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Enhancement of Sindbis Virus Self-Replicating RNA Vaccine Potency by Linkage of Mycobacterium tuberculosis Heat Shock Protein 70 Gene to an Antigen Gene

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Recently, self-replicating RNA vaccines (RNA replicons) have emerged as an effective strategy for nucleic acid vaccine development. Unlike naked DNA vaccines, RNA replicons eventually cause lysis of transfected cells and therefore do not raise the concern of integration into the host genome. We evaluated the effect of linking human papillomavirus type 16 E7 as a model Ag to Mycobacterium tuberculosis heat shock protein 70 (HSP70) on the potency of Ag-specific immunity generated by a Sindbis virus self-replicating RNA vector, SINrep5. Our results indicated that this RNA replicon vaccine containing an E7/HSP70 fusion gene generated significantly higher E7-specific T cell-mediated immune responses in vaccinated mice than did vaccines containing the wild-type E7 gene. Furthermore, our in vitro studies demonstrated that E7 Ag from E7/HSP70 RNA replicon-transfected cells can be processed by bone marrow-derived dendritic cells and presented more efficiently through the MHC class I pathway than can wild-type E7 RNA replicon-transfected cells. More importantly, the fusion of HSP70 to E7 converted a less effective vaccine into one with significant potency against E7-expressing tumors. This antitumor effect was dependent on NK cells and CD8+ T cells. These results indicated that fusion of HSP70 to an Ag gene may greatly enhance the potency of self-replicating RNA vaccines. The Journal of Immunology, 2001, 166: 6218–6226.

RNA replicon vaccines can be derived from α virus vectors, such as Sindbis virus (1), Semliki Forest virus (2, 3), or Venezuelan equine encephalitis virus (4) vectors. These vaccines are self-replicating and self-limiting and may be administered as either RNA or DNA, which is then transcribed into RNA replicons in transfected cells or in vivo (5, 6). Self-replicating RNA is capable of replicating in a diverse range of cell types and allows the expression of the Ag of interest at high levels (7). Additionally, self-replicating RNA eventually causes lysis of transfected cells (8). These vectors therefore do not raise the concern associated with naked DNA vaccines of integration into the host genome. This is particularly important for vaccine development targeting proteins that are potentially oncogenic, such as the human papillomavirus (HPV)4 E6 and E7 proteins.

In the past few years, studies have demonstrated that immunization with heat shock protein (HSP) complexes isolated from tumor or virus-infected cells are able to induce potent antitumor (9) or antiviral immunity (10). Immunogenic HSP-peptide complexes can also be reconstituted in vitro by mixing the peptides with HSPs (11), and HSP-based protein vaccines can also be administered by fusing Ags to HSPs (12). We have recently demonstrated that linkage of HPV-16 E7 Ag to Mycobacterium tuberculosis HSP70 leads to the enhancement of DNA vaccine potency (13, 14). These investigations have made HSPs attractive for use in immunotherapy. The HSP vaccines that have been tested thus far have been in the form of peptide/protein-based vaccines or DNA-based vaccines. To date, HSPs have not been applied in the form of self-replicating RNA vaccines.

In this study, we chose HPV-16 E7 as a model Ag for vaccine development because HPV, particularly HPV-16, are associated with most cervical cancers. HPV oncogenic proteins, E6 and E7, are coexpressed in most HPV-containing cervical cancers and are important in the induction and maintenance of cellular transformation. Therefore, vaccines targeting E6 or E7 proteins may provide an opportunity to prevent and treat HPV-associated cervical cancers. HPV-16 E7 is a well-characterized cytoplasmic/nuclear protein that is more conserved than E6 in HPV-associated cancer cells and has been applied in a variety of HPV vaccines. We therefore chose to use HPV-16 E7 as our model Ag.

In our current study, we investigated whether genes linking HSP70 to full-length E7 can enhance the potency of self-replicating Sindbis RNA vaccines. We showed that a Sindbis RNA vaccine linking HSP70 to E7 significantly increased expansion and activation of E7-specific CD8+ T cells and NK cells, bypassing the CD44 arm and resulting in potent antitumor immunity against E7-expressing tumors. We also found that the Sindbis E7/HSP70 RNA vaccine could induce apoptotic death of transfected cells. Our in

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4 Abbreviations used in this paper: HPV, human papillomavirus; HSP, heat shock protein; DCs, dendritic cells; GFP, green-fluorescent protein; BHK, baby hamster kidney; β-gal, β-galactosidase; LDH, lactate dehydrogenase.

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vitro studies indicated that E7 Ag from E7/HSP70 RNA replicon-transfected cells can be processed by bone marrow-derived dendritic cells (DCs) and presented more efficiently through the MHC class I pathway than wild-type E7 RNA replicon-transfected cells. Our study may have important implications for vaccine development.

Materials and Methods

Plasmid DNA constructs and preparation

The generation of pcDNA3-HSP70, pcDNA3-E7, and pcDNA3-E7/HSP70 has been described previously (15). For the generation of pcDNA3-GFP, a DNA fragment encoding the green-fluorescent protein (GFP) was first amplified with PCR using pEGFPN1 (Clontech, Palo Alto, CA) as a template and a set of primers: 5’-atcggatccatggtgagcaagggcgaggag-3’ and 5’-gg gaagcctttacttgtacagctcgtccatg-3’. The amplified product was further cloned into the BamHI/HindIII cloning sites of pcDNA3 vector. For the generation of pDNA3-E7/GFP, a DNA fragment encoding HPV-16 E7 was first amplified with PCR using pcDNA3-E7 as a template and a set of primers: 5’-atggaagctttacttgtacagctcgtccatg-3’ and 5’-gg gaagcctttacttgtacagctcgtccatg-3’. The amplified product was further cloned into the EcoRI/BamHI cloning sites of pcDNA3-GFP. The Sindbis virus RNA replicon vector, SINrep5 (17), and SINrep5-E7 (18) have been described previously. For the generation of SINrep5-HSP70 and SINrep5-E7/HSP70, DNA fragments encoding M. tuberculosis HSP70 and cimeric E7/HSP70 were isolated from pcDNA3-HSP70 and pcDNA3-E7/HSP70, respectively, and further cloned into the corresponding XhoI and Pmel sites of the SINrep5 vector to generate SINrep5-HSP70 and SINrep5-E7/HSP70 constructs. For the generation of SINrep5-E7/GFP, DNA encoding E7/GFP was isolated from pcDNA3-E7/GFP and further cloned into XhoI/Pmel sites of SINrep5. The accuracy of these constructs was confirmed by DNA sequencing.

In vitro RNA preparation

The generation of RNA transcripts from SINrep5-HSP70, SINrep5-E7, SINrep5-E7/GFP, SINrep5-E7/HSP70, and SINrep5 was performed using the protocol as previously described (18). Briefly, SpeI was used to linearize DNA templates for the synthesis of RNA replicons from SINrep5-HSP70, SINrep5-E7, SINrep5-E7/HSP70, SINrep5-E7/GFP, and SINrep5 constructs. RNA vaccines were transcribed in vitro and capped using SP6 RNA polymerase and capping analogue from T3 in vitro transcription kit (Life Technologies, Gaithersburg, MD) according to vendor’s manual. After synthesis, DNA was removed by digestion with DNase I. Synthesized RNA was quantified and analyzed using denaturing formaldehyde agarose gels (19). The purified RNA was divided into aliquots to be used for vaccination in animals and for transfection of a baby hamster kidney (BHK21) cell line. The protein expression of the transcripts was assessed by transcription of the RNA into BHK21 cells using electroporation.

Cell lines

BHK21 cells were obtained from the American Type Culture Collection (Manassas, VA) and grown in Glasgow MEM supplemented with 5% FBS, 10% tryptose phosphate broth, 2 mM glutamine, and antibiotics. Cells were kept at 37°C in a humidified 5% CO2 atmosphere and were passaged every 2 days. The production and maintenance of TC-1, an HPV-16 E7-expressing tumor cell line, have been described previously (20). On the day of tumor challenge, TC-1 cells were harvested by trypsinization, washed twice with 1× HBSS, and finally resuspended in 1× HBSS to the designated concentration for injection.

Mice

Female C57BL/6 mice (6 to 8 wk old) from the National Cancer Institute (Frederick, MD) were purchased and kept in the oncology animal facility of The Johns Hopkins Hospital (Baltimore, MD). All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals.

RNA vaccination

All SINrep5 RNA vaccines were generated using in vitro transcription as described above. RNA concentration was determined by OD at 260 nm. The integrity and quantity of RNA transcripts were further checked using denaturing gel electrophoresis. Mice were vaccinated i.m. with 10 µg/mouse SINrep5-HSP70, SINrep5-E7, SINrep5-E7/HSP70 mixed with SINrep5-E7/HSP70, SINrep5-E7/GFP, or SINrep5 RNA vaccines in the right hind leg while SINrep5-E7/HSP70 was administered in doses of 0.1, 1, and 10 µg/mouse.

CTL assays

Cytolysis was determined by quantitative measurements of lactate dehydrogenase (LDH) using CytoTox96 nonradioactive cytotoxicity assay kits (Promega, Madison, WI) according to the manufacturer’s protocol. Briefly, splenocytes were harvested and pooled 2 wk after RNA vaccination. Five mice were used for each vaccinated group. Splenocytes were cultured with 1 µg/ml E7 peptide (aa 30–67, RATYNYTIF) in a total volume of 2 ml RPMI 1640, supplemented with 10% (v/v) FBS, 50 U/ml penicillin/streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids in a 24-well tissue culture plate for 6 days as effector cells. For controls, mAb GK1.5 (21) was used for CD4 blocking, and mAb 2.43 (22) was used for CD8 blocking. mAb 2.43 or mAb GK1.5 was added to the cultured splenocytes at a concentration of 50 µg/ml on day 5 and incubated overnight. CTL assays were performed on day 6. TC-1 tumor cells were used as target cells. The TC-1 cells mixed with splenocytes at various E:T ratios. After 5 h incubation at 37°C, 50 µl of the cultured medium were collected to assess the amount of LDH in the cultured medium according to the manufacturer’s protocol. The percentage of lysis was calculated from the equation: 100 × [(A − B)/(C − D)], in which A is the reading of experimental-effector signal value, B is the effector spontaneous background signal value, C is maximum signal value from target cells, D is the target spontaneous background signal value.

ELISA

For the determination of IFN-γ in the supernatant of cultured splenocytes, splenocytes were harvested 2 wk after vaccination and cultured with 1 µg/ml E7 peptide (aa 49–57) containing the MHC class I epitope (RAHYNIVTF) (23) or 10 µg/ml E7 peptide (aa 30–67) containing the class II epitope (DSSEDEIDGFAPQADRAYHYNIVTFCCDKDSTLLR) (24) in a total volume of 2 ml RPMI 1640, supplemented with 10% (v/v) FBS, 50 U/ml penicillin and streptomycin, 2 mM l-glutamine, 1 mM sodium pyruvate, and 2 mM nonessential amino acids in a 24-well tissue culture plate for 6 days. As a control, mAb 2.43 (22) was used for CD8 blocking. mAb 2.43 (50 µg/ml) was added to the cultured splenocytes during the incubation period. The supernatants were harvested and assayed for the presence of IFN-γ using ELISA kits (Endogen, Woburn, MA) according to the manufacturer’s protocol. Splenocytes from mice vaccinated with Sig/E7/LAMP-1 RNA vaccine (18) were pulsed with E7 peptide and used as a positive control for the ELISA.

For the determination of E7 protein from BHK21 cells transfected with SINrep5-E7 or -E7/HSP70 RNA, an indirect ELISA method was used as described previously (18). The ELISA plate was read with a standard ELISA reader at 450 nm.

In vivo tumor protection experiments

For the tumor protection experiment, mice (5 per group) were immunized i.m. with 10 µg/mouse SINrep5 RNA, SINrep5-HSP70, SINrep5-E7, SINrep5-E7/GFP, or 0.1, 1, or 10 µg/mouse SINrep5-E7/HSP70 RNA. Fourteen days after immunization, mice were injected i.v. with 1 × 109 cells/mouse TC-1 tumor cells in the tail vein. Three weeks after tumor challenge, mice were euthanized. The number of tumor nodules on the lung surface in each mouse was evaluated and counted by experimenters blinded to sample identity.

In vivo Ab depletions

The procedure for in vivo Ab depletion has been described previously (18, 25). In brief, mice were vaccinated with 1 µg/mouse self-replicating SINrep5-E7/HSP70 RNA i.m. and challenged with 1 × 106 cells/mouse TC-1 tumor cells via tail vein injection. Depletions were started 1 wk before tumor challenge. Isotype IgG2a Ab (PharMingen, San Diego, CA) was used as a nonspecific control. mAb GB1.5 (21) was used for CD4 depletion, mAb 2.43 (22) was used for CD8 depletion, and mAb PK136 (26) was used as a nonspecific control. mAb GK1.5 (21) was used for CD4 depletion. Cell surface marker staining and flow cytometry analysis

Splenocytes removed from naive or vaccinated groups of mice were immediately treated with cell surface marker staining using a previously described protocol (18). Cells were then washed once in FACScan buffer and stained with PE-conjugated monoclonal rat anti-mouse NK1.1 Ab and PE-Cy7-conjugated monoclonal rat anti-mouse CD3 Ab (PharMingen). The population of NK cells was NK1.1+ and CD3−. The percentage of NK cells in mice immunized with various self-replicating RNA vaccines was analyzed using flow cytometry.
In vitro cell death analysis

BHK21 cells \((1 \times 10^7)\) were transfected with 4 \(\mu\)g SINrep5, SINrep5-E7, SINrep5-HSP70, or SINrep5-E7/HSP70 RNA transcripts. We used cells transfected with SINrep5-b-gal to determine transfection efficiency. The SINrep5-b-gal transfected cells were fixed and stained for lacZ expression using 5-bromo-4-chloro-3-indolyl-D-galactoside (27). In general, the transfection efficiency in our electroporation was consistent and measured to be \(\sim 30\%\). Unmodified BHK21 cells and electroporated BHK21 cells without RNA were used as controls. BHK21 cells were collected and assessed every 24 h, until h 72. The percentage of apoptotic BHK21 cells was analyzed using annexin V apoptosis detection kits (PharMingen) according to the manufacturer's protocol, followed by flow cytometry analysis. The percentage of apoptotic cells was corrected for transfection efficiency.

CTL assay using DCs pulsed with BHK21 cells transfected with various RNA transcripts

We performed CTL assays using DCs pulsed with BHK21 cells transfected with various RNA transcripts using a protocol similar to what has been described by Albert et al. (28, 29) with modifications. DCs were generated by culture of bone marrow cells in the presence of GM-CSF as described previously (18). BHK21 cells \((1 \times 10^7)\) were transfected with 4 \(\mu\)g various self-replicating SINrep5 RNA constructs via electroporation as described above. BHK21 cells were collected 16–20 h after electroporation. The levels of E7 protein expression in BHK21 cells transfected with SINrep5-E7 or SINrep5-E7/HSP70 RNA transcripts were determined by ELISA as described above. Transfected BHK21 cells \((3 \times 10^5)\) were then cocultivated with \(1 \times 10^5\) bone marrow-derived DCs at 37°C for 48 h. These DCs were used as target cells and D\(^8\)-restricted E7-specific CD8\(^{+}\) T cells \((30)\) were used as effector cells. CTL assays were performed with effector cells and target cells \((1 \times 10^5/well)\) mixed together at various E:T ratios \((1:1, 3:1,\) and \(9:1)\) in a final volume of 200 \(\mu\)l. After 5 h incubation at 37°C, 50 \(\mu\)l of the cultured medium were collected to assess the amount of LDH in the cultured medium as described above. DCs cocultivated with untransfected BHK21 cells, transfected BHK21 cells alone, untreated DCs alone, and CD8\(^{+}\) T cell line alone were included as negative controls.

Results

Construction and characterization of self-replicating RNA constructs

Generation of plasmid DNA constructs and subsequent preparation of self-replicating SINrep5 RNA constructs was performed as described in Materials and Methods. The SINrep5 vector contains the genes encoding Sindbis virus RNA replicase and the SP6 promoter (17). A schematic diagram of SINrep5, SINrep5-HSP70, SINrep5-E7, SINrep5-E7/GFP, and SINrep5-E7/HSP70 RNA transcripts using SP6 RNA polymerase is shown in Fig. 1. An ELISA was performed to demonstrate E7 protein expression in BHK21 cells transfected with various self-replicating RNA constructs. SINrep5-E7 and SINrep5-E7/HSP70 expressed comparable amounts of E7 protein (data not shown).

Vaccination with self-replicating SINrep5-E7/HSP70 RNA enhances an E7-specific cytotoxic immune response

CD8\(^{+}\) T lymphocytes are one of the most crucial effectors for inducing antitumor immunity. To determine the quantity of E7-specific CD8\(^{+}\) T cell responses generated by the SINrep5-E7/
HSP70 RNA vaccine, CTL assays were performed. Splenocytes from vaccinated mice were cultured with E7 peptide (aa 49–57) containing the MHC class I epitope and served as effector cells. TC-1 tumor cells were used as target cells. As shown in Fig. 2A, vaccination with SINrep5-E7/HSP70 generated a significantly higher percentage of specific lysis compared with vaccination with other SINrep5 RNA vaccines \((p < 0.001,\text{ one-way ANOVA})\). Furthermore, CTL activity appeared to be CD8 specific because blocking with CD8-specific Ab led to a significant loss of specific lysis (Fig. 2B). Interestingly, vaccination with SINrep5-E7 RNA resulted in a percentage of specific lysis only slightly higher than background. This finding was consistent with our previous observation that vaccination with naked wild-type E7 DNA (13) or SINrep5-E7 RNA (18, 25) did not generate strong E7-specific CD8+ T cell immune responses, suggesting that wild-type E7 is a weak Ag.

We also performed an ELISA to determine the concentration of IFN-γ in the supernatant of cultured splenocytes. As shown in Fig. 3, splenocytes from mice vaccinated with SINrep5-E7/HSP70 RNA secreted the highest concentration of IFN-γ compared with splenocytes from mice vaccinated with other SINrep5 RNA vaccines \((p < 0.001,\text{ one-way ANOVA})\). The increase of IFN-γ secretion is likely due to CD8-specific T cells because blocking with CD8-specific Ab during the in vitro peptide stimulation led to a significant decrease in IFN-γ concentration (Fig. 3). These results also indicated that fusion of HSP70 to E7 did not significantly enhance IFN-γ-secreting E7-specific CD8+ T cell activity.

To determine whether vaccination with the self-replicating SINrep5-E7/HSP70 RNA could protect mice against E7-expressing TC-1 tumours, mice were inoculated with TC-1 cells (Fig. 4). Mice vaccinated with SINrep5-E7/HSP70 RNA were less susceptible to tumor growth than mice vaccinated with the other RNA vaccines \((p < 0.001,\text{ one-way ANOVA})\). Vaccination with self-replicating SINrep5-E7/HSP70 RNA does not enhance IFN-γ-secreting E7-specific CD4+ T cell activity or anti-E7 Ab titers

Vaccination with self-replicating SINrep5-E7/HSP70 RNA does not enhance IFN-γ-secreting E7-specific CD4+ T cell activity or anti-E7 Ab titers

To assess the E7-specific CD4+ T cell immune responses generated by self-replicating SINrep5-E7/HSP70 RNA, an ELISA was performed to determine the concentration of IFN-γ in the supernatant of cultured splenocytes. Splenocytes obtained from mice vaccinated with various self-replicating SINrep5 RNA vaccines were cultured in vitro with E7 peptide (aa 30–67) containing the MHC class II epitope (24) for 6 days. As a negative control, an ELISA was also performed without peptide. In addition, we used splenocytes from Sig/E7/LAMP-1 RNA-vaccinated mice (18) pulsed with E7 peptide as a positive control for the ELISA to ensure the success of this assay. We have previously shown that mice vaccinated with Sig/E7/LAMP-1 RNA vaccine generated a significant increase of IFN-γ-secreting CD4+ E7-specific T cells (18). As shown in Fig. 4, splenocytes from mice vaccinated with SINrep5-E7/HSP70 RNA did not generate a significant increase in IFN-γ concentration compared with splenocytes from mice vaccinated with other SINrep5 RNA vaccines. These results suggested that fusion of HSP70 to E7 did not significantly enhance IFN-γ-secreting E7-specific CD4+ T cell activity.

The quantity of anti-HPV-16 E7 Abs in the sera of the vaccinated mice was determined using a direct ELISA 2 wk after vaccination. Mice vaccinated with SINrep5-E7/HSP70 did not exhibit higher titers of E7-specific Abs in the sera of mice than mice vaccinated with the other RNA vaccine constructs (data not shown).

**FIGURE 3.** ELISA for IFN-γ secreted by E7-specific CD8+ T cells. Mice were immunized with various self-replicating RNA vaccines via i.m. injection. Splenocytes were collected 14 days after vaccination. Splenocytes obtained from mice vaccinated with various self-replicating SINrep5 RNA vaccines were cultured in vitro with E7 peptide (aa 49–57, RAHYNIHTF) containing the MHC class I epitope or without any peptide for 6 days. The supernatants of the culture medium were collected to detect IFN-γ concentration using an ELISA. Results from the ELISA are from one representative experiment of three performed.

**FIGURE 4.** ELISA for IFN-γ secreted by E7-specific CD4+ T cells. Splenocytes from mice vaccinated with various self-replicating RNA vaccines were cultured in vitro with E7 peptide containing the MHC class II epitope (aa 30–67, DSSEEDIEIDGPAGQAEPRDRAHYNIHTFCCCKC DSTLRL) or no peptide (control). The supernatant of the culture medium was collected to detect IFN-γ concentration using an ELISA. We used IFN-γ-secreting splenocytes from Sig/E7/LAMP-1 RNA-vaccinated mice (18) as a positive control for CD4+ IFN-γ-secreting T cells to ensure the success of this assay. Results from the ELISA are from one representative experiment of three performed.
tumors, an in vivo tumor protection experiment was performed as described in Materials and Methods. As shown in Fig. 5A, fewer pulmonary tumor nodules were identified in mice vaccinated with the self-replicating E7/HSP70 RNA vaccines (0.1, 1, and 10 μg) compared with mice vaccinated with the other RNA vaccines \((p < 0.001, \text{one-way ANOVA})\). Representative gross photographs of the lung tumors are shown in Fig. 5B. Our results demonstrated that self-replicating SINrep5-E7/HSP70 RNA vaccines protected mice from i.v. tumor challenge even at the low dosage of 0.1 μg, whereas mice vaccinated with 10 μg of the other SINrep5 RNA vaccines developed numerous lung nodules from TC-1 tumor challenge. Our data also showed that linkage of E7 to an irrelevant protein such as GFP does not generate a significant antitumor effect and that the enhancement of antitumor effect by HSP70 requires physical linkage of HSP70 to E7.

**FIGURE 5.** Tumor protection experiments using various SINrep5 self-replicating RNA vaccines. Mice were immunized with various SINrep5 self-replicating RNA vaccines and challenged with TC-1 tumor cells as described in Materials and Methods. A, The mean number of tumor nodules on the lung surface of the vaccinated mice was used as a measurement of the effectiveness of the various self-replicating RNA vaccines in controlling HPV-16 E7-expressing tumor growth. There were fewer mean pulmonary nodules in mice vaccinated with self-replicating E7/HSP70 RNA vaccines (0.1, 1, and 10 μg) than in mice vaccinated with the other RNA vaccines \((10 \mu g; p < 0.001, \text{one-way ANOVA})\). The tumor protection experiments were repeated three times, yielding similar results. B, Representative gross photographs of the lung tumors in each vaccinated group. There are multiple grossly visible lung tumors in unvaccinated control mice and mice vaccinated with SINrep5 or SINrep5-E7 RNA vaccines. Lung tumors in the SINrep5-E7/HSP70 RNA-vaccinated group cannot be seen at the magnification provided in this figure.

\[CD8^+ \text{ T cells and NK cells are important for the antitumor effect generated by vaccination with SINrep5-E7/HSP70 RNA vaccines}\]

To determine the subset of lymphocytes that are important for protection against E7-expressing tumor cells, we performed in vivo Ab depletion experiments. Ab depletion was initiated 1 wk before tumor challenge and terminated on day 21 after tumor challenge. As shown in Fig. 6, the mean number of pulmonary nodules from mice depleted of CD8\(^+\) T cells or NK1.1 cells was significantly higher than that observed in mice treated with the control IgG2a isotype Ab. Similar results were observed in mice treated with control IgG2a isotype Ab and in mice without Ab depletion. Furthermore, depletion of NK1.1 cells resulted in a higher mean number of tumor lung nodules than did CD8\(^+\)
depletion. In comparison, the mean number of pulmonary nodules from mice depleted of CD4\(^+\) T cells resembled results obtained from the mice receiving IgG2a isotype Ab, indicating that CD4\(^+\) T cells were not critical in generating this effect. These results suggest that both CD8\(^+\) T cells and NK cells were essential for the Ag-specific antitumor immunity generated by SINrep5-E7/HSP70 RNA vaccine. CD8\(^+\) T cells and NK cells have also been found to be important in generating an antitumor effect in mice treated with autologous tumor-derived heat shock protein preparations (9).

To investigate whether NK cells were significantly expanded in mice vaccinated with various RNA vaccines, we performed flow cytometry analysis of CD3\(^-\)NK1.1\(^+\) cells. We found that the presence of NK cells was markedly increased in all constructs (E7/HSP70, E7, HSP70, and control plasmid) relative to naive mice, indicating that the expansion of NK cells is not limited to the E7/HSP70 RNA vaccine (Fig. 7). Because our data indicated that the various RNA replicon-based vaccines each produced a similar number of NK cells, NK cells alone are probably not sufficient to account for the antitumor effect generated by the E7/HSP70 RNA vaccine.

**Self-replicating RNA vaccines induce apoptosis**

Self-replicating RNA vaccines have been shown to induce apoptotic changes after uptake by cells (3). We therefore evaluated the percentage of apoptotic cells in BHK21 cells transfected with various RNA vaccines. Because transfection efficiency was only 30%, the percentages of apoptotic BHK21 cells were corrected for transfection efficiency. As shown in Fig. 8, all of the BHK21 cells transfected with various RNA vaccines generated a higher percentage of apoptosis compared with the two control groups (untransfected or electroporated without RNA vaccines). We observed no

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**FIGURE 6.** In vivo Ab depletion experiments to determine the effect of lymphocyte subsets on the potency of self-replicating SINrep5-E7/HSP70 RNA vaccine. Mice were immunized with 1 \(\mu\)g/mouse self-replicating SINrep5-E7/HSP70 RNA via i.m. injection. Two weeks after vaccination, mice were challenged with \(1 \times 10^6\) TC-1 cells/mouse i.v. via tail vein. Depletions were initiated 1 wk before tumor challenge and lasted for 28 days. Three weeks after tumor challenge, the mice were sacrificed. The number of pulmonary tumor nodules in mice depleted of CD8\(^+\) T cells and NK1.1 cells was significantly higher than those in mice depleted of CD4\(^+\) T cells or receiving control IgG2a isotype Ab.

**FIGURE 7.** Flow cytometry analysis of NK cells in mice immunized with various self-replicating SINrep5 RNA vaccines. Mice were immunized, and splenocytes were collected as described in Materials and Methods. Splenocytes were stained for CD3 and NK1.1 immediately without any stimulation. A, Flow cytometry analysis to demonstrate the number of NK cells in mice immunized with various self-replicating RNA vaccines. The percentage of NK cells in splenocytes is indicated in the upper left corner. B, Histogram demonstrating the percentages of NK cells in vaccinated mice. The percentage of NK cells from the splenocytes of mice immunized with self-replicating RNA vaccines was higher than those in mice receiving no immunization. There was no significant difference in the percentage of NK cells generated by the various self-replicating RNA vaccines. Data from the NK cell surface marker staining shown here are from one representative experiment of two performed.
fected BHK21 cells as target cells and E7-specific CD8+ T cells. We used bone marrow-derived DCs coincubated with transfectants in a Sindbis virus RNA vector led to enhancement of E7-specific CD8+ T cell-mediated immunity and an impressive antitumor effect. Similarly, we previously demonstrated that E7/HSP70 generated potent immunological and antitumor effects in a naked conventional DNA vector (13) and in a DNA-based self-replicating RNA replicon vector, also known as “suicidal DNA” (14). Our data are consistent with prior reports using HIV-1 p24 (12), OVA (31), or influenza nucleoprotein (32) as model Ags linked to HSP70. Fusion to HSP70 increased the immunogenicity of these Ags. Although chimeric HSP70 fusions have been shown to work well in these models, at least one study found that gp96, another member of heat shock proteins, fused with β-gal did not lead to activation of β-gal-specific T cells in vivo (33). Thus, the strategy of linking HSP to Ag to enhance vaccine potency may depend on the type of HSP used for the linkage.

We observed that SINrep5-E7/HSP70 significantly enhanced E7-specific CD8+ T cell responses compared with SINrep5-E7 RNA vaccines in vivo. It seems unlikely that the observed enhancement of E7-specific CD8+ T cell responses in vivo occurs through improvement of direct MHC class I presentation of E7 to CTLs by cells expressing E7/HSP70, a process known as “direct priming.” Intramuscular delivery of RNA replicons probably delivers RNA into muscle cells, which are not ideal professional APCs because they lack costimulatory molecules that are important for efficient activation of T cells. Even if the various SINrep5 constructs are delivered to cells other than muscle cells, self-replicating RNA eventually induces apoptosis (8). The initially transfected cells are thus unlikely to directly present Ag in an efficient manner.

**FIGURE 8.** Flow cytometry analysis to determine apoptotic death of host cells induced by self-replicating RNA vaccines. BHK21 cells transfected with various RNA vaccines were stained with annexin V-FITC and propidium iodide followed by flow cytometry analysis. Unmodified BHK21 cells and electroporated BHK 21 cells without RNA were used as controls. The percentages of apoptotic cells revealed a statistical decline when transfected with SINrep5 RNA 24–72 h after electroporation (representative with SIN-E7/HSP70: 70.3 ± 3.6% for 24 h, 49.3 ± 4.2% for 48 h, 18.0 ± 3.1% for 72 h; p < 0.001, one-way ANOVA). No statistical difference could be found in the percentages of apoptotic cells transfected with various SINrep5 RNA vaccines. This experiment was repeated twice, yielding similar results.

**FIGURE 9.** CTL assays to demonstrate enhanced MHC class I presentation of E7 in bone marrow-derived DCs pulsed with BHK21 cells transfected by SINrep5-E7/HSP70 RNA. Bone marrow-derived DCs were cocultured with various RNA-transfected BHK21 cells and used as target cells. DPα-restricted E7-specific CD8+ T cells were used as effector cells (30). Cytolysis was determined by quantitative measurements of LDH as described in Materials and Methods. The self-replicating E7/HSP70 RNA vaccines generated significantly higher percentages of specific lysis (E:T ratios, 3:1 and 9:1) compared with the other RNA vaccines (p < 0.001). CTL assays shown here are from one representative experiment of two performed.
Enhancement of E7-specific CD8+ T cell responses by chimeric SINrep5-E7/HSP70 in vivo is likely generated by presentation of exogenous proteins through the MHC class I pathway. The exogenous chimeric E7/HSP70 protein may be taken up and processed by other APCs via the MHC class I-restricted pathway (34–36). HSP70 complexes have been shown to enter professional APCs by binding specifically to the surface of these APCs followed by receptor-mediated endocytosis (37). More recent investigations have focused on identifying receptors for HSPs. For example, one recent study has successfully identified CD91 as the receptor for a member of the HSP family (gp96) on APCs (38).

Our study suggests that transfection of cells by the SINrep5-E7/HSP70 vector led to apoptosis of cells. However, we cannot rule out the possibility that secondary necrosis occurred in our experimental conditions. It is not clear whether DCs process apoptotic or necrotic cells containing E7/HSP70 protein or whether they process E7/HSP70 protein when it is released after apoptosis or secondary necrosis of transduced cells. Because various SINrep5 constructs can induce cell apoptosis at similar levels (Fig. 8), the distinct enhancement in E7-specific CD8+ T cell activity was most likely due to the linkage of E7 with HSP70. Thus, our results suggested that the linkage of HSP70 to E7 was capable of enhancing MHC class I presentation of the linked E7 Ag through an exogenous pathway.

Another important factor for the enhancement of Ag-specific CD8+ T cell activity by chimeric E7/HSP70 may be the biology of professional APCs, such as DCs. One recent study reported that necrotic but not apoptotic cell death leads to release of HSPs and induces expression of Ag-presenting and costimulatory molecules on the DCs (39), and others have found that HSPs fused to Ag may stimulate DCs to up-regulate expression of MHC class I and II and costimulatory molecules (40). Thus, induced maturation of DCs by HSP70 linked to Ag may augment T cell activity generated by the chimeric E7/HSP70 RNA vaccine.

A comparison of the current study with our previous studies revealed that different forms of nucleic acid vaccines may activate different subsets of effector cells in the vaccinated host and thereby have different mechanisms for the generation of an antitumor effect. For example, our study indicated that NK cells played an essential role in the antitumor effect mediated by E7/HSP70 RNA replicon-based vaccines. In contrast, NK cells were not as important for the anti-tumor effect generated by the E7/HSP70 naked DNA vaccine (13) or suicidal DNA vaccine (14); depletion of NK1.1+ cells in mice vaccinated with naked E7/HSP70 DNA or suicidal DNA did not abolish antitumor immunity (13, 14). In vivo Ab depletion experiments demonstrated that CD8+ T cells were important for the antitumor effects induced by vaccination with either E7/HSP70 DNA or E7/HSP70 RNA replicon-based vaccines. Thus, different forms of nucleic acid vaccines may activate different subsets of effector cells in the vaccinated host and have different mechanisms for mediating an antitumor effect.

The apoptotic changes generated by the self-replicating RNA vaccine raise potential safety concerns. We observed increased apoptotic changes and inflammatory responses localized at the injected sites of RNA replicon-based vaccines in mice (data not shown). However, we performed pathological examination of the vital organs in the E7/HSP70-vaccinated mice and did not observe any significant pathological changes. There are also potential risks associated with the presence of HPV-16 E7 protein in host cells. E7 is a viral oncoprotein that disrupts cell cycle regulation by binding to tumor suppressor pRB protein in nuclei (41), potentially leading to the accumulation of genetic aberrations and eventual malignant transformation in the host cells. The usage of self-replicating RNA vectors may ease the concern for oncogenicity of E7 protein because transfected cells eventually undergo apoptosis.

In summary, our results revealed that fusion of the gene encoding M. tuberculosis HSP70 to HPV-16 E7 gene in RNA replicon can generate significant E7-specific CD8+ T cell-mediated immune responses and antitumor effects against HPV-16 E7-expressing murine tumors. Our study also indicated that fusion of HSP70 to an Ag gene may greatly enhance the potency of RNA replicon-based vaccines and can potentially be applied to other cancer systems with known tumor-specific Ags or other infectious diseases.

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References


