

# Mouse Homologue of a Novel Human Oncofetal Antigen, Glypican-3, Evokes T-Cell–Mediated Tumor Rejection without Autoimmune Reactions in Mice

Tetsuya Nakatsura,<sup>1</sup> Hiroyuki Komori,<sup>1,2</sup>  
Tatsuko Kubo,<sup>3</sup> Yoshihiro Yoshitake,<sup>1</sup>  
Satoru Senju,<sup>1</sup> Toyomasa Katagiri,<sup>4</sup>  
Yoichi Furukawa,<sup>4</sup> Michio Ogawa,<sup>2</sup>  
Yusuke Nakamura,<sup>4</sup> and Yasuharu Nishimura<sup>1</sup>

Departments of <sup>1</sup>Immunogenetics, <sup>2</sup>Surgery II, and <sup>3</sup>Molecular Pathology, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, and <sup>4</sup>Laboratory of Molecular Medicine, Human Genome Center, Institute of Medical Science, The University of Tokyo, Tokyo, Japan

## ABSTRACT

**Purpose and Experimental Design:** We recently identified glypican-3 (GPC3) overexpressed specifically in human hepatocellular carcinoma, as based on cDNA microarray analysis of 23,040 genes, and we reported that GPC3 is a novel tumor marker for human hepatocellular carcinoma and melanoma. GPC3, expressed in almost all hepatocellular carcinomas and melanomas, but not in normal tissues except for placenta or fetal liver, is a candidate of ideal tumor antigen for immunotherapy. In this study, we attempted to identify a mouse GPC3 epitope for CTLs in BALB/c mice, and for this, we set up a preclinical study to investigate the usefulness of GPC3 as a target for cancer immunotherapy *in vivo*.

**Results:** We identified a mouse GPC3-derived and K<sup>d</sup>-restricted CTL epitope peptide in BALB/c mice. Inoculation of this GPC3 peptide-specific CTL into s.c. Colon26 cancer cells transfected with mouse *GPC3* gene (C26/GPC3) led to rejection of the tumor *in vivo*, and *i.v.* inoculation of these CTLs into sublethally irradiated mice markedly inhibited growth of an established s.c. tumor. Inoculation of bone marrow-derived dendritic cells pulsed with this peptide prevented the growth of s.c. and splenic C26/GPC3 accompanied with massive infiltration of CD8<sup>+</sup> T cells into tumors.

Evidence of autoimmune reactions was never observed in surviving mice that had rejected tumor cell challenges.

**Conclusions:** We found the novel oncofetal protein GPC3 to be highly immunogenic in mice and elicited effective antitumor immunity with no evidence of autoimmunity. GPC3 is useful not only for diagnosis of hepatocellular carcinoma and melanoma but also for possible immunotherapy or prevention of these tumors.

## INTRODUCTION

Primary hepatocellular carcinoma is one of the common malignancies throughout the world. Because of the global pandemic of hepatitis B and C infections, the incidence of hepatocellular carcinoma is rapidly on the rise in Asian and Western countries (1). This trend is expected to continue for the next 50 years because of the long latency between infection and development of hepatocellular carcinoma. The prognosis of advanced hepatocellular carcinoma remains poor, and effective treatment strategies are urgently needed.

The report of the cloning human melanoma antigen, *MAGE* gene, stated that the human immune system can recognize cancer as a foreign body and can exclude it (2). This genetic approach of T-cell epitope cloning led to identification of a many genes encoding for tumor antigens and antigenic peptides recognized by tumor-reactive CTLs, thereby enhancing the possibility of antigen-specific cancer immunotherapy (3–6). Recently, >1500 types of candidates of tumor antigens have been identified with the SEREX method (7, 8). We also reported cancer antigens identified with this method (9–11). cDNA microarray technology, by which investigators can obtain comprehensive data with respect to gene expression profiles, is rapidly progressing. Studies have shown the usefulness of this technique for identification of novel cancer-associated genes and for classification of human cancers at the molecular level (12–16). We have recently succeeded in identification of a novel cancer rejection antigen specifically expressed in esophageal cancer with cDNA microarray technology (17).

To identify candidates of ideal hepatocellular carcinoma antigen for tumor immunotherapy, which is strongly expressed in almost all hepatocellular carcinomas but not in normal adult tissues, except for immune privilege tissues such as testis and placenta or fetal organs, we used two kinds of data of cDNA microarrays containing 23,040 genes. One is a comparison of expression profiles between 20 hepatocellular carcinomas and their corresponding noncancerous liver tissues (18) and the other is that of various normal human tissues (19). When using these data, we identified *glypican-3* (*GPC3*) overexpressed specifically in hepatocellular carcinoma, and we reported that GPC3 is a novel tumor marker for human hepatocellular carcinoma (20) and melanoma (21). Not only the amino acid sequences but also the expression patterns of human and mouse

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**Requests for reprints:** Yasuharu Nishimura, Department of Immunogenetics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto 860-8556, Japan. Phone: 81-96-373-5310; Fax: 81-96-373-5314; E-mail: mxnishim@gpo.kumamoto-u.ac.jp.

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GPC3 protein were very similar. GPC3 is an oncofetal protein overexpressed in almost all human hepatocellular carcinomas and melanomas (20). Both human and mouse GPC3 are expressed in normal tissues, including placenta and fetal liver, but not in other normal adult tissues. In the present study, we set up preclinical studies to investigate the usefulness of GPC3 as a target for cancer immunotherapy *in vivo*, and we found this oncofetal protein to be highly immunogenic in mice in that it elicited effective antitumor immunity with no evidence of autoimmunity.

## MATERIALS AND METHODS

**Cell Lines.** A subline of BALB/c-derived colorectal adenocarcinoma cell line Colon26, C26 (C20) (22) was provided by Dr. Kyoichi Shimomura (Fujisawa Pharmaceutical Co., Osaka, Japan). B16 and HepG2 were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging, and Cancer Tohoku University (Sendai, Japan). T2K<sup>d</sup> was provided by Dr. Paul M. Allen of Washington University School of Medicine (St. Louis, MO). These cells were maintained *in vitro* in RPMI 1640 or DMEM supplemented with 10% FCS. Expression of H-2K<sup>d</sup> was examined with fluorescence-activated cell sorting analysis and an antimouse H-2K<sup>d</sup>-specific antibody and a subsequent FITC-labeled antimouse antibody.

**Transfection of GPC3 Gene into Cells.** Plasmids, including full-length murine *GPC3* cDNA clones, were purchased (Invitrogen, Osaka, Japan). A cDNA fragment encoding for GPC3 protein was inserted into pCAGGS-IRES-neo-R, a mammalian expression vector containing the chicken  $\beta$ -actin promoter and an internal ribosomal entry site (IRES)-*neomycin N-acetyltransferase* gene cassette. We used the empty pCAGGS-IRES-neo-R plasmid as a control. These cDNAs were transfected into C26 (C20) cells by lipofection, as previously described (10), and selected with G418.

**Mice.** Female 7-week-old BALB/c mice (H-2<sup>d</sup>), purchased from Charles River Japan (Yokohama, Japan), were kept in the Center for Animal Resources and Development of Kumamoto University and handled in accordance with the animal care policy of Kumamoto University.

**Identification of a CTL Epitope in BALB/c Mice.** Peptides were purchased from biologica (Tokyo, Japan), and their purity, as estimated by high-performance liquid chromatography, was >95%. The immunizations were done as follows: we primed the mice with 50  $\mu$ g of each 12 kinds of GPC3-derived peptides emulsified in 50  $\mu$ L of complete Freund's adjuvant (Sigma, Tokyo, Japan) diluted with 50  $\mu$ L of saline s.c. into the left flank and boosted these mice with the same peptides emulsified in incomplete Freund's adjuvant by the same method used for priming 7 days after priming. Splenocytes removed from mice 7 days after the last immunization were harvested, depleted of RBCs by hypotonic lysis, and cultured in 24-well culture plates ( $2.5 \times 10^6$ /well) in 45% RPMI/45% AIMV/10% FCS supplemented with recombinant human interleukin 2 (100 units/mL), 2-mercaptoethanol (50  $\mu$ mol/L), and each peptide (10  $\mu$ mol/L). Then, 5 days later, cytotoxicity of these cells directed against target cells was assayed in a standard 6-hour <sup>51</sup>Cr release assays (10). We purified CD8<sup>+</sup> T cells from bulk CTLs with the

MACS system with antimouse CD8 $\alpha$  (Ly-2) monoclonal antibody, and these CD8<sup>+</sup> CTLs were used for adoptive transfer into BALB/c mice.

**Bone Marrow-derived Dendritic Cell (BM-DC) Vaccine.** BM-DCs were generated as follows: BM cells ( $2 \times 10^6$ ) were cultured in RPMI 1640 supplemented with 10% FCS, together with granulocyte macrophage colony-stimulating factor (5 ng/mL) for 7 days in 10-cm plates, and these BM-DCs were pulsed with GPC3-8 peptide (10  $\mu$ mol/L) at 37°C for 2 hours and used as GPC3-8 peptide-pulsed BM-DC vaccine.

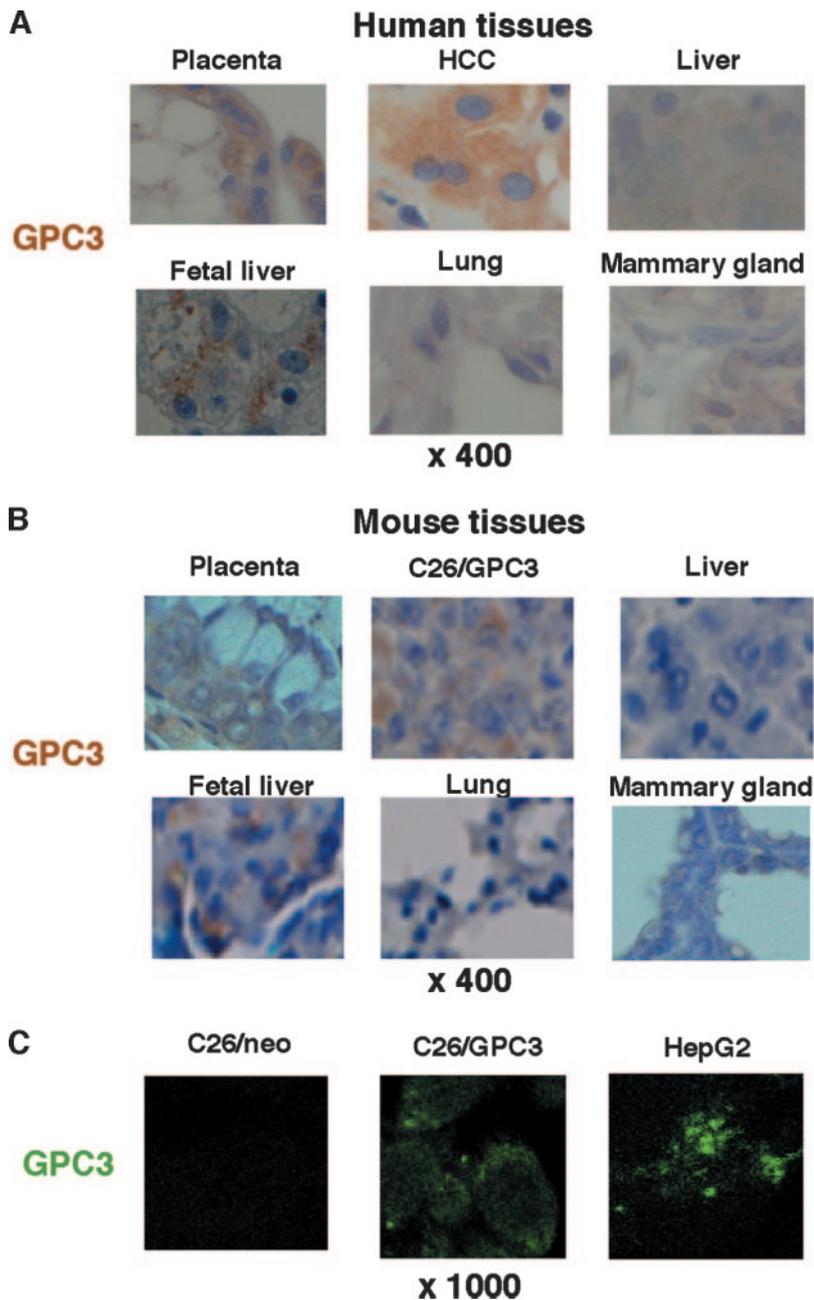
***In vivo* Depletion of CD4<sup>+</sup> and CD8<sup>+</sup> T Lymphocytes.** The mice were given a total of six i.p. transfers (days -18, -15, -11, -8, -4, and -1) of the ascites (0.1 mL/mouse/transfer) from hybridoma-bearing nude mice. The mAbs used were rat antimouse CD4 (clone GK1.5) and rat antimouse CD8 (clone 2.43). Normal rat IgG (Sigma, St. Louis, MO; 200  $\mu$ g/mouse/transfer) was used as control. Depletion of T-cell subsets by treatment with monoclonal antibodies was confirmed by flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

**Histologic and Immunohistochemical Analysis.** Immunohistochemical (23) and immunocytochemical (24) detections of GPC3 were done, as described previously. We purchased Human, Normal Organs, and Cancers, Tissue Array, BC4 (SuperBioChips Laboratories, Seoul, Korea) and Human Fetal Normal Multi Tissue Slide (BioChain, Hayward, CA) for immunohistochemical analysis. H&E staining and standard methods were used. Immunohistochemical staining of CD8 was done, as described previously (25). For the terminal deoxynucleotidyl transferase-mediated nick end labeling method, we used ApopTag Fluorescein *In Situ* Apoptosis Detection kits (Serological Corporation, Norcross, GA).

**Statistical Analysis.** We analyzed all data with the StatView statistical program for Macintosh (SAS, Inc., Cary, NC) and evaluated the statistical significance with unpaired *t* test. The percentage of overall survival rate was calculated with the Kaplan-Meier method, and statistical significance was evaluated with the Wilcoxon test.

## RESULTS

**Limited Expression of GPC3 Protein in Both Human and Mouse Fetal Tissues.** We and other investigators found GPC3 to be overexpressed in hepatocellular carcinoma (20, 26–31) and melanoma (21), so we did an immunohistochemical analysis of GPC3 with various human and mouse tissues (Fig. 1 and Table 1). The expression patterns of human and mouse GPC3 protein were very similar. GPC3 protein was expressed in placenta and fetal liver, but no or only an expression was observed in all normal adult human and mouse tissues tested, including brain, lung, heart, liver, kidney, mammary gland, spleen, and thymus (Fig. 1, A and B). The mouse colorectal cancer cell line Colon26 (C26) did not express GPC3, but after stable transfection of mouse *GPC3* genes, GPC3 protein was expressed in C26/GPC3 (Fig. 1C). This C26/GPC3 tumor inoculated s.c. into BALB/c mice expressed GPC3 as evidenced in our immunohistochemical analysis (Fig. 1B). The expression level of GPC3 protein in C26/GPC3 is not higher than that of human hepatocellular carcinoma (Fig. 1A) or the human hepa



*Fig. 1* Expression of GPC3 protein, the candidate of an ideal target for immunotherapy of hepatocellular carcinoma (HCC) and melanoma, in human and mouse tissues and cells. *A* and *B*, expression of GPC3 protein detected by immunohistochemical analysis in various human (*A*) and mouse (*B*) tissues. Objective magnification was 400 $\times$ . *C*, expression of GPC3 protein detected by FITC-conjugated anti-GPC3 antibodies in HepG2 and GPC3-null mouse colon cancer cell line Colon26 transfected with control vector (C26/neo) or *GPC3* gene (C26/GPC3). Objective magnification was 1000 $\times$ .

tocellular carcinoma cell line HepG2 (Fig. 1C). As a result, the expression levels of GPC3 protein in the human hepatocellular carcinoma, human melanoma, and C26/GPC3 tumor were evidently much higher than those in all adult normal tissues of both human and mouse, including lung and mammary gland, except for placenta and fetal liver (Table 1).

**Identification of a GPC3-derived and K<sup>d</sup>-restricted CTL Epitope in BALB/c Mice.** Structural motifs of peptides bound to human HLA-A24 and mouse K<sup>d</sup> are similar. The amino acid sequences of human and mouse GPC3 have a 95% homology. We searched for GPC3-derived peptides of which amino acid sequences were completely shared between

human and mouse GPC3. Among these peptides, we selected those carrying binding motifs to both HLA-A24 and K<sup>d</sup> molecules, as previously described (10), and prepared 12 different synthetic peptides GPC3-1~12 (Fig. 2A). When we tested these peptides for their potential to induce tumor-reactive CTLs *in vitro* from spleen cells derived from mice immunized with GPC3 peptides, only GPC3-8 EYILSLEEL peptide-induced CTLs showed specific cytotoxicity against C26/GPC3 (GPC3+, H-2<sup>d</sup>) and T2 cells transfected with the H2-K<sup>d</sup> gene (T2K<sup>d</sup>) pulsed with GPC3-8 but not against C26 (GPC3-, H-2<sup>d</sup>), B16 (GPC3+, H-2<sup>b</sup>), and T2K<sup>d</sup> cells pulsed with GPC3-7 (Fig. 2, A and B). These findings indicate

**Table 1** The expression levels of GPC3 protein determined by immunohistochemical analysis in various human and mouse tissues

| +++*                           | ++          | +, +/-        | -        | -               | -         |
|--------------------------------|-------------|---------------|----------|-----------------|-----------|
| Human hepatocellular carcinoma | Placenta    | Lung          | Liver    | Spleen          | Ovary     |
| Human melanoma                 | Fetal liver | Mammary gland | Brain    | Thymus          | Uterus    |
|                                |             |               | Heart    | Stomach         | Prostate  |
|                                |             |               | Kidney   | Small intestine | Testis    |
| C26/GPC3 tumor                 |             |               | Pancreas | Colon           | C26 tumor |

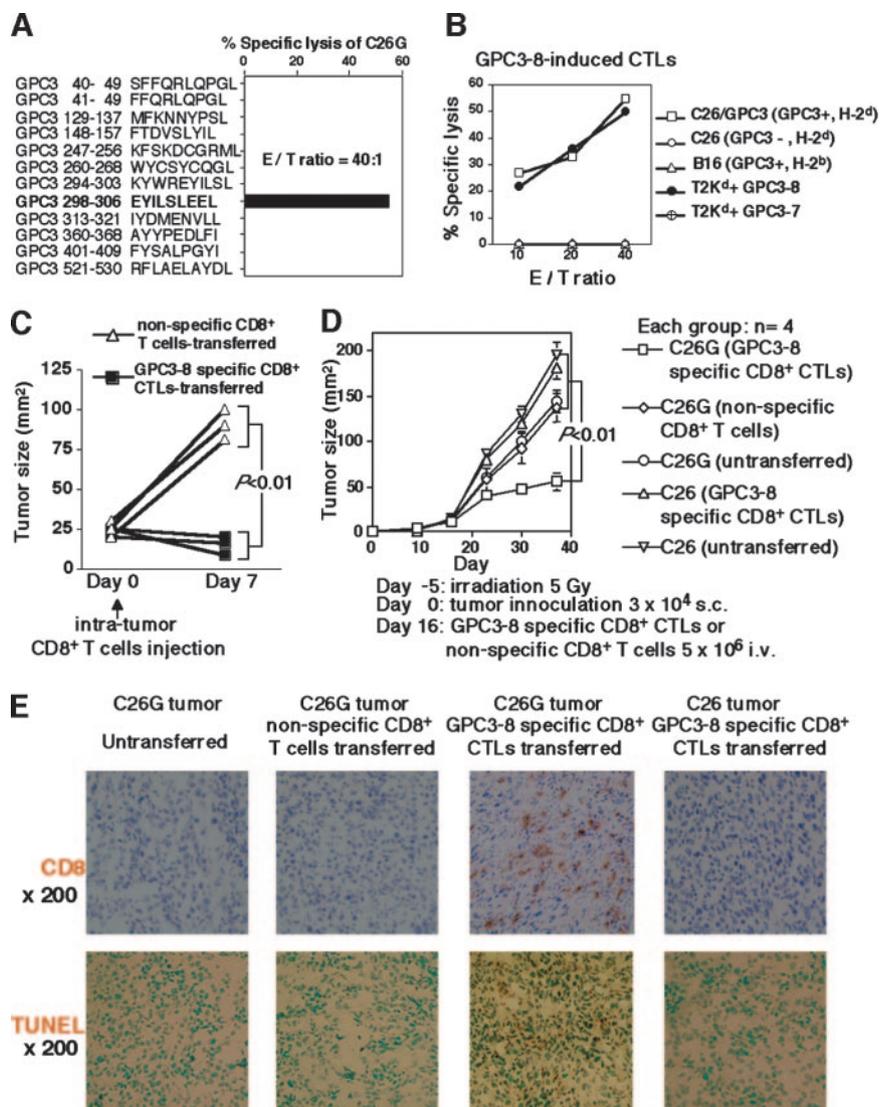
\* Expression levels of GPC3 protein determined by immunohistochemical analysis: +++, very strong; ++, strong; +, +/-, weak, -, no or very weak expression.

that this GPC3-8 peptide has the capacity to induce tumor-reactive CTLs and that peptide vaccination primed CTLs reactive to this peptide *in vivo*.

**CTL Inoculation Reduced the Growth of C26/GPC3 Tumor in Mice.** We determined if these GPC3-8 peptide-induced CTLs were effective against C26/GPC3 tumors inoculated s.c. into BALB/c mice. We separated CD8<sup>+</sup> T cells from

these GPC3-8 peptide-induced CTLs or from nonspecific cells cultured with interleukin 2, without peptide and injected each of these CD8<sup>+</sup> T cells ( $1 \times 10^7$ ) into each three C26/GPC3 tumors with a diameter of 5 mm ( $24.2 \pm 1.5$  mm<sup>2</sup>). After 7 days, all three tumors treated with GPC3-8-specific CD8<sup>+</sup> CTLs became smaller ( $15.0 \pm 3.2$  mm<sup>2</sup>), whereas three tumors treated with nonspecific CD8<sup>+</sup> T cells became larger ( $92.3 \pm 9.6$  mm<sup>2</sup>).

**Fig. 2** Identification of a GPC3-derived and K<sup>d</sup>-restricted CTL epitope, GPC3-8 EYILSLEEL, and adoptive CTL transfer therapy in BALB/c mice. **A.** BALB/c mice were immunized with 12 GPC3 peptides. Sensitized spleen cells, stimulated *in vitro* with each GPC3 peptide ( $10 \mu\text{mol/L}$ ) and cultured for 5 days with 100 units/mL interleukin 2, were examined for CTL activity against GPC3-expressing C26/GPC3 cells to identify GPC3-8 EYILSLEEL epitopic peptide. Values represent percent specific lysis calculated based on mean values of triplicate assays. **B.** cytotoxicity of GPC3-8-induced CTLs against various target cells. **C.** CD8<sup>+</sup> CTLs ( $1 \times 10^7$ ) isolated from GPC3-8-induced cells generated as described in A or nonspecific cells cultured with interleukin 2, without peptide, were injected into the C26/GPC3 tumor with a diameter of 5 mm in each three mice. The comparison of the tumor size (mm<sup>2</sup>) of each three GPC3-8-specific CD8<sup>+</sup> CTL-treated tumor and nonspecific CD8<sup>+</sup> T-cell-treated tumor was indicated. **D.** suppression of the growth of GPC3-expressing C26/GPC3 tumor inoculated s.c. into sublethally irradiated (5 Gy) mice adoptively transferred with GPC3-8-specific CD8<sup>+</sup> CTLs or nonspecific CD8<sup>+</sup> T cells. Data are representative of two independent and reproducible experiments. Tumor area was calculated as a product of width and length. Data are presented as mean area of tumor  $\pm$  SE, and we evaluated the statistical significance with unpaired *t* test. **E.** Immunohistochemical analysis of CD8 or terminal deoxynucleotidyl transferase-mediated nick end labeling-positive cells in specimens of C26/GPC3 or C26 tumor on 21 days after adoptive CTL transfer as done in D.



There was a statistical significance ( $P < 0.01$ ) in difference of tumor growth between these two groups (Fig. 2C). The results indicate that the GPC3-8 peptide-specific CD8<sup>+</sup> CTLs reduced the growth of tumors expressing GPC3.

**Sublethal Irradiation of Mice Elicited Effective Antitumor-adoptive Immunity.** Antitumor responses could be augmented by T-cell homeostatic proliferation in the periphery, involving expansion of T cells recognizing MHC/tumor antigenic peptide ligands (32–34). To investigate tumor growth in a homeostatic CTL proliferation model, we inoculated C26/GPC3 or C26 cells ( $3 \times 10^4$ ) s.c. into BALB/c mice 5 days after sublethal irradiation (5 Gy). We injected i.v.  $5 \times 10^6$  of GPC3-8–induced CD8<sup>+</sup> CTLs or nonspecific CD8<sup>+</sup> T cells derived from spleen cells cultured with interleukin 2, without peptide for 5 days on day 16 after tumor inoculation, when C26/GPC3 or C26 tumors grew to a diameter of 3 to 4 mm ( $11.9 \pm 0.8$  mm<sup>2</sup>). Mice were placed into five groups: (a) C26/GPC3 (GPC3-8–induced CD8<sup>+</sup> CTLs); (b) C26/GPC3 (nonspecific CD8<sup>+</sup> T cells); (c) C26/GPC3 (untransferred); (d) C26 (GPC3-8–induced CD8<sup>+</sup> CTLs); and (e) C26 (untransferred). Measurement of tumor size was continued for 37 days after inoculation of the tumor cells when one untreated mouse died (Fig. 2D). Each group included four mice, and we obtained reproducible results in two separate experiments. Mean tumor size on day 37 in C26/GPC3 (CTL) group ( $51.0 \pm 6.0$  mm<sup>2</sup>) was significantly smaller than that in the other four groups ( $137.2 \pm 16.1$ ,  $145.3 \pm 12.1$ ,  $176.2 \pm 10.1$ , and  $195.1 \pm 10.2$  mm<sup>2</sup>;  $P < 0.01$ ). Weight of spleen ( $0.23 \pm 0.03$  or  $0.25 \pm 0.05$  g) and spleen cell number ( $1.20 \pm 0.40 \times 10^8$  or  $1.25 \pm 0.25 \times 10^8$ ) of GPC3-8–induced CD8<sup>+</sup> CTLs or nonspecific CD8<sup>+</sup> T-cell–transferred groups, C26/GPC3 (GPC3-8–induced CD8<sup>+</sup> CTLs) and C26/GPC3 (nonspecific CD8<sup>+</sup> T cells), were larger than those ( $0.12 \pm 0.03$  g,  $0.23 \pm 0.03 \times 10^8$ ) of untransferred mice, C26/GPC3 (untransferred) on day 37. These differences were statistically significant ( $P < 0.01$ ), indicating that homeostatic proliferation of T cells had occurred. GPC3-8–induced CD8<sup>+</sup> CTLs, but not nonspecific CD8<sup>+</sup> T cells, could infiltrate the C26/GPC3 tumor, but not the C26 tumor, and induced apoptosis of C26/GPC3 tumor cells (Fig. 2E). Thus, sublethally irradiated lymphopenic mice transfused with syngeneic GPC3-8–reactive CTLs showed tumor growth inhibition for established C26/GPC3 tumors.

**Vaccination of GPC3-8 Peptide-pulsed BM-DCs Induced Complete Rejection of C26/GPC3 Tumor Challenge in Mice.** The capacity of GPC3-8 peptide-pulsed BM-DCs to prime GPC3-8–specific T cells *in vivo* was analyzed with a s.c. tumor injection model (Fig. 3B–G) and an intrasplenic tumor injection model (Fig. 4, A and B). The protocol of DC vaccination in this study is shown (Fig. 3A). In the s.c. tumor injection model, mice were placed into five groups: (a) C26/GPC3 (BM-DC+GPC3-8); (b) C26/GPC3 (BM-DC); (c) C26/GPC3 (untreated); (d) C26 (BM-DC+GPC3-8); and (e) C26 (untreated). GPC3-8 peptide-pulsed or unpulsed BM-DCs ( $5 \times 10^5$ ) were injected i.p. into BALB/c mice twice at 7-day intervals. Death never occurred during the vaccination period. Subcutaneous inoculation of C26/GPC3 or C26 cells ( $3 \times 10^4$ ) into the right flank was given 7 days after the last vaccination. In groups 2 to 5, s.c. tumor appeared 13 days after the inoculation (Fig. 3B). Measurement of tumor size was continued until 38 days after

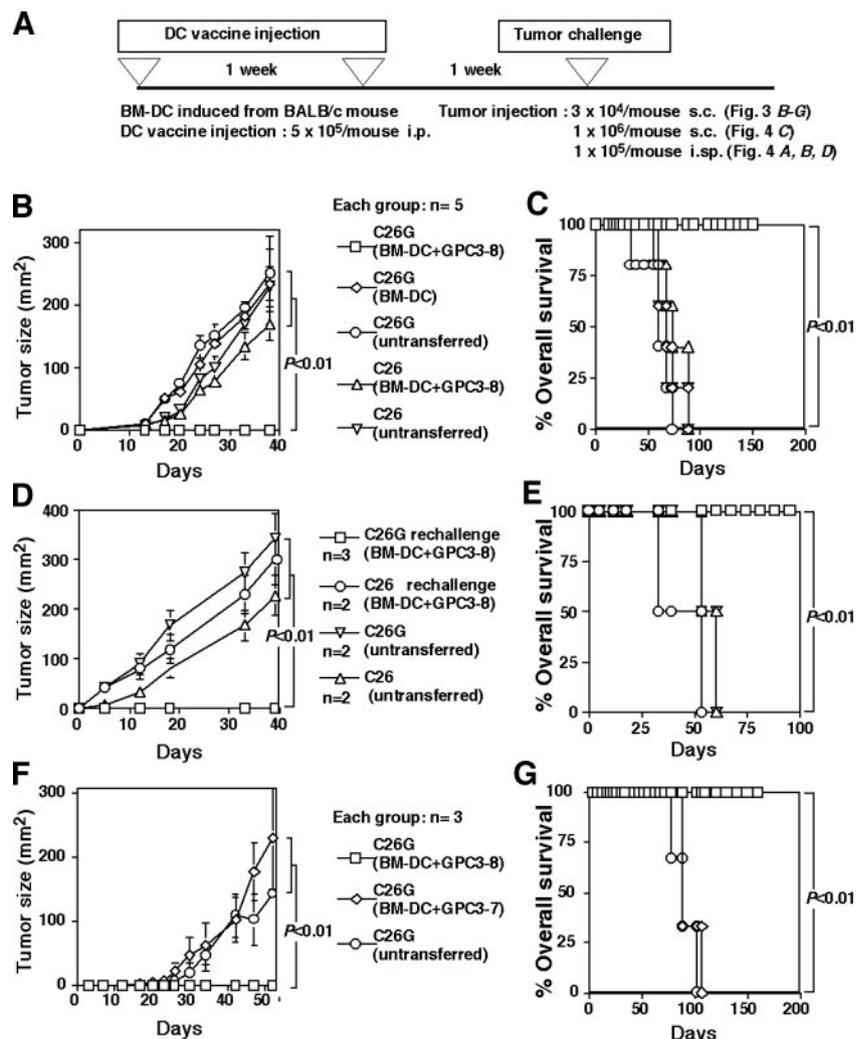
inoculation of the tumor cells when one untreated mouse died. All five mice in group 1 completely rejected  $3 \times 10^4$  of C26/GPC3 cells but not  $3 \times 10^4$  of C26 cells. Mean tumor size on day 38 in group 1 mice (0 mm<sup>2</sup>) was significantly smaller than that in the other four groups 2 to 5 ( $234.0 \pm 28.4$ ,  $251.0 \pm 60.0$ ,  $170.3 \pm 26.1$ , and  $229.0 \pm 64.2$  mm<sup>2</sup>, respectively,  $P < 0.01$ ). All mice in groups 2 to 5 died within 88 days after inoculation of the tumor cells (Fig. 3C). In group 1, a tumor was not detected in all five mice 150 days after the inoculation. A statistical significance ( $P < 0.01$ ) of difference was found between group 1 and groups 2 to 5. This experiment was repeated with similar results. However, the transfer of GPC3-8 peptide-pulsed BM-DCs showed no efficacy against the established C26G tumor (data not shown). Therefore, the GPC3-8 peptide-pulsed BM-DC therapy has the potential to prevent growth of tumors expressing GPC3 but could not induce regression of an established tumor.

We also inoculated C26/GPC3 or C26 cells s.c. into three surviving mice that completely rejected the first challenges of C26/GPC3 cells by vaccination with BM-DC+GPC3-8 (Fig. 3, D and E). These mice also rejected rechallenges of C26/GPC3 cells but not C26 until >150 days after the first challenge. In mean tumor size on day 39 and overall survival, the differences between the C26/GPC3-rechallenged group and the other three groups were statistically significant ( $P < 0.01$ ). These results showed that effects of vaccinations with GPC3-8 peptide-pulsed BM-DCs continued for a long time and that the vaccination can prevent recurrence of GPC3-expressing tumors.

Furthermore, we repeated experiment with another control, BM-DC+GPC3-7 (Fig. 3, F and G). Binding affinity to K<sup>d</sup> of GPC3-8 and that of GPC3-7 is predicted to be similar with Bioinformatics & Molecular Analysis Section.<sup>5</sup> As a result, we obtained similar data with experiments with BM-DCs not pulsed with any peptide.

We next analyzed the effect of the vaccination on an intrasplenic tumor injection model (Fig. 4, A and B). In this model, mice were placed into two groups: (a) C26/GPC3 (BM-DC+GPC3-8) and (b) C26/GPC3 (untransferred). Each group included five mice, and the results were reproducible in two separate experiments. Seven days after the last vaccination, inoculation of C26/GPC3 cells ( $1 \times 10^5$ ) into the spleen was done after laparotomy. Eighteen days after the inoculation, we observed the spleens (Fig. 4A) and livers (Fig. 4B). Tumor nodules appeared in spleens of all five untreated mice, and multiple metastases appeared in two livers (40%) of such mice. On the contrary, all five vaccinated mice completely rejected  $1 \times 10^5$  of C26/GPC3 cells inoculated into the spleen, and liver metastasis was nil. Differences in weights of spleen and liver and the rates and the numbers of appearance of tumor nodules in spleen were statistically significant among these two groups. Hence, GPC3-8 peptide-pulsed BM-DCs have the capacity to prevent growth in the spleen and possibly liver metastasis of GPC3-expressing tumors.

<sup>5</sup> Internet address: [http://bimas.dcrf.nih.gov/molbio/hla\\_bind/](http://bimas.dcrf.nih.gov/molbio/hla_bind/).

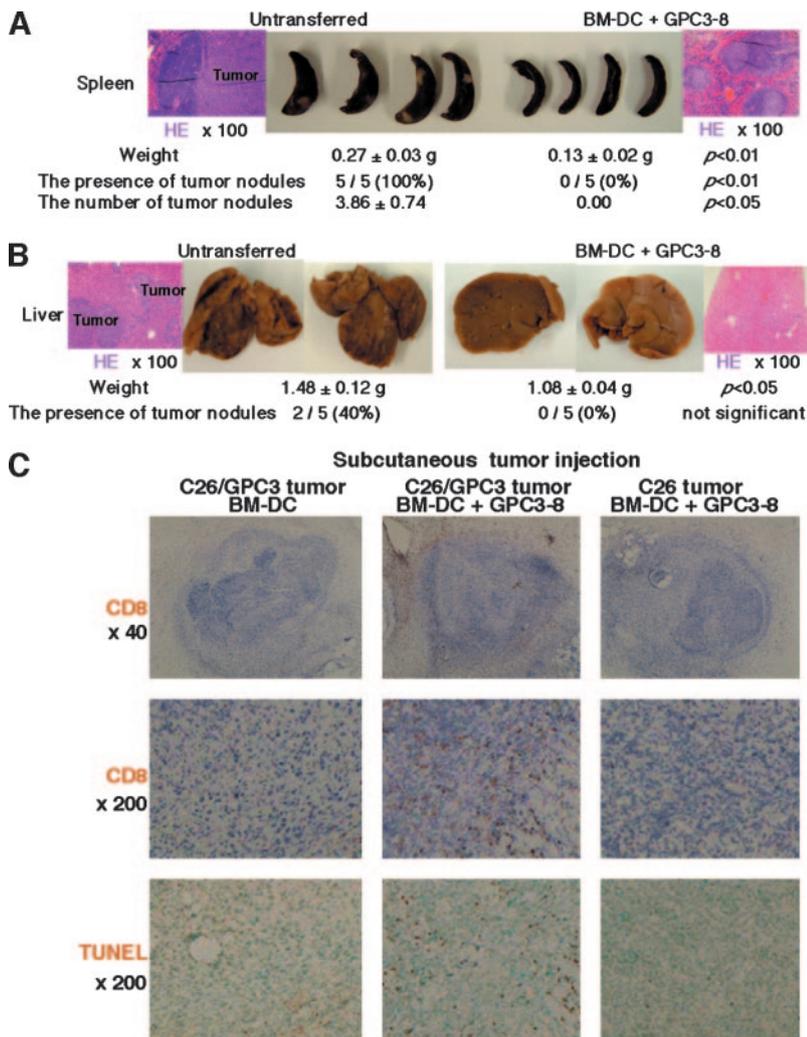


**Fig. 3** Mice vaccinated with GPC3-8 peptide-pulsed BM-DCs completely rejected C26/GPC3 tumor challenge. **A**, protocol of peptide-pulsed BM-DC vaccination. **B–G**, s.c. tumor injection model at the first challenge (**B**, **C**, **F**, and **G**) and the rechallenge (**D** and **E**). **B**, **D**, and **F**, suppression of the growth of GPC3-expressing C26/GPC3 tumor inoculated s.c. in mice vaccinated with GPC3-8 peptide-pulsed BM-DCs. Data are representative of two independent and reproducible experiments. Tumor area was calculated as a product of width and length. Data are presented as mean area of tumor  $\pm$  SE, and we evaluated the statistical significance using unpaired *t* test. **C**, **E**, and **G**, percentage of overall survival was calculated with the Kaplan-Meier method, and the statistical significance of differences between each group was evaluated with the Wilcoxon test.

**Vaccination of GPC3-8 Peptide-pulsed BM-DCs Induced Infiltration of CD8<sup>+</sup> T Cells into C26/GPC3 Tumor Cells and Apoptosis of Tumor Cells *In vivo*.** In the s.c. tumor injection model, all mice immunized with the BM-DC+GPC3-8 vaccine completely rejected challenges of C26/GPC3 cells ( $3 \times 10^4$ ). To ascertain that these rejections were induced by CD8<sup>+</sup> CTLs, s.c. inoculation of C26/GPC3 or C26 cells ( $1 \times 10^6$ ) into the right flank was done 7 days after the last vaccination. After tumor formation, we removed the tumor and immunohistochemically stained it with anti-CD8 antibody and the terminal deoxynucleotidyl transferase-mediated nick end labeling method (Fig. 4C). Infiltrations of CD8<sup>+</sup> T cells into C26/GPC3 tumors and apoptosis of C26/GPC3 tumor cells were observed only in mice vaccinated with GPC3-8 peptide-pulsed BM-DCs but never in mice vaccinated with unpulsed BM-DCs. We also evaluated spleens immunohistochemically with the intrasplenic tumor injection model (Fig. 4D). Eighteen days after tumor inoculation, there were fewer CD8<sup>+</sup> T cells and terminal deoxynucleotidyl transferase-mediated nick end labeling-positive apoptotic tumor cells in C26/GPC3 tumor nodules in spleens of untreated mice. On the contrary, there were many

CD8<sup>+</sup> T cells in the considerably enlarged white pulp and some terminal deoxynucleotidyl transferase-mediated nick end labeling-positive apoptotic tumor cells in spleens of mice immunized with the BM-DC+GPC3-8. These results suggest that GPC3-8 peptide-pulsed BM-DCs have the potential to prime a many GPC3-specific CTLs to kill C26/GPC3 tumor cells.

**Involvement of CD8<sup>+</sup> T Cells in Protection against C26/GPC3 Induced by GPC3-8 Peptide-pulsed BM-DC Vaccination.** To determine the role of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in protection against tumor cells induced by GPC3-8 peptide-pulsed BM-DC vaccination, we depleted mice of CD4<sup>+</sup> or CD8<sup>+</sup> T lymphocytes by treatment with anti-CD4 or anti-CD8 monoclonal antibody *in vivo*, respectively. With this treatment, >90% of CD4<sup>+</sup> and CD8<sup>+</sup> T cells were depleted (data not shown). During this procedure, mice were immunized with GPC3-8 peptide-pulsed BM-DCs and challenged with C26/GPC3 cells (each group:  $n = 4$ ). Depletion of CD8<sup>+</sup> T cells totally abrogated the protective immunity induced by GPC3-8 peptide-pulsed BM-DCs but that of CD4<sup>+</sup> T cells did not do so (data not shown). These results suggest that CD8<sup>+</sup> T cells play



**Fig. 4** Vaccination with GPC3-8 peptide-pulsed BM-DCs induced infiltration of CD8<sup>+</sup> T cells into C26/GPC3 tumor cells, but not into normal tissues, and induced the apoptosis of C26/GPC3 tumor cells. **A** and **B**, intrasplenic tumor injection model. Eighteen days after inoculation of C26/GPC3 cells ( $1 \times 10^5$ ) into spleens, the spleens (**A**) and livers (**B**) were observed macroscopically and histologically. **C**, Subcutaneous C26/GPC3 or C26 tumors were analyzed with immunohistochemical staining with anti-CD8 monoclonal antibody and terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) methods 4 days after inoculation of  $1 \times 10^6$  tumor cells. **D**, Spleens were analyzed with immunohistochemical staining with anti-CD8 monoclonal antibody and TUNEL method 18 days after inoculation of  $1 \times 10^5$  of C26/GPC3 cells. **E**, The normal tissues of BM-DC+GPC3-8-vaccinated or CTL-treated mice were pathologically and immunohistochemically examined. Objective magnification was 200 $\times$ . **F**, Placenta and mammary gland of BM-DC+GPC3-8-vaccinated female mice and fetal livers were immunohistochemically analyzed. Objective magnification was 200 $\times$ .

critical roles in antitumor immunity induced by GPC3-8 peptide-pulsed BM-DCs.

**No Evidence of Autoimmune Reactions in Surviving Mice that had Rejected Tumor Cell Challenges.** GPC3 expression in normal adult mice was not evident in all tissues tested, which suggests a low risk of damage to normal tissue as a result of immune responses to the GPC3 protein. To evaluate the risk of autoaggression by immunization against GPC3-8, the tissues of BM-DC+GPC3-8 immunized or CTL-treated mice were pathologically examined. Mice shown in Fig. 2, **C** and **D**, were sacrificed at 7 and 21 days after CD8<sup>+</sup> T cells transfer, respectively. In addition, mice shown in Figs. 3 and 4A–C were sacrificed at >150, 25, and 14 days after the last BM-DC+GPC3-8 vaccination, respectively. All mice were apparently healthy without abnormality, suggesting autoimmunity, such as dermatitis, arthritis, or neurologic disorder. The brain, liver, lungs, and heart of these mice were critically scrutinized, and findings were compared with those in normal mice. These tissues had normal structures and cellularity in each of the two mice of groups examined, and pathological changes caused by immune response, such as lymphocyte infiltration or tissue

destruction and repair, were nil (Fig. 4E). There were no CD8<sup>+</sup> T cells in these tissues, which had been immunohistochemically stained (Fig. 4E). Although CD8<sup>+</sup> T cells infiltrate in the C26/GPC3 tumor 21 days after CD8<sup>+</sup> T cells transfer (Fig. 2E) and at 14 and 25 days after the last BM-DC vaccination (Fig. 4, **C** and **D**), infiltration of CD8<sup>+</sup> T cells was not observed in all adult normal tissues examined at 7 and 21 days after CD8<sup>+</sup> T cells transfer (data not shown) and at >150, 25, and 14 days after the last BM-DC vaccination (Fig. 4E). These results indicate that lymphocytes stimulated with the GPC3 peptide did not recognize normal self-cells that could express GPC3 at physiologically low levels.

**Vaccination of GPC3-8 Peptide-pulsed BM-DCs Induced GPC3-specific CTLs, but did not Induce Damage of Placenta and Fetal Liver Expressing GPC3.** In murine tissues, GPC3 protein is expressed in placenta and fetal liver (Fig. 1B). To evaluate the risk of autoimmunity against placenta and fetal liver by immunization with BM-DC+GPC3-8, we carried out cross-breeding of BM-DC+GPC3-8-vaccinated female mice with normal male mice and compared events with normal mice pairs. To ascertain induction of GPC3-8-specific CTLs in

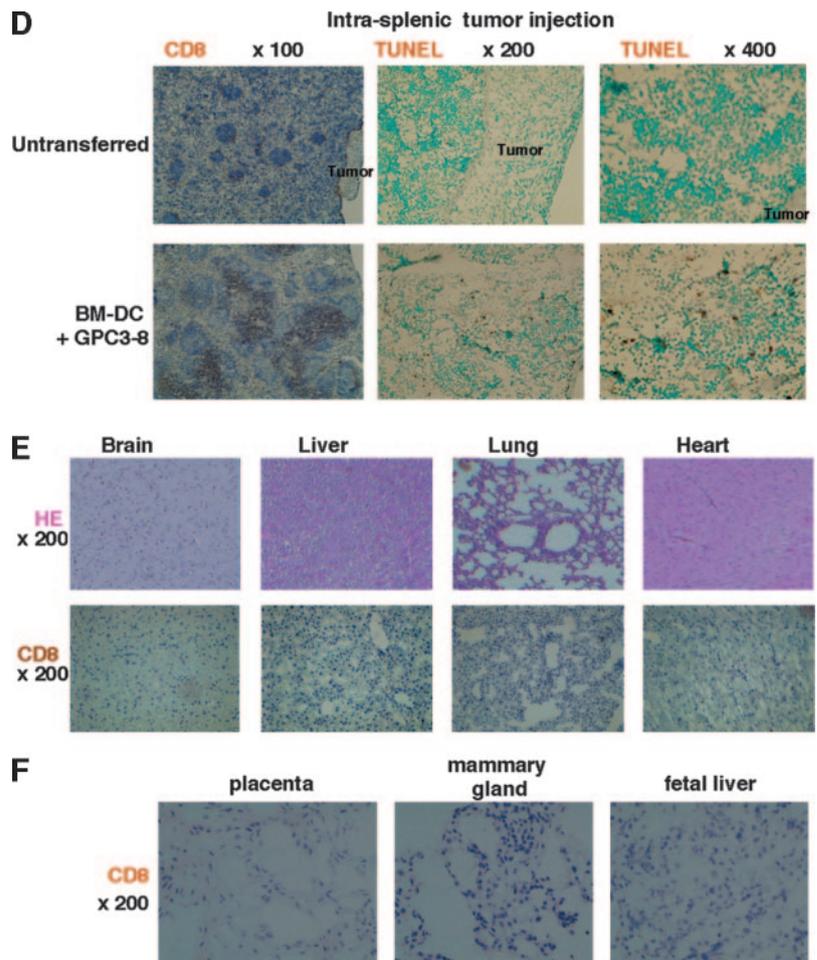


Fig. 4. Continued.

BM-DC+GPC3-8–vaccinated female mice not used for breeding, s.c. inoculation of C26/GPC3 or C26 cells ( $3 \times 10^4$ ) into the right flank of these female mice was done. All these vaccinated mice completely rejected  $3 \times 10^4$  of C26/GPC3 cells 100 days after inoculation but not C26 cells (data not shown). Pregnancy and births in vaccinated female mice were normal. Six mice were born from each untreated and vaccinated three female mice, and all neonates were normal. Placenta and mammary gland of BM-DC+GPC3-8–vaccinated female mice and fetal livers had normal structures and cellularity with no pathological changes caused by immune responses, such as lymphocyte infiltration or tissue destruction and repair. There were no CD8<sup>+</sup> T cells in these tissues, which had been immunohistochemically stained (Fig. 4F). Furthermore, to ascertain induction of GPC3-8–specific CTLs in vaccinated female mice that gave birth, s.c. inoculation of C26/GPC3 or C26 cells ( $1 \times 10^6$ ) into the right flank of these mice was done. After tumor formation, we removed the tumor and immunohistochemically stained it with anti-CD8 antibody. Infiltration of CD8<sup>+</sup> T cells into C26/GPC3 tumors but not into C26 tumors, as observed in Fig. 4C, was evidenced (data not shown). These results indicate that in this vaccination model GPC3-8–specific CD8<sup>+</sup> CTLs do not attack placenta and fetal livers expressing GPC3.

## DISCUSSION

In 1996, Pilia *et al.* (35) reported that *GPC3* is mutated in patients with Simpson-Golabi-Behmel syndrome. This syndrome is an X-linked disorder characterized by pre- and postnatal overgrowth (36). *GPC3*-deficient mice have several abnormalities found in Simpson-Golabi-Behmel syndrome patients, including overgrowth and cystic and dysplastic kidneys (37). Some reports indicated that *GPC3* expression is down-regulated in tumors of different origin. They showed that, although *GPC3* is expressed in the normal ovary, mammary gland, and mesothelial cells, the expressions are undetectable in a significant proportion of ovarian, breast cancer, and mesothelioma cell lines (38). In all cases where *GPC3* expression was lost, the *GPC3* promoter was hypermethylated, and mutations were nil in the coding region. Collectively, these data suggest that *GPC3* can act as a negative regulator of growth in these cancers.

On the contrary, in the case of hepatocellular carcinoma, tumors originating from tissues that express *GPC3* only in the embryo, *GPC3* expression tends to reappear with malignant transformation. Whether reexpression of *GPC3* plays a role in progression of these tumors is unknown. Why is *GPC3* up-

regulated only in hepatocellular carcinoma and melanoma? We are investigating to determine whether GPC3 is involved in the oncogenesis of melanoma and hepatocellular carcinoma.

The genetic approach of T-cell epitope cloning method (2–6) and SEREX method (7–11) led to identification of a many genes encoding for tumor antigens and antigenic peptides recognized by tumor-reactive CTLs, thereby enhancing the possibility of antigen-specific cancer immunotherapy (2–8). MAGE (2) and NY-ESO-1 (8) represent cancer-testis antigen, and MART-1 (39), gp100 (40, 41), and tyrosinase (42) represent melanocyte-differentiation antigen. Cancer-testis antigens, expressed only in tumor cells and not in normal adult tissues, except for immune privilege tissues, including testis, ovary, and placenta, are ideal targets for tumor immunotherapy. One can prevent development of autoimmune diseases by vaccination with cancer-testis antigens, and many cancer-testis antigens are expressed in a variety of cancers (43). However, the rates of expression of cancer-testis antigens in cancers are at most 50% (43), and expression often shows heterogeneity within the same tumor (43, 44).

On the contrary, melanocyte-differentiation antigens are expressed homogeneously in almost all melanoma cells, so one can use these antigens for immunotherapy of melanoma patients. However, autoimmunity, such as vitiligo and uveitis, developed after vaccination with these antigens because these antigens are expressed in normal melanocytes (33). cDNA microarray technology is rapidly progressing (12–16). We have recently succeeded in identifying a novel cancer rejection antigen specifically expressed in esophageal cancer with cDNA microarray technology (17). In the present study, we found that GPC3 is highly immunogenic to stimulate eradication by T cells of tumor expressing GPC3 in mice. GPC3 is an ideal candidate antigen useful for immunotherapy of hepatocellular carcinoma, and vaccination against GPC3 is not expected to induce autoimmune diseases because it has unique tissue specificity regarding protein expression. Our study is the first to show that cDNA microarray technology is useful for identifying ideal cancer antigens.

The *HLA-A24* is the most common *HLA class I* allele in the Japanese population, and 60% of Japanese (95% of whom are genotypically *A\*2402*), 20% of Caucasians, and 12% of Africans are positive for *HLA-A24* (45, 46). It is important for especially Japanese to identify *HLA-A24*-restricted CTL epitope peptides. Structural motifs of peptides bound to human *HLA-A24* and *BALB/c* mouse  $K^d$  are similar (47–49), and the amino acid sequences of human and mouse GPC3 have a 95% homology. We searched for GPC3-derived peptides with a common sequence in both and selected peptides carrying binding motifs to both *HLA-A24* and  $K^d$  molecules, as previously described, and we prepared 12 different synthetic peptides GPC3-1~12. GPC3-derived and  $K^d$ -restricted CTL epitope identified in *BALB/c* mice will be applicable to human *HLA-A24*-restricted CTLs. Therefore, we selected *BALB/c* mice in this study. Although we wanted to use hepatocellular carcinoma or melanoma cell lines, these cell lines derived from *BALB/c* mouse were not available. Furthermore, we found no GPC3-expressing murine tumor cell lines originating from *BALB/c* mice, and we had already established a system for analysis of mouse tumor immunity *in vivo* with Colon26. Anyway, in this

study, with Colon26/GPC3, we could prove that GPC3 could be a cancer rejection antigen in mice. We recently found out that mouse melanoma cell line B16 expressed GPC3, so we are planning to investigate GPC3-mediated antitumor immunity with B16 in *C57BL/6* mouse.

We observed the effects of adoptive transfer of highly selected tumor-reactive T cells directed against GPC3-8 peptide, even against established tumors, without causing autoimmune-related destruction. These results show the possibility of therapy of adoptive transfer of GPC3-specific CTLs for both hepatocellular carcinoma and melanoma. We also showed that GPC3-8 peptide-pulsed BM-DCs can prime GPC3-8-specific T cells *in vivo*, and growth of C26 expressing GPC3 was prevented without inducing autoimmune destruction in both s.c. and intrasplenic tumor injection models. The transfer of GPC3-8 peptide-pulsed BM-DCs showed no efficacy against the established C26G tumor (data not shown). On the contrary, vaccination of mice with GPC3-8 peptide emulsified in complete Freund's adjuvant could prime GPC3-8-specific T cells *in vivo*, but could not reject the challenge of C26/GPC3 (data not shown). These results show that GPC3-8 peptide-pulsed BM-DC therapy prevented the appearance or recurrence of tumors, yet this procedure did not induce regression of an established tumor. We suggest that adoptive transfer of GPC3-specific CTLs is a novel treatment strategy for patients with hepatocellular carcinoma or melanoma, and we also suggest that GPC3-derived peptide-pulsed DC vaccination is a novel strategy for prevention of hepatocellular carcinoma or melanoma in patients treated surgically, in patients with liver cirrhosis and chronic hepatitis who are at high risk for development of hepatocellular carcinoma, and in humans at high risk for development of melanoma. Whether GPC3 is an ideal target for immunotherapy in human hepatocellular carcinoma and melanoma, naturally expressing GPC3, will continue to be investigated in our laboratory.

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