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RESEARCH ARTICLE

The mouse chorionic gonadotropin β-subunit-like (muCGβl) molecule produced by tumor cells elicits the switch of T-cell immunity response from T_H2 to T_H1 in mice immunized with DNA vaccine based on rhesus monkey homologous CGβ (rmCGβ)

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Abstract

Background CGβ is expressed not only in placenta, but also in a wide range of tumors. To study DNA vaccine based on xenogeneic CGβ for cancer immuno-therapy, we investigated whether rhesus monkey CGβ (rmCGβ) DNA vaccine could induce protective T-cell responses and humoral responses in mouse.

Methods We constructed a plasmid containing the rmCGβ coding sequence. Two cloned syngeneic SP2/0 myeloma cell lines that stably express muCGβl (SP2/0-muCGβl) and HN (SP2/0-HN) protein were established. Inoculation of these cell lines was made into mice that had been immunized with DNA vaccine. Specific IgG and IgG type were measured by ELISA and the cytokine expression was detected with RT-PCR. To measure the lymphocyte metabolic activity, the MTS assay was used.

Results After injection of SP2/0-muCGβl into mice that had been immunized with DNA vaccine, a significant increase in the IgG2a specific to the antigen ($p < 0.05$) and a decrease in the specific IgG1 ($p < 0.05$) were measured. The expression of T_H1 but not T_H2 cytokines, including IFN-γ and IL-2, were detected in the splenocytes. However, injection of tumor cells expressing irrelevant or mock molecules into immunized mice could not induce these changes. The survival rate of vaccine-immunized mice injected with SP2/0-muCGβl was as high as 58.3% after 55 days.

Conclusions The rmCGβ DNA vaccine has proved to be a potential strategy for protection against tumors with homologous molecules. The muCGβl produced by tumors is able to elicit an immunity switch from T_H2 to T_H1 in vaccinated mice. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords CGβ; tumor vaccine; T-cell immunity; switch; immuno-protection

Introduction

Chorionic gonadotropin (CG) is a placenta-derived hormone that plays an important role in successful implantation and early pregnancy establishment in primates. It is comprised of non-covalently linked α and β

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subunits. The β subunit is responsible for most of the biological functions. In addition to production in the placenta, CG and its individual subunits, especially the β subunit, are overexpressed in a wide range of gonadal and non-gonadal tumors [1]. Due to the marked overexpression of CGβ in neoplastic cells in tumors of differing histological origin, CGβ has become an ideal target molecule for immunogene cancer therapy.

DNA inoculation technology has been recently proved to be a potent means to generate both humoral and cellular immune responses in various animals and human. In 1997, Geissler *et al*. reported their results from trials on the human chorionic gonadotropin β subunit (hCGβ) anti-tumor DNA vaccine [2]. After inoculation of the cell line had expressed hCGβ into BALB/c mice, larger tumors were induced in 2 weeks; however, mice immunized with hCGβ vaccine showed a marked reduction in tumor size and weight compared with animals immunized with empty plasmid. During the evolutionary process, many genes were highly conserved, which was characterized by varying degrees of gene similarity among different species. As Wei *et al*. reported in 2001 [3], immunogene therapy of tumors with vaccine based on xenogeneic homologous vascular endothelial growth factor (VEGF) as a model antigen could overcome immune tolerance of the growth factors associated with tumor growth. They observed that the anti-tumor activity and production of VEGF-specific autoantibodies significantly elevated IgG1 and IgG2b, and the specific autoantibodies could be abrogated by the depletion of CD4+ T lymphocytes.

In a previous study, we first cloned the rmCGβ (GenBank Accession No. AY011 015). rmCGβ shares 79.5% homology to hCGβ at a cDNA level [4]. To study the feasibility of an anti-tumor DNA vaccine by using the xenogeneic CGβ gene, we chose the rmCGβ vaccine to detect the immune responses and anti-tumor effect, and a powerful eukaryotic expression vector pCR3.1-rmCGβ was constructed. In this paper, we report the results of immune responses and anti-tumor effect in BALB/c mice immunized with pCR3.1-rmCGβ after inoculation with the cloned syngeneic SP2/0 myeloma cell line that constitutively expresses the muCGβl protein (SP2/0-muCGβl). To measure whether the anti-tumor activity induced by the vaccine was specific to CGβ expressed by tumor cells, SP2/0 transfected with mock plasmid (SP2/0-mock) and Newcastle disease virus HN gene were also established. In a mice tumor model, we found that the rmCGβ DNA vaccine could produce protection responses against the tumors expressing homologous protein.

The signals that regulate T_H1 or T_H2 commitment of CD4+ T cells have been intensely investigated. More and more pathways that regulate T_H responses were found besides dendritic cells, antigen dose and costimulation [5]. In scientific research, to assess the T_H response type induced by cancer vaccine, people usually detect the IgG type in sera or cytokine expression in lymphocytes *in vitro* after vaccine immunization, but before tumor cells challenge. However, in our present study, we measured the IgG type and the cytokine expression before and after tumor inoculation. The results showed that CGβ produced by tumor cells was able to elicit the switch of T-cell immunity response from T_H2 to T_H1 in mice immunized with pCR3.1-rmCGβ vaccine.

Materials and methods

Mice

The female BALB/c mice were purchased from the Institute of Genetics, Chinese Academy of Sciences. All experiments were approved by the Animal Care and Use Committee at the Institute of Zoology, Chinese Academy of Sciences.

Plasmid construction and cell lines

The full-length rmCGβ cDNA was cut from the pCMV$_4$-rmCGβ vector (conserved by our laboratory) by HindIII and XbaI (Promega), then inserted into the same site of the eukaryotic expression vector pCR3.1 (Invitrogen). The desired recombinant was characterized by restriction analysis with HindIII/XbaI and HindIII/PstI, then by sequencing. The correct recombinant pCR3.1-rmCGβ was transformed into the *E. coli* DH5α strain. The pCR3.1-rmCGβ plasmid was purified with an endotoxin-free plasmid mega kit (Qiagen). To identify the specification of anti-tumor activity, the pCR3.1-muCGβl and pCR3.1-HN vector were constructed. The two recombinants were also confirmed by restriction digestion and DNA sequencing.

The HeLa and CHO cells were transiently transfected with the pCR3.1-rmCGβ to access the expression *in vitro*. The SP2/0 cell line was stably transfected with the pCR3.1-muCGβl to measure the anti-tumor effect of the vaccine. The HepG2 cells were transfected with pCR3.1-muCGβl for cell supernatants containing free muCGβl. All cell lines were obtained from the cell bank of the Chinese Medical Academy of Sciences.

rmCGβ expression in cultured HeLa and CHO cells

The HeLa and CHO cells were seeded onto 25-cm^2 flasks with Dulbecco's modified Eagle's medium (DMEM; Gibco/BRL), supplemented with 10% fetal bovine serum (FBS; Hyclone), and were incubated at 37 °C in a 5% CO_2/95% air humidified incubator until 60% confluence. The purified pCR3.1-rmCGβ and pCR3.1 mock plasmid were transfected with Lipofectamine (Gibco/BRL) by its technical illustration. The cultured medium and the transfected cells were collected at 24 or 48 h.

Detection of rmCGβ protein expression *in vitro*

Confocal microscopy and Western blotting were used to detect the protein expression of rmCGβ *in vitro*.

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Since the rmCGβ and hCGβ cDNA sequences share 79.5% homology, mouse anti-hCGβ monoclonal antibody (PharMingen) was used as the first antibody and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC; Promega) was used as the second antibody. The process was performed according to a previous report [6]. The cells were counterstained with propidium iodide (Sigma) for visualizing nuclei and were analyzed for expression using a confocal microscope (Leica, TCS-NT).

The total proteins in transfected CHO cells were extracted by Trizol reagent (Invitrogen). rmCGβ and other proteins were separated by 5–12.5% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred onto a nitrocellulose (NC) membrane (Amersham Pharmacia) at 200 mA for 2 h. Anti-hCGβ monoclonal antibody (1 : 1000 with PBST) was used as the first antibody and incubated with the NC membrane for 2 h at 37 °C. The membrane was washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Sant Cruz) for 1 h at 37 °C, washed four times, each time for 10 min, then developed with ECL reagent (Pierce).

Animal model of tumor growth

To evaluate anti-tumor activity in BALB/c mice, cloned syngeneic SP2/0 myeloma target cell lines were established after transfection with muCGβl. The stably transfected cell line was selected by medium containing G-418. The SP2/0 cell lines transfected with pCR3.1 and HN recombinant plasmid were also established as the control in the same way. The expression of muCGβl and HN in the SP2/0 cell lines was detected by confocal microscopy and Western blotting as described above. The first antibody for detecting the HN protein in the SP2/0 cell lines was chicken anti-HN serum (China National Virus Center) and the second antibody was anti-chicken IgG-HRP (KPL).

Animals were challenged with SP2/0-mock, SP2/0-muCGβl and SP2/0-HN 5 weeks after the last immunization, and the sizes of tumors were measured every 3 days.

Plasmid DNA immunization

Each experimental mouse received 20 μg pCR3.1-rmCGβ vaccine in the leg muscle by three-spot injection. All mice were injected with 100 μl 0.25% burpivacaine-HCl (Sigma) 24 h before plasmid inoculation. Mock plasmid control mice were injected in the same way with an equal volume of pCR3.1. Mice were boostered twice at 1-week intervals with the same amount of plasmid DNA. Sera were obtained every week after animals received the last injection of plasmid. Three weeks after the last immunization, the control mice ($N = 4$) and the experimental mice ($N = 4$) were sacrificed for immunological assays. For different tumor challenge experiments, the remaining experimental mice were divided into three groups which were injected with SP2/0-mock, SP2/0-HN or SP2/0-muCGβl.

Preparation of spleen lymphoid cells

Mice were anesthetized with isoflurane 2 weeks before and after tumor inoculation. The spleen cells were harvested with the lymphocyte separation medium and then treated with 8.3% NH_4Cl/0.17 M Tris (pH 7.4) for 10 min at 37 °C. The lymphocytes were diluted to 2×10^6 cells/ml in RPMI-1640 medium (containing 10% FBS, 50 μM β-mercaptoethanol) after viability determination with trypan blue.

Lymphocyte metabolic activity assay

50 μl per well of RMPI-1640 (supplemented with 5% FBS and 50 μM 2-ME) containing 5 or 1 μg/mL hCGβ standard antigen (PharMingen) were added to 96-well culture plates (Costar). Bovine serum albumin (BSA; Promega) at 5 μg/ml was set as a non-specific antigen control. After adding 50 μl (approximately 10^5 cells/well) single-splenocyte suspension (lymphocytes from mock plasmid and vaccine-immunized mice 3 weeks after the last immunization) per well, the plate was incubated in a humidified CO_2 incubator at 37 °C for 2 days. A total of 20 μl of CellTiter 96 Aqueous One Solution reagent (Promega) per well was added into the plate, and the plate was incubated for 4 h at 37 °C in a humidified CO_2 incubator. Then the absorbance of samples at 490 nm was measured (Bio-Rad, 3550). The results were expressed as stimulation index (SI) (ratio of OD490nm with CGβ antigen and negative control).

Analysis of IgG1 and IgG2a before and after tumor inoculation

Flat-bottomed 96-well microtiter plates (Costar) were coated overnight at 4 °C with 100 μl (1 μg/ml) per well of hCGβ antigen (PharMingen) diluted in 0.05 M bicarbonate buffer (pH 9.6), washed three times in PBST (0.1% Tween-20 and 0.01% Triton-X-100 in phosphate-buffered saline (PBS)), and blocked with 1% BSA/PBS at room temperature for 0.5 h. After washing, 100 μl of 1 : 100 diluted serum collected from immunized mice 2 weeks before and after tumor inoculation were added to each well. After washing three times in PBST, anti-mouse IgG1 and IgG2a conjugated with horseradish peroxidase (HRP) (Sigma) diluted 1 : 1000 in PBS were added. A 10-mg TMB tablet was dissolved in 0.025 M phosphate-citrate buffer and added to the solution in each well for color development for 30 min. The color development was stopped by adding 2 M H_2SO_4, and the plates were read using a plate reader at 450 nm (Bio-Rad, 3550).

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Analysis of the cytokine expression in lymphocytes before and after tumor inoculation

Mice were anesthetized with isoflurane 2 weeks before and after tumor inoculation. The spleen cells were harvested with lymphocyte separation medium and then treated with 8.3% NH_4Cl/0.17 M Tris (pH 7.4) for 10 min at 37 °C. Lymphocytes were cultured in RMPI-1640 containing 10% FBS. A part of lymphocytes of the mice before tumor inoculation were stimulated with the cell culture supernatants of the transfected HepG2 cells containing 2.2 μg/ml of free muCGβl measured by immunoradiometric assay for 24 or 48 h. The total RNA of lymphocytes with or without stimulation was isolated and purified by Trizol reagent (Invitrogen). Reverse transcription polymerase chain reaction (RT-PCR) was adopted to detect the expression of cytokines in the spleen cells. The reaction was performed by using the RT-PCR kit according to the protocol provided (Promega). The primers of m-IL2 (Cat: 5462-3), m-TNFα (Cat. no.: 5468-3), m-TGFβ (Cat. no.: 5478-3), and m-GMCSF (Cat. no.: 4473-3) were purchased from Clontech (USA). The primers of m-IFNγ and m-IL4 were synthesized by Retrogen (USA). The RT reaction was allowed to proceed at 48 °C for 45 min. The reverse transcriptase was inactivated at 95 °C for 5 min prior to the PCR reaction. The PCR amplification program was 30 cycles for 1 min at 94 °C, 1 min at 60 °C, and 1 min at 68 °C, followed by 10 min at 68 °C. The purified PCR product (5 μl) was detected by electrophoresis in 1.5% agarose gel. The expression of β-actin was used as the control.

Statistical analysis

Values are reported as the mean ± SEM. Significant differences were assessed by one-way ANOVA (SPSS). The difference between groups was considered statistically significant when the p value was lower than 0.05.

Results

Construction of rmCGβ eukaryotic expression vector

Following DNA sequencing to confirm the correct coding sequence of pCR3.1-rmCGβ, the desired recombinant expression vector pCR3.1-rmCGβ, inserted into the full-length cDNA rmCGβ, was confirmed by restriction enzyme analysis (Figure 1A). pCR3.1-muCGβl and pCR3.1-HN were also identified by restriction digestion (Figure 1B).

Expression of rmCGβ *in vitro*

The results from confocal microscopy and Western blotting showed that the eukaryotic cells transfected with recombinant vector could express the rmCGβ protein (Figure 2). No spontaneous rmCGβ protein was detected in cells transfected with mock plasmid.

Establishment of the tumor cell lines producing muCGβl and HN

Confocal microscopy showed that all SP2/0-muCGβl could express the muCGβl, and SP2/0-HN could express the HN protein. No spontaneous similar proteins were detected in control cells. Western blotting results also supported this claim (Figure 3).

Lymphocyte metabolic activity assay

Activation and proliferation of T_H lymphocytes play a critical role in both the CD4+ T lymphocytes and the

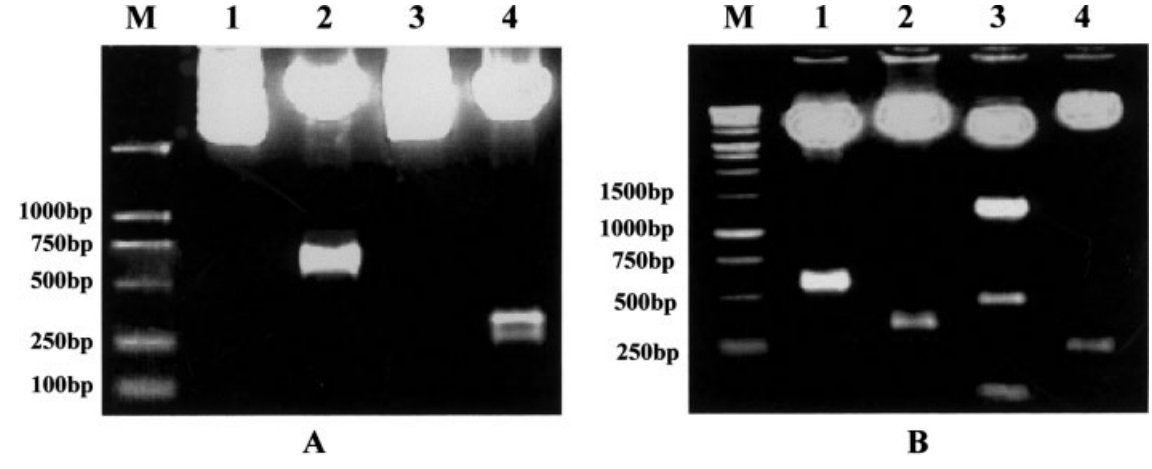

Figure 1. Identification of the pCR3.1-rmCGβ, muCGβl and HN constructs by restriction digestion before sequencing. (A) Analysis of pCR3.1-rmCGβ. M: 2-kb; marker 1: pCR3.1 digested by HindIII/XbaI; 2: pCR3.1-rmCGβ digested by HindIII/XbaI; 3: pCR3.1 digested by HindIII/PstI; 4: pCR3.1-rmCGβ digested by HindIII/PstI. (B) Analysis of pCR3.1-muCGβl and HN. M: 1-kb; marker 1: pCR3.1-muCGβl digested by EcoRI; 2: pCR3.1-muCGβl digested by PstI; 3: pCR3.1-HN digested by EcoRI; 4: pCR3.1-HN digested by PstI

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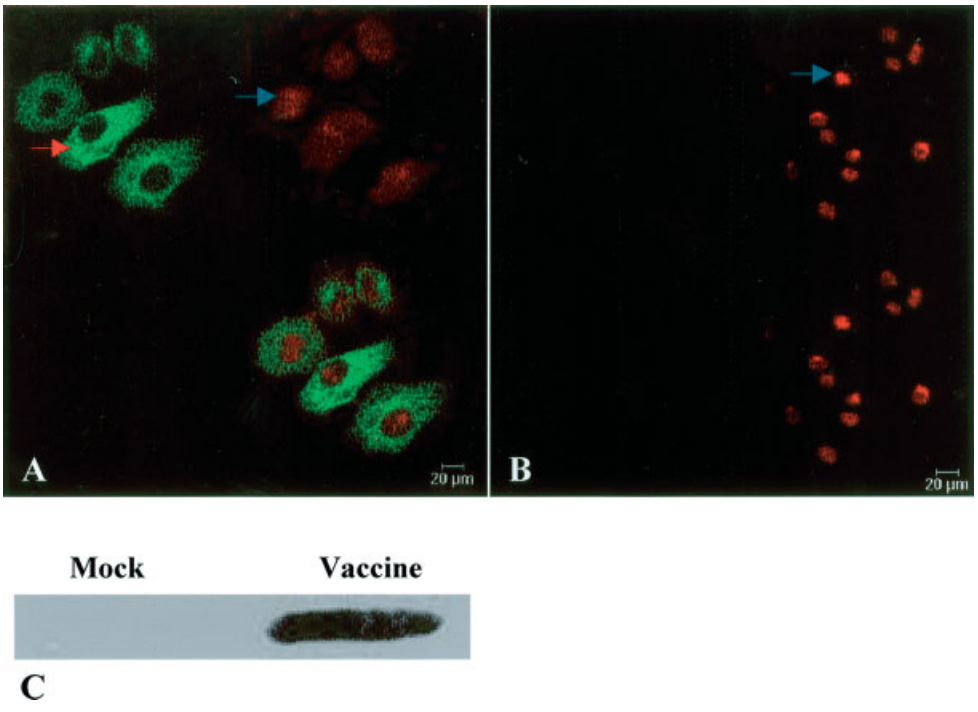

Figure 2.

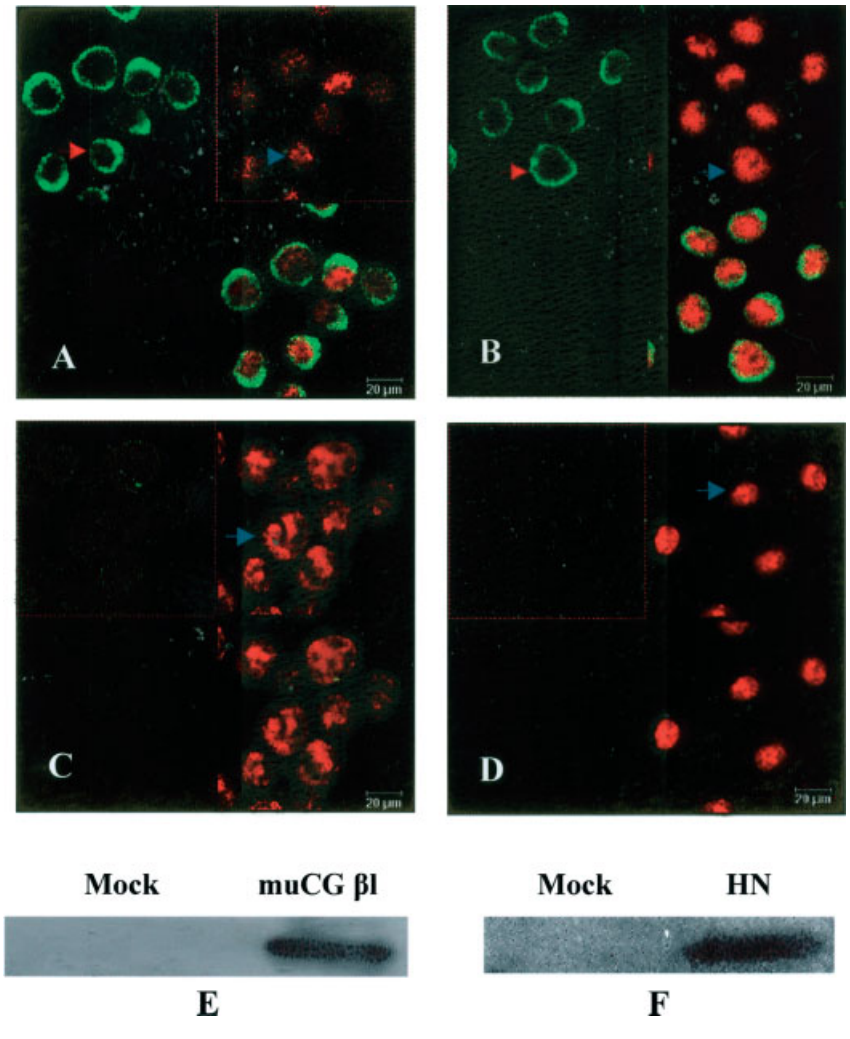

Figure 3.

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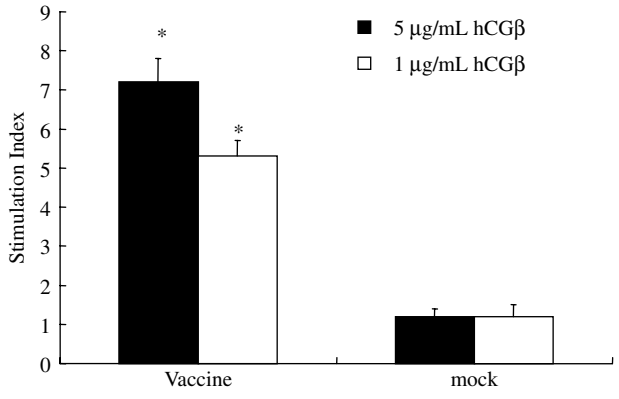

Figure 4. Lymphocyte metabolic activity assay. The lymphocytes from mice of each group immunized with pCR3.1-rmCGβ and mock were harvested as described in Materials and methods, and were stimulated with hCGβ protein antigen at a final concentration 5 or 1 μg/ml. Lymphocytes stimulated with 5 μg/mL BSA were used as negative control. After stimulation for 2 days, the test was performed according to the protocol provided with the CellTiter 96 Aqueous One Solution kit. The absorbance at 490 nm was read. The results are expressed as stimulation index (ratio of OD490nm with stimulation antigen and negative antigen) *$p < 0.05$, compared with the group immunized with mock plasmid

CD8+ cytotoxic T lymphocytes. The splenocytes from mock and vaccine plasmid immunized mice were prepared and tested for lymphocyte metabolic activity as described in Materials and methods. As shown in Figure 4, the higher level of activity of T lymphocytes stimulated by 5 or 1 μg/mL hCGβ was observed from the mice immunized with 20 μg pCR3.1-rmCGβ three times (SI: 7.2 ± 0.6; 5.3 ± 0.4) compared with that of control mice (SI: 1.1 ± 0.3, 1.1 ± 0.3).

Analysis of T_H1 and T_H2 immune response

The IgG1 and IgG2a type antibodies in the sera samples at 1 : 100 dilution were detected by standard ELISA. The results are shown in Figure 5. There was a switch of CGβ-specific antibody type elicited through inoculation of the tumor cells with muCGβl in mice that had been immunized with CGβ DNA vaccine. The antibody type in recombinant immunized mice biased towards IgG2a and IgG1 before tumor inoculation. After the tumor challenge, the volume of IgG1 in vaccine-immunized mice injected with SP2/0-muCGβl was lower ($p < 0.05$), while the volume of IgG2a of those animals increased ($p < 0.05$). The increase of IgG2a in mice protected completely was more significant than that in mice protected only partly ($p < 0.05$). In contrast, no significant switch was detected in mock DNA-immunized mice and mice immunized with vaccine but challenged with SP2/0-mock or SP2/0-HN.

The mRNA expression of mIL-2, mIL-4, mIFN-γ, mTNF-α, mTGF-β, and mGM-CSF cytokines in lymphocytes

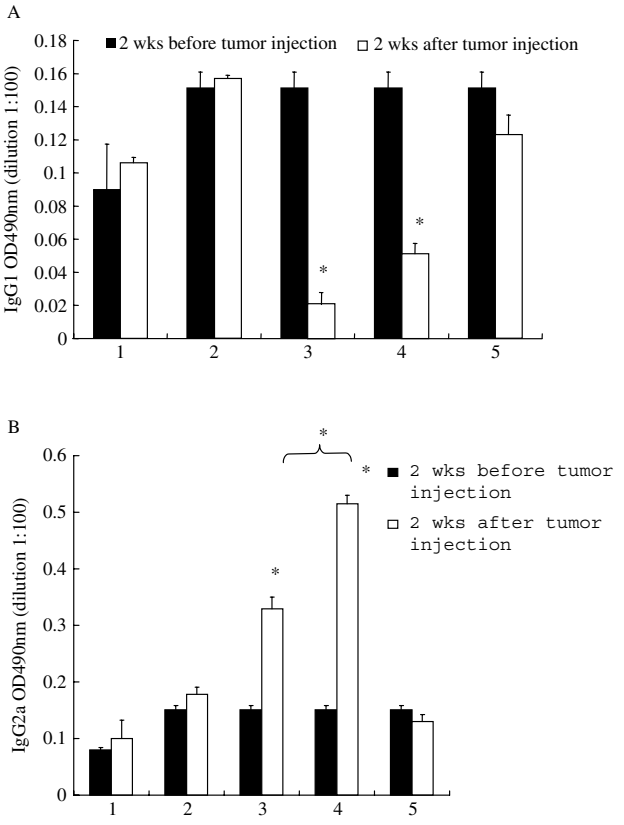

Figure 5. The IgG1 (A) and IgG2a (B) type antibodies specific to the CGβ 1 : 100 diluted sera collected from the mice 2 weeks before and after tumor challenge were tested by the standard sandwich ELISA. *$p < 0.05$. The BALB/c mice group. 1: mock + SP2/0-muCGβl; 2: pCR3.1-rmCGβ + SP2/0-mock; 3: pCR3.1-rmCGβ + SP2/0-muCGβl, displayed a small tumor; 4: pCR3.1-rmCGβ + SP2/0-muCGβl, displayed no tumor; 5: pCR3.1-rmCGβ + SP2/0-HN

Figure 2. Detection of the expression of rmCGβ protein in transfected HeLa cells by immuno-fluorescent assay (bar = 20 μm) and Western blotting. (A) pCR3.1-rmCGβ-transfected HeLa cells. (B) pCR3.1 plasmid control transfected HeLa cells. (C) Western blotting detected the expression of rmCGβ in transfected CHO cells. The total protein in the transfected cells was extracted with Trizol reagent and separated by SDS-PAGE, then transferred to a NC membrane. The membrane was probed with anti-CGβ monoclonal antibody, then incubated with anti-mouse IgG-HRP, and developed using the ECL reagent. In all figures, green fluorescence (red arrow) represents the protein expression, red fluorescence (blue arrow) denotes the nuclei of cells

Figure 3. Detection of the muCGl and HN protein expression in SP2/0. The SP2/0 cells stably transfected with the muCGl and HN plasmid after selected were detected by immuno-fluorescent assay (bar = 20 μm) and Western blotting. Anti-CGβ monoclonal antibody was used as the first antibody to detect the muCGl expression, and anti-HN sera was used to detect HN expression. (A) SP2/0 stably transfected with pCR3.1-muCGl; (B) SP2/0 stably transfected with pCR3.1-HN; (C) SP2/0 transfected with pCR3.1 mock plasmid; (D) SP2/0 transfected with pCR3.1 mock plasmid; (E) Western blotting detected the muCGβl expression in SP2/0 cells; and (F) detection of the HN protein in SP2/0 cells using Western blotting. Results of one representative of three experiments

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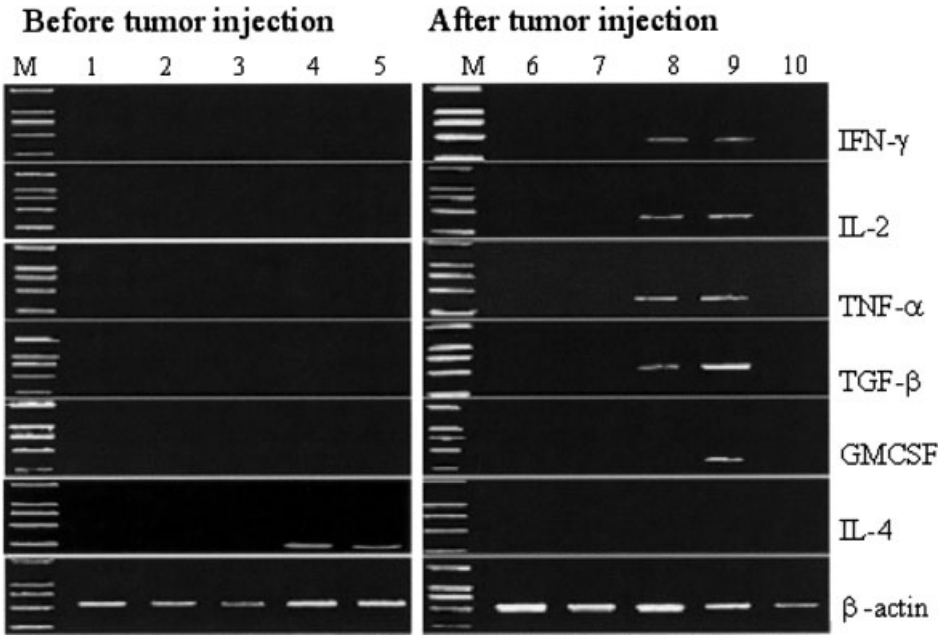

Figure 6. Detection of the expression of cytokines in splenocytes by RT-PCR. The splenocytes were recovered 3 weeks after the last immunization but 2 weeks before the tumor inoculation, and half of them were stimulated in the medium containing the free muCGβl for 24 or 48 h. The lymphocytes were harvested 2 weeks after the tumor challenge. The total RNA was isolated and analyzed by RT-PCR to detect the expression of the T_H1 or T_H2 type cytokines. M: 2 Kb; marker. 1: splenocytes from the mice immunized with the pCR3.1 mock plasmid without stimulation; 2: splenocytes from the mice immunized with pCR3.1-rmCGβ without stimulation; 3: lymphocytes from the mice immunized with the pCR3.1 mock plasmid stimulated; 4: lymphocytes from the mice immunized with pCR3.1-rmCGβ and stimulated for 24 h; 5: lymphocytes from the mice immunized with pCR3.1-rmCGβ and stimulated for 48 h; 6: splenocytes from the group mock + SP2/0-muCGβl; 7: splenocytes from the group vaccine + SP2/0-mock; 8: splenocytes from vaccine + SP2/0-muCGβl, displaying a small tumor; 9: splenocytes from the group vaccine + SP2/0-muCGβl, displaying no tumor; 10: splenocytes from the group vaccine + SP2/0-HN. All experiments were conducted three or four times, and results of a representative experiment are shown

without stimulation and stimulated in medium containing 2.2 μg/mL of free muCGβl for 24 and 48 h before tumor inoculation and cytokine expression in splenocytes after tumor inculation was detected by RT-PCR (Figure 6). No mRNA expression of mIL-2, mIFN-γ, mTNF-α, mTGF-β, or mGM-CSF was detected in lymphocytes stimulated or unstimulated before tumor inoculation. The expression of T_H2 cytokine mIL-4 was observed in activated lymphocytes from the vaccinated group before tumor inoculation. After the tumor challenge, mRNA expression of mIL-2, mIFN-γ, mTNF-α, and mTGF-β was detected in splenocytes from both the completed and partly protected mice injected with SP2/0-muCGβl, and TGF-β in splenocytes of completely protected mice was more than that in splenocytes of partially protected mice. What is interesting is that the mRNA expression of mGM-CSF was only observed in splenocytes from pCR3.1-rmCGβ-immunized mice that displayed no tumor growth, but not from the mice in which small tumor growth was observed. In contrast, no expression of these cytokines was detected in mock DNA-immunized mice and vaccine-immunized mice but challenged with SP2/0-mock or SP2/0-HN.

Protection against tumor growth induced by vaccine *in vivo*

The tumor cell growth of pCR3.1-rmCGβ-immunized mice challenged with SP2/0-muCGβl was either completely inhibited or substantially reduced when the right flanks were injected with 1×10^6 tumor cells within 14 days. All mock DNA-immunized mice developed larger tumors at injection sites, and all vaccine-immunized mice challenged with SP2/0-mock or SP2/0-HN also developed larger tumors within 14 days. The mice photos were taken 26 days after tumor inoculation, as shown in Figure 7A.

The tumor size of DNA vaccine-immunized mice injected with SP2/0-muCGβl was significantly reduced in comparison with vaccinated mice challenged with SP2/0-mock and SP2/0-muCGβl (Figure 7B). All mock DNA-immunized mice receiving SP2/0-muCGβl died within 55 days. Similarly, all pCR3.1-rmCGβ-immunized mice injected with SP2/0-mock and SP2/0-HN died from massive tumor growth. In contrast, the survival rate of vaccine-immunized mice injected with SP2/0-muCGβl was 58.3% after 55 days, and the survival rate of mice which had complete protection was 25%.

Discussion

hCG and its individual subunits, especially the β subunit (hCGβ), are produced in a wide range of tumors, and the free hCGβ protein is a candidate antigen for active immuno-therapy of tumor cells that express and secrete this molecule. It has been shown that hCGβ genetic vaccine was able to elicit immune responses in mice and

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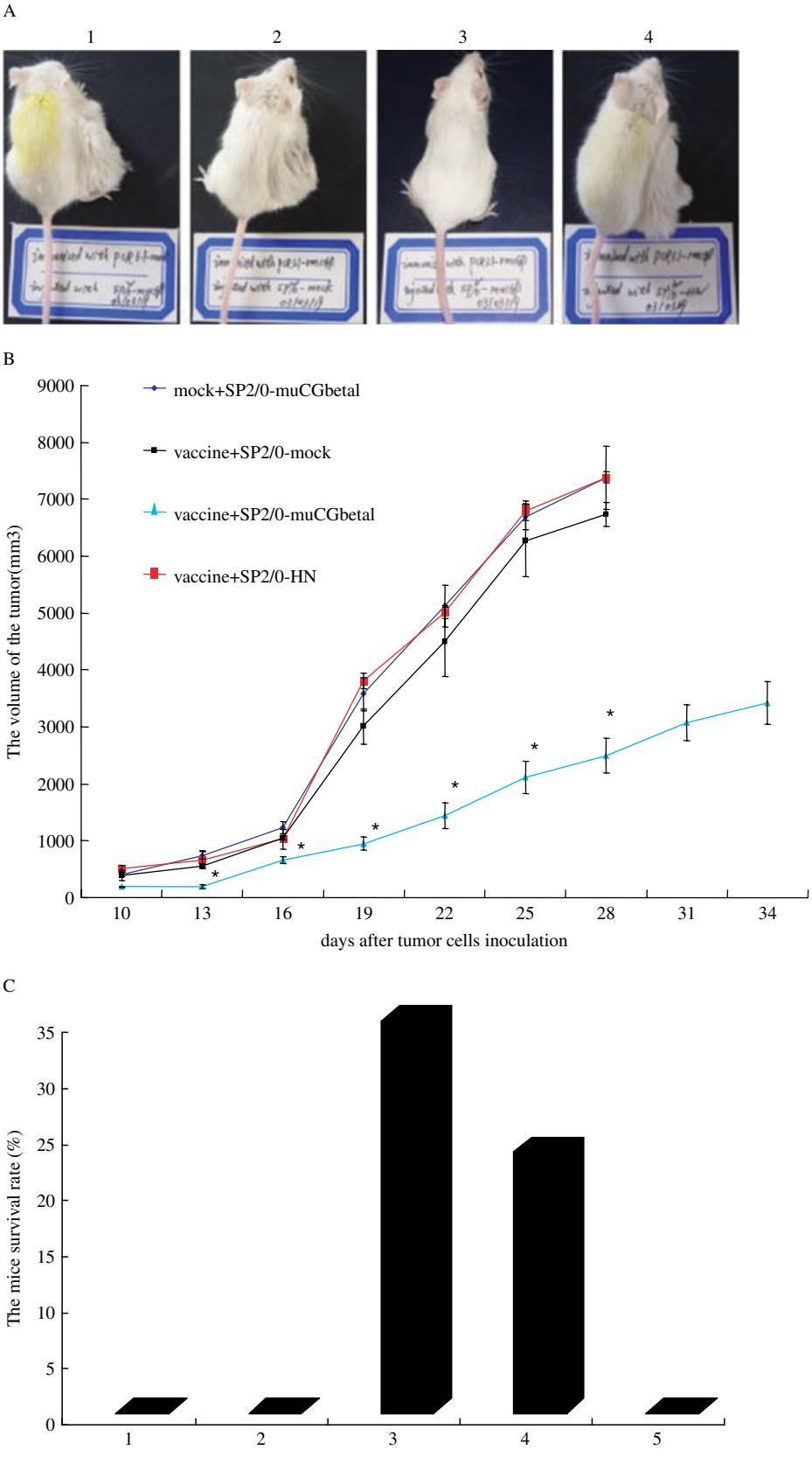

Figure 7. Tumor volume and survival rate after inoculation of tumor cells. (A) Photographs of mice 26 days after inoculation with 1 × 10^6 tumor cells into the right flank. Representative examples of mice demonstrated the anti-tumor effect of the vaccine. 1: mock + SP2/0-muCGβl; 2: vaccine + SP2/0-mock; 3: vaccine + SP2/0-muCGβl; 4: vaccine + SP2/0-HN. (B) The tumor size was measured every 3 days, and the volume was calculated ($4/3\pi$ × length/2 × (width/2)^2). The results are expressed as mean ± SEM. The asterisks (*) indicate a significant difference compared with the three control groups. (*$p < 0.05$). (C) The survival rate of mice 55 days after inoculation with tumor cells. 1: mock + SP2/0-muCGβl; 2: vaccine + SP2/0-mock; 3: vaccine + SP2/0-muCGβl, and the mice had partial protection; 4: vaccine + SP2/0-muCGβl, and the mice had complete protection; 5: vaccine + SP2/0-HN

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protect the animals from challenge with tumor cells. In our present study, we found that rmCGβ as a model antigen is able to induce both the B-cell and T-cell anti-tumor immunity specific to CGβ. We rule out the possibility that the anti-tumor activity with the rmCGβ may result from the nonspecifically augmented immunity against tumor growth in mice, because the mice immunized with mock plasmid and the rmCGβ-immunized mice injected with SP2/0-mock or SP2/0-HN showed no anti-tumor activity.

The response against CGβ may be provoked by the immunization of rhesus monkey CGβ. This suggestion is supported by the tumor size and mice survival rate after the mice were challenged with tumor cells. Before the injection of SP2/0-muCGβl into the vaccine-immunized mice, the antibody type produced in response to immunization was IgG1 and IgG2a, whereas, after injection of SP2/0-muCGβl into the mice, the main IgG type was IgG2a. Detection of IgG type showed that the volume of IgG2a was increased but the volume of IgG1 was decreased during this process. Since IgG2a response is promoted by IFN-γ and is consistent with T_H1-type response [7], we suppose that the CGβl expressed by established tumor cells could shift T-cell responses from T_H2 to T_H1 in mice that had been immunized with the rmCGβ DNA vaccine. The expression of cytokines in the splenocytes also demonstrated the switch. Before the inoculation of the SP2/0-muCGβl, only type 2 cytokine IL-4 [8] was detected in the lymphocytes stimulated with antigen from the vaccine-immunized mice. After SP2/0-muCGβl injection, type 1 cytokines IFN-γ, IL-2, and TNF-α [8,9] were detected in the splenocytes from the mice immunized with vaccine. Meanwhile, IL-4 could not be detected. No expression of these cytokines was detected in control groups. Why could the CGβ not induce the expression of the T_H1-type cytokines in splenocytes from immunized mice *in vitro* as described previously [2]? The difference between hCGβ and rmCGβ as immuno-antigens used in this study and the previous study should be an important reason. The vector backbones used in our experiments and Geissler's are also different [2], and it has been reported that different vector backbones could elicit different types of immuno-responses [10]. Why was the muCGβl produced by the tumor cells *in vivo* but not by the muCGβl protein *in vitro* able to initiate the T_H1-type cytokines expression in lymphocytes? The reasons for this difference are unclear and also attract our interest: one possibility is the difference between *in vitro* and *in vivo*. Furthermore, the initiation of the T_H-type switch needs an appropriate condition apart from the antigen as the stimulant, dendritic cell type and costimulation.

From the results of cytokine expression obtained by RT-PCR, the expression of the GM-CSF and high expression of the TGF-β in completely protected mice compared with partly protected mice was notable. It has been reported that GM-CSF can trigger maturation and activation of murine dendritic cells, as well as enhance CTL and T_H1 responses to the antigen against cancer [7,11,12]. TGF-β is a pleitropic cytokine which performs multiple regulatory functions in the immune system. One of the roles of TGF-β is the inhibition of naive T-cell differentiation into effector cells. Gorelik *et al*. reported that TGF-β inhibited differentiation of CD4+ cells into T_H2 cells by blocking the expression of GATA-3 [13]. Apoptosis could be induced by TGF-β in various cancer cell types, including primary hepatocytes, hepatoma cell lines, prostate epithelial cancer cells, B cells and hematopoietic cells [14]. Our findings demonstrated that GM-CSF and TGF-β could enhance the T_H1 responses in this mouse model and may play roles in the process of protection against tumors. The interesting finding in this study is that, in the same group, some of the DNA vaccine-immunized mice got complete protection, while the others failed. In our opinion, the tumor escape mechanism is responsible for this failure [15].

In conclusion, our data suggest that a DNA vaccine based on rmCGβ is an approach to prevent against tumors which overexpress CGβ. The vaccine can induce humoral and cellular protective immuno-responses in mice. CGβl produced by the mouse tumor cells *in vivo*, but not the stimulation with CGβl *in vitro*, is able to trigger the immunity switch from T_H2 to T_H1. GM-CSF and TGF-β may enhance the anti-tumor activity and protect the animals from tumor challenge. Most importantly, these results provide a potential strategy for the molecular therapy of tumors marked with CGβ.

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