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Two oral HBx vaccines delivered by live attenuated *Salmonella*: Both eliciting effective anti-tumor immunity

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Abstract

Live attenuated bacteria have great potential for use in vaccine development due to several unique advantages, including stable antigen expression, effective antigen presentation, convenient and inexpensive delivery, and low cost of vaccine production. In this study, we expressed hepatitis B virus x gene (HBx) on mouse melanoma cells as the target antigen and constructed *Salmonella*-based HBx vaccines by two strategies, i.e., recombinant eukaryotic plasmid encoding HBx and a recombinant prokaryotic plasmid encoding Type III secretion system effector-HBx fusion protein. Both HBx constructs elicited significant levels of CTL reaction and IFN-γ secreting T cells. When mice were challenged with melanoma cells expressing HBx, tumor growth rates in immunized animals were significantly slower than controls. Tumor sizes and tumor weight indices of immunized mice were also significantly lower than controls. We conclude that both strategies described in this study may lead to novel approaches of tumor vaccines.
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1. Introduction

Live attenuated bacteria have been used extensively in vaccine development due to their unique advantages as vaccine carriers: (1) stable *in vivo* antigen expression; (2) effective antigen presentation; (3) components of bacterial cells serving as effective immunological adjuvants; (4) convenient and inexpensive vaccine delivery with no associated trauma; and (5) low cost of vaccine production. Attenuated strains of *Salmonella* have all of these characteristics and have been well studied [1,2].

Salmonella can survive and multiply in macrophages and other antigen presenting cells (APCs) [3,4], which is a very useful feature of *Salmonella* as antigen carriers for vaccine development. There are reports indicating that live attenuated *Salmo-*

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nella bacterial cells in macrophages could release plasmids into eukaryotic cytoplasm by some unknown mechanism [5]. This finding has inspired the study and development of several vaccines with antigen sequence inserted in eukaryotic plasmids, which are then transformed into live attenuated *Salmonella* [5,6]. On the other hand, researchers are also exploring the bacterial type III secretion system (TTSS) for use in vaccine development. TTSS is a needle-like structure expressed on bacterial cell-surface [7,8]. It consists of two types of proteins: structural proteins and effectors. Effectors can be secreted and translocated into host cells via the TTSS needle complex. This translocation is mediated by the N- terminal amino acid sequence of effectors; changes in the C-terminal region of effectors do not interfere with translocation [9–11].

In *Salmonella*, there are two types of TTSSs, encoded by *Salmonella* Pathogenicity Island-1 (SPI-1) and *Salmonella* Pathogenicity Island-2 (SPI-2) and denoted as SPI-1 TTSS and SPI-2 TTSS, respectively [12–14]. The SPI-1 TTSS is expressed and assembled in the early stages of infection, during which host cell invasion by bacteria is facilitated by SPI-1 TTSS–host cell interaction [4,12]. In contrast, SPI-2 TTSS is mainly assembled in systemic infection stage, during which bacteria reside within phagocytic vacuoles of the host cells. SPI-2 TTSS ensures the necessary communication between bacteria and host cells [12]. Both SPI-1 TTSS and SPI-2 TTSS have their unique effectors, such as SopE, SopE2, and SptP for SPI-1, and SspH2 and SifA for SPI-2.

TTSS effectors, or effector-antigen fusion proteins, are secreted into the cytosol of macrophages so that they can be processed to initiate MHC-I restricted immune reactions [15–19]. In addition, these proteins may also start MHC-II restricted reactions via different mechanisms. Recently, Russmann and colleagues utilized SspH2-fusion proteins to immunize mice and achieved both significant CD8+ and CD4+ T cell responses [20]. In their studies, these researchers found that SspH2 works better than SopE2 or SifA in the fusion proteins in eliciting CD8+ or CD4+ T cell responses [20]. However, despite these exciting early results and some additional TTSS-based vaccine studies [15–19], no any such vaccine has been successfully developed to date.

Hepatocellular carcinoma (HCC) is one of the most prevalent forms of human cancer worldwide [21] and its development is strongly associated with infections with hepatitis viruses, including hepatitis B virus (HBV). HBx, an HBV-encoded protein with multiple functions in cellular signal transduction and HBV replication [22], has been recognized as an important oncogene, closely relevant with the carcinogenesis and development of HBV-induced HCCs [23–25]. Several cytotoxic T lymphocyte (CTL) epitopes have been mapped wiin HBx sequence [26,27]. Vaccines based on HBx full-length sequence or specific epitopes could elicit significant immune reactions [28]. However, immunogenicity of cancer antigens has been low, imposing a long-existing hurdle in anti-tumor immunology. Therefore, augmentation of the immunogenicity of cancer antigens has been a long sought goal in the development of effective vaccines. In this study, we attempted to take the advantages of *Salmonella* as a vaccine vector to elevate the immunogenicity of cancer antigens, using HBx as a representative tumor antigen in the experiments. We cloned HBx into *Salmonella* by two strategies, one into a eukaryotic plasmid and one into TTSS expressed as a protein fused to SspH2 (SspH2-HBx). When delivered orally to the animal, both vaccine strains elicited significant levels of protective immunity against the transplanted melanoma tumor that expressed the HBx antigen.

2. Materials and methods

2.1. Bacteria strains and cell lines

Salmonella typhimurium SL1344 and SL3261 were obtained from *Salmonella* Genetic Stock Center, SGSC (www.ucalgary.ca/~kesander). Bacterial strains were all cultured in LB broth or on LB-agar plates [29]. B16 C57BL/6J mouse melanoma cells (ATCC Nos. CRL-6475, H2-K^b) and RAW264.7 mouse macrophage cells (ATCC No. TIB-71) were generously provided by Dr. Hongyan Pang (Department of Pharmacology, Institute of Materia Medica, Chinese Academy of Medical Sciences & Peking Medical College, China). B16 cells were cultured in RPMI 1640 (Invitrogen) + 10% calf serum (Invitrogen), and RAW264.7 cells were cultured in DMEM (Invitrogen) + 10% calf serum.

2.2. HBx gene amplification, cloning, and transfection

HBV-carrying serum was collected from a female patient with acute hepatitis B and was kindly provided by Dr. Jia Wang (Department of Microbiology, Peking University Health Science Center, China). HBV genomic DNA was isolated using DNAout reagent (Biolife Biotechnology Co., Ltd., Beijing, China), and HBx was

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