核酸疫苗由於其穩定性和簡便在產生特殊抗原免疫中扮演著引人注目的角色, 但仍然需要提高其 DNA **疫苗的效力. 近年來在特定的抗原呈現細胞中, 尤其是在樹狀突細胞裡**, Flt3-ligand **已經被鑑定出是一種重要的組織介素. 在一重組分子連接** Flt3-ligand **到抗原的模型上時, 可能其目標是將抗原連結到樹狀突襲細胞上. 使用** HPV-16E7 **唯一抗原模型利用基因槍的方式, 把子宮頸癌有關的人類乳突病毒**(HPV-E7)**的蛋白質直接送入人體內. 在動物實驗方面, 把** HPV-16E7 **連接** Flt3 **這種組織介素的** DNA **後, 送入老鼠體內不僅可以增加動物體內特殊抗** E-7 T **細胞的量, 同時可以達到抑制攜帶** E7 **的腫瘤細胞的擴散. 因此可以考慮將以此種疫苗作人類第一階段臨床試驗**.

Enhancement of DNA Vaccine Potency By Linkage of Antigen Gene to a FLT3-Ligand Gene

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ABSTRACT

Nucleic acid vaccines represent an attractive approach for generating antigen-specific immunity because of their stability and simplicity of delivery. However, there is still a need to enhance the potency of DNA vaccines. Recently, Flt3-ligand has been identified as an important cytokine in the generation of professional antigen presenting cells, particularly dendritic cells. A recombinant molecule linking Flt3-ligand (FL) to a model antigen may potentially target the antigen to dendritic cells. Using HPV-16 E7 as a model antigen, we evaluated the effect of linkage to Flt3-ligand on the potency of antigen-specific immunity generated by naked DNA vaccines administered intradermally via gene gun. We found that vaccines containing chimeric FL/E7 fusion genes dramatically increased the frequency of E7-specific $CD8^+$ T cells relative to vaccines

containing the wild-type E7 gene. *In vitro* studies indicated that cells transfected with FL/E7 DNA were capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than cells transfected with wild-type E7 DNA. Furthermore, bone marrow-derived dendritic cells pulsed with FL/E7 fusion protein are capable of presenting E7 antigen through the MHC class I pathway in a more efficient manner than dendritic cells pulsed with wild-type E7 protein. More importantly, this fusion converted a less effective vaccine into one with significant potency against established E7-expressing metastatic tumors. Interestingly, FL-E7 fusion vaccines mainly targeted CD8+ T cells and antitumor effects were completely CD4-independent. These results indicate that fusion of Flt3-ligand gene to an antigen gene may greatly enhance the potency of DNA vaccines via CD8-dependent pathways.

INTRODUCTION

Antigen-specific cancer immunotherapy has recently emerged as a promising approach for controlling cancer because it is capable of developing specific immunity against neoplastic cells while not attacking normal cells. Increasing evidence suggests that professional antigen presenting cells (APCs), particularly dendritic cells (DCs), are the central players for mediating cancer immunotherapy. An effective vaccine most likely requires a strategy that targets antigen to professional APCs to activate antigen-specific T cells.

Recently, DNA vaccines have become an attractive approach for generating antigen-specific immunotherapy. Naked DNA is safe, has low immunogenicity, and can be repeatedly administered. Furthermore, DNA vaccines can be easily prepared in large scale at lower relative cost with high purity and stability compared to proteins [Robinson, 1995 #2917; Pardoll, 1995 #2830; Donnelly, 1997 #2242]. One of the concerns about DNA vaccines is their potency, since they do not have the intrinsic ability to amplify *in vivo* as viral vaccines do. We reason that a DNA vaccine encoding a fusion antigen that is can be directed to sites of immune induction, such as dendritic cells may enhance vaccine potency (Boyle *et al.*, 1998). One such molecule is GM-CSF. It has been demonstrated that the linkage of GM-CSF gene to antigen gene has enhanced the potency of DNA vaccines. For example, fusion of GM-CSF with antigen in a DNA vaccine can lead to enhancement of immune responses and protection against HIV [Lee, 1999 #11] and hepatitis C [Lee, 1998 #22]. Although the specific mechanism for such enhancement of DNA vaccine potency is not clear, it is believed that chimeric GM-CSF/antigen may act

as an immunostimulatory signal to DCs, inducing differentiation from an immature DC form to a mature form and mobilizing active dendritic cells at the onset of a cell-mediated immune response [Banchereau, 1997 #14][Steinman, 1988 #44]. Since DCs and their precursor cells express high levels of GM-CSF receptors (GM-CSFR), chimeric GM-CSF/antigen may target and concentrate the linked antigen to DCs and further improve DNA vaccine potency. Another important molecule that also possesses a growth-stimulatory effect on DCs and to be capable of generating large numbers of DCs *in vivo* is Flt3-ligand [Maraskovsky, 1996 #45; Shurin, 1997 #230]. Flt3-L has emerged as an important molecule for the development of tumor vaccines that augment the function and quantity of DCs *in vivo.* Flt3 (fms-like tyrosine kinase 3), a murine tyrosine kinase receptor, was first described in 1991 [Matthews, 1991 #237; Rosnet, 1991 #238] and found to be a member of the same family of receptors as c-kit and c-fms receptors - the type III receptor kinase family (for review see [Lyman, 1998 #319]). In hematopoietic tissues, the expression of flt3 is restricted to the CD34-positive progenitors. Flt3 has been used to identified and subsequently clone the corresponding ligand, Flt3-L [Lyman, 1993 #240; Hannum, 1994 #239]. The predominant form of Flt3-L is synthesized as a transmembrane protein from which the soluble form is generated, presumably by proteolytic cleavage [Lyman, 1995 #241]. These proteins function by binding to and activating unique tyrosine kinase receptors. Expression of the Flt3 receptor is primarily restricted, among hematopoietic cells, to the most primitive progenitor cells, including dendritic cell precursors. To date, flt3-ligand have not been used in the form of chimeric DNA vaccines.

We chose human HPV-16 E7 as a model antigen for vaccine development because HPVs, particularly HPV-16, are associated with most cervical cancers. The HPV oncogenic proteins, E6 and E7, are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers. Vaccines or immunotherapies targeting E7 and/or E6 proteins may provide an opportunity to prevent and treat HPV-associated cervical malignancies. In our current study, we investigated whether genes linking full-length E7 to flt3-ligand can enhance the potency of DNA vaccines. We compared DNA vaccines containing wild type HPV-16 E7 with DNA vaccines containing full-length E7 fused to flt3-ligand for their immune response generation and their ability to protect animals against the HPV-16 E7-expressing murine tumors [Lin, 1996 #37]. We show that linkage of E7 to flt3-ligand dramatically increases expansion and activation of E7-specific CD8+ T cells, completely bypassing the CD4 arm. This enhanced CD8 response results in potent antitumor immunity against established tumors.

DISCUSSION

In this study we demonstrated that FL can significantly enhance the potency of HPV-16 E7-expressing DNA vaccines. DNA vaccines with FL fused to HPV-16 E7 elicited strong E7-specific cellular immunity and generated significant $CD8^+$ T cell-dependent preventive effects against HPV-16 E7-expressing murine tumors. Furthermore, chimeric FL/E7 DNA vaccine was capable of controlling lethal lung metastasis.

Our data demonstrated that FL can preferentially enhance $CD8^+$ T cell responses of E7 DNA vaccines. In contrast, $CD4^+$ T cell responses were not detectably enhanced by FL linkage. The most plausible mechanisms for the enhancement of $CD8^+$ T cell responses in vaccinated miceis not clear. However, our data also indicated that the linkage of FL to E7 has led to the enhanced MHC class I presentation of E7 compared to wild type E7 in transfected cells (Figure 9). Therefore, the linkage of FL to E7 may facilitate the precessing of E7 into the MHC class I presentation pathway. Ballistic DNA delivery can introduce DNA directly into dermal dendritic cell precursors. The FL/E7 expressing DCs thereby may present E7 through MHC class I pathway more efficiently than E7 expressing DCs.

Another important mechanism that may contribute to the enhanced E7-specific CD8+ T cell immune responses *in vivo* is related to so call "cross-priming" effects of FL/E7 complexes where the chiemeric FL/E7 can lead exogenous proteins to the MHC-I restricted antigen presentation pathway. Our data suggested that chimeric FL/E7 protein can be processed by bone marrow-derived dendritic cells and presented more efficiently through MHC class I pathway than wild type E7 protein (Figure 10). However, the "cross-primimg" of chimeric FL/E7 probably does not play a major role in the gene gun-mediated FL/E7 DNA vaccines. It has been shown that direct priming of CD8+ T cells by gene-transfected DCs is the key event in gene gun-mediated DNA immunization [Porgador, 1998 #147; Akbari, 1999 #198], while cross-priming of DCs is not an major mechanism for gene gun-mediated DNA vaccination [Porgador, 1998 #147; Akbari, 1999 #198]. However, we can not completely rule out the possibility of cross-priming, because FL/E7 might be released from other cell types, such as keratinocytes (which

were also transfected by gene gun vaccination), and then enter the DCs via the cross-priming mechanism.

In this study, we failed to detect significant E7-specific IFN-γ or IL 4-secreting $CD4^+$ T cells in FL/E7 vaccinated mice (Figure 3). The observed enhancement of E7-specific CD8+ T cell immune responses was most likely not facilitated by E7-specific $CD4^+$ T helper cells. However, if the FL/E7 DNA vaccine generates FL-specific CD4+ T cells, these cells may contribute to the generation and expansion of E7-specific $CD8^+$ T cells. These FL-reactive T helper cells can exert a strong helper effect by reacting to conjugated peptides [Del Giudice, 1994 #57]. This may also contribute to the increase in E7-specific $CD8^+$ T cell precursors observed in mice vaccinated with the FL/E7 DNA vaccine.

While FL/E7 generates potent CD8+ T cell responses through enhanced MHC class I presentation, other constructs that target antigen to MHC class II presentation pathways may provide enhanced CD4+ T cell responses. This realization raises the notion of co-administration of vaccines that directly enhance MHC class I and class II restricted pathways. We have previously developed a chimeric Sig/E7/LAMP-1 DNA vaccine that uses the LAMP-1 endosomal/lysosomal targeting signal for enhancing the MHC class II presentation pathway of E7 [Wu, 1995 #42]. The FL/E7 vaccine described here in conjunction with a MHC class II-targeting vaccine such as Sig/E7/LAMP-1 may activate multiple arms of the immune system in a synergistic fashion, leading to significantly enhanced CD4+ and CD8+ T cell responses and potent antitumor effects.

While the FL/E7 vaccine holds promise for mass immunization, three safety issues need to be resolved. First, the DNA may integrate into the host genome, resulting

in the inactivation of tumor suppresser genes or the activation of oncogenes. This may lead to malignant transformation of the host cell. Fortunately, it is estimated that the frequency of integration is much lower than that of spontaneous mutation and integration should not pose any real risk [Nichols, 1995 #112]. The second issue concerns potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is an oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei [Lukas, 1994 #119]. Thus, the presence of E7 in host cells may lead to accumulation of genetic aberrations and eventual malignant transformation in the host cells. The oncogenicity of E7 can be eliminated by introducing mutations into E7 DNA so that the resulting E7 protein cannot bind with pRB [Heck, 1992 #122] but still maintains most of its antigenicity. The third issue is the concern over the generation of autoimmunity that may be caused when FL lead to excessive dendritic cells *in vivo.* In our study, we performed pathological examination of the vital organs in the FL/E7-vaccinated mice, including the intestines. We did not observe any significant pathology, indicating that FL/E7 is a potent vaccine with minimal malevolent side effects.

In summary, our results indicate that fusion of FL to HPV-16 E7 gene can generate stronger E7-specific CD8+ T cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors generated by E7 DNA vaccines. Our results indicate that fusion of FL to an antigen gene may greatly enhance the potency of DNA vaccines and can potentially be applied to other cancer systems with known tumor-specific antigens.