 (PD)	[79,81]
Breast cancer	Autologous DC/autologous tumor	10	2 (PR), 1 (SD) and 7 (PD)	[80]
Renal cell carcinoma	Autologous DC/autologous tumor	13	5 (SD) and 8 (PD)	[80]
Renal cell carcinoma	Allogeneic DCs/autologous tumor	20	2 (PR), 8 (SD) and 10 (PD)	[77]

CR: Complete response; DC: Dendritic cell; MR: Mixed response; PD: Progressive disease; PR: Partial response; rh: Recombinant human; SD: Stable disease.

has been conducted in a variety of tumors safely without any serious side effects. Fusion-cell vaccination was first reported in patients with melanoma [110,111]. Among 16 patients with advanced stage metastatic melanoma, one complete and one partial remission, and six cases of stable disease were observed. Interestingly, stable disease could be maintained by repeated booster injections for more than 24 months in some patients. Vaccination of 17 melanoma patients with fusions of autologous tumor cells and autologous DCs were also reported [112]. One had a partial response with decrease in size of all evaluable tumor manifestations. In one patient, some of the metastases were regressed despite an overall progressive disease, and one patient achieved disease stabilization for 6 months. In our initial clinical report on fusion-cell vaccine, eight patients with malignant glioma were vaccinated with fusions of autologous DCs and autologous tumor cells. In all six cases analyzed, the concentration of IFN-γ in the T-cell culture supernatant increased after vaccination. However, clinical responses were not observed [78]. Therefore, we had conducted a Phase I/II clinical study for the safety profile of vaccination with DC/tumor fusion cells and recombinant human (rh) IL-12 in patients with malignant brain tumor, breast cancer, gastric cancer, colorectal carcinoma, ovarian carcinoma and melanoma [79,81]. No serious adverse effects were observed. Three out of 12 patients with malignant brain tumor (25%) achieved a partial response and one patient had a mixed response, but other patients showed no tumor regression. A total of 13 out of 16 patients with brain tumor (81%) showed cutaneous delayed typed hypersensitivity responses. Vaccinations of fusion cells and rhIL-12 provide good therapeutic responses in some of the patients with brain tumor. In this clinical trial, we vaccinated patients with extremely small amounts of fusion cells and IL-12, but this resulted in immunologic and clinical responses in a subset of patients with malignant brain tumor. On the other hand, patients with metastatic breast or renal cancer were also treated with fusions of autologous tumor and autologous DCs [80]. In this study, 23 patients were vaccinated by fusion cells and no significant treatment-related toxicity was observed. In a subset of patients, vaccination resulted in immunological responses. Interestingly, two patients with breast cancer exhibited disease regression, including a near complete response of a large chest wall mass. Five patients with renal carcinoma and one patient with breast cancer had disease stabilization. Recently, they also assessed the vaccination with fusions of allogeneic DCs and autologous tumor cells in patients with metastatic renal cell carcinoma [77]. Vaccination resulted in antitumor immune responses in ten (48%) of 21 evaluable patients. Two patients demonstrated a partial clinical response and eight patients had stabilization of their disease. However, DC/tumor fusion cell-based vaccine may work more effectively in patients in the early stage of the disease with low tumor burden after surgery, chemotherapy or irradiation, and patients with a still uncompromised immune system are expected to respond best to this vaccine.

Methods for generation of the fusion cells

Although the chemical agent PEG [11], electroporation [23,28,38,54,85], and many viruses [26,27] have been used for the fusion strategy, the fusion process of these methods is different. We have used PEG to fuse DCs and tumor cells. In this approach, DCs are mixed with tumor cells at a

ratio of 10:1 in serum-free prewarmed RPMI-1640. Mixed-cell pellet are gently resuspended in prewarmed 50% PEG solution for 3–5 min at room temperature. Subsequently, the PEG are diluted by slow addition and mixing of 1, 2, 4, 8 and 16 ml of serum-free prewarmed RPMI medium until 50 ml. Cell pellets are resuspended in RPMI-1640 supplemented with 10% autologous heat-inactivated serum, GM-CSF and IL-4 and cultured in a 5% CO_2 atmosphere at 37°C for 3–5 days. By this time, each DC/tumor-fusion cell are integrated into a single entity and are loosely adherent to the culture dish. Unfused tumor cells grow firmly attached to the plates, whereas DC/tumor fusion cells are loosely adherent in the culture wells. DC/tumor fusion cells can be selected and purified by gentle pipetting, and firmly attached tumor cells are discarded. There is no standardized method for the selection of DC/tumor fusion cells. Some studies have reported enriching DC/tumor fusion cells by FACS cell sorting [24,113] and a method to transfect DCs with Tyr-green fluorescent protein reporter virus [27]. On the other hand, short-term culture of fusion-cell preparations can also promote DC/tumor fusion efficiency and reduce cell aggregates [93]. Therefore, it is not necessary to enrich DCs/tumor fusion cell preparations using special methods.

Conclusion

The DC/tumor fusion strategy has been generated and successfully used as a vaccine in mice models. Tumor-specific immune responses were also successfully induced in patients by DC/tumor fusion-cell vaccine; however, the overall rate of clinical response remains low. Thus, a deeper understanding of human cancer pathogenesis is critical to improve the design of novel cancer vaccine. Immune systems of mice and humans differ in many aspects. Research with cancer patients' samples is fundamental to understand human diseases and to design novel therapies. Tumor cells use a variety of immunosuppressive mechanisms to defeat potentially effective immune responses through elaboration of immunosuppressive cytokines (TGF-β and IL-4, -6 or -10), the production of immunosuppressive immune cells (Tregs and myeloid-derived suppressor cells [MDSCs], or tumor-associated macrophages (TAMs), and disruption in cell signaling (MHC class I loss, degradation of T-cell receptor, STAT-3 signaling loss in T cells, or induction of indoleamine-2, 3-dioxygenase) [114]. Impaired antitumor immunity is also associated with the maturation stage of DCs that produce proinflammatory cytokines (IL-6 and TNF-α). Immature DCs give rise to Th2-type $CD4^+$ T cells that produce IL-4, -6 and -13 [56]. This immunosuppressive environment also promotes generation of Tregs and accumulation of MDSCs and TAMs. Importantly, tumor tissues comprise not only of tumor cells but also of tumor-associated fibroblasts, vascular endothelial cells and extracellular matrix, all of which are key regulators in tumorigenesis [115,116]. Therefore, tumor rejection also can be achieved by modulation of tumor-stromal fibroblasts or by disturbance of the network [117]. Indeed, fusion of mouse DCs and endothelial cells results in induction of endothelial cell-specific $CD4^+$ and $CD8^+$ T-cell responses, which markedly inhibit angiogenesis and eradiate tumor [118]. These evidences explain, at least in part, why different therapeutic efficacy of fusion-cell vaccination has been achieved in mice compared with human studies. Patients early in the course of the disease with low tumor burden and still an uncompromised immune system are expected to respond best to clinical responses by fusion-cell vaccination.

Future perspective

Despite tremendous progress in basic immunological research, effective vaccines for most types of cancer are still lacking. Fusion-cell vaccine alone may be insufficient to have a significant contribution to treat advanced caner patients. DC vaccines may be combined with other immunotherapy or conventional therapy. In a few murine studies, the combination therapy of fusion-cell vaccine and adoptive immunotherapy is very effective against poorly immunogenic carcinoma [36,119,120]. Interestingly, when adoptive immunotherapy was combined with nonmyeloablative lymphodepleting chemotherapy, 18 (51%) of 35 treated patients experienced objective clinical responses, including three ongoing complete responses and 15 partial responses [121]. This improvement of clinical responses is most likely achieved by the elimination of Tregs and MDSC. A $CD25^{high}$ targeting immunotoxin (denileukin diftitox) can deplete $FoxP3^+$ Treg, decrease Treg function and enhance antigen-specific T-cell responses *in vitro*. It indicates the potential for combining Treg depletion with cancer vaccines to enhance tumor antigen-specific immune responses [122]. Moreover, DC vaccination can be also combined with chemotherapy, radiotherapy, hormonal therapy, monoclonal antibodies or photodynamic therapy to reduce Treg and enhance CTL responses. Combined, these approaches have enormous potential to improve the current outcomes from conventional cancer therapy. Cytotoxic chemotherapy

not only affects the tumor but also depletes Treg, potentially enhancing immune responses. Post-chemotherapy immune system reconstitution may provide a unique opportunity for therapeutic intervention by shaping the repertoire towards reactivity to tumor antigens [123,124]. Although many technical and theoretical challenges remain, the next decade will see the first clinical trials testing whether Treg-based therapies are effective in cancer patients.

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Executive summary

- Fusions of dendritic cells (DCs) and tumor cells express a broad spectrum of tumor-associated antigens, including those known and unidentified that are processed endogenously and presented by MHC class I and II pathways in the context of costimulatory signals.
- The fusion cell facilitates the entry of tumor-associated antigens that are synthesized *de novo* in the heterokaryon into the DC endogenous pathway of antigen-processing and -presentation machinery.
- Fusions of autologous or allogeneic tumor cells with autologous DCs result in antigen presentation through MHC class I and/or II pathways, stimulation of both $CD4^+$ and $CD8^+$ T cells, and induction of antigen-specific polyclonal cytotoxic T lymphocyte (CTL).
- Fusions of impaired DCs and tumor cells also stimulate the expansion of T regulatory cells (Tregs), while Toll-like receptor (TLR) agonist-stimulated fusion cells may promote the expansion of CTLs.
- Synergism between TLR-stimulated DCs and heat-treated tumor cells enhances the immunogenicity of fusion cells.
- Fusion-cell vaccine can be combined with other immunotherapy, chemotherapy, radiotherapy, hormonal therapy, monoclonal antibodies or photodynamic therapy to provide promising means of inducing therapeutic antitumor immunity.

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IX. 肝癌の治療

腫瘍因子からみた治療戦略

多発肝細胞癌

Treatment strategy of multiple hepatocellular carcinoma

古瀬純司

Key words：多発肝細胞癌，肝内転移，多中心性発生，肝動脈化学塞栓療法，化学療法

はじめに

肝細胞癌(hepatocellular carcinoma)は肝内に複数の病変を認める多発肝細胞癌の病態を示す場合が多く，病変数は治療戦略上大きな要素となる．肝細胞癌に対する治療選択のガイドラインとして，我が国では肝癌診療ガイドラインが作成され，単発，2または3個，および4個以上で治療選択が異なってくる．更に肝細胞癌では多発の病態として肝内転移と多中心性発生があり，治療選択や予後も異なる．

本稿では，多発肝細胞癌の概念や診断，治療選択について概説する．

1. 概念・定義

日本肝癌研究会の全国原発性肝癌追跡調査報告(第17回；2002-2003)では，16,187例中，単発57.9％，2個以上42.1％であり，ほぼ半数が多発肝細胞癌である[1]．肝内の多発病変については，その成因として，主病巣からの肝内転移(intrahepatic metastasis)と多中心性発生(multicentric occurrence)があり(**表1**)[2]，治療選択と予後の推定に大きくかかわってくる．

肝細胞癌の多中心性発生の診断として，分子

表1　多発肝細胞癌の種類(文献[2]より引用)

肝内転移	1) 門脈腫瘍栓(portal vein tumor thrombus)あるいは，これを基盤として増殖したと考えられる癌病変 2) 最大の癌腫の近傍に多く，離れるに従って数が少なくなるような癌病変群 3) 孤立性の癌病変でも，最大の癌腫の近傍にあり，それに比して明らかに小さく，かつ組織型がそれと同様か，分化度が低い癌病変
多中心性発生	肝細胞癌の複数病変がみられ，以下のその場で発生し増殖しつつある病変が強く推定される病変 1) 腺腫様過形成(adenomatous hyperplasia)や既存の肝構築を保つ早期肝細胞癌 2) 中分化，あるいは低分化癌組織の辺縁に高分化癌組織の存在を認める肝細胞癌

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生物学的手法や遺伝子解析による試みが行われている．B型肝炎ウイルス(hepatitis B virus: HBV)関連の肝細胞癌において，HBV DNAの組み込みパターンを解析することにより，多中心性発生が証明されている．Sakamotoらは，1人の患者から切除された2つの離れた病変において，Southern blot法を用いてHBV DNAのclonalityを解析し，それぞれ異なった組み込みパターンが認められたことから，これらが独立して発生した肝細胞癌であると報告している[3]．また，Sheuらは，DNA fingerprinting法により9人中7人の肝細胞癌患者において，異なったHBV DNAのclonalityをもつ複数の病変があることを確認している[4]．Odaらは，p53癌抑制遺伝子の変異を検索することにより肝細胞癌の多段階発育や多中心性発生の診断が可能と報告している[5]．

しかし，実際の臨床では，原発巣(多中心性発生)か転移巣(肝内転移)か診断が困難な場合が多く，画像診断での特徴や臨床経過が大きな判断根拠となる．

2. 診　　断

多発肝細胞癌の診断としては，複数の病変の検出すなわち数の診断と，それが肝内転移によるものか多中心性発生によるものかの病態診断に分けられる．多くの肝細胞癌では動脈から血流が供給されており，造影早期で濃染され，後期でwash outによる欠損像として描出される．早期の高分化型肝細胞癌では動脈血流を受けることが少なく，腫瘍内血流を欠く場合が多い．一部では門脈支配の優位を示す病変もみられる．中分化癌あるいは低分化癌では動脈血流が増大し，最終的には動脈血のみの支配となる[6]．それぞれの病変が異なる画像所見を示し，動脈血流の少ない病変あるいはそれを含む病変では多中心性発生を示唆し，動脈相で濃染する均一な病変は肝内転移を考える．

画像診断の発達により，極めて小病変の検出が可能となり，かなり正確な診断が行われるようになってきた．現在，数の診断として，超音波，dynamic CT，MRI，血管造影，動脈造影CTが行われている．超音波は最も簡便な検査法として最初に行われることが多く，他の検査法で検出されない早期の小肝癌が描出されることも多い．造影超音波検査により腫瘍内血流の診断や造影後期でのfilling defectによる小病変の検出能も上がっている．CTはmulti-detector-row CT(MDCT)が主流であり，空間分解能，時間分解能の高い画像データ収集により小病変の検出が可能となっている．超音波や造影CT(dynamic CT)の造影パターンにより，腫瘍の性状が把握され，多中心性発生か肝内転移かの診断の補助となる．MRIの画像も急速に向上しており，更に拡散強調画像など新しい診断法が行われている．動脈造影CTは肝動脈から造影してCT撮影を行うCT during hepatic arteriography(CTA)と経上腸間膜動脈的に門脈造影を行って撮影するCT during arterial portography(CTAP)が行われる．肝細胞癌の病変はCTAで高吸収域として描出され，CTAPでは非腫瘍部の肝組織が門脈血流により染まり，腫瘍部は門脈血流を欠くため低吸収域となる．しかし，CTやMRIの診断能が発達しており，血管造影を用いた診断としての意義は少なくなっている．

3. 臨床的特徴と予後

多発肝細胞癌では同時性多発と異時性多発の2つの面を考える必要がある．肝細胞癌は比較的早期から経門脈性の肝内転移をきたしやすい．根治的な治療が行われても，検出されない微小転移巣が既に存在し，早期に多発性の再発を認めることも少なくない．また，多くの肝細胞癌の背景にHBVあるいはC型肝炎ウイルス(HCV)の感染による慢性炎症を伴っており，異時性の多中心性発生も考慮する必要がある．Aizawaらは，C型肝炎の肝線維化の違いによりsevere fibrosisとmild fibrosisでは累積肝細胞癌発症率はそれぞれ8年で28％，6％，13年で45％，23％であり(年間発癌率3.5％，0.75％)，有意差を認めたと報告している(relative risk: 3.48，95％CI: 1.51-8.06，p=0.004)[7]．

単発例と多発例においては，当然予後に差が

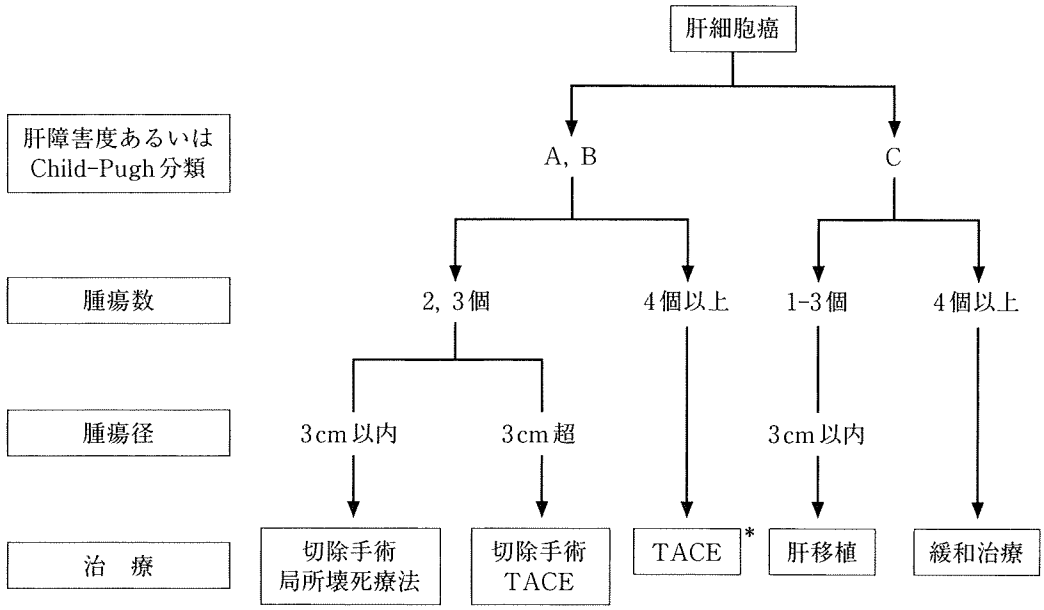

図1　多発肝細胞癌の治療戦略(肝癌診療ガイドライン'肝細胞癌治療アルゴリズム'[9]を基に作成)

TACE: 肝動脈化学塞栓療法. *TACEの無効例, 血管侵襲, 肝外転移例では今後, 全身化学療法が適応となる.

みられる. 日本肝癌研究会の全国原発性肝癌追跡調査報告によると, 肝切除において単発, 2個, 3個以上と腫瘍数の増加に伴い5年生存率もそれぞれ59.2%, 46.4%, 30.0%と低下している[1]. 多中心性発生例と肝内転移例の予後の差については, 正確な診断が困難な場合が多く実際の臨床では難しいが, Okusakaらは, 根治的に切除された多発肝細胞癌において, 原発性肝癌取扱い規約の基準に基づき, 多中心性発生例と肝内転移例に分類し, その予後を検討している[8]. それによると3年無再発生存率と3年生存率はそれぞれ23%, 8%と92%, 51%であり, 多中心性発生例で有意に良好な予後が得られたとしている[8].

4. 治　　療

肝細胞癌の治療においては, 腫瘍数, 大きさ, 腫瘍塞栓の有無などの進行度と肝障害度に応じた治療選択が必要である. また, 同じ多発癌でも多中心性発生例と肝内転移例では治療戦略も異なってくる. 多中心性発生ではそれぞれが局所にとどまっていることから, 効果的な局所治療により根治を目指した治療が選択されるべきである. 一方, 肝内転移では顕在病変の治療だけでなく, 微小転移巣を考慮した治療を選択するということが基本的な考え方となり, 初回治療あるいは再発時において, 肝動脈化学塞栓療法(transcatheter arterial chemoembolization: TACE)など経動脈的治療を組み合わせた併用療法が必要となる. 実際の臨床では3個以内であれば多中心性発生の可能性が高く, 切除やラジオ波焼灼療法などの局所壊死療法が適応となり, 4個以上であれば肝内転移の関与が高いと判断され, TACEが適応となるというのが一般的なコンセンサスである.

多発肝細胞癌の治療戦略として, 我が国の肝癌診療ガイドライン'肝細胞癌治療アルゴリズム'では, 肝障害度A, Bかつ2, 3個であれば, 腫瘍径3cm以内なら切除あるいは局所壊死療法, 3cm超なら切除あるいはTACEが推奨されている(**図1**)[9]. これは日本肝癌研究会の追跡調査に基づいており, 腫瘍径2cm以内では腫瘍数2個および3個での治療成績は肝切除, 局所壊死療法(エタノール注入)で差はなく, また腫瘍径2-5cmでも2個あるいは3個での治療成績は両者で有意差はないと報告されている[10].

また切除，エタノール注入療法(PEI)，TACEの後ろ向き比較試験では腫瘍径3cm以下，3個以内，肝機能良好例で切除とPEIでは差がなかったものの，TACEでは有意に予後不良であったと報告されている[11]．局所壊死療法は一般に腫瘍径3cm以下で適応されており，上記のアルゴリズムのような治療選択となる．一方，肝障害度Cの肝機能不良例ではこれらの治療は原則として適応にならない．3個以内，腫瘍径3cm以下(単発は5cm以内)であれば肝移植が，それ以外は症状緩和治療が勧められる．

肝障害度A，Bかつ4個以上の場合はTACEが選択される．多発肝細胞癌におけるTACEの有用性はランダム化比較試験およびメタアナリシスで検証されている．Llovetらは，切除不能肝細胞癌を対象(65-77％が多発例)にgelatin spongeとdoxorubicinによるTACE，gelatin spongeのみの塞栓療法(TAE)および保存的治療のみ(対照群)の3群によるランダム化比較試験を行い，2年生存率がTACE 63％，TAE 50％，対照群27％と対照に比べTACEで有意に予後が良好であったと報告している[12]．また，6つのランダム化比較試験のメタアナリシス(503例)でも塞栓療法による2年生存率の有意な改善が示されている(オッズ比0.53，p=0.017)[13]．

門脈腫瘍塞栓や肝静脈腫瘍塞栓を伴う多発肝細胞癌や巨大腫瘍の場合，TACEの適応はなく，標準的な治療法は確立していない．そのような例に対して動注化学療法が行われることが多い．動注化学療法では高濃度の抗癌剤を肝細胞癌に直接投与することにより，局所濃度を高め，全身への影響を抑えることが治療の根拠となっている．cisplatin単独，5-FU+cisplatin，5-FU+interferonなどのレジメンが主に行われ，全身化学療法に比べ高い奏効率が得られている．しかし，生存に関する有用性は十分に検証されておらず，肝癌診療ガイドラインでも十分な科学的根拠がないとされている．

全身化学療法は，切除，局所壊死療法，TACEなどの局所療法が適応とならない，あるいは無効の場合に適応となる．これまで多くの臨床試験が行われてきたが，生存期間に寄与する標準治療は確立していなかった．近年，癌の進展，増殖にかかわる様々なシグナル伝達が明らかとなり，それらをターゲットとした分子標的薬の開発が行われてきている．その中で，上皮成長因子レセプター(EGFR)の下流であるRAFキナーゼとVEGFR-1-3，PDGFR-βなどを標的とするマルチキナーゼ阻害薬sorafenibを用いて，placebo controlのランダム化比較試験が行われた[14]．その結果，全生存期間中央値はsorafenib群10.7カ月，control群7.9カ月，ハザード比0.69(95％CI: 0.55-0.87，p<0.001)とcontrol群に比べsorafenib群で有意な生存期間の改善が確認され[14]，欧米を中心に多くの国で肝細胞癌に適応が承認されている．我が国でも保険適応の承認が待たれており，局所治療の適応とならない多発肝細胞癌(肝外転移を含む)に対する標準治療として確立していくものと考えられる．更に現在，肝細胞癌に対し，多くの分子標的薬が開発されており[15]，今後，多発肝細胞癌の予後改善につながるものと期待される．

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