**IMPORTANCE**  In conjunction with chemotherapy, immunotherapy with dendritic cells (DCs) may eliminate minimal disease burden by generating cytotoxic T lymphocytes. Enhanced cytosolic bioavailability of tumor-specific antigens improves access to human leukocyte antigen (HLA) class I molecules for more efficient cytotoxic T lymphocyte generation. Various cell-penetrating domains (CPDs) are known to ferry covalently linked heterologous antigens to the intracellular compartment by traversing the plasma membrane.

**OBJECTIVE**  To determine whether generating melanoma antigen family A, 3 (MAGE-A3), a tumor-specific cancer-testis antigen, as a fusion protein with CPD will enhance the cytosolic bioavailability of MAGE-A3.

**DESIGN**  MAGE-A3 was amplified by polymerase chain reaction using complementary DNA from renal tissue and cloned in frame with a CPD (YARKARRQARR) at the amino-terminal end and hexahistidine at the carboxy-terminal end to generate CPD–MAGE-A3 in a pQE-70 expression vector. Cultures were grown in *Escherichia coli* BL21 Star (DE3-pLysS) cells followed by nickel-nitrilotriacetic acid affinity purification of recombinant proteins.


**RESULTS**  Media composition and isopropyl-β-thiogalactosidase induction were optimized to achieve high levels of protein expression followed by purification. Western blot analysis with MAGE-A3 antibodies recognized both MAGE-A3 and CPD–MAGE-A3 proteins, while CPD antibodies recognized only CPD–MAGE-A3. Purified CPD–MAGE-A3 exhibited more efficient DC membrane penetration than did MAGE-A3 alone as confirmed by immunofluorescence analysis. High-level expression of several unique DC markers (CD80, CD83, CD86, and HLA-DR) by flow cytometry was consistent with a mature DC phenotype, indicating that pulsing with CPD–MAGE-A3 did not alter specific cell-surface antigens required for T-cell activation.

**CONCLUSIONS AND RELEVANCE**  We have demonstrated for the first time, to our knowledge, that cloning and purification of MAGE-A3 with CPD enhances its cytosolic bioavailability in DCs without altering cell-surface antigens, potentially making it a more potent therapeutic cancer vaccine compared with existing MAGE-A3 protein and peptide vaccines.
The immune system has the ability to detect and eliminate tumor cells through the generation of cytotoxic T lymphocytes (CTLs), a process known as immune surveillance.1 However, cancer cells may evade this process, and immunotherapy with dendritic cells (DCs) is a relatively nontoxic alternative that may overcome elements of host immune incompetence.

Dendritic cells are antigen (Ag)-presenting cells that endocytose exogenous Ags and play a central role in both initiating and modulating the immune response leading to stimulation of naive T cells.2 Although tumor cells are potential immunogens because they express tumor-specific Ags (TSAs), they are generally not capable of initiating therapeutically useful immune responses owing to their immune inhibitor properties.3 In contrast, DCs can capture TSAs and process them into peptides that bind to human leukocyte antigen (HLA) class I and II molecules. The DCs then migrate to lymph nodes where they interact with both CD8+ and CD4+ T lymphocytes to generate CTLs and helper T lymphocytes, respectively, to shape the adaptive immune response, making them ideally suited for cancer immunotherapy.2

First-generation vaccines based on exogenous pulsing of DCs with tumor lysates, RNA, or peptides have all demonstrated limited success.4 This is because the exogenously pulsed TSA enters the cytoplasm to access the HLA class I pathway by the incompletely understood and inefficient process of cross-priming unique to DCs, where endocytosed TSAs are leaked into the cytosol.5 The process of cross-priming requires high TSA concentrations, providing the rationale for development of better methods for generating CTLs, and accounts for the relative ineffectiveness of prior immunotherapy attempts.6

A nonimmunogenic, 11-amino acid motif on the HIV-1 transactivator of transcription protein, known as the protein transduction domain or cell-penetrating domain (CPD) (YGRKKRRQRRR), that can ferry large, covalently linked heterologous proteins in and out of cells is responsible for this phenomenon.7 Various synthetic CPDs are capable of delivering Ags through the plasma membrane into the cellular compartment.8

Cancer-testis Ags are a family of TSAs whose expression is restricted to immunoprivileged gonadal germ cells, thereby making them ideal targets for tumor immunotherapy. Melanoma antigen family A, 3 (MAGE-A3) is a cancer-testis Ag that has attracted particular attention as a candidate for cancer immunotherapy because it is expressed by a wide variety of human cancer types, with several HLA class I- and II-restricted epitopes. These characteristics suggest that MAGE-A3-based vaccines would be immunogenic and that the resulting immune responses are unlikely to target healthy tissues.

Clones of MAGE-A3 CTLs have been generated and shown to lyse cancer cell lines.9 In one large clinical study, MAGE-A3 protein–based tumor vaccine administered in the adjuvant setting prevented disease relapse of non–small cell lung cancer.10 Unlike peptide vaccines, recombinant protein vaccines have the potential to induce a broad array of immune responses because they possess a number of HLA class I and II peptides.4

We sought to address the problem of inadequate cytoplasmic TSA expression by using synthetic CPDs to create fusion proteins that penetrate through the plasma membrane. We hypothesize that MAGE-A3 Ags generated as fusion proteins in-frame with CPDs will gain access to the HLA class I pathway in the cytosol, eventually leading to robust CTL responses. Adequate CTL responses may increase tumor cell killing and achieve durable tumor regression.

Methods

Construction of Cloning and Expression Vectors

Protein expression vectors for MAGE-A3 and CPD-MAGE-A3 were constructed as follows. We performed polymerase chain reaction to amplify MAGE-A3 from kidney tissue complementary DNA and both subcloned it into a pDRIVE T vector (Qiagen) and further cloned it in-frame with YARKARRQARR sequence in a CPD vector. Both MAGE-A3 and CPD-MAGE-A3 were excised and cloned into a pQE-70 protein expression vector (Qiagen) in-frame with the downstream hexahistidine sequence to generate pQE-70-MAGE-A3 and pQE-70-CPD-MAGE-A3.

Induced Expression of Recombinant Proteins in Escherichia coli

BL21 Star (DE3-pLysS)–competent cells (Invitrogen) designed for protein expression were transformed with pQE-70–CPD-MAGE-A3 and pQE-70–MAGE-A3 and cultivated in 250 mL of 1 of 5 different commercial media obtained from the pEX Protein Expression Media Optimization Kit (US Biological): (1) Turbo Broth, (2) Superior Broth, (3) Power Broth, (4) Hyper Broth, and (5) Luria-Bertani Broth (Miller), each containing 100-μg/mL ampicillin. In addition, 2XYT Broth (AMRESCO) used. Induction of protein expression was performed when the optical density reached 1.0 at 600 nm by adding increasing concentrations of isopropyl-D-thiogalactosidase (IPTG) from 0.1mM to 2.0mM. Cultures were further incubated at 30°C and 37°C for various times ranging from 5 to 12 hours. Bacterial cells were centrifuged at 5000g for 10 minutes, and the pellet was suspended in 5 volumes of Bacterial PE (G-Biosciences). The suspension was then vortexed for 1 minute and incubated on ice for 5 minutes. Five microliters of lysozyme (G-Biosciences) containing DNase and RNase was added, and the suspension was incubated at 37°C for 30 minutes followed by centrifugation at 20 000g, 4°C, for 30 minutes.

Affinity Purification of Recombinant Proteins

Cell lysates were passed through a 0.45-μm filter, equilibrated with 50mM sodium phosphate (pH 7.0), 0.5M sodium chloride, 10mM imidazole, and 1% Triton X-100, and left at 4°C for 1 hour with occasional mixing. The resin was set in a small hexahistidine-tag purification column by nickel–nitrilotriacetic acid (Qiagen) (0.5 × 2.5 cm) and washed with 50mM sodium phosphate (pH 7.0) and 40mM imidazole. The adsorbed recombinant proteins were eluted with 0.5M sodium chloride containing 150mM imidazole buffer (pH 7.0) and collected in 0.5-mL fractions.
Electrophoresis and Western Blotting
Total protein was resolved on 4% to 12% polyacrylamide gel and transferred onto a polyvinylidene fluoride membrane by Western blotting. The detection of recombinant proteins using our custom-made CPD sequence (YARKARRQARR) (Biocarta) was performed at 1:10,000 dilution. We used MAGE-A3 monoclonal antibody to identify both MAGE-A3 and CPD-MAGE-A3 protein bands by chemiluminescence assay.

Immunofluorescence Microscopy
Peripheral blood mononuclear cells were collected from healthy individuals after approval from the Wayne State University Institutional Review Board and written informed consent were obtained. Monocyte-derived DCs were generated as previously described. Cytospin slides were incubated with MAGE-A3 mouse anti-human antibody at room temperature for 1 hour followed by 2 washes with phosphate-buffered saline (pH 7.2) containing 0.05% octylphenoxypolyethoxyethanol (IGEPAL CA-630; Sigma). The cells were further incubated with goat antimouse Alexa 488 (Molecular Probes), washed, fixed, and analyzed using a confocal microscope.

Flow Cytometry
Cells were collected during the logarithmic phase, digested with resuspended medium, and added to a 6-well plate (10^5 cells/well). We added CPD–MAGE-A3 or MAGE-A3 at a concentration of 10^7 cells/well. The cells were incubated at 37°C for 24 hours according to our previous protocol with minor modification. The cells were then stained with fluorescein isothiocyanate to detect surface antigens, including HLA-DR, CD83, CD80, and CD86 (R&D Systems). For intracellular detection of MAGE-A3 or CPD–MAGE-A3, DCs were stained with either the custom-made CPD–MAGE-A3 or anti-MAGE-A3 antibody. Isotype-specific negative controls designed for both extracellular and intracellular staining were used in all experiments.

Results

Construction of Bacterial Expression MAGE-A3 Vector
With CPD
We used polymerase chain reaction to amplify MAGE-A3 from kidney tissue complementary DNA, subcloned it into a pDRIVE T vector (Figure 1A) (Qiagen), and further cloned it in-frame with CPD sequence YARKARRQARR in a CPD vector (Figure 1B). Both MAGE-A3 and CPD–MAGE-A3 were excised for further cloning into expression vectors (Figure 1C).

Induction of Recombinant Protein Expression
Both MAGE-A3 and CPD–MAGE-A3 were digested from the pDRIVE T vector and ligated into the pQE-70 protein expression vector in-frame with downstream hexahistidine (Figure 2A and B). Identity of the vectors was confirmed by restriction digestion analysis and DNA sequencing (data not shown). The pQE-70 expression vectors are designed to express a target protein under the regulation of promoter Ts/ lac in E coli BL21 Star (DE3 pLysS) cells that are specifically designed for efficient lysis and induction of recombinant protein. Three clones of each recombinant protein were selected, and protein induction was attempted at various times and temperatures in Luria-Bertani Broth. The temperature shift from 37°C to 33°C after a 5-hour IPTG induction improved recombinant protein expression, with the highest expression observed in clone 3 of MAGE-A3 (Figure 2C) and clone 1 of CPD–MAGE-A3 (Figure 2D).

Effect of Media Composition, Time, and IPTG Concentration on Protein Induction
We investigated the effect of different media composition on the growth of E coli carrying CPD–MAGE-A3 and the induction of protein expression (Figure 3A). While Luria-Bertani Broth and Power Broth showed the slowest biomass production as well as low rates of induction of recombinant protein, we observed a 3-fold increase in biomass production in 2XYT Broth and a 2-fold increase in Superior Broth after a 5-hour induction. Protein expression was analyzed at various concentrations of IPTG in 2XYT Broth, with maximal induction achieved at 1.0mM (Figure 3B).

Purification and Characterization of MAGE-A3 and CPD–MAGE-A3 Recombinant Proteins
Recombinant proteins were purified from the selected high-expression clones using nickel-charged resin affinity chromatography. The MAGE-A3 and CPD–MAGE-A3 proteins were effectively eluted at imidazole concentrations of 150mM and 200mM, respectively, although there was minor contamination in both cases. The purified MAGE-A3 and CPD–MAGE-A3 proteins were further characterized by Western blotting. The detection of recombinant proteins using our custom-made CPD sequence (YARKARRQARR) (Biocarta) was performed at 1:10,000 dilution. We used MAGE-A3 monoclonal antibody to identify both MAGE-A3 and CPD–MAGE-A3 protein bands by chemiluminescence assay.
proteins each showed as a single band at approximately 35 kDa and 38 kDa, respectively, which were similar to their calculated molecular mass (Figure 4A). Although more than 90% of the expressed protein was produced in the insoluble frac-
tion as inclusion bodies, approximately 0.1 mg of both recombinant proteins was purified from the soluble fraction of the cell lysate. Western blot analysis with MAGE-A3 antibodies recognized both MAGE-A3 and CPD–MAGE-A3 proteins (Figure 4B), while CPD antibodies recognized only CPD–MAGE-A3 (Figure 4C).

**Efficient DC Penetration of CPD–MAGE-A3**

We compared the ability of CPD–MAGE-A3 vs MAGE-A3 to access the cytoplasm of DCs by pulsing the proteins on day 6 of culture with 3mM MAGE-A3 control and 3mM CPD–MAGE-A3. We observed very little fluorescent staining in MAGE-A3-pulsed DCs after 2 hours (Figure 5A). In contrast, CPD–MAGE-A3 penetrated the DCs within 5 minutes after pulsing. This clearly demonstrates efficient DC penetration and a rapid way to introduce CPD–MAGE-A3 into the cytoplasm. Studies using deconvolution and confocal microscopy confirmed that CPD–MAGE-A3 was localized to the DC cytosol (Figure 5B and C).

**CPD–MAGE-A3 Entry Did Not Alter the Phenotypic Characteristics of DCs**

To understand whether the CPD of CPD–MAGE-A3 changed the surface antigen expression of DCs, we analyzed several unique DC markers by flow cytometry. The persistent, high-level expression of CD80, CD83, and CD86 in DCs pulsed with CPD–MAGE-A3 is consistent with a mature phenotype, indicating that this process did not interfere with the expression of critical monocyte-derived DC surface molecules (Figure 6).

**Discussion**

Although chemotherapy remains one of the mainstays of cancer treatment, cancer vaccines are being increasingly recognized as an important component of combinatorial therapy.12,13 Some of the key advantages of DC-based immunotherapy are its excellent safety profile and its negligible adverse effects, which may positively affect a patient’s quality of life.

Various methods have been used to formulate DC vaccines to generate tumor-specific CTLs. In ex vivo pulsing, the tumor protein, lysate, and peptide preparation is pulsed with DCs that are able to internalize, process, and present Ags.14 First-generation vaccines with exogenous pulsing showed limited success. Even when these ex vivo strategies were complemented with addition of either cytokines4 or Toll-like receptors15 that enhance immune responses and induce DC maturation, the rate of tumor regression did not exceed that observed with standard chemotherapy. Along these lines, several immune-targeted clinical trials have correlated marginally improved overall survival with the presence of CTLs, but only when concomitant chemotherapy was administered.16

The CPD method described here to pulse DCs may represent a significant improvement over prevailing techniques to generate therapeutic vaccines. Dendritic cells transduced with CPD-ovalbumin induced both CD4+ and CD8+ T-lymphocyte responses and repressed ovalbumin-expressing tumors in mice.17 Vaccination of DCs with the transactivator of transcription–CPD of carcinoembryonic antigen has been shown to efficiently inhibit tumor growth when compared with DCs expressing carcinoembryonic antigen alone.18 These results demonstrate that the use of CPD-recombinant TSA is more effective in generating tumor cell lysis than TSA alone. The 16-amino acid peptide pentratin from the DNA-binding domain of the *Drosophila* antennapedia gene more efficiently presented the immunodominant ovalbumin epitope to generate CTLs when compared with a control construct and also generated an interferon-γ response in C57BL/6 mice.19 Growth of melanoma in mice was prevented when challenged with DCs transduced with transactivator of transcription–CPD–Trp2 protein, but Trp2 protein-pulsed DCs alone were shown to be ineffective.20

MAGE-A3 has been shown to be immunogenic and its peptides are presented by HLA class I and II molecules, generat-
ing both helper T lymphocytes and CTLs. These attributes make it an ideal candidate for immunotherapy. Indeed, it has been successfully used as an immunotherapy target in melanoma and non–small cell lung cancer. More recently, MAGE-A3 protein has been incorporated into the first US Food and Drug Administration–approved DC vaccine, APC 8015 (sipuleucel-T), to be used in combination with chemotherapy for the treatment of prostate cancer. We have demonstrated that the addition of CPD to MAGE-A3 increases its cytosolic bioavailability and thereby may further facilitate anti-tumor CTL responses compared with prior studies.

Expression of proteins within tumor cells demonstrates both interindividual and intraindividual variation as the cancer progresses. Although most patients with cancer express high levels of MAGE-A3 protein, some have low-level expression, which is due to hypermethylation of the promoter and will impair recognition by DC vaccine–generated CTLs. The DNA hypomethylating agent 5-aza-2′-deoxycytidine has been shown to increase the expression of MAGE-A3 in Mel 313 melanoma cells expressing low levels of the protein, but not in Mel 275 melanoma cells already expressing high levels of MAGE-A3. These observations indicate a possible role for 5-aza-2′-deoxycytidine as an adjunct to immunotherapy to up-regulate tumor-specific MAGE-A3 expression in patients who have low expression levels, thereby potentially increasing the pool of patients eligible for treatment.

**Conclusions**

We have demonstrated for the first time, to our knowledge, that cloning and purifying MAGE-A3 with CPD enhances its cytosolic bioavailability in DCs without altering specific CD antigens required for T-cell activation, potentially making it a more potent therapeutic cancer vaccine compared with existing MAGE-A3 protein and peptide vaccines. Further, we show that bacterial-recombinant proteins can easily be engineered to purify large amounts of CPD–MAGE-A3. Use of full-length proteins circumvents the need to define HLA class I allele binding before vaccination and increases the number of epitopes recognized by CTLs when compared with peptide-pulsed DCs. Finally, the use of proteins rather than plasmids or viral vectors for in vitro DC vaccine preparation avoids the practical and theoretical safety concerns regarding genomic modification.

**ARTICLE INFORMATION**

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Enhanced Intracellular Targeting of Tumor-Specific Antigens You Can Lead a Horse to Water, But...

David H. Berger, MD, MHCM

Dendritic cell (DC)-based cancer vaccines can be prepared with relative ease and have been shown to induce therapeutic antitumor immune responses both in experimental models and, more recently, in the clinic for treatment of hormone-resistant prostate cancer and other malignant neoplasms.1 However, DC vaccination protocols are clearly not yet optimized2 and the generation of more effective regimens continues to be an area of active research. One of the key hurdles in developing an effective DC vaccine is poor access of tumor-specific antigen (TSA) to the intracellular human leukocyte antigen class I pathway, which is required to generate tumor-reactive cytotoxic T lymphocytes (CTLs).3

Batchu et al4 have described a method to overcome this important barrier by facilitating intracellular entry of the widely expressed TSA melanoma antigen family A, 3 (MAGE-A3) via linkage with a novel cell-penetrating domain (CPD) that facilitates intracellular entry of the MAGE-A3 antigen.5

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trials by cloning CPD in-frame with established TSAs or (2) enabling use of new TSAs coexpressing CPD to effectively deliver immunotherapy, the authors have not yet demonstrated the next steps in the pathway ultimately leading to enhanced tumor cell killing. These include demonstration of binding of MAGE-A3 peptides to human leukocyte antigen class I antigen, interaction of DCs with naive T cells, CTL generation, and, finally, tumor cell lysis. In addition, the authors have not addressed whether protein transduction has any negative effects on DC viability and functionality apart from looking at the few cell-surface receptors involved in initiating the immune response. These issues are ripe for further study and require clarification before this exciting method can be translated to the clinical arena.

ARTICLE INFORMATION

Author Affiliations: Department of Surgery, Baylor College of Medicine, Houston, Texas; Operative Care Line, Michael E. DeBakey Veterans Affairs Medical Center, Houston, Texas.

Corresponding Author: David H. Berger, MD, MHCM, Department of Surgery, Baylor College of Medicine, 1977 Butler Blvd, Ste 5.191, Houston, TX 77030 (dhb@bcm.edu).


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