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Review article

Immunotherapy for advanced or relapsed cervical cancer

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ABSTRACT

Cervical cancer is the third most commonly diagnosed cancer and the fourth leading cause of cancer deaths worldwide. Conventional approaches by surgery, radiotherapy, and/or chemotherapy cannot provide satisfied therapeutic outcomes for advanced or relapsed cervical cancer. Thus, immunotherapy or biotherapy for these patients has been the mode of treatment. A number of studies on immunotherapy for cervical cancer in animals has been published. This time, we summarized our experiences and concepts with regard to the therapeutic approach for advanced cervical cancer in humans.

Introduction

Human papillomavirus (HPV) infects a large number of women worldwide and presents in more than 99% of all cervical cancer patients.[1] However, cervical cancer is still a major health problem in the world. A recent study revealed that there are approximately 470,000 cases of cervical cancer are diagnosed annually worldwide.[2] Cervical cancer is also the second leading cause of cancer-related deaths among women worldwide.[3]

About 30% of cervical cancer patients will experience failure after definitive treatment, including surgery, radiotherapy, and/or chemotherapy. The prognosis for patients with tumor recurrence is dismal. The 5-year survival rates of patients with advanced cervical cancer treated by palliative therapy reportedly range from 5% to 15%.[4–7] Patients who suffer from recurrent cervical cancer will have a poor prognosis in the conventional modality of treatment. The 1-year survival rates ranged between 15% and 20%. Therefore, novel approaches for the prevention or treatment of recurrent cervical cancer are necessary.

The initial stage at cervical cancer diagnosis is still the best predictor of the disease prognosis. Although early-stage cervical cancer is curable in most patients by surgery and/or radiotherapy, the survival rates decrease sharply in a patient with a locally advanced stage. The 5-year survival rate is 35–65% in stage IIB cervical cancer, 28–35% in stage IIIb, and 10–15% in stage IVA.[8] Although surgical intervention combined with chemotherapy or radiotherapy has been well developed in recent decades; even now a large number of patients with advanced cervical cancer fail following conventional therapy. Thus, in the past few years, we have used integrated therapy, including conventional therapy and adjuvant agents, to enhance host immunosurveillance to make cancer cells dormant. Immunotherapy is a reliable treatment decision in cancers with poor therapeutic outcome.

The cervical cancer vaccine is based on the notion that the immune system could possibly mount a rejection strength response against the neoplastic cell conglomerate. In the case of cervical cancer, the therapeutic targets are easily identified: HPV xenoantigens are readily recognized by the immune system, and their targeting has shown great promise in preclinical models of therapeutic vaccination and clinical studies of preventive vaccination. These advances will also be applicable to vulvar or vaginal cancers caused by HPV.

Furthermore, as E6 and E7 are required for the induction and maintenance of the malignant phenotype,[9] cervical cancer cells are unlikely to evade an immune response through antigen loss. Thus, although care must be taken, given the oncogenic nature of these genes, E6 and E7 proteins represent good targets for developing antigen-specific immunotherapeutics or vaccines for cervical cancer.

How to create effective antitumor response for HPV-16 related lesions

In a previous study, our preclinical therapeutic vaccination has shown promising results.[10–16] Various forms of HPV vaccines, such

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as vector-, peptide-, protein-, deoxyribonucleic acid (DNA)-, chimeric/*in vivo* adjuvant pulse Virus-like particle (VLP)-, and cell-based vaccines, have been described in experimental systems targeting HPV-16 E6 and/or E7 proteins. Dendritic cells (DCs) are the most effective antigen-presenting cell (APC) population in inducing activation and proliferation of naïve T cells. T cells, especially CD8+ cytotoxic T lymphocytes (CTLs), are major effector cells in tumor immunotherapy. Immunotherapy using the heterologous prime-boost regimen has emerged as an effective approach for generating robust antigen-specific T-cell-mediated immune responses against tumors and infectious diseases. In our previous study, we characterized the contribution of $CD4^+$ T cells in the induction of $CD8^+$ T-cell immune responses and generation of long-term antitumor effects by vaccination with SINrep5-E7/HSP70 and booster with vac-E7/HSP70.[12] Vaccination with CD4 depletion significantly reduced the number of E7-specific $CD8^+$ T cells in mice. In addition, $CD4^+$ T cells play an important role in the generation of long-term antitumor effect.[12,13] Thus, CD4+ T cells are found to be important in the generation of $CD8^+$ T cell immune responses and long-term antitumor effect in vaccinated mice with established tumors.

It is well-known that activation-induced cell death (AICD) plays an important role in the decline of active antigen-specific T cells following vaccination or infection.[17] In general, AICD may contribute to the regulation of cellular immune responses. It may serve as an important mechanism for limiting immune responses through the killing of antigen-activated T cells upon the encounter of the specific antigen. This is an important mechanism for our immune system to regulate our adaptive immune responses and to preserve the homeostasis of our immune system.[17] Thus, the use of repeated booster immune adjuvants may circumvent immunosuppressive factors such as TGF-beta, FASL and prostaglandins, break T cell tolerance to tumor antigens, ensure adequate T cell costimulation, and/or provide signals to enhance particular cytokine responses that initiate antitumor immunity and maintain long-term memory/effector CTLs.

DNA vaccines, which are simple and reproducible agents, have emerged as an attractive approach for generating antigen-specific immunotherapy. These agents will enable host immune cells to recognize xenoantigens (ex. HPV) and play a central role in antitumor response. Then, activated T helper cells will make antigen-specific response to target antigens. The signaling requirements for this activation have been described on the basis of two distinct signals that are generated in the T cells and induced by APCs.

In addition, DNA vaccines may enhance antigen-specific CD8+ T-cell immune responses. The HPV oncoproteins E6 and E7 are consistently expressed in HPV-associated cancer cells and are responsible for their malignant transformation. Therefore, HPV E6 and E7 are ideal target antigens for developing vaccines and immunotherapeutic strategies against HPV-associated neoplasms.

In addition, CD4 (+) T cells play an important role in controlling HPV-associated lesions. This finding resulted from immune deficiency patients who have a higher possibility of having HPV-associated disorders. Thus, CD4 (+) T cells are important in tumor immunity and maintain long-term tumor antigen-specific memory responses.

Adjuvant agents induce *in vivo in situ* immune activation

The initiation of the immune response occurs in the lymph nodes that drain the vaccination site through intradermal Gene Gun administration or the injection of adjuvant agents. At this time, DCs play a critical role in the transport of antigen to the lymph node and in presenting or activating naïve antigen-specific T cells. These adjuvant agents will induce naïve Langerhan cells (LCs) to immature DCs. The maturation of DCs are highly efficient in the capture and uptake of antigens, pinocytosis and phagocytosis. The ultimate goal of cancer immunotherapy is the eradication of tumor cells by the immune system. Both the innate and the adaptive arm of the immune system can contribute to the eradication of tumor cells, with natural killer (NK) cells and T cells, respectively, as key players.

DNA vaccination has emerged as a promising strategy for cancer immunotherapy. However, since DNA vaccines have low immunogenicity, various strategies have been developed to enhance the potency of DNA vaccines. The linkage of IL-2 to HPV-16 E7 antigen significantly enhances the DNA vaccine potency against E7-expressing tumors.[14]

In the adaptive immune response against tumor cells, it is crucial to activate CD8+ cytotoxic T lymphocytes (CTLs). This strategy will help in exploiting their cytotoxic potential against tumor cells after recognition of tumor-associated antigens (TAA) or antigen-specific immunization (ASI). Adding cluster gene gun plasmid E7-HSP70DNA vaccine offered a simple solution in restoring the efficacy of the prime-boost vaccination with viral vectors and has potentially significant clinical applications. Gene gun plasmid E7-HSP70DNA vaccine will trigger skin LCs. Following the uptake of antigens and in the presence of stimulatory signals, the DCs migrate to the draining lymph node via afferent lymph vessels. During their migration, the cells process the specific antigen and undergo a maturation process. This process will result in DCs maturation and is highly efficient in presenting antigenic peptides to T cells. Then, the mature DCs will activate the T cells by providing costimulatory signals. These activated T cells will directly differentiate into appropriate effector cells.[18] Thus, the activation of naive CD8+ cells occurs via APCs, with DCs considered as the most powerful APCs. These activated T cells will capture and process TAAs, and then present the epitopes on their membranes in complex with major histocompatibility complex (MHC) molecules. Therefore, the maturation of APCs by danger signals is essential for the presentation of epitopes (E6/E7) in a stimulatory way to T cells.

Cancer immunoprevention and immunotherapy

Immunoprevention is a fresh approach to cancer protection and/or adjuvant immunotherapy based on the stimulation of host immunosurveillance following surgery, chemotherapy, and/or radiotherapy in cancer treatment. Although vaccination with preventive HPV vaccines can generate high titers of serum neutralizing antibodies in animals and humans, this immunization may not be able to generate significant therapeutic effects for established or breakthrough HPV infections, especially the escaped antibody-mediated neutralization. Therapeutic vaccines should induce specific cell-mediated immunity, prevent the development of lesions, and eliminate pre-existing lesions or malignant tumor cells.

In our opinion, DCs have potential to recognize foreign antigen, process it into small peptides for presentation onto MHC molecules to the TCR, and provide the essential costimulatory molecules for activation of naïve CD4+ and CD8+ T cells. Right now, aluminum-containing adjuvants continue to be widely used as adjuvants. Therefore, we think that VLPs such as Gardasil (Merck) or Cervarix (GSK) will serve as a potential peptide for cervical cancer therapy. Thus, biological therapy is a option for patients with advanced cervical cancer. It uses the body's immune system, either directly or indirectly, to flight cancer cells and lessen the side effects that may be caused by conventional cancer treatments.[19]

Cytokines and/or chemokines in cancer immunotherapy

Individual immune cells, including innate cells and adaptive cells, maintain personal health. Therefore, immune dysfunction will contribute to persistent inflammation and/or some infectious diseases. Chronic inflammation may result in persistent cell damage, which is hard to repair. Long-term cell damage may induce the formation of oncogenic cancer cells.

It is found that many cytokines and chemokines show the ability to stimulate immune systems. Thus, cytokines such as interferon (IFN)-α, IFN-β, or IFN-γ, interleukin (IL)-2 and IL-12 achieved by a "Th2 to Th1 switch" are believed to contribute in the proliferation of specific populations of leukocytes involved in antitumor immunity. These have been used in numerous clinical trials with consistent results. Stimulation results in the secretion of cytokines, such as type-I IFNs (IFN-α, IFN-β, or IFN-γ), tumor necrosis factor-α (TNF-α), IL-6, and IL-12, and these cytokines will upregulate some surface molecules such as intercellular adhesion molecule 1 (ICAM-1), CD40, B7-1, and B7-2, and MHC classes I and II. Type-I IFNs have demonstrated complex antitumor properties, including the direct induction of cancer cell apoptosis and potent enhancement of antitumor immune responses through the stimulation of DCs.[20,21]

In summary, both immune and nonimmune cells can be used for cytokine cancer therapy, including (1) T cells: T cells have ability to enhance the development, proliferation and/or function of either endogenous or exogenous adoptively transferred effector T cells. (2) Natural killer (NK) cells: The NK cells have the ability to enhance NK activity and improve antibody-dependent cell-mediated cytotoxicity. (3) Tumor cells: Tumor cells can upregulate antigen and MHC expression. It will induce an antiproliferative effect. (4) DCs and APCs: DCs and APCs can be generated and matured *in vitro*, and operated *in vivo*.[22]

Rational biotherapy for cervical cancer

Advanced stage cervical cancer has a high possibility of local regional relapse or distant metastasis, even after radiation and/or chemotherapy. There is an urgent requirement to develop an affordable clinical therapeutic agent against advanced cervical cancer. The DCs are potent antigen-presenting cells, which play central roles in bridging between innate and acquired immunity via direct cell–cell interaction and/or cytokine production[18] OK-432 (Picibanil), a penicillin-sensitive *Streptococcus pyogenes*, has been reported with potent immunomodulation properties in cancer treatment by stimulating the maturation of DCs and secretion of Th-1 type cytokines. OK-432 followed by TC-1 lysate pulsing thrice can generate markedly increased immune responses of E7-specific $CD4^+$ T cells and a moderate increase in the production of NK cells, as well as a satisfactorily protective and therapeutic antitumor effect.[13] These findings warrant OK-432 combination with tumor lysate will be an effective and safe vaccine in future clinical applications of advanced cervical cancer therapy.

DCs are considered to be the principal APCs, whose main function is to identify microbial structures and present them to naïve T cells,[18,23,24] and clinical application of DC has been initiated as cellular immunotherapy against cancer.[25] The interaction of T cells with DCs is crucial for directing T cell differentiation towards Th1, Th2, or Th17.[26,27] Cytokines, secreted by DCs during initial T cell stimulation, play an important role in the subsequent differentiation of effector T cells. Th1 cells, through IFN-γ production, regulate antigen presentation and immunity against intracellular pathogens.[9,28]

Current alternative therapy for cancer therapy

Even with optimum treatment, 40% of patients with invasive cervical cancer are likely to relapse and die. Therefore, alternative strategies to reduce the global mortality and morbidity from invasive cervical cancer are necessary. HPV 16 and 18 are the most common oncogenic HPV strains and account for 70–85% of cervical malignant tumors. Clinical physicians can use several cancer therapeutic drugs such as cytokines (aldesleukin, IFN-α, IFN-β, and IFN-γ, Ok-432), Aldara (Imiquimod), intravenous immunoglobulin (IVIG), macrophage or granulocyte colony stimulating factor, and monoclonal antibodies such as Avastin and Herceptin to confront advanced cervical cancer. Chemopreventive drugs, such as Vitamin D3, combined with conventional therapeutic tools (surgery, chemotherapy, and/or radiotherapy) are also a therapeutic regimen for advanced cervical cancer. Thus, we orchestrate good cellular immune functional to eradicate HPV-related cervical lesions or invasion cervical cancer through cluster triggers. Then, APCs will uptake tumor lysate to generate personal immunization.

Preventive cervical cancer virus-like particles (VLPs) vaccine for cervical cancer therapy

Recently, several VLPs have been shown to prime CTL responses when delivered to animals as exogenous antigens.[29–33] The stimulation of an MHC-I-restricted specific CTL response by exogenous, immunogenic material has been known for many years. DCs are essential for induction of an immune response. DCs are professional APCs because they can capture antigens in the periphery and subsequently migrate to the lymphoid tissues to prime and modulate a specific cellular response. They have regained interest because they seem to be involved in CTL priming by tumor cells, DNA vaccination, and autoantigens. Interactions between T cells and APCs regulate the induction, amplification, and differentiation of cellular immune responses. HPV 16 VLP-based vaccine formulations have been shown to induce strong humoral and cell-mediated immunity against HPV 16-related squamous intraepithelial lesions. Although the efficiency of HPV VPL vaccine to treat advanced cervical cancer is still questioned, this finding indicates that HPV VLP has similar antigenic determinants as naïve HPV capsids and that vaccine antigens are efficiently presented to the immune system.

OK-432 will be used as an adjuvant agent to trigger skin LCs to become immature DCs that are capable to uptake HPV 16 VLP (Gardasil or Cervarix). Then, it will generate CD4+,CD8+, NK and NKT cell response after contacting with HPV 16 VLP. These VLPs can activate DCs and upregulate MHC class I or II molecules, the costimulatory molecules CD28/CD80, CD86 and CD83.[34] Thus, HPV L1 VLPs activate DCs, resulting in the upregulation of costimulatory molecules and secretion of IL-6 and IL-8, whereas high VLP concentrations also induce IL-10 and IL-12.[35] The innate recognition of HPV 16 L1 VLP signals APCs to express costimulatory molecules and secrete cytokines. These APC-derived signals can induce the polarization of naïve CD4+ helper T cells toward Th2 or Th1 phenotype. Th1 cells produce IFN and the TNF to regulate B cells to produce antigen-specific IgG2a. Th2 cells express interleukin-4 (IL-4), IL-5, IL-9, and IL-13 and can promote IgG1 and IgE class switching.[36]

Integrated therapy – Immunotherapy, Chemotherapy, and Radiotherapy

Current therapeutic approaches to patients with locally advanced stage cervical cancer are conventional *in situ* ablative

modalities, including cisplatin-based chemotherapy and radiation therapy. The 5-year survival rate of patients with nonresectable cervical cancer is dismal. In our institute, locally advanced staged (stage IIb or advanced) 5-year relapse-free survival rate was 0–40% (IIB to IVA).[8] Over 99% of cervical cancer is caused by persistent infection with an oncogenic strain of HPV-16, and viral oncoproteins E6 and E7 are functionally required for disease initiation and persistence. HPV-targeted immune strategies present a compelling opportunity in which to demonstrate proof of principle. The prophylactic vaccines, while effective, present the same barriers to uptake as those for screening and early treatment.

As the tumor microenvironment has so many immunosuppressive factors, including myeloid-derived suppressor cells (MDSCs), Treg (CD4+CD25+T regulatory cells), chemokines, and cytokines regulated host immune homeostasis. The CD4+CD25+ T cells, also called naturally occurring Tregs, are found to be important in immune suppressors in the cancer microenvironment. However, CD8+ T cells are major effector cells in cancer immunotherapy. The IFN-γ production in the tumor microenvironment may be important to the enhanced antitumor effect generated. Recently, Lugade et al have demonstrated that radiation is capable of inducing IFN-γ production in the tumor microenvironment. IFN-γ presents in the tumor microenvironment and upregulates MHC class I molecules in the tumor cells. This will result in increasing tumor cell target recognition by the antigen-specific CD8+ T cells and increased T cell infiltration. All these factors may potentially contribute to the radiation-induced enhancement of antitumor immunity.[37,38]

Alternations to the tumor microenvironment may influence the effectiveness of subsequent immunotherapy. Radiation is an important treatment for the local control of cancer based on its ability to directly kill tumor cells. However, there is increasing evidence that localized irradiation of the tumor may also modify the tumor microenvironment and generate inflammatory cytokines, which can increase the robustness of immune response. Low-dose radiation can increase adhesion molecules (VCAM-1, ICAM-1, MHC class I and/or II, IFN-γ, and MIG and IP-10) or enhance chemokine or cytokine secretion and antigen release.[37] Radiation will initiate APCs to uptake, reconstruct, and enhance the recognition between host cells and tumor cells interaction. It is well accepted that the infiltration of effector T cells into tumors is required for successful antitumor response. Therefore, immunomodulatory agents can be used in recombinant signal 1 (MHC/Ag/TCR) and signal 2 (costimulatory molecules or adhesion molecules), and/or signal 3 (chemokines or cytokines) and generate effective immunization.

Topple cancer cells through integrated therapy

The optimal activation of naïve T cells requires both antigen-specific signaling and induction of costimulatory pathways. In the absence or deficiency of costimulatory signaling, naïve T cells fail to recognize antigens and become tolerant to cancer cells. In order to break this immune tolerance and elicit effective antitumor response, many strategies have been used. The expression of costimulatory molecules B7.1 and B7.2 or CD40+APCs will block inhibitory signaling by anti-CTLA-4 antibody (ipilimumab) and grafting T cells with the stimulating receptor CD28 in adoptive immunity.

Tumor rejection plays a significant role in a cell-mediated immune response. One is CD28, which when engaged by CD80 (B7.1), triggers a stimulatory signal to activate naïve T cells. Memory CD8+ T cells are activated by IL-2 and IL-4 which secreted by CD4+ T cells. Type 1 CD8+ T cells (Tc1) secrete IFN-γ, whereas type 2 CD8+ T cells (Tc2) secrete IL-4 and IL-5. Tc1 and Tc2 effector

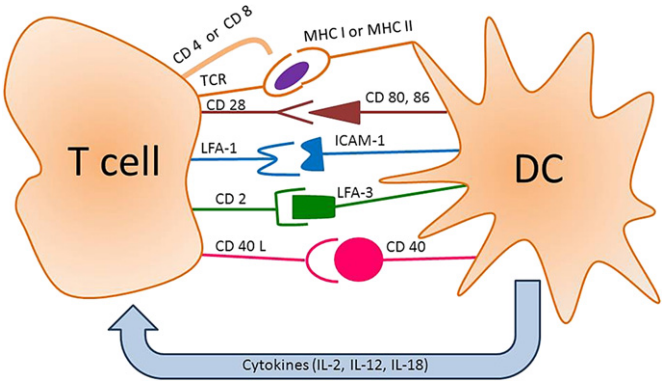

Fig. 1. Dendritic cells as a key player of immune response. Signal 1 is the cognate signal delivered to T cells by peptide/MHC class I or II complexes on the surface of APCs. Signal 2 triggers costimulatory molecules such as CD28 binding to CD80 and CD86 or the CD40/CD40L pathway. signal 1 or signal 2 activation will induce cell-cell interaction to generate signal 3 (cytokines). Abbreviation: DC Dendritic cell; ICAM-1 Intercellular Adhesion Molecule 1; MHC Major Histocompatibility Complex; LFA Lymphocyte Function Associated Antigen.

cell therapy elicits long-term tumor immunity and results in complementary endogenous type 1 antitumor responses.[39]

Completely effective immunity, including cellular mediated immunity (CMI) or humoral mediated immunity (HMI), is cooperated. Effective T cells activation depends upon signal transmission through antigen specific T cell receptors and costimulatory receptors (Fig 1).

Monitoring of a response against a specific protein and/or partially undefined antigens through immune score

In protein/peptide vaccine trials, antibody responses, CD4+ T cell responses, and CD8 T-cell responses may be induced, signifying a higher level of complexity. In case of vaccination with modified tumor cells, tumor-cell lysates, or DC/tumor cell fusions a variety of antigens may differentially induce immune responses, including antibody responses, CD4, and CD8 T-cell responses. Cytokine flow cytometry (CFC) assays are a potentially powerful tool for analyzing antigen-specific T-cell responses in a quantitative manner. The ability to quantitate frequencies of functional antigen specific T cells has enabled investigators to assess the relationship between the strength of CD4+ and CD8+ T-cell responses and immune protection and/or therapy in a number of disease models.

Conclusion

The recognition of oncogenic HPV as the primary etiological agent for cervical cancer and its precursor lesions has paved the way for the development of the control of cervical cancer and other HPV-associated malignancies. Clinical HPV vaccines trials provide a unique opportunity to identify the characteristics and mechanisms of the immune response that best correlates with clinical vaccine potency. Successful vaccination against circulating tumor cells and/or metastatic cancer cells is critically dependent on inducing an appropriate immune response.

In the future, several strategies can be developed to achieve adjuvant biotherapy for advanced cervical cancer. (1) Achieve superior *in vivo* activation of APCs. (2) Create an inflammatory environment at the tumor site and promote the homing of effector lymphocytes to the tumor. (3) Enhance CD4+ T cells' activation. (4) Downregulate the suppressive effects of regulatory T (Treg) cells and tumor cells. (5) Enhance the immunogenicity of tumor cells. (6) Prevent angiogenesis. We hope that further studies on

immunotherapy and biotherapy for advanced or relapsed cervical cancer can provide highly effective therapy and improve patients survival rate and life quality.

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