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Full Length Research Paper

Protective antitumor activity induced by a fusion vaccine with murine beta-defensin2 and VE-cadherin in mouse models

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Targeting angiogenesis is an effective strategy for anticancer therapy. The vascular endothelial-cadherin (VE-cad) regulated angiogenesis is a potential target for anti-angiogenesis. Here, we develop a fusion vaccine plasmid DNA pSec-MBD2-VE-cad from VE-cad and murine beta defensin2 (MBD2) to induce immunity for cancer therapy. The expression and biological activity of fusion protein were detected *in vitro*. Anti-tumor effects and inhibition of angiogenesis via pSec-MBD2-VE-cad were investigated in mice model. The anti-VE-cad antibodies and cytotoxic T lymphocyte (CTL) responses were analyzed. Inhibition of tumor-induced angiogenesis and prolonged survival were shown in mice challenged with murine colon adenocarcinoma (CT26) or Murine fibrosarcoma cell line (MethA) after immunization with the fusion vaccine. Moreover, VE-cad-specific antibodies and specific T cell cytotoxicity were detected. The fusion vaccine based on self immune peptide Murine beta defensin2 (MBD2) and self antigen mVE-cad could induce autoimmunity and inhibit tumor growth, and thus there may be potential applications in cancer therapy.

Key words: Fusion vaccine, murine beta defensin2 (MBD2), murine vascular endothelial-cadherin (mVE-cad), antigen targeting, anti-angiogenesis.

INTRODUCTION

Cancer causes millions of deaths every year, and seriously affects the quality of people's lives. With the development of molecular biology and a better understanding of the mechanisms for manipulating the immune system, tumor immunotherapy, especially, targeting angiogenesis therapy, has become known as one of the novel treatments along with surgery, radiation therapy, and chemotherapy (Folkman, 2006; Hou et al., 2004). Targeting angiogenesis therapy attempts either to block signal transduction between angiogenic growth factors and their corresponding receptors or to increase angiogenic inhibittors within tumors (Hou et al., 2004). The vascular endothelial-cadherin (VE-cad), originally called cadherin-5 and selectively expressed in endothelial cells (ECs), plays a pivotal role in endothelium integrity and in the control of vascular permeability (Suzuki et al., 1991; Breviario, 1995; Carmeliet et al., 1999; Crosby et al., 2005). Increasing evidences have indicated that VE-cad may be a molecular target for anti-angiogenic therapy.

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Abbreviations: mVE-cad, murine vascular endothelial cadherin; **MBD2**, murine beta-defensin2; **pMBD2-mVE-cad**, plasmid DNA encoding fusion gene with MBD2 and mVE-cad; **pmVE-cad,** plasmid DNA encoding mVE-cad; **pMBD2,** plasmid DNA encoding MBD2; **iDC,** immature dendritic cell; **CTL,** cytotoxic T lymphocyte.

Furthermore, most of the previously reported trials of anti-angiogenesis therapy by targeting VE-cad involved passive immunization using monoclonal antibody (Corada et al., 2001, 2002; Liao et al., 2000, 2002; Menon et al., 2006; Tzima et al., 2005). However, at higher doses the mAb BV13, directed to mouse VE-cad, increased significantly vascular permeability in the lung and heart, thus inducing toxic effects (Corada et al., 1999). Therefore, active immunity by breaking immune tolerance against VE-cad may be considered a useful approach to anti-angiogenesis therapy.

Recent studies have shown that a small antimicrobial peptide Murine beta-defensin 2 (MBD2) provided a critical link between innate and adaptive immune response (Biragyn et al., 2002). MBD2 modulates adaptive immune response not only by recruiting immature dendritic cells (iDCs) through chemokine receptor CCR6 but also by up-regulating dendritic cells (DCs) maturation through a pattern recognition receptor (PRR), Toll-like receptor 4 (TLR-4)(Biragyn et al., 1999, 2002; Lillard et al., 1999; Tani et al., 2000; Yang et al.,1999). In addition, a receptor-mediated process has been shown for MBD2-mediated antigen cross-presentation, which induces protective, T-cell–dependent antitumor immunity (Biragyn et al., 1999; Ma et al., 2006). We previously reported that a fusion vaccine based on MBD2 and murine VEGFR2 (mFlk-1) induced autoimmunity against endothelial cells, resulting in inhibition of tumor growth (Wang et al., 2007). Given that cadherin has been also observed over-expression or co-expression on tumor vessels or tumor cells (Arnold et al., 1999; Cavallaro et al., 2006; Saha et al., 2007, 2008), we speculated that VE-cad, as another important target, could also be used to formulate a fusion vaccine to provide an effective protection against tumors.

Here, we construct a fusion DNA vaccine with MBD2 and mVE-cad through a linker peptide [(G_4S) ×3] sequence and established cationic nano-liposomes-DNA delivery system for transfection. The effects of resulting vaccine and underlying mechanism were investigated in two mice models.

MATERIALS AND METHODS

Animals and cell lines

Female BALB/C mice at the age of 6-8 weeks were purchased from the West China Experimental Animal Center. Murine colon carcinoma cell line CT26, Murine fibrosarcoma cell line MethA and cell line COS-7 were purchased from ATCC. Both CT26 and MethA were cultured in growth media RPMI 1640 (FBS; Gibco BRL) and COS-7 cells in DMEM (Gibco BRL). The growth media were supplemented with 10% (vol/vol) fetal bovine serum (FBS; Gibco BRL), 2 mM L-glutamine and 0.1 mg/ml of amikacin. The cells were incubated at 37°C with 95% air humidity and 5% CO_2 in air.

Construction and preparation of pMBD2-mVE-cad

The mature sequence MBD2 and mVE-Cad were amplified by polymerase chain reaction (PCR) from previous constructs in our lab and constructed into the pSecTag B/CMV vector (Invitrogen). The PCR primers for the sub-cloning are as follows: MBD2-forward: 5'-GAACTTGACCACTGCCACACCAATG-3'; MBD2-reverse: 5'-TCAT TTCATGTAC TTGCAACAGGGGTTC-3'; mVE-cad-reverse: 5'- CTA CTGGGCTGCCATCTCCTC ACAGAAGG-3'; mVE-cad-forward: 5'-GACTGGATTTGGAATCAAATGCAC-3'. MBD2-mVE-cad-linker-reverse: 5'-CTACTGGGCTGCCATCTCCTCACAGAAGG-3'; MBD2-mVE-cad-linker-forward: 5'-GAACTTGACCACTGCCACACCAATG-3'; MBD2 and mVE-cad were fused with a 15-amino acid [(G_4S) ×3] linker by overlap PCR. The recombinants pMBD2-mVE-cad, pMBD2 and pmVE-cad were confirmed by DNA sequencing (Invitrogen). All the constructs for treatments *in vivo* and *in vitro* were extracted and purified using EndoFree Kits (Qiagen).

Expression and biofunctional assay of MBD2-mVE-cad

The recombinants pMBD2-mVE-cad, pmVE-cad or empty vector pSecTag B were respectively transfected into COS-7 cells with LipofectAMINE 2000 reagent (Invitrogen) as per the manufacturer's instruction 48 h post transfection. The supernatants of the three groups of transfectant COS-7 cells (1×10^6 cells/mL) were collected, and then concentrated by super filter (Minipore, 5kd). Recombinant proteins, MBD2-mVE-cad and mVE-cad, were analyzed by Western blot. The bioactivity of the recombinant proteins was evaluated by measuring the ability of their corresponding supernatants to chemoattract iDCs. The migration of iDCs was assessed using a 96-well microchamber chemotaxis plate (5 μm; Neuro Probe) following published methods (Howard et al., 2005). Briefly, Murine iDCs were isolated from bone marrow and were added to the upper chamber of a 96-well chemotaxis plate filter. The supernatants (30 μl) were added to the lower compartment; interferon-gamma (IFN-gamma) inducible protein10 (IP10) was used as a positive control. The chemotaxis plate was incubated for 4 h at 37ºÇsupplemented with 5% CO_2 and then at 4ºÇ for 10 min. After the membrane was removed, cells were fixed in 70% methanol, and stained for 5 min in Wright's staining solution. The number of cells that migrated to the lower surface was counted microscopically in six randomly chosen high power fields microscopically. Each transfection was in triplicate and the as-says were performed three times.

Immunization and tumor models

Mice were immunized with 100 μg per mouse per injection of cationic liposome-encapsulated DNA vaccine (plasmid : nano-liposome = 1:3), using pMBD2-mVE-cad, pMBD2, pmVE-cad, pSecTag B vector, Liposome or normal saline (N.S, non-immunized mice). Immunization was delivered to all groups by intramuscular injection in both hind quadricepses once a week for 4 weeks. The mice were then challenged with s.c. injection of 3×10^5 tumor cells in the right flank on the 7th day after the last immunization. Tumor width (W) and length (L) were measured every 3 days by calipers. The tumor volume (Tv) was calculated according to the formula:

$$Tv = 0.52\times L\times W^2$$

The mice treated for immunohistochemical analysis were sacrificed on day 20 after tumour cell injection.

Immunohistochemistry and alginate-encapsulate tumor cell assay

Immunohistochemical analysis was performed. In brief, frozen sections were fixed in acetone and incubated with an anti-CD31 antibody (R and D) and a biotin labeled anti-IgG antibody. The sections were then stained with labeled streptavidin-biotin reagents

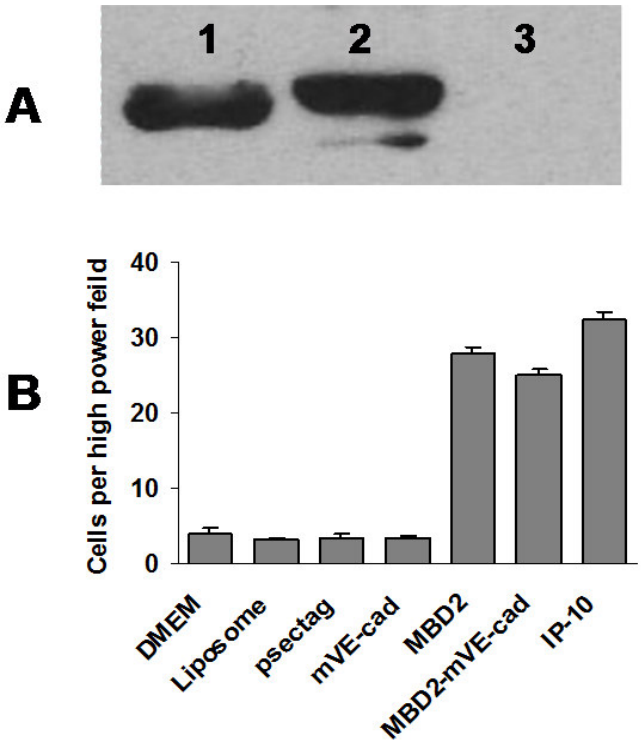

Figure 1. Expression and biological activity of pMBD2-mVE-cad *in vitro*. The Supernatants of COS-7 cells transfected by pMBD2-mVE-cad, pmVE-cad or pSecTag B with LipofectAMINE 2000 reagent, 48 h post transfection, were harvested and concentrated by super filter (Minipore, 5 kd) for western blot analysis and chemotaxis assay. A, MBD2-mVE-cad and mVE-cad in the supernatants were detected by western blot analysis with anti-mVE-cad mAb. 1.Transfected with pmVE-cad; 2, Transfected with pMBD2-mVE-cad; 3, Transfected with pSecTag B. B, Induction of iDCs migrated by culture supernatants. Chemotaxis for iDCs was assessed by the number of cells that migrated to the lower surface counted as described in Materials and methods. MBD2-mVE-cad and MBD2 show not only chemotaxis ability similar to IP-10 but also significant difference in comparison with other groups. The data were expressed as mean±S.D; n=10. Three replicates were done for each treatment.

(Dako). Subsequently, sections were re-stained by hematoxylin. The number of microvessels was counted in six randomly chosen high power fields in each section.

Alginate-encapsulate tumor cell experiment was performed according to described methods (He et al., 2003). Briefly, alginate beads formed and contained about 1×10^5 tumor cells per bead. Four beads were implanted subcutaneously into both the dorsal sides of the immunized mice. On the 13th day post bead implantation, mice were injected intravenously with 0.1 ml of 100 mg/kg fluorescein isothiocyannate (FITC)-dextran (Sigma) solution. Alginate beads were exposed surgically, photographed, and then immediately removed after FITC-dextran injection. The uptake of FITC-dextran was measured.

ELISA

Briefly, 96-well plates were coated with prokaryotic expressed mVE-cad in coating buffer (carbonate bicarbonate, pH 9.6) overnight at 4ºC. Diluted mice sera were added and followed by horseradish peroxidased (HRP) anti-mouse IgG. Enzyme activity was developed using 2, 2'-azino-bis [3-ethylbenzthiazoline-6-sulphonic acid] (ABTS, Sigma) as a substrate, and absorbance was measured at 405 nm with an ELISA reader (Bio-Rad).

Evaluation of T-Cell response

The possible cytotoxic T lymphocytes (CTLs)-mediated mVE-cad cytotoxicity was determined by a standard ^{51}Cr release assay. Spleens were collected on day 7 after the last vaccination. Next, T lymphocytes (TLs) isolated from single-cell suspensions with Nylon Fiber Column T (L-Type, Wako) were used as CTL effector cells and CT26 transfected with pVE-cad as target cells, the untreated CT26 cells as a control. Effector cells and target cells were seeded into a 96-well microtiter plate in different E: T ratios. The CTL activity was calculated by the formula:

$$\%\ \text{lysis} = \frac{\text{Experimental release–spontaneous release}}{\text{Maximum release–spontaneous release}} \times 100$$

Statistical analysis

Spss11.5 was used for statistical analysis. The statistical differences between the groups were tested by performing ANOVA, and an unpaired Student's t-test. Survival curves were compared using the log-rank test. All data were regarded as significant if $p<0.05$.

RESULTS

In vitro expression of MBD2-mVE-cad and its biological activity

Culture supernatants of COS-7 cells respectively transfected with pSec-MBD2-mVE-cad, pSec-mVE-cad and pSecTag B were analyzed 48 h post transfection by Western-blot with an mVE-cad specific mAb. Recombinant proteins MBD2-mVE-cad and mVE-cad were detected in the culture supernatants of the corresponding transfections (Figure 1A). Then, to investigate the biological activities of secreted MBD2 and MBD2-mVE-cad, their ability to chemoattract murine iDCs were tested with the culture supernatants. The results showed that the supernatant of COS-7 transfected with pMBD2-mVE-cad chemoattracted iDCs as did the supernatant of pMBD2 or IP10, whereas supernatant of pmVE-cad, pSecTag B and Lipsome or NS-transfected cells did not exhibit chemotaxis towards the iDCs (Figure 1B).

Protective effects elicited by pMBD2-mVE-cad fusion vaccine

To investigate whether DNA vaccination with pMBD2-mVE-cad can induce a specific immune response *in vivo*, Mice were immunized using pMBD2-mVE-cad, pMBD2, pmVE-cad, pSecTag B vector, Lipsome and NS, respectively, and then challenged with CT26 cells or MethA cells. There were significantly protective effects in mice

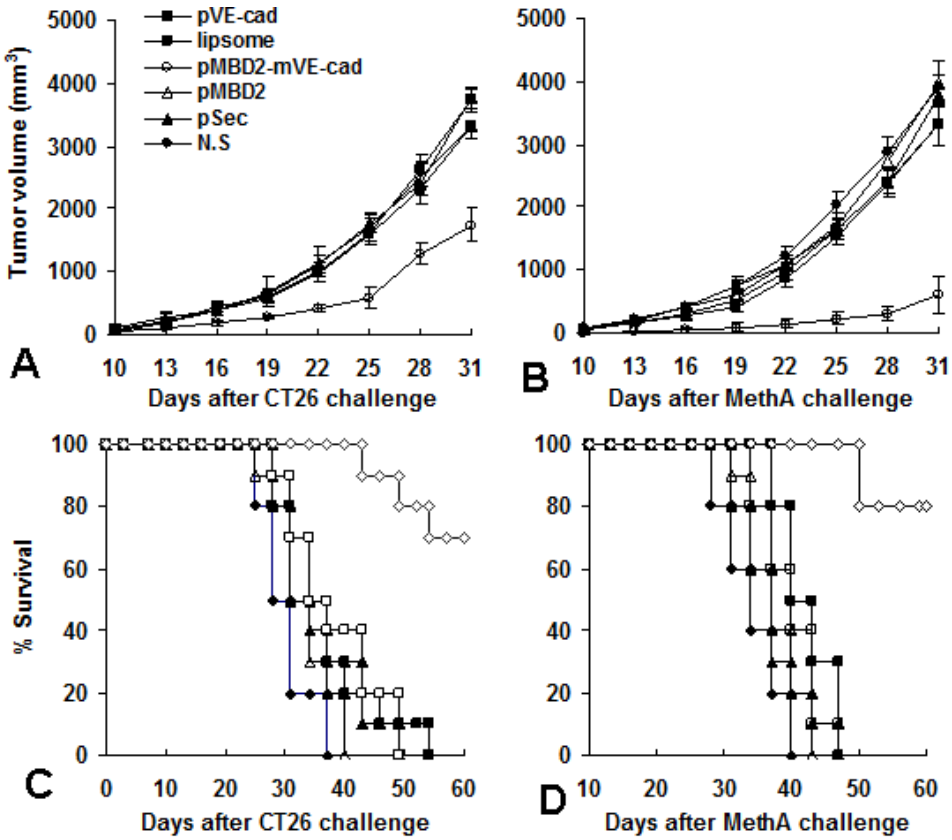

Figure 2. Induction of protective anti-tumor immunity by pMBD2-mVE-cad. Mice were immunized with 100 µg of pMBD2-mVE-cad, pmVE-cad, pMBD2 Lipsome, pSecTag B or normal saline once a week for 4 weeks and then challenged with CT26 cells (A, C) and MethA cells (B and D) on day 7 after the last immunization. There was a significant difference in tumor volume (p=0.01) and mice survival (p=0.01, by log-rank test) between pMBD2-mVE-cad immunized and other control groups in CT26 model. The data were expressed as mean±S.D; n=10. Each group experiments were done twice.

immunized with pMBD2-mVE-cad, compared with control groups in both two models (Figure 2). Moreover, neither the pmVE-cad nor pMBD2 immunization alone showed protective effects. The results suggested that MBD2 fusion played an important role in the functioning of the fusion vaccine.

Inhibition of angiogenesis

To further verify whether pMBD2-mVE-cad mediated anti-tumor effect is depended primarily on inhibition of angiogenesis, the microvessel staining by immunohistochemistry and alginate-encapsulate tumor cell assay were performed. Compared with control groups, immunohistochemical staining analysis of the tumor tissue from pMBD2-mVE-cad immunized mice with an anti-CD31 antibody showed significantly decreased microvessel density (Figure 3Aa-d), and the number of microvessels also showed significant difference (Figure 3C). The inhibition of angiogenesis was assayed by the alginate-encapsulate tumor cell assay (Figure 3Ba-d). New blood vessels in alginate beads from pMBD2-mVE-cad immunized mice were apparently sparse. In addition, FITC-dextran uptake was significantly decreased from mice treated with pMBD2-mVE-cad, in comparison to control groups (Figure 3D). These data suggest that angiogenesis was inhibited in tumors; therefore, the growth of tumors was restrained.

Induction of specific antibody and cytotoxic T lymphocyte response by pMBD2-mVE-cad

To investigate whether anti-angiogenic immunity is the possible mechanism underlying the pMBD2-mVE-cad-orchestrated anti-tumor activity *in vivo*, the sera antibody response and the killing activity of CTLs were examined. The elevated antibody level against mVE-cad was detected by ELISA (Figure 4A). T cells isolated from spleens of pMBD2-mVE-cad-treated mice exhibited higher cytotoxicity to VE-cad-positive CT26 tumor cells than those

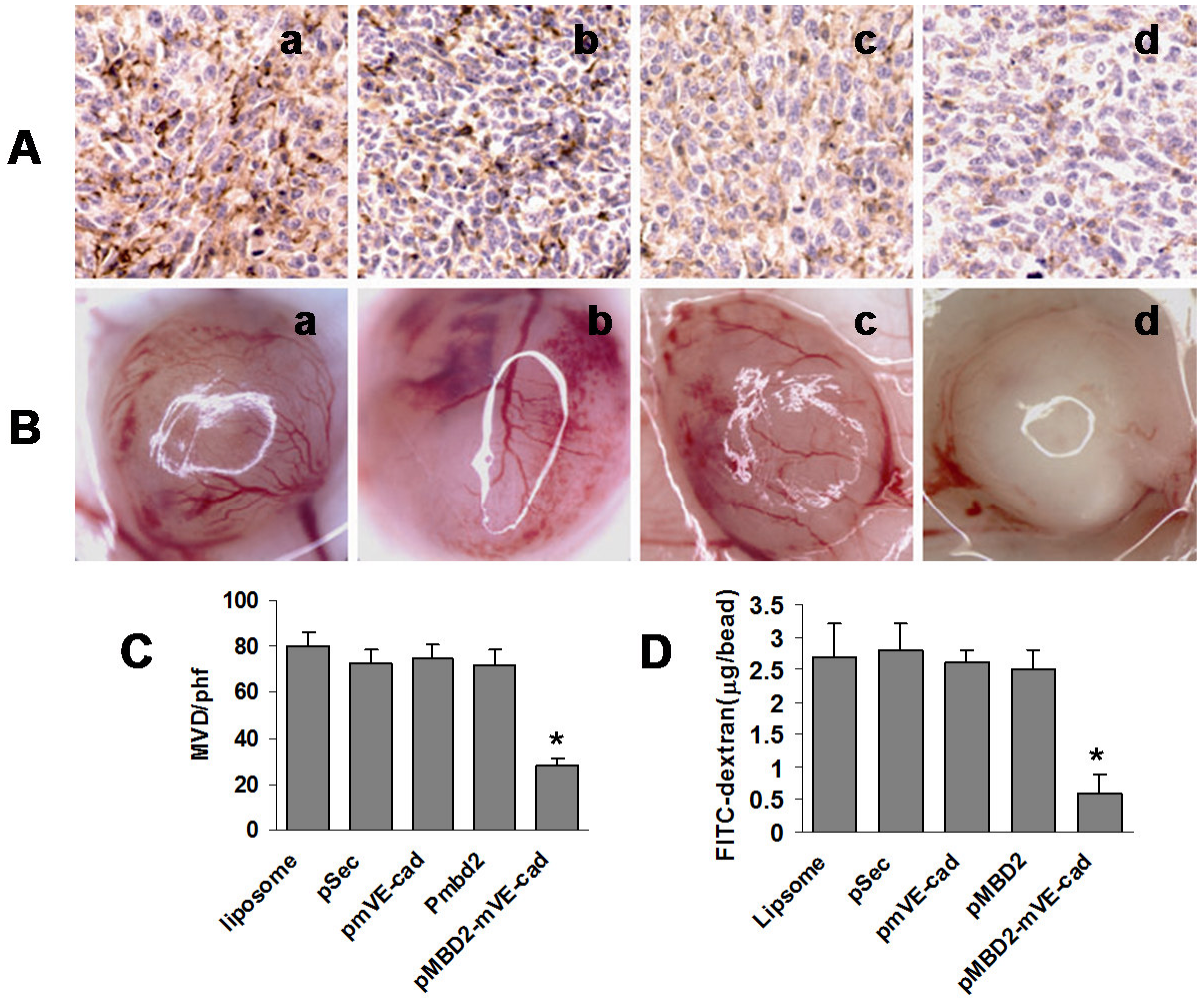

Figure 3. Inhibition of angiogenesis within tumors. A. Immunhistochemical analysis using an anti-CD31 antibody. Balb/c mice were immunized and challenged with 3×10^5 CT26 cells. Frozen sections of tumor tissues from mice immunized by pSecTag B (a), pmVE-cad (b), pMBD2 (c), pMBD2-mVE-cad (d), lipsome or normal saline (data not shown) were tested. B. The alginate-encapsulate tumor cell assay. Mice were immunized with 100 μg of pMBD2-mVE-cad, pmVE-cad, pMBD2, Lipsome, pSecTag B once a week for 4 weeks. Alginate beads containing 1×10^5 CT26 cells per bead were then implanted subcutaneously into the backs of mice 7 days after last immunization. Beads were surgically removed twelve days later. C. Vessel density of tumor tissues from pMBD2-mVE-cad immunized mice indicated a significant decrease, compared with control groups (p=0.01). The data were expressed as mean ± S.E. D. FITC-dextran uptake of beads from pMBD2-mVE-cad immunized mice showed a significant decrease compared with control groups (p=0.01). The data were expressed as mean ±S.D.

from control groups (Figure 4B), however, they did not show cytotoxicity to VE-cad-negative CT26 tumor cells (Figure 4C). These findings indicate the fusion vaccine induce specific immunity against VE-cad-positive cells. Furthermore, the cytotoxicity may be blocked by anti-CD8 or anti-major histocompatibility complex (MHC) class mAbs, but not by anti-CD4 *in vitro* (Figure 5), which suggests that the killing activity observed may primarily result from MHC class I-dependent $CD8^+$ CTL activity, and not from MHC class II-dependent $CD4^+$ T cells. Taken together, these findings indicate pMBD2-mVE-cad functions dependent on both humoral and cellular immunity.

DISCUSSION

Angiogenesis plays a central role in the growth and metastasis of primary solid tumors. It is a complex biological process regulated by several components of the extracellular matrix, including VE-cad. Endothelial cells play a critical role in a large number of physiological and pathological processes, especially in tumorigenesis (Cines et al., 1998). The expression of VE-cad in new vessels shows relative specificity, which suggests its potential as targets of cancer immunotherapy (Cavallaro et al., 2006). However, it is difficult to elicit the immunity to mVE-cad because of the immune tolerance acquired during the

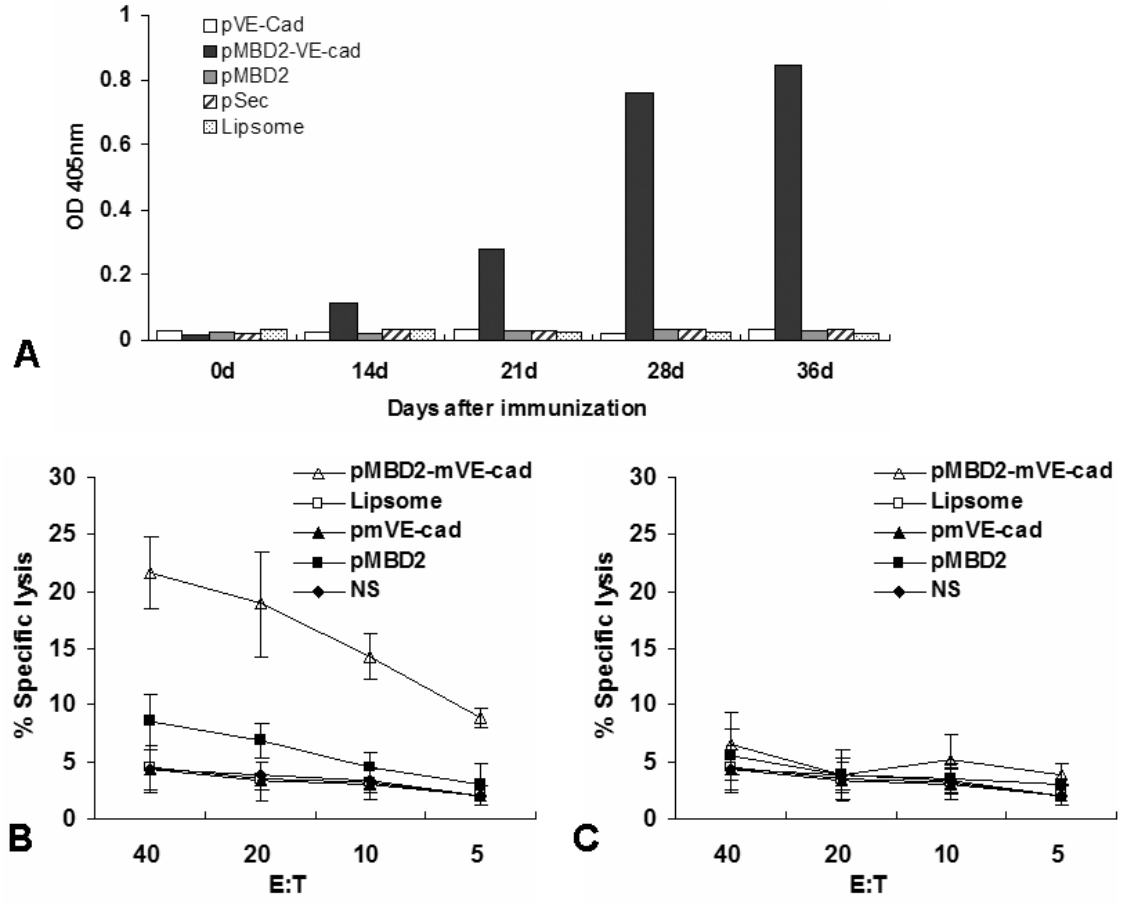

Figure 4. Specific antibody and cytotoxic T lymphocyte responses induced by MBD2-mVE-cad. To determine whether MBD2-mVE-cad could induce specific antibody responses and CTL activity *in vivo*, the antibodies against mVE-cad and the tumor cell killing activity of CTLs were examined. The elevated antibody level against mVE-cad was detected by ELISA (A). T cells isolated from spleens of pMBD2-mVE-cad immuned mice exhibited higher cytotoxicity to mVE-cad-positive CT26 cells than that of control groups (B, p=0.01), but did not show cytotoxicity to mVE-cad-negative CT26 cells (C).

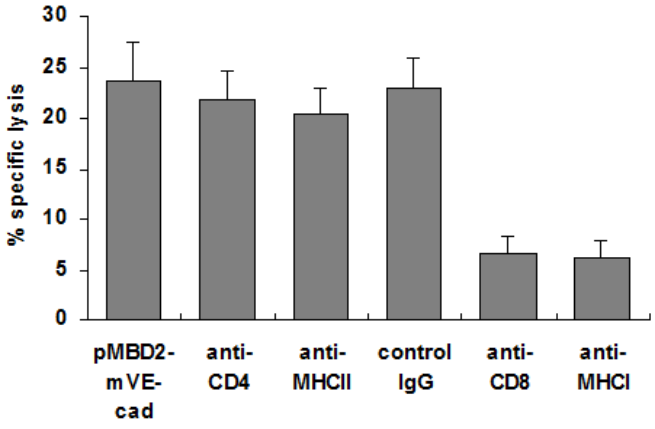

Figure 5. pMBD2-mVE-cad functions depend on both $CD4^+$ and $CD8^+$ T cells. The cytotoxicity could be almost completely blocked by anti-CD8 or anti-MHC class I (anti-H-2Kb/H-2Db) mAb. These data indicated that the killing activity may result mainly from MHC class I-dependent $CD8^+$ CTL activity. The data were expressed as mean ±S.D.

natural development of the immune system. In this study, we report that the fused genetic vaccine based on self or syngeneic MBD2 and mVE-cad genes is able to break the immune tolerance to mVE-cad and thus generate a protective effect against tumors by inducing anti-angiogenic immunity.

Several observations have been made in the present study concerning the fusion vaccine. The fusion protein MBD2-mVE-cad showed a similar capacity of chemotaxsis compared to MBD2 and IP10, which implies that the fusion protein was able to recruit and target APCs and then deliver an activation signal to promote adaptive immune responses. The fusion vaccine pMBD2-mVE-cad generated significant anti-tumor effects in both two mice models. Our data further revealed that pMBD2-mVE-Cad generated significant inhibition of angiogenesis; this might be responsible for inhibition of tumor growth. Furthermore, T cells isolated from mice immunized with pMBD2-mVE-Cad showed increased cytotoxicity against mVE-Cad–positive CT26 cells. Interestingly, it was noted that the

effects of the fusion vaccine were restrained by the anti-$CD4^+$ or $CD8^+$ antibodies. $CD4^+$ helper T lymphocytes and MHC-II play a key role in both humoral and cellular immune responses. Anti-CD4 and MHC-II antibodies impaired protective immune responses. Likewise, the diminished function of pMBD2-mVE-cad by anti-$CD8^+$ and MHC-I antibodies indicated that $CD8^+$ T lymphocyte immune response also played an important role. Based on these findings, we can conclude that both $CD4^+$ and $CD8^+$ T lymphocytes are involved in the pMBD2-mVE-cad-mediated immune response against tumor growth in host mice.

DCs, the most important APCs play a vital role in inducing immune responses. In addition, accumulating evidences have shown that genetic fusion vaccines targeting to DCs could significantly amplify immunological recognition and further enhance specific immune responses. MBD2 could mediate antigen cross-presentation via targeting DCs by CCR6, which induced protective, T-cell dependent anti-tumor immunity (Biragyn et al., 2007; Wang et al., 2007). On the other hand, MBD2 functions as a ligand of TLR4 to activate iDCs. TLR4 can modulate both innate and adaptive immunities. Activation of TLR4 pathways through their ligands can enhance immune responses to vaccines by acting as an adjuvant (Andreani et al., 2007; Kanzler et al., 2007)). In our study, MBD2 was fused with mVE-cad to help the antigen internalization into APCs, which actually contributed to antigen cross-presentation; further, this is responsible for provocation of both humoral and cellular immunity.

To date, several studies demonstrate that VE-cad is involved in angiogenesis (Breviario et al., 1995; Carmeliet et al., 1999; Crosby et al., 2005; Matsumura et al., 1997). The human VE-cadherin promoter is activated in tumour angiogenesis (Prandini et al., 2005). Antibody therapy by targeting VE-cad has proven to be an effective strategy to inhibit angiogenesis (Corada et al., 2001, 2002; Liao et al., 2000, 2002; Menon et al., 2006; Tzima et al., 2005). However, active immunization with the fusion vaccine may afford the opportunity to elicit a lasting immune response. More importantly, the fusion vaccine may generate a specific cellular immunity against VE-cad-positive endothelial cells. VE-cad plays a pivotal role in endothelium integrity and in the control of vascular permeability; this is different from some tumor-related growth factors pathway. Therefore, the fusion vaccine based on VE-cad may be considered an alternative for cancer therapy. Furthermore, since cadherin has been found over-expressed in some tumors and related to clinical prognosis (Calvisi et al., 2004; Chan et al., 2008; Heimann et al., 2000; Kuniyasu et al., 2000; Labelle et al., 2008; Saha et al., 2008).It is rational to speculate that pMBD2-mVE-cad-mediated immunity is as effective as other cadherin-positive tumor cells as it is to VE-cad-positive CT26 cells. In this study, VE-cad-positive CT26 cells were used as target cells to perform the ^{51}Cr release assay, and the specific cytotoxicity was observed.

In summary, our study show that the fusion vaccine with MBD2 and VE-cad induced both humoral immunity and cellular immunity against VE-cad and VE-cad-positive cells; this subsequently generated significant anti-tumor effects. Our results indicate that the fusion vaccine, pMBD2-mVE-cad, should be explored as a vaccine can-didate in cancer gene therapy.

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