Regression of HPV-Positive Tumors Treated With a New Listeria monocytogenes Vaccine

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**Background:** Human papillomavirus (HPV) has been implicated in the pathogenesis of 15% to 23% of head and neck squamous cell carcinomas as well as most oropharyngeal carcinomas. The viral oncoproteins E6 and E7 are expressed in HPV-positive tumor cells and therefore provide ideal targets for tumor immunotherapy. Because of its unique ability to induce a cellular immune response, the intracellular bacteria *Listeria monocytogenes* has been studied as a potential HPV-positive tumor vaccine.

**Objective:** To present a new recombinant strain of *L. monocytogenes* that is effective in treating HPV-positive tumors in a murine model.

**Design:** A new recombinant *L. monocytogenes* vaccine, Lm-ActA-E7, was designed by transforming an attenuated *Listeria* strain with an E7 expression cassette. The cassette consists of the HPV-16 E7 sequence fused to the *Listeria* protein ActA. The resultant strain of bacteria secretes E7 antigen as a fusion protein with ActA.

**Methods:** Tumors were established in C57BL/6 mice with a syngeneic HPV-positive cell line prior to treatment with vaccine.

**Intervention:** The Lm-ActA-E7 vaccine was administered intraperitoneally to the mice 5 days after tumors were established. A booster dose was administered 7 days after the first dose. Tumor progression was measured in 2 dimensions periodically after the vaccination.

**Results:** In C57BL/6 mice, the administration of Lm-ActA-E7 caused the complete regression of HPV-positive tumors in 6 of 8 mice tested. A cytotoxic T-lymphocyte assay revealed that administration of the vaccine caused the generation of cytotoxic T cells specific for E7.

**Conclusion:** Our results demonstrate the ability of a new *Listeria*-based vaccine to generate a specific antitumor T-cell response and cause the regression of HPV-positive tumors in a murine model.

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QUAMOUS CELL CARCINOMA OF the head and neck (HNSCC) remains a worldwide health problem. Recent estimates indicate more than 360 000 new cases of HNSCC worldwide annually, and more than 200 000 annual deaths.1 In the United States, an estimated 37 200 new cases will be diagnosed in 2003, with 11 000 deaths.2 Despite technological advances in surgery, radiation, and chemotherapy, 5-year survival rates remain less than 50% worldwide.1

New therapies to treat HNSCC are being developed and tested, including immunotherapeutic strategies that attempt to boost the immune response to eliminate malignant cancer cells.3,5 An ideal cancer immunotherapeutic strategy would target a known tumor antigen expressed by the tumor cells but not by normal cells. The ideal therapeutic agent would significantly enhance the host immune response to the antigen so that partial or complete tumor regression is achieved.

In this study we describe a novel immunotherapeutic strategy for the treatment of 1 subset of HNSCC. The targeted tumor antigen is the human papillomavirus (HPV) protein E7. Human papillomavirus DNA is present in roughly 15% to 23% of HNSCC,6 and in over 50% of oropharyngeal SCCs.6 The protein E7 is an ideal candidate for immunotherapeutic vaccine approaches because it is constitutively expressed in HPV-transformed tissues and is thought to be necessary to maintain the transformed state of these cells.9 In addition, E7 has been shown to be immunogenic in humans.11,12

Central to the development of a therapeutic vaccine for HPV-associated tumors is the identification of a vector that provokes a strong cell-mediated immune response. The intracellular bacteria *Listeria monocytogenes* has been examined as a potential vector for HPV-positive tumor vaccine. L. monocytogenes is a facultatively intracellular bacterium that is capable of inducing a T-cell response and has been used as a vector for the delivery of cancer antigens in murine models.13,14

The objective of this study was to present a new recombinant strain of *L. monocytogenes* that is effective in treating HPV-positive tumors in a murine model.
response. Intracellular bacteria are among the potential vaccine vectors under investigation. These bacteria, such as Listeria, Salmonella, and bacillus Calmette-Guerin, have the ability to generate strong cell-mediated immunity in murine models.13 They also produce toxins that could serve as an adjuvant in generating effective innate immunity and cytokine release.13,22 Bacillus Calmette-Guerin is currently used as an immunotherapeutic agent for bladder cancer.13,16

Among the intracellular bacteria, Listeria monocytogenes is the focus of our laboratory studies. It is a B-hemolytic, gram-positive, facultative bacterium that infects many human cells, including antigen-presenting cells such as dendritic cells and macrophages. This strain of bacteria has an unusual life cycle that leads to the activation of the 2 main T-cell populations: cytotoxic (CD8+) and helper (CD4+) T cells (Figure 1).17 Because of its unique immunologic properties, Listeria has been studied as a vaccine vector by several different laboratories for the treatment of different infectious and neoplastic diseases.18-23

Herein we describe a recombinant strain of L monocytogenes engineered to express and secrete HPV-16 E7. In this strain, Lm-ActA-E7, E7 is secreted as a fusion protein with ActA, an L monocytogenes protein necessary for the assembly of the actin filaments that propel Listeria to the periphery of an infected cell. We investigate the ability of this vaccine vector to cause tumor regression and to elicit an E7-specific immune response in a murine model.

METHODS

MICE

Our experimental mice were 6- to 8-week-old C57BL/6 mice purchased from Charles River Laboratories (Wilmington, Mass). All animal experiments were performed under approved protocols from the institutional animal care and use committee of the University of Pennsylvania.

CELL LINES

The EL4 cell line (provided by T. C. Wu, MD, PhD, Johns Hopkins, Baltimore, Md)24 was derived from C57BL/6 mice lung epithelial cells. The cells were immortalized with HPV-16 E6 and E7 and transformed with the c-Ha-ras oncogene. They express both E6 and E7 and are highly tumorigenic.25 The EL4 cell line is established from a lymphoma induced in a C57BL mouse. The EL4/E7 cell line was derived from the EL4 cell line that expresses the E7 protein (provided by Robert Tindle, PhD, Royal Children’s Hospital, Brisbane, Queensland, Australia).25 All of these cell lines were grown in culture with RPMI 1640, 10% fetal calf serum, 2mM l-glutamine, 100 U/mL of penicillin, 100 µg/mL of streptomycin, 100µM nonessential amino acids, and 1mM sodium pyruvate at 37°C and 10% carbon dioxide.

LISTERIA MONOCYTOGENES STRAINS AND PROPAGATION

The 2 Listeria strains used in this study were Lm-ActA-E7 and Lm-LLO-NP. The latter construct, also known as DP-L2028, has been previously described.26 Lm-ActA-E7 refers to a recombinant strain of L monocytogenes that carries a plasmid that expresses the E7 protein fused to the ActA protein.

Figure 1. Antigens secreted by Listeria monocytogenes are presented to both CD4+ and CD8+ T cells. After phagocytosis into an antigen-presenting cell (APC), Listeria are either destroyed within the phagolysosome (top pathway within the cell), where peptides are loaded onto major histocompatibility complex class II molecules and presented to CD4+ T cells, or they escape into the cytoplasm of the cell (bottom pathway). Here, L monocytogenes secretes antigens (black dots) that will be transported to the endoplasmic reticulum (ER) for loading onto class I molecules and presentation to CD8+ T cells.

The actA-E7 DNA insert was generated by gene splicing by overlap extension in several steps. First, the promoter (phLy) and signal sequence from the L monocytogenes gene hly was amplified via polymerase chain reaction (PCR) from pGG55 (pLLO-E7, a construct from our laboratory previously described26) using primer 5’-GGGGTCTAGACCTCTTTTGATTAGATA TTC-3’ (XhoI site is underlined) and primer 5’-ATC TTCGCTATCTGTCGCCGGCGGCGGTCTTCGACT TGTTGGCCG-’3 (NotI site is underlined; primer sequence shown in bold is the actA gene overlap).

The actA gene was PCR amplified from the wild-type L monocytogenes 10403s genome using primer 5’-GGCG CAACAAAACGTGAACAGCGCGCCGGCGACAGATA GCCAGAGT-3’ (NotI site is underlined; primer sequence shown in bold is the phLy overlap) and primer 5’-ATG TGGTGTACTTCATGGTCCAGATCGGCGAT CAAITTC-3’ (XhoI site is underlined; primer sequence shown in bold is the actA gene overlap). These primers amplify the first 420 amino acids of the ActA protein.

The E7 gene was PCR amplified from pGG55 (pLLO-E7) using primer 5’-GGAAATTGATCGCCCTAGCTCTGTG GGAGCATGGAGATACACCTAC-3’ (XhoI site is underlined; primer sequence shown in bold is the actA gene overlap) and primer 5’-AAACGGATTTATTTAGATCCCG GGTATGTTTCTGAGAAACA-3’ (XhoI site is underlined; primer sequence shown in bold is the prfA gene overlap). The prfA gene was PCR amplified from the wild-type L monocytogenes 10403s using primer 5’-TGTTCCTCAAGAA CGCATGGGAGATAACCTACA-3’ (XhoI site is underlined; primer sequence shown in bold is the prfA gene overlap).

The phLy fused to the actA gene (phLy-actA) was PCR generated and amplified from purified phLy DNA and purified actA DNA using the phLy primer (upstream) 5’-GGGGTCTAGACCTCTTTTGATTAGATA TTC-3’ (XhoI site is underlined) and actA primer (downstream) TGTAGGTTGTATCTCCATGCTCGAGACTGGCATCAAITTC-3’ (XhoI site is underlined).

The E7 gene fused to the prfA gene (E7-prfA) was PCR generated and amplified from purified E7 DNA and purified prfA DNA using the E7 primer (upstream) GGAATTGATCGCCCTAGCTCTGTGGAGCATGGAGATAACACCC-3’ (XhoI site is underlined).
The plasmid was used to transform the *Listeria monocytogenes* strain XFL-7 to create Lm-ActA-E7. It includes the promoter (*phly*) and signal sequence (ss) from the *hly* gene, the actA gene, the human papillomavirus 16 E7 gene, and the transcription factor prfA. XFL-7 is a prfA-negative strain of *L monocytogenes*. Thus, only bacteria that retain the plasmid will replicate in vivo.

A schematic representation of the plasmid pActA-E7. The plasmid was used to transform the *Listeria monocytogenes* strain Lm-ActA-E7. It includes the promoter (*phly*) and signal sequence (ss) from the *hly* gene, the actA gene, the human papillomavirus 16 E7 gene, and the transcription factor prfA. XFL-7 is a prfA-negative strain of *L monocytogenes*. Thus, only bacteria that retain the plasmid will replicate in vivo.

**Figure 2.** A schematic representation of the plasmid pActA-E7. The plasmid was used to transform the *Listeria monocytogenes* strain XFL-7 to create Lm-ActA-E7. It includes the promoter (*phly*) and signal sequence (ss) from the *hly* gene, the actA gene, the human papillomavirus 16 E7 gene, and the transcription factor prfA. XFL-7 is a prfA-negative strain of *L monocytogenes*. Thus, only bacteria that retain the plasmid will replicate in vivo.

**RESULTS**

**CONSTRUCTION OF Lm-ActA-E7**

Lm-ActA-E7 is a new recombinant strain of *L monocytogenes* that uses a multicopy episomal expression system to secrete the HPV protein E7 fused to the *Listeria* protein ActA. Lm-ActA-E7 was generated by introducing a recombinant plasmid vector, pActAE7, into the *L monocytogenes* strain XFL-7. pActAE7 carries a DNA insert that contains the following genes: (1) the 310-bp promoter from the *hly* gene and the *hly* signal sequence; (2) the 1171-bp actA gene; (3) the 300-bp E7 gene; (4) the 1019-bp prfA gene; and (5) a chloramphenicol resis-
tance gene (CAT). The promoter that drives the expres-
sion and secretion of this fusion protein is derived from
the \textit{hly} gene, an \textit{L monocytogenes} gene that encodes the
protein listeriolysin O (LLO). Using gene splicing by over-
lap extension PCR methods, this promoter was spliced
to \textit{actA}, a \textit{Listeria} gene that encodes the protein ActA,
which is a surface protein necessary for actin assembly.

The \textit{E7} gene encodes the HPV protein that is the target
of our immunotherapeutic strategies. \textit{prfA} is a transcrip-
tion factor without which the \textit{L monocytogenes}
strain \textit{XFL-7} will not replicate. Thus, during in vivo propaga-
tion only those bacteria that contain the DNA insert will
reproduce. This recombinant strain is highly attenu-
ated, with an LD_{50} of \(2.5 \times 10^9\) cfu, compared with the
wild-type \textit{L monocytogenes} strain 10403s, which has an
LD_{50} of \(5 \times 10^8\) cfu (data not shown).

\textbf{Lm-ActA-E7 SECRETES A FUSION ActA-E7 PROTEIN}

We predicted that, driven by the \textit{pHly}, our recombinant
\textit{Listeria} vaccine vector would produce a fusion protein
58 kDa in size (ActA, 46 kDa; E7, 12 kDa). Owing to the
presence of the signal sequence, our vector should also
secrete this protein. Western blot analysis of the super-
natant of pelleted bacteria using an anti-E7 monoclonal
antibody confirmed that Lm-ActA-E7 expresses and se-
cretes a 58-kDa fusion protein (Figure 3).

\textbf{Lm-ActA-E7 INDUCES COMPLETE REGRESSION
OF ESTABLISHED TC-1 TUMORS}

The HPV-positive syngeneic cell line TC-1 was used to
establish tumors in the left flank of C57BL/6 mice. Seven
days later, most tumors had reached a palpable size of 4
to 5 mm in diameter. The mice were treated with 2 doses,
1 week apart, of either Lm-ActA-E7 or a negative control
vector, Lm-LLO-NP. For each experiment, 1 group was
left untreated. While Lm-LLO-NP had little effect on tumor growth
compared with the untreated group, those mice treated
with Lm-ActA-E7 had significantly smaller tumors after
28 days (\(P<.005\) and \(P<.001\), respectively). Depicted is 1 experiment
representative of 4.

\textbf{Lm-ActA-E7 INDUCES E7-SPECIFIC CYTOTOXIC
T-LYMPHOCYTE ACTIVITY}

To determine whether the Lm-ActA-E7 was able to in-
duce an E7-specific cytotoxic T-lymphocyte (CTL) re-
sponse, a CTL assay was performed. C57BL/6 mice were
vaccinated with 0.1 LD_{50} of Lm-ActA-E7 or the negative
control vector, Lm-LLO-NP, or left untreated. One week

\begin{figure}[h]
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\caption{Lm-ActA-E7 secretes E7 as a fusion protein. Two bacterial clones
that retained the plasmid pActAE7, Lm-ActA-E7-2.5.3 and Lm-ActA-E7-2.5.4,
were grown overnight at 37°C in Luria-Bertoni medium. Bacteria were
pelleted by centrifugation, and each supernatant was precipitated with
trichloroacetic acid. E7 expression was analyzed by Western blot. The blot
was probed with anti-E7 monoclonal antibody followed by horseradish
peroxidase-conjugated antimouse antibody (Amersham Pharmacia Biotech,
Little Chalfont, England). The blot was developed using enhanced
chemiluminescence detection reagents (Amersham). Lm-ActA-E7-2.5.4 was
used in all subsequent experiments.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure4.png}
\caption{Lm-ActA-E7 causes regression of established TC-1 tumors.
C57BL/6 mice received \(2 \times 10^9\) TC-1 cells subcutaneously on the left flank.
Tumors grew to 5 mm after 7 days. The mice were then treated with 0.1
median lethal dose of Lm-ActA-E7 or Lm-LLO-NP as a negative control
on day 7, and a booster dose was given on day 14. The third and final group
was left untreated. The average tumor diameter was measured with calipers
and is shown for each mouse. The difference in tumor sizes between the
Lm-ActA-E7 group and either control group at days 20 and 28 is statistically
significant (\(P<.005\) and \(P<.001\), respectively). Depicted is 1 experiment
representative of 4.}
\end{figure}
later, booster immunizations were administered. Splenocytes were then harvested 7 days after the second vaccination and incubated with irradiated TC-1 feeder cells. After 7 days of in vitro stimulation, a standard 
$^{51}$Cr release assay was performed using EL4 or EL4/E7 as the target cells and the harvested splenocytes as the effector cells. The results showed that the target cells that expressed E7 were effectively lysed by splenocytes from Lm-ActA-E7–vaccinated mice but not by splenocytes from the control and untreated mice (Figure 5A). The target cells that do not express E7 were not lysed by splenocytes from any of the treatment groups (Figure 5B).

The clinical manifestations most often include fever and gastroenteritis.\(^ {31}\) Immunocompromised persons and pregnant women and their fetuses are more susceptible than the population at large. In these susceptible patients listeriosis can have more severe consequences, such as meningitis or sepsis.\(^ {29}\)

Despite the potential for listeriosis, we believe that use of an attenuated \textit{L monocytogenes} vector is feasible for human trials. Oral inoculation of the bacteria in adult volunteers has been investigated and found to be safe.\(^ {32}\) Though work presented here is based on parenteral administration, the mice tolerated the vaccine well with no signs of toxic effects or illness throughout the course of the experiments (data not shown). Certainly, a clinical trial would exclude immunocompromised individuals and women of childbearing age. Our recombinant strain of \textit{Listeria} is highly attenuated compared with wild type, and \textit{Listeria} remains exquisitely sensitive to standard antibiotic therapy in the event of clinical listeriosis.\(^ {33}\)

Because HPV DNA is not present in all head and neck cancers, this particular vaccine strategy is not feasible for all patients with HNSSC. However, most patients with oropharyngeal HNSSC will have HPV-associated tumors. Because surgical treatment of tumors in this region often results in severe functional morbidity, nonsurgical treatments are often used. Our vaccine strategy would complement existing modalities, providing specific eradication of malignant cells without damage to normal tissues. Local intratumoral injections or oral inoculations of Lm-ActA-E7 are also potential methods of administering the vaccine in oropharyngeal tumors. Animal studies investigating the efficacy of these routes of administration are currently under investigation in our laboratory.

In summary, Lm-ActA-E7 is a new cancer immunotherapeutic agent capable of treating HPV-positive tumors. Given that many head and neck tumors are associated with HPV infection, this cancer vaccine has a potential role to play in the treatment of HNSSC.

Figure 5. A, Lm-ActA-E7 induces E7-specific cytotoxic T-lymphocyte (CTL) activity. C57BL/6 mice were immunized with 0.1 median lethal dose of Lm ActA-E7 or Lm-LLD-NP. A separate group of mice was left untreated. A booster immunization was administered 7 days later. Splenocytes were harvested 7 days after the booster and established in primary culture with irradiated TC-1 cells for 7 days. Following the primary culture, CTL activity was assayed via chromium \$^{51}$Cr release from EL4-E7 cells. The CTL activity was significantly higher in those mice that were vaccinated with Lm-ActA-E7 than in controls (P < .05). Results are expressed as the mean of triplicate cultures. These results are representative of 3 experiments. B, In a control experiment, splenocytes harvested from mice immunized with Lm-ActA-E7 or Lm-LLD-NP and untreated were cultured with EL4 target cells that do not express E7. A standard \$^{51}$Cr assay was performed after culture with irradiated TC-1 cells for 7 days. Negligible CTL activity against EL4 cells was detected. Results are expressed as the mean of triplicate cultures. These results are representative of 2 separate experiments.

\textbf{COMMENT}

In this study we describe the creation of a new recombinant \textit{L monocytogenes} tumor vaccine, Lm-ActA-E7. In a murine model, the administration of Lm-ActA-E7 results in the induction of E7-specific CTLs and the regression of HPV-positive tumors. The induction of tumor-specific CTLs is crucial to any therapeutic vaccine strategy because these lymphocytes are capable of eliminating cells that express foreign peptide in the context of major histocompatibility complex class I molecules.

Lm-ActA-E7 secretes E7 in the context of a fusion protein, including both ActA and E7. One reason a fusion protein strategy was developed is because previous studies showed that different fusion protein constructs were more effective than vaccines that secreted tumor antigen alone.\(^ {30}\) The reasons that the fusion proteins generate a more potent immune response are unclear and are under investigation.

\textit{Listeria monocytogenes} is a ubiquitous gram-positive bacterium that can be readily isolated from soil, water, and vegetation.\(^ {27}\) It has been shown that humans incidentally ingest \textit{Listeria} via contaminated food or water frequently.\(^ {28,29}\) Pathologic human \textit{Listeria} infection, or listeriosis, is rare, estimated to occur at a rate of less than 1 per 100 000 population per year in North America.\(^ {30}\)
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