Composite peptide-based vaccines for cancer immunotherapy (Review)

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Abstract. The use of peptide-based vaccines as therapeutics aims to elicit immune responses through antigenic epitopes derived from tumor antigens. Peptide-based vaccines are easily synthesized and chemically stable entities, and of note, they are absent of oncogenic potential. However, their application is more complicated as the success of an effective peptide-based vaccine is determined by numerous parameters. The success thus far has been limited by the choice of tumor antigenic peptides, poor immunogenicity and incorporation of strategies to reverse cancer-mediated immune suppression. In the present review, an overview of the mechanisms of peptide-based vaccines is provided and antigenic peptides are categorized with respect to their tissue distribution in order to determine their usefulness as targets. Furthermore, certain approaches are proposed that induce and maintain T cells for immunotherapy. The recent progress indicates that peptide-based vaccines are preferential for targeted therapy in cancer patients.

Contents

1. Introduction

Cancer, particularly malignant tumors, is the leading cause of mortality in developed countries and the second leading cause of mortality in developing countries (1). For decades, advancements have been made in traditional cancer treatment regimens, chiefly surgery, chemotherapy and radiation. Although these treatment regimens have had certain success, they are not entirely adequate or optimal for tumors that have migrated to areas that are inaccessible by surgery or chemotherapy/radiation is not permissible. Therefore, immunotherapy, which may produce fewer side-effects and prevent metastasis in comparison to traditional treatments, has become of increasing interest in the area of cancer treatment.

In the field of immunotherapy, increasing attention has been focused on the use of cancer vaccines that activate T cells to treat growing tumors (2). The development of peptide-based vaccines has taken >20 years. A vaccine specific for tumor antigens may have wide application and utility in the prevention of the recurrence in numerous different malignancies. In cancer patients, the body masks tumor antigens by the addition of carbohydrate moieties to avoid an uncontrolled autoimmune response; however, in the process, the elimination of threatening tumor cells is also impeded (3). Therefore, the development of peptide-based vaccines that directly stimulate the immune system would be highly significant.

Peptides, which are composed of several amino acids and are absent of oncogenic potential, are antigenic epitopes derived from tumor-associated antigens (TAA) or tumor-specific antigens (TSA) (3,4). Peptide-based vaccines are designed to elicit specific T cells against antigens selectively expressed by tumor cells (5). In comparison to traditional treatment, peptide-based vaccines significantly prolonged the overall survival rate and spare normal tissue due to its low toxic effect (6-8). Evidently, there is a large number of cancer patients requiring the development of novel approaches for immunotherapy.

2. Mechanism of antitumor immunity by peptide-based vaccines

The application of peptide-based vaccines is based on three distinct steps to create a specific antitumor immune response. To initiate immunity, dentritic cells (DCs), which are taken up exogenously as part of a therapeutic vaccine (9), differentiate into immunogenic mature DCs (10). DCs enable the presentation of peptides on major histocompatibility complex (MHC) class I and II molecules (Fig. 1). Previously, the majority of peptide-based vaccines target MHC class I peptides to

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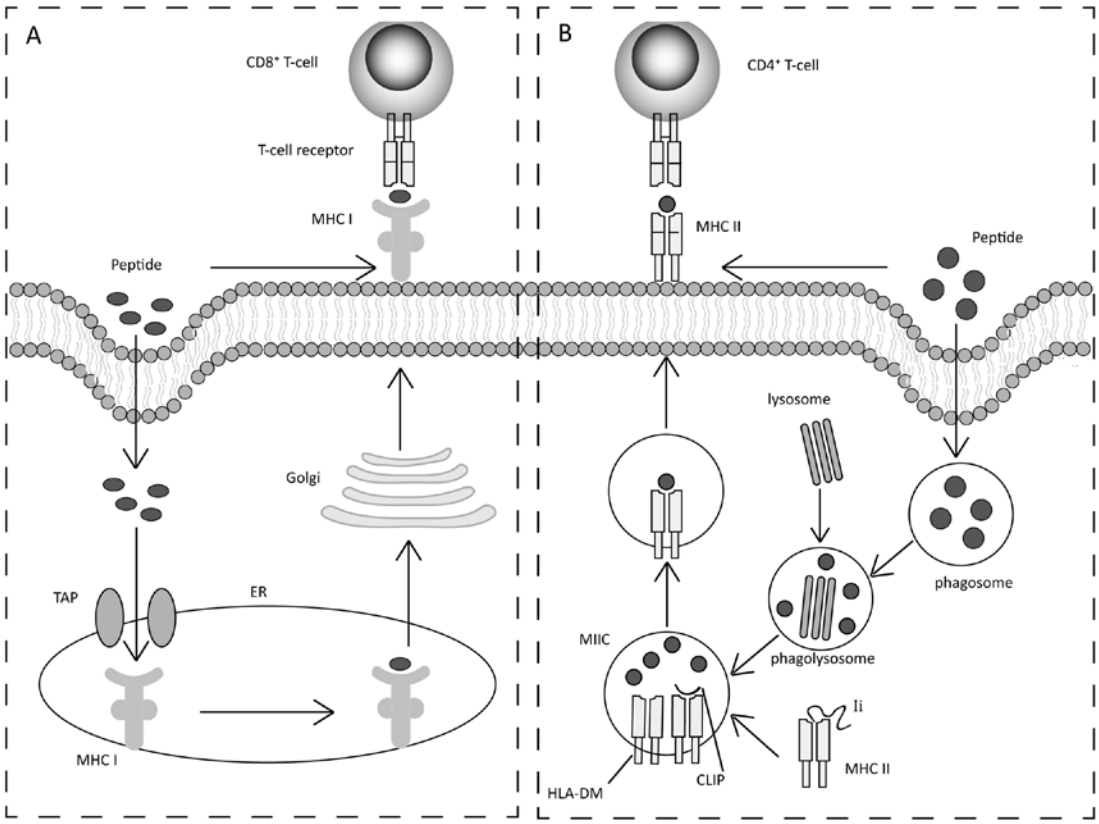

Figure 1. Entry of peptide-based vaccines through the MHC I or MHC II pathway. High affinity peptides may load onto the MHC molecules directly at the cell surface of DCs. The exact mechanism of the peptide-based vaccine uptake may vary depending on the peptide sequence. MHC, major histocompatibility complex; DC, dentritic cells; CD, cluster of differentiation; TAP, transporter associated with antigen presentation; ER, endoplasmic reticulum; MIIC, MHC class II compartment; HLA, human leukocyte antigen; CLIP, class II-associated Ii peptide.

stimulate cytotoxic T lymphocyte (CTL) responses. MHC class I binds with peptides that are ~8-12 amino acids in length (Fig. 1A) (11). Peptides are transported into the endoplasmic reticulum (ER) by a transporter associated with antigen presentation (12). Subsequently, peptide-MHC class I complexes go through the Golgi and are delivered to the cell surface for recognition by cluster of differentiation 8^+ ($CD8^+$) CTLs (13). Concurrently, a small proportion of MHC class II-restricted peptides stimulate $CD4^+$ T helper cells. MHC class II-restricted peptides are generally 12-20 amino acids in length (Fig. 1B) (3). Extracellular peptides are taken up by antigen-presenting cells and placed into phagosomes, which fuse with lysosomes to form phagolysosomes (14). MHC class II assembled in the ER associates with the invariant chain (Ii), and the Ii-MHC class II complex is transported to phagolysosome and is known as the MHC class II compartment (MIIC). In the MIIC, Ii is degraded, leaving a residual class II-associated Ii peptide in the peptide-binding groove (12). MHC class II requires human leukocyte antigen (HLA)-DM (H2-DM in mice) to completely expose the peptide-binding groove, binding with a specific peptide (15). Peptide-MHC class II complexes are delivered to the cell surface for recognition by $CD4^+$ T helper cells.

Subsequently, in lymphoid organs the peptide-loaded DCs trigger specific T-cell responses (10). These T cells, such as $CD4^+$ T helper cells and $CD8^+$ T cells, show potent cytotoxic effects by two signals (16). One signal is from the T cell receptor (TCR) interacting with peptide-MHC complexes on the DCs (17). The other signal is from the interaction of DC surface receptor CD80/CD86 with T-cell co-stimulatory molecule, CD28 (17,18).

Finally, specific T cells must migrate to the tumor microenvironment to cause the cytotoxic response. Considerable knowledge has been obtained on $CD8^+$ CTLs that have been identified as the most powerful effector cells (19). $CD4^+$ T helper 1 cells (Th1) secrete several cytokines, such as IFN-γ, TNF-α and interleukin-2 (IL-2) (20,21). These cytokines exert direct antitumor immunity and antiangiogenic effects (22). Notably, specific $CD4^+$ T helper cells have been found to enhance $CD8^+$ CTL recruitment and proliferation (21).

3. Peptide selection

During the past two decades, a majority of antigenic peptides have been discovered. In principle, there are two types of tumor antigens. One type is from TSAs, which are expressed exclusively by tumors. TSAs generally arise from viral infections or genetic mutations (23). The second more common type is derived from TAAs. TAAs are found on malignant and normal cells, but in a significantly higher number in the former.

From TSAs. Peptide-based vaccines targeting viral oncogene products are ideal candidates to elicit strong immune responses without generating autoimmunity. As these viral oncoproteins are not expressed in normal cells and their expression is required to maintain the malignant phenotype, the viral protein is considered as a potential target for cancer immunotherapy. Recently,

Table I. Antigens from genetic mutation recognized by HLA class I and class II restricted T cells.

Antigen	Tumor	Refs.
HLA class I-restricted		
β-catenin	Melanoma	(69)
CDK-4	Melanoma	(70)
MART-2	Melanoma	(71)
MUM-1/2	Melanoma	(72)
MUM-3	Melanoma	(73)
HSP70-2	Renal cancer	(74)
Caspase-8	Head/neck cancer	(75)
p21/ras	Pancreatic, colorectal, lung cancer	(76)
HLA class II-restricted		
p53	Head/neck cancer	(77)
TPI	Melanoma	(78)
CDC27	Melanoma	(79)

HLA, human leukocyte antigen; CDK, cyclin-dependent kinase; MART, melanoma antigen recognized by T cells; MUM, melanoma ubiquitously mutated; HSP, heat-shock protein; TPI, triose-phosphate isomerase ; CDC, cell division cycle.

a variety of viral oncoproteins in virus-associated cancers have been used as vaccines to induce T-cell responses. For instance, injection of human papillomavirus type 16 E5 peptide + CpG resulted in strong T-cell immunity and inhibited tumor growth, whilst prolonging the survival time in animals with cervical cancer (24). Similarly, two recombinant Epstein-Barr virus antigenic peptides, EBNA1 fused with LMP2, boosted T-cell immunity and was proven to be safe and had low immunogenicity in the phase I clinical trial for nasopharyngeal carcinoma (7).

The antigens occurring in a number of different proteins expressed by tumor cells are the result of genetic mutations. A controversy exists over the idea that a single human tumor, as in a mouse system, can express multiple mutated antigens and generate new ones during progression, thereby making their characterization even more complex. However, in the last few years, the situation has slowly changed. Several studies (69-79) have described such antigens as peptide epitopes recognized by T cells in combination with MHC class I and II in human tumors, such as melanoma, non-small cell lung cancer, renal cancer and head/neck cancer (Table I). The presumed advantages of mutated antigens are based on the potential to be recognized as non-self by the immune system and their potential resistance to negative selection if the mutated protein participates in cell survival (25).

From TAAs. TAAs can be divided into four major categories: i) Differentiation antigens; ii) cancer/testis antigens shared by germ and tumor cells; iii) overexpressed antigens, such as normal proteins whose expression is upregulated in tumor cells; and iv) universal tumor antigens.

Differentiation-antigens. These antigens are expressed by the normal tissue and tumor originating from these tissues. The majority of these antigens have been applied to treat melanoma, such as melanoma Ag recognized by T cells (MART-1)/Melan A, gp100, tyrosinase, tyrosinase-related protein (TRP-1) and TRP-2. Gp100, which were initially identified, were reported as non-mutated differentiation antigens expressed by a melanocytic lineage, including normal melanocytes, pigmented retinal cells and melanomas, but not in other normal tissues or non-melanoma tumors. In a current clinical trial with tumor-free lymph nodes of stage I to III melanoma patients, immunization with modified $gp100_{209\text{-}2M}$ peptide without co-administration of $CD4^+$ cell-restricted antigens induced the effective expansion of tumor-reactive memory $CD8^+$ T cells with high proliferation potential (26). In another clinical study, the response rate was higher and the progression-free survival rate was longer with gp100:209-217 (210 M) peptide vaccine plus IL-2 compared to IL-2 alone in patients with metastatic melanoma (27). These studies demonstrate that differentiation proteins may be suitable targets for immunotherapy.

Cancer/testis (CT) antigens. Expression of these antigens is restricted to human germ cells within the testis and trophoblasts, and is also expressed on a variety of types of human cancers. The antigens in testis do not induce an immune response, as testis cells do not express MHC class I. Since CT antigens are not expressed in normal tissue, these antigens may be potentially useful targets for tumor-specific immunotherapy. More than 40 antigens have been identified as CT antigens, including melanoma antigen (MAGE), B melanoma antigen, New York oesophageal squamous cell carcinoma 1 (NY-ESO-1) and G antigen 1. The first CT antigen was discovered from a patient with melanoma who was identified as having cytotoxic T cells that recognized autologous tumor cells (28). Through DNA-cloning methodology, the gene encoding the tumor antigen MZ2-E was cloned and was termed MAGE1. Fujie *et al* (29) proposed that through using the MAGE-1-encoded peptide it was possible to immunize an increased number of patients by means of such peptide-based immunotherapeutic approaches to MAGE-1-positive malignant tumors. Thus far, NY-ESO-1 is the most studied due to its strong capacity to induce a tumor-specific immune response (30). Previously, a completed clinical study using co-administration of CpG 7909 and Montanide ISA-51 with peptide NY-ESO-1 p157-165 showed the vaccine to be capable of inducing CTLs, resulting in an extended survival time in the majority of vaccinated patients (31). Currently, a number of clinical trials treating various cancers are being performed using antigenic peptide NY-ESO-1 combined with differing adjuvants.

Overexpressed-antigens. In healthy tissues, these antigens are expressed at low levels on the surface of normal cells. Conversely, in the majority of cancers these antigens are overexpressed but with no preferential expression on certain tumor types, involving human epidermal growth factor receptor-2 (HER-2), human mucin 1 (MUC1) and cyclin B1. In humans, the HER-2 protein is expressed during fetal development but is weakly detectable in the epithelial cells of a number of normal tissues in adults. Overexpression of the HER-2 protein has been identified in numerous types of

human cancers, such as breast, ovarian and non-small-cell lung cancer. Immunizing patients with peptides derived from HER-2/neu protein admixed with granulocyte-macrophage colony-stimulating factor (GM-CSF) have been indicated to result in the generation of T-cell immunity specific for the HER-2/neu (32). In addition, MUC1 has been studied as a target for immunotherapy following a long developmental phase. Transmembrane glycoprotein MUC1 is expressed on the apical surface of polarized epithelial cells. However, in the majority of epithelial malignancies, MUC1 is overexpressed and loses its polarity of expression (33). In pre-clinical studies using primates, MUC1 tandem repeat peptide administered with LeIF elicited T helper cells and CTL responses (34). This study showed the peptide-based vaccine to be safe and to possibly be a vaccine that induces MUC1-specific immune responses in patients with cancer.

Universal tumor antigens. Over the past decade, numerous TAAs have been reported. However, for any particular TAA, expression is restricted to several tumor types. To circumvent this, a new category of TAAs, known as 'universal tumor antigens,' has been described. Such universal tumor antigens are highly expressed in tumor cells of different tissue origins with minimal expression in normal counterparts. Survivin and telomerase have been reported to be suitable as target universal tumor antigens for active immunization of cancer patients. In a previous study, telomerase was expressed in 85-90% of cancer patients and was an attractive universal tumor antigen (35). Telomerase helps to mediate functional telomeres, maintaining at the end of chromosomes, and prevent cells from going into senescence, particularly in cancer cells. The majority of human cells do not express telomerase activity, but the majority of human tumors exhibit strong activity (36). In 2006, Brunsvig *et al* (37) conducted a phase I/II clinical study in patients with non-small cell lung cancer (NSCLC), and the results demonstrated that intradermal injections of GV1001 (hTERT: 611-626) was immunogenic, safe and induced strong specific immune responses. Based on these initial encouraging results, the study reported further clinical studies of GV1001 in NSCLC patients. Vaccination with GV1001 was indicated to exhibit low toxicity, induced considerable immune response rate and established durable T-cell memory (38).

4. Strategies to induce and maintain T cells

Peptide-based vaccines present certain objective limitations. Free peptides have poor immunogenicity or no tertiary structure, and thus are rapidly degraded by tissue and serum peptidases prior to being loaded onto DCs. Recent studies indicate that optimal strategies to induce and maintain T cells include adjuvants, cytokines, HLA class II-restricted helper epitopes, immune-modulating antibodies and low-affinity peptides combined with high-affinity peptides, which are described in the following.

Adjuvants. Peptide-based vaccines require additional adjuvant to elicit efficient immunological response. Conjugates of peptides with heat shock proteins (HSPs) (39,40) or ligands of toll-like receptors (TLRs) (41-43) have been applied to a broad range of vaccines as adjuvants to enhance the immunogenicity of peptides. In 2000, the study by Cho *et al* (44) reported that HSP65 fusion proteins stimulated DCs to increase expression of MHC (class I and II) and co-stimulatory (B7.2) molecules. This study suggested a mechanism in which the HSP fusion proteins induced CTLs to peptides without requiring exogenous adjuvants or the participation of $CD4^+$ T cells (44). However, TLRs may improve vaccination efficacy through activating DC maturation, thereby upregulating the expression of MHC molecules and enhancing antigen uptake. Khan *et al* (45) reported that TLR ligand-peptide conjugates improved intracellular trafficking and processing pathways, triggering optimal antigen presentation and T cells priming.

Cytokines. Cytokines, such as GM-CSF and IL-12, are used as an adjuvant in vaccines. GM-CSF increases the number of immature DCs and migration, and it induces MHC class II expression and activation by macrophages. In melanoma patients, subcutaneous injection with GM-CSF modestly increased the immune response against peptide vaccines (46). The cytokine IL-12 augments antitumor immunity through promoting Th1 cell differentiation and stimulating the production of IFN-γ from $CD4^+$ Th1 and $CD8^+$ T cells (47). In preclinical and clinical studies, a significant proportion of patients with resected melanomas experienced an improved performance of the peptide vaccines when it was combined with properly dosed IL-12 therapy (48).

HLA class II-restricted helper epitopes. HLA class II-restricted helper epitopes enhance specific CTLs and generate T-cell memory (49-51). $CD4^+$ T helper cells can activate DCs to enhance antigen presentation, resulting in the secretion of IL-2 and other cytokines from DCs that may help to direct the immune response. Furthermore, cytokines secreted by Th1, such as IL-2 and IFN-γ, are required for the generation of CTLs, as well as in promoting $CD8^+$ memory T-cell development. IL-2 induces CTL activation and proliferation (52). Simultaneously, IFN-γ controls the migration of CTLs (53). IL-2 is essential for programming the ability of $CD8^+$ memory T cells to re-expand upon secondary infection *in vivo* (52,54). Knutson *et al* (55) evaluated whether active immunization with HER-2/neu helper peptides generated $CD4^+$ and $CD8^+$ T-cell responses in patients. Following vaccination, HER-2/neu-specific $CD8^+$ T-cells increased in the majority of patients. Additionally, the specific T cells were able to lyse tumors and the immunity was long-lived. Subsequently, Gritzapis *et al* (56) indicated that in comparison to Her-2 (435-443) CTL peptide alone, mice vaccinated with Her-2 (435-443) plus Her-2 (776-790) exhibited longer lasting antitumor responses and induced memory immunity. Thus, there is a rationale for induction of $CD4^+$ T cells with peptide-based vaccines, either in combination with stimulation of $CD8^+$ T cells or on their own.

Immune-modulating antibodies. Combining peptide-based vaccines with immune-modulating antibodies may be a novel strategy to overcome immune suppression (17,57,58). Several antibody therapies that are either agonistic or inhibit receptors have shown benefits in cancer treatment (59-61). Specific recognition by T cells is a two-step process. In addition to the interaction of TCRs with MHC-peptide complexes as the first signal for T cell recognition, a second signal co-stimulates

the receptors that determine whether the T cell will become activated or anergic. These surface co-stimulatory receptors transmit agonistic or inhibitory signals through engagement of specific ligands. The co-stimulatory activity of these receptors can be mimicked by antibodies modulating T-cell proliferation, cytokine secretion and cytolysis (62). There are a number of known agonistic receptors, including 4-1BB (CD137), OX40, CD27, GITR and CD28 (62-64). The immune response against a peptide vaccine combined with the systemic delivery of anti-4-1BB antibodies resulted in considerably improved antitumor therapeutic activity through CTLs, NK cells, neutrophils and IFN-γ (65). Receptors that serve as targets for inhibitory antibodies include CTLA-4, PD-1 and BTLA. In one study, a combination of peptide-based vaccines with blocking co-inhibitory receptor signaling, resulted in antibody blockage of the co-inhibitory receptors, CTLA-4 and PD-1, decreased T cell anergy and allowed specific T cells to carry out their effector function (66).

Low-affinity peptides combined with high-affinity peptides. Peptides that bind to MHC molecules with high affinity usually induce high-avidity T cells. Administration of high-affinity peptides of exogenous antigens (such as viruses) is necessary for their elimination. However, if the antigen is autologous, high-affinity peptides possibly lead to tolerance. Thus, for a peptide-based vaccine the most appropriate peptides may be low-to-medium-affinity peptides (67). However, low-affinity peptides are difficult to identify. In order to overcome this difficulty, attempts have been made to raise the affinity of peptides for the MHC through binding with high-affinity peptides. For example, synthetic peptides, such as $TERT_{578-592}$ combined with two peptides derived from TERT, as high-affinity forms strongly stimulated antitumor immune responses (22). In a parallel study, Disis *et al* (68) synthesized four peptides from HER-2/neu protein and two were shown to be avid binders to HLA-A2.1, whereas the other two may be shown to elicit peptide-specific CTL *in vivo.*

5. Conclusions

The significant advantage of peptide-based vaccines is that they are easily synthesized, chemically stable entities and notably, are absent of oncogenic potential. Antigenic peptides offer a simple and flexible way to deal with the complexity of tumor antigens through bypassing the requirements for antigen processing.

As discussed previously, several promising preclinical and clinical studies for peptide-based vaccines are currently being carried out. Thus far however, there is no peptide-based vaccine currently available on the market. There are drawbacks that may hinder the peptide vaccine therapy. First, tumor cells can downregulate MHC molecules or disable other components of the antigen processing machinery. Second, peptides are HLA class I and II restricted and, consequently, restrict the treatment of patients in the clinical trials. The majority of previous cancer vaccines target MHC class I-restricted peptides to stimulate CTL responses. However, the clinical effect of CTL peptide-based vaccines remains modest. Third, tumor cells may upregulate surface ligands (such as PD-L1), which engage inhibitory receptors on the surfaces of activated T cells (PD-1), to mediate T-cell anergy. These drawbacks emphasize the requirement for significant changes in the applications of peptide-based vaccines. Various combinational approaches have been carried out to raise the efficacy of peptide-based therapies.

In conclusion, there is evidence that peptide-based vaccines have increased responses and prolonged survival rates in patients with cancer. Firstly, designing a peptide-based vaccine for cancer immunotherapy is challenging, involving the selection of appropriate antigenic peptides. Strategies to increase the effects to generate antitumor $CD4^+$ cells that recognize MHC class II-restricted peptides may have impact due to the importance of $CD4^+$ cells in enhancing activation and survival of $CD8^+$ effector cells, as well as generating $CD8^+$ memory T cells. Synthetic peptides that have antigenic low-affinity combined with high-affinity peptides raise the affinity of the peptides for the MHC and may significantly enhance antitumor response. Furthermore, increasing numbers of peptide-based vaccines with the co-administration of adjuvants, cytokines or immunomodulatory antibodies have been shown to induce and maintain immune responses. Finally, further studies are required to focus on the synergy of peptide vaccination with chemotherapy, involving larger studies providing evidence to evaluate the curative effects *ex vivo* and *in vivo.*

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