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Prophylactic, therapeutic and anti-metastatic effects of an HPV-16 mE6Δ/mE7/TBhsp70Δ fusion protein vaccine in an animal model

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Abstract

Human papillomaviruses (HPVs), particularly HPV-16, are not only causally linked to cervical cancers but also play an important role in the development of other cancers. The oncoproteins, E6 and E7, are consistently coexpressed in the majority of HPV-containing carcinomas and their metastatic lesions, and are critical to the induction and maintenance of malignant phenotype, and also can cause tumor metastasis. Therefore, E6 and E7 represent ideal tumor-specific antigens for the development of immunotherapy to prevent and treat HPV-associated cancers and their metastases. The powerful antigenic nature of *Mycobacterium tuberculosis* heat shock protein 70 (TBhsp70) is emphasized by evidence that mammals are capable of recognizing murine and human multiple B and T cell epitopes in this protein, and therefore allows it to be used as an adjuvant-free carrier to stimulate the immune response to a covalently linked fusion partner. In our present study, we developed a recombinant TBhsp70Δ protein expression vector that permits the production of other protein fused to TBhsp70Δ. A recombinant HPV-16 mE6Δ/mE7/TBhsp70Δ fusion protein was expressed and purified, and immunization with the fusion protein in the absence of adjuvant was capable of providing strong protection to C57BL/6 mice against challenge and rechallenge with TC-1 cells, but not HPV negative Lewis lung cancer cells, and induced established TC-1 tumor regression and led to long-term survival. Consistent with the in vivo results, the fusion protein immunization in the absence of adjuvant induced cytolytic T lymphocytes recognized specifically TC-1 tumor cells in vitro. We also demonstrated that immunization with the fusion protein in the absence of adjuvant was effective in both preventing and treating TC-1 metastatic lesions in the lung metastasis model. In particular, immunization with the fusion protein caused regression of established lung metastatic lesions in 50% of immunized animals. This study represents an instance of tumor therapy with a TBhsp70Δ fusion protein and provides the scientific basis for the clinical application of the HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein in the treatment of HPV-associated cancers and their metastases.

Keywords: HPV-associated cancers; Heat shock protein; E6 and E7; Immunotherapy; Metastasis

1. Introduction

Human papillomaviruses (HPVs) are the predominant etiologic agent of cervical cancer and carries three transforming oncogenes, E5, E6 and E7. More than 99% of cervical cancers and their precursor lesions contain HPV DNA [1,2]. In addition, increasing evidence has indicated that HPVs are also associated with the development of lung cancer [3], head and neck cancer [4], esophageal cancer [5], gastric cardiac adenocarcinoma [6], non-melanoma skin cancer [7], breast cancer [8], transitional cell carcinoma of urinary bladder [9], colon cancer [10], ovarian malignancy [11], prostate cancer [12] and penile cancer [13] and so on. Among the approximately 100 different genotypes of HPVs, it is the presence of the high-risk HPV type 16 (HPV-16) that is most frequently associated with the appearance of these malignancies [1]. Furthermore, two HPV-16 oncoproteins, E6 and E7, are consistently coexpressed in the majority of HPV-containing carcinomas and metastatic lesions by the target cell upon viral integration, and are critical to the induction and maintenance of malignant transformation and malignant phenotype [1,14], and also can cause tumor metastasis [15]. Moreover, E6 and E7 are highly conserved in amino acid

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sequence among HPV genotypes [16] and are antigenic in man [17,18], and are completely foreign viral proteins, thus they may harbor more antigenic peptides/epitopes than a mutant cellular protein. Therefore, E6 and E7 represent ideal tumor-specific antigens for the development of immunotherapy to prevent and treat HPV-associated cancers and their metastases [19,20]. Several lines of evidence suggest that specific cell-mediated immune responses are important in controlling established HPV infections as well as HPV-associated neoplasms [19]. (1) The incidence of HPVs infection with high-risk types such as HPV16 far exceeds the number of individuals who present with high-grade squamous intraepithelial lesions (HSILs), and fewer still progress to invasive cancer. It is believed that the induction of cell-mediated immunity by the host contributes to limiting the progression from infection and low-grade SILs (LSILs) to HSILs and cancer. (2) The prevalence of HPV-related diseases (infections and neoplasms) is increased in patients with impaired cell-mediated immunity, including transplant recipients and human immunodeficiency virus-infected patients. (3) Animals immunized with non-structural viral proteins are protected from papillomavirus infection or the development of neoplasia. Immunization also facilitates the regression of existing lesions. (4) Infiltrating $CD4^+$ (T-helper cells) and $CD8^+$ (cytotoxic T lymphocyte, CTL) cells have been observed in spontaneously regressing warts. Various forms of HPV vaccines [19], including vector-based vaccines, peptide-based vaccines, protein-based vaccines, DNA-based vaccines, chimeric VLP-based vaccines and cell-based vaccines, have been described in experimental systems. In the past, most researchers focused on E7. Since E6 represents another important target for potential vaccines to control HPV-associated lesions, it is also crucial to develop vaccines targeting E6 [21]. It will be of interest in future studies to develop vaccines encoding both E6 and E7 and compare their efficacies with those of E6 or E7 alone vaccines in vitro and in vivo.

Many vaccines under current development are composed of synthetic, recombinant or highly purified sub-unit vaccines. Such vaccines are often considered safer than classical vaccines, but may be less immunogenic. Adjuvant formulations are an attractive approach to enhancing their immune response [22]. But adjuvants, including aluminum salts, oil-based emulsions, liposomes and others, such as lipid A, that are lipid soluble, are foreign substances to the body and may evoke side effects in addition to the desired immune stimulation. Some of the real and theoretical risks from adjuvants [22] include toxicity, local inflammation, nodule, cross-reaction with human antigens, immune suppression, carcinogenesis, teratogenesis and so on. Many types of adjuvants are complex groups of compounds that have theoretical, technical, manufacturing or safety issues that must be considered [23].

The lack of optimal adjuvants has been problematic in vaccine development and the use of *Mycobacterium tuberculosis* heat shock protein 70 (TBhsp70) may be a practical alternative [24,25]. Heat shock proteins (HSPs) are among the most abundant constituents of bacteria, even when bacteria are not subject to stress. They are also highly conserved; for example, hsp70 protein from one bacterial species is generally about 50% identical to that of any other bacterial species [26]. This combination of abundance and conservation may explain why the HSPs are frequent targets of the immune response to bacterial infection. The especially powerful antigenic nature of TBhsp70 is emphasized by evidence that mammals are capable of recognizing murine and human multiple B and T cell epitopes in this protein, and therefore allows it to be used as an adjuvant-free carrier to stimulate the humoral and cellular immune response to a covalently linked fusion partner [24,27,28]. Various forms of the hsp70 vaccines that have been tested thus far include peptide vaccines (influenza virus NP) [29], protein vaccines (HIV-1 p24, chicken ovalbumin and HPV-E7) [24,27,28,30], DNA vaccines (HPV-E7) [31–33] and vector vaccines (recombinant AAV encoding E7 CTL epitope) [34]. These studies have demonstrated that hsp70 fusion vaccines can dramatically increase expansion and activation of fusion partner-specific $CD8^+$ T cells in the absence of adjuvant, completely bypassing the CD4 arm, and that this function resides in half of the ATP-binding domain of TBhsp70 (aa161–370) [28], and made HSPs more attractive for use in immunotherapy.

Protein-based vaccines have become an appealing approach to generating antigen-specific immunotherapy because of their simplicity, safety, efficacy and capacity for repeated administration. A large protein fragment would be an especially rich source of many different naturally processed peptides. Peptide mixtures of this kind, derived from specific antigens of interest, would be particularly suitable for forming intracellular peptide–MHC complexes with the highly diverse MHC proteins found in different individuals of genetically outbred populations. Recently, we have demonstrated that the HPV-16 mE6Δ/mE7 fusion protein vaccine with incomplete Freund's adjuvant (IFA) could elicit potent CTL responses in vitro and efficient anti-tumor immunity in a mouse model [35]. Based on these and other results, in the present study, we developed a recombinant TBhsp70Δ protein expression vector that permits the production of other protein fused to TBhsp70Δ. We produced and purified a recombinant HPV-16 mE6Δ/mE7/TBhsp70Δ fusion protein, and found that it could dramatically increase expansion and activation of specific CTLs, and prevent and treat HPV-associated cancers and their metastases when administered to mice in the absence of adjuvant. Our data may help to develop a safe and effective vaccine against HPV-associated cancers and their metastases, and provide a scientific basis for the use of HPV16 mE6Δ/mE7/TBhsp70Δ fusion protein in future clinical trials.

2. Material and methods

2.1. Recombinant plasmid construction

2.1.1. pET70Δ

The DNA fragment containing the truncated TBhsp70, half of the ATP-binding domain of TBhsp70 (aa161–370), coding sequence was polymerase chain reaction (PCR) amplified from recombinant plasmid pUC57 (containing the TBhsp70 coding sequence synthesized by Sangon Co., Shanghai, China) using the following primers. The forward primer (5′-