induction of their differentiation to DCs. By sporadic selection with a selection drug, ES cell clones transfected with genes can be propagated while maintaining the capacity to express gene products after their differentiation to DCs. Therefore, one can use ES cell transfectants as an infinite source for genetically modified DCs.

In the present study, using this method, we generated DCs expressing chemokine along with a model Ag, OVA. We determined whether coexpression of T cell-attracting chemokine with antigenic protein by DCs enhanced the capacity to prime Ag-specific CTLs upon in vivo transfer. We also examined the potency of the genetically modified DCs to elicit antitumor immunity against tumor cells expressing OVA. Among T cell-attracting chemokines, we selected SLC, Mig, and Lptn, and comparatively evaluated their effects.

Materials and Methods

Mice

CBA and C57BL/6 mice were obtained from CLEA (Tokyo, Japan) or Charles River Breeding Laboratories (Hamamatsu, Japan) and kept under specific pathogen-free conditions. Male CBA and female C57BL/6 mice were mated to produce (CBA crossed with C57BL/6) F_1 (CBF$_1$) mice and all in vivo experiments were done with the F_1 mice at 6–8 wk of age.

Cell lines

The ES cell line TT2, derived from (CBA crossed with C57BL/6) F_1 blastocysts (17), were maintained as described (18). The T cell hybridoma RF33.70 (19), recognizing OVA$_{257-264}$ in the context of K^b, and the M-CSF-defective bone marrow-derived stromal cell line, OP9 (20), have been reported. MO4 (21) was generated by transfection of C57BL/6-derived melanoma B16 with the pAc-neo-OVA plasmid, as described (22). The procedure for induction of differentiation of ES cells into DCs has been reported (16), and ES-DCs recovered after a 14-day culture in bacteriological petri dishes were used for in vivo and in vitro assays.

Peptide, cytokines/chemokines, and anti-chemokine Ab

The K^b-binding peptide OVA$_{257-264}$, SIINFEKL, were synthesized using the F-MOC method on an automatic peptide synthesizer (PSSM8; Shimadzu, Kyoto, Japan) then purified by HPLC. Recombinant mouse GM-CSF was provided by Kirin Brewery (Tokyo, Japan). Recombinant mouse SLC, Mig, and Lptn, were purchased from DACO JAPAN (Kyoto, Japan). Goat anti-mouse SLC and Mig Abs and biotinylated goat anti-mouse SLC and Mig Abs were also purchased from DACO JAPAN. Rabbit anti-mouse Lptn Ab was purchased from eBioscience (San Diego, CA), and was biotinylated using a MiniBiotin-XX Protein Labeling kit (F-6347; Molecular Probes, Portland, OR).

cDNA array analysis of chemokine gene expression

BM-DCs were generated from bone marrow cells of CBF$_1$ mice, as described (23, 24). Total RNA was extracted from BM-DCs on day 12 and ES-DCs on day 14 of culture in bacteriological petri dishes, using RNeasy mini kits (Qiagen, Studio City, CA). Total RNA (3 μg) from each sample was reverse transcribed into cDNA with Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI) in the presence of [α-^{32}P]dCTP (Amersham Pharmacia Biotech, Piscataway, NJ). The resulting cDNA probes were hybridized to cDNA fragments spotted on GEArray membranes (SuperArray, Bethesda, MD). Hybridization and wash of the membranes were done following the manufacturer's instructions. The intensity of radioactive signaling from the hybridized probes was analyzed on a BAS-2000 (Fujifilm, Tokyo, Japan). The signal from expression of each chemokine gene was normalized to the signal derived from β-actin on the same membrane and expressed as arbitrary units calculated using the formula: Chemokine mRNA arbitrary units = (chemokine signal − background signal)/(β-actin signal − background signal) (25).

Plasmid construction

A cDNA fragment encoding for OVA protein was transferred to pCAG-IP (26), a mammalian expression vector containing the chicken β-actin promoter and an internal ribosomal entry site (IRES)-puromycin N-acetyltransferase gene cassette, to generate pCAG-OVA-IP. To obtain pCAGGS-IRES-neo-R, a DNA fragment containing IRES-neomycin-resistant (neo-R) was inserted into a mammalian expression vector pCAGGS (27). A cDNA fragment coding for chemokine protein was inserted into pCAGGS-IRES-neo-R. SLC cDNA was obtained by RT-PCR using murine spleen cells as the RNA source and PCR primers, AACCCTCTAGCCCGCCGCCAC-CATGGCTCAGAGATG ACTCT (forward) and AACCCGGATCCAGGCGGGCTACTACTGGCT ATCC (reverse). Mig cDNA was obtained by RT-PCR using murine spleen cells stimulated for 24 h with IFN-γ as the RNA source and the PCR primers, AACCCTCTAGACCCGCCGCCACCATGAAGTCCGCTGTTCTTTTCC (forward) and AACCCGGATCCAGGGTGCTTGTTGGTAAAG (reverse). Lptn cDNA was obtained by RT-PCR, using murine spleen cells as the RNA source stimulated for 24 h with PMA and A23187 and the PCR primers AAC CCTCTAGACCCGCCGCCACCATGAGACTTCTCCTCCTGAC (forward) and AACCCGGATCCCTGGAGGCTGTTACCCAGTC (reverse). The design of these primers results in cloning of chemokine cDNA downstream of the Kozak sequence (28). The PCR products were cloned into a plasmid vector (pGEM-T easy; Promega), confirmed by sequencing analysis, and then transferred to the expression vector.

Transfection of ES cells and generation of ES-DCs expressing chemokine along with OVA

To generate OVA-transfected ES cell clones, TT2 ES cells were introduced with pCAG-OVA-IP by electroporation and selected with puromycin using the reported procedure (16). OVA-transfected ES cell clones were differentiated to ES-DCs, and an ES cell transfectant clone highly expressing OVA after differentiation to DCs (ES-OVA) was selected, based on the capacity to stimulate RF33.70, the OVA-reacting T cell hybridoma. The selected ES cell clone was transfected with one of three kinds of chemokine expression vectors or pCAGGS-IRES-neo-R (mock). Transfected ES cells were cultured on neo-R primary embryonic fibroblasts feeder layers and selected with G418 (500 μg/ml), and drug-resistant colonies were picked up. Double-transfectant ES cell clones producing high amounts of chemokine after differentiation to DCs were selected. To determine chemokine levels in culture supernatants, ELISAs were done as we reported (29).

T cell hybridoma assay for detection of OVA peptide-K^b complexes

Graded numbers of ES-DCs as stimulators were seeded into 96-well flat-bottom culture plates together with RF33.70 as responders (5 × 10^4 cells/well in final volume of 200 μl). After 24 h of culture, the supernatant (50 μl/well) was collected and added to culture of the IL-2-dependent cell line, CTLL-20 (5 × 10^3/100 μl/well), in 96-well flat-bottom culture plates. After 16 h, [^{3}H]thymidine (248 MBq/mmol) was added (37.5 KBq/well) and cells were incubated for a further 8 h. The incorporation of [^{3}H]thymidine by CTLL-20 was measured by scintillation counting.

In vitro survival assay of ES-DCs

ES-DCs recovered from 14-day culture in petri dishes were cultured again in petri dishes (1.2 × 10^5/90 mm dish) under several conditions. After 7 days, cells were recovered by pipetting, stained with trypan blue and microscopically counted. Some recovered cells were also stained with propidium iodide (10 μg/ml) and analyzed on a flow cytometer (FACScan, BD Biosciences, San Jose CA) to detect dead cells.

Assay of the migration of DCs in vivo

DCs (2 × 10^6) labeled with 1 μM CFSE (Molecular Probes, Oss, The Netherlands) in serum-free medium for 10 min at 37°C, were i.p. transferred into the CBF$_1$ mouse. After 40 h, 5-μm frozen sections of the spleen were made and examined under a fluorescence microscope (Olympus, Melville, NY) or stained with H&E. ^{111}In-labeled DCs (1 × 10^6) were i.p. transferred into mice. After 40 h, several organs were isolated and the radioactivity in each organ was measured on a gamma counter as described by Eggert et al. (6) and Morse et al. (9). The radioactivity was expressed as the percentage of injection dose per 0.1 gram of tissue, so that the values were adjusted to 0.1 g of tissue to correct for weight differences of each organ.

Induction of OVA-specific CTLs in vitro and cytotoxicity assay

ES-DCs (4 × 10^5/well) or BM-DCs (4 × 10^5/well) were cocultured with T cells (2.5 × 10^6/well) purified with a nylon wool column from spleen cells of unprimed CBF$_1$ mice in 24-well culture plates in RPMI 1640 supplemented with 10% FCS. In some experiments, ES-DCs were killed before use by treatment at 70°C for 20 min. BM-DCs were prepared as described (23) then pulsed with OVA peptide (10 μM) for 4 h, washed twice, and used as stimulators. After 5 days of culture, cells were recovered

and used as effector cells in cytotoxicity assay using peptide-pulsed EL-4 cells as target cells, as described (16).

Induction of OVA-specific CTLs in vivo

Genetically modified ES-DCs, viable or heat-killed, or OVA protein (50 μg) were injected i.p. to mice twice at 7-day intervals, and 7 days after the second transfer, the mice were killed and spleen cells were isolated. Whole spleen cells were cultured in vitro in the presence of OVA peptide (0.1 μM) for 5 days and OVA-specific CTL activity was analyzed as described (16).

Tumor prevention experiments

In tumor prevention experiments and survival studies, 2×10^4 or 3×10^3 genetically modified ES-DCs were transferred i.p. into mice. Transfers were done twice at 7-day intervals, and 7 days after the second transfer, MO4 cells were challenged s.c. in the shaved left flank region. Tumor sizes were determined biweekly in a blinded fashion and survival rate was monitored. Tumor index was calculated as: Tumor index (in millimeters) = square root (length × width).

In vivo depletion of $CD4^+$ and $CD8^+$ T lymphocytes

Mice were transferred i.p. twice with 3×10^3 ES-DC-OVA/mock or ES-DC-OVA/SLC at 7-day intervals, and 7 days after the second transfer, the mice were challenged s.c. with 3×10^6 MO4 cells (day 0). The mice were given a total of six i.p. transfers (days −18, −15, −11, −8, −4, −1) of the ascites (0.1 ml/mouse/transfer) from hybridoma-bearing nude mice. mAbs used were rat anti-mouse CD4 (clone GK1.5) and rat anti-mouse CD8 (clone 2.43). Normal rat IgG (Sigma-Aldrich, St. Louis, MO; 200 μg/mouse/transfer) was used as control. Tumor measurements were made 15 days after tumor challenge. Results are expressed as tumor index + SD. Each group included eight mice. Depletion of T cell subsets by treatment with mAbs was confirmed by flow cytometric analysis of spleen cells, which showed a >90% specific depletion.

Histological analysis of tumor tissues

Freshly excised tumor tissues were immediately frozen and embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN). Serial 5-μm sections were made using cryostat and underwent immunochemical staining with mAbs specific to CD4 (L3T4; BD PharMingen, San Diego, CA) or CD8 (Ly-2; BD PharMingen) and N-Histofine Simple Stain Mouse MAX PO (Nichirei, Tokyo, Japan).

Statistical analysis

Two-tailed Student's t test was used to determine the statistical significance of differences in lytic activity of spleen cell preparations and tumor growth, and between treatment groups. A value of $p < 0.05$ was considered significant. The Kaplan-Meier plot for survivals was assessed for significance using the Breslow-Gehan-Wilcoxon test. Statistical analyses were made using StatView 5.0 software (Abacus Concepts, Calabasas, CA).

Results

Profile of chemokine gene expression in ES-DCs

We recently established a culture method to generate DCs from mouse ES cells. ES-DCs have the capacity to stimulate T cells comparable to BM-DCs (16). At the beginning of the present study, to determine the profile of chemokine gene expression by ES-DCs, we analyzed chemokine mRNAs by cDNA macroarray hybridization analysis, comparing ES-DCs and BM-DCs. The gene expression of DC-derived chemokines and chemokines that chemoattract T cells is shown in Fig. 1. The analysis revealed that chemokine gene expression profile of ES-DCs was somewhat different from that of BM-DCs. However, both DCs expressed C10, and expression of T cell-attracting chemokines produced by cells other than DCs such as SLC, Lptn, Mig, or stromal cell-derived factor 1α were rarely detected in both types of DCs generated in vitro. Therefore, we presumed augmentation of the immunomodu-
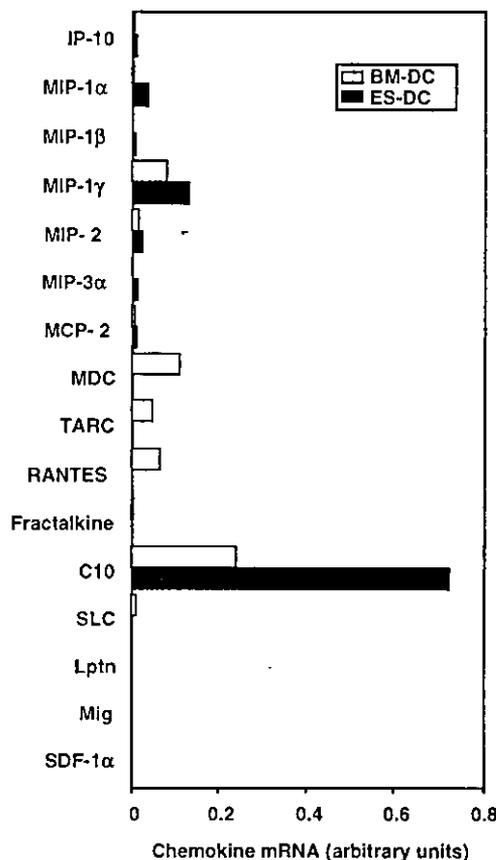
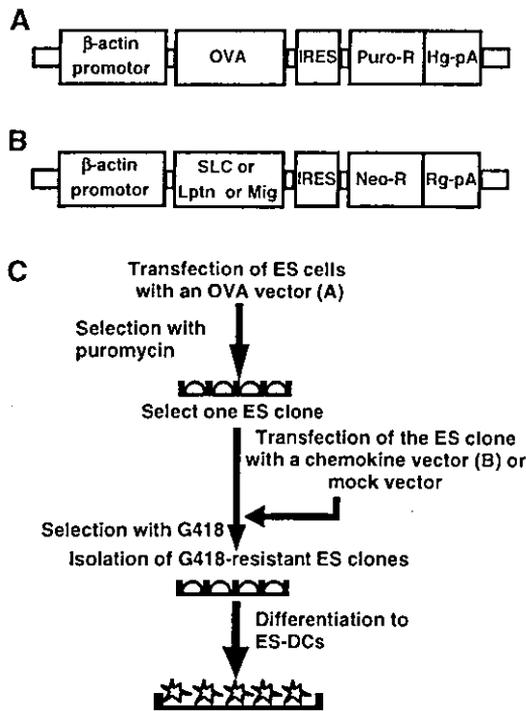

FIGURE 1. Profile of chemokine gene expression in ES-DCs and BM-DCs. Radiolabeled cDNA generated from ES-DCs and BM-DCs were hybridized to chemokine gene-specific 44 cDNA fragments spotted on nylon membranes. The hybridization signals were normalized to the signal derived from β-actin on the same membrane. Data for DC-derived chemokines and chemokines with T cell-attracting property are shown.

lating capacity by in vivo transferred DCs through genetic modification of DCs to express such T cell-attracting chemokines.

Generation of ES-DCs expressing chemokine along with antigenic protein

Using the expression vector driven by the β-actin promoter and containing the IRES-drug-resistant marker gene (Fig. 2, A and B), we can generate ES cell transfectant clones expressing the gene products after their differentiation to DCs. Using this system, we first prepared an ES cell transfectant clone highly expressing OVA after differentiation to DCs. Subsequently, we introduced chemokine expression vectors or mock vector into the ES cell clone expressing OVA (ES-OVA) (Fig. 2C). We selected one double-transfectant ES cell clone for each chemokine gene or mock vector transfection and generated four kinds of ES-DCs expressing chemokine along with OVA or OVA alone, and designated them ES-DC-OVA/SLC, ES-DC-OVA/Lptn, ES-DC-OVA/Mig, and ES-DC-OVA/mock. Therefore, the four double transfectant ES cell clones used in this study originated from the same ES cell clone transfected with the OVA gene.

We acquired DCs from these ES cell transfectant clones and compared their capacity to stimulate the $OVA_{257-264}$-specific and K^b-restricted T cell hybridoma, RF33.70. As shown in Fig. 3A, ES-DC-OVA/SLC, ES-DC-OVA/Lptn, ES-DC-OVA/Mig, ES-DC-OVA, and ES-DC-OVA/mock could stimulate RF33.70 with a comparable efficiency. The amounts of chemokine produced by the
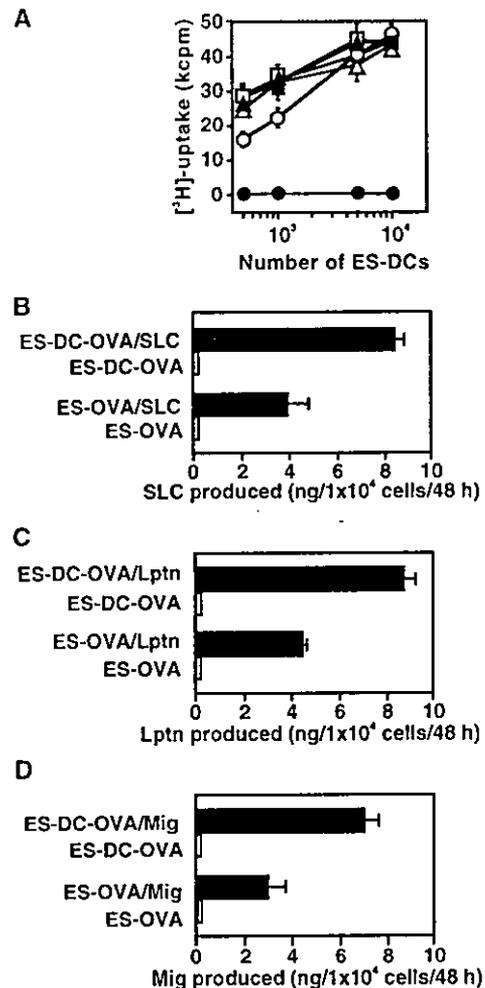
A

β-actin promotor | OVA | IRES | Puro-R | Hg-pA

B

β-actin promotor | SLC or Lptn or Mig | IRES | Neo-R | Rg-pA

C

Transfection of ES cells
with an OVA vector (A)

Selection with
puromycin

Select one ES clone

Transfection of the ES clone
with a chemokine vector (B) or
mock vector

Selection with G418
Isolation of G418-resistant ES clones

Differentiation to
ES-DCs

FIGURE 2. Generation of ES-DCs expressing chemokine simultaneously with OVA. A, Structure of OVA protein expression vector, pCAG-OVA-PI. The expression of this gene is driven by the chicken β-actin promoter. The OVA protein coding sequence is followed by the IRES-puromycin N-acetyltransferase gene (Puro-R) and the polyadenylation signal sequence of human growth hormone (Hg-pA). B, Structure of chemokine expression vector, pCAGGS-chemokine-IRES-neo-R. Expression of this gene is driven by the chicken β-actin promoter. The chemokine coding sequence is followed by the IRES-neo-R gene (Neo-R) and a polyadenylation signal sequence of rabbit β-globin poly(A) (Rg-pA). C, TT2 ES cells were transfected with pCAG-OVA-PI. Puromycin-resistant colonies were picked up and expanded. An ES cell clone highly expressing OVA was selected and transfected with one of three kinds of pCAGGS-chemokine-IRES-neo-R or with a mock vector. G418-resistant colonies were picked up and expanded. One ES clone expressing large amounts of chemokine after DC differentiation was selected for each chemokine and used in the described experiments.

three kinds of chemokine gene-transfected cells used in this study are shown in Fig. 3, B–D. Both ES cells and differentiated ES-DCs produced transgene-derived chemokines, and comparable protein amounts of chemokines were produced by the three chemokine gene-transfected ES-DCs. Morphology and surface phenotypes of chemokine gene-transfected ES-DCs were not significantly different from ES-DC-TT2 (DCs derived from parental TT2 ES cells) (data not shown). These results suggest that the forced expression of OVA protein and the chemokines by gene transfer to ES cells do not affect their differentiation to DCs.
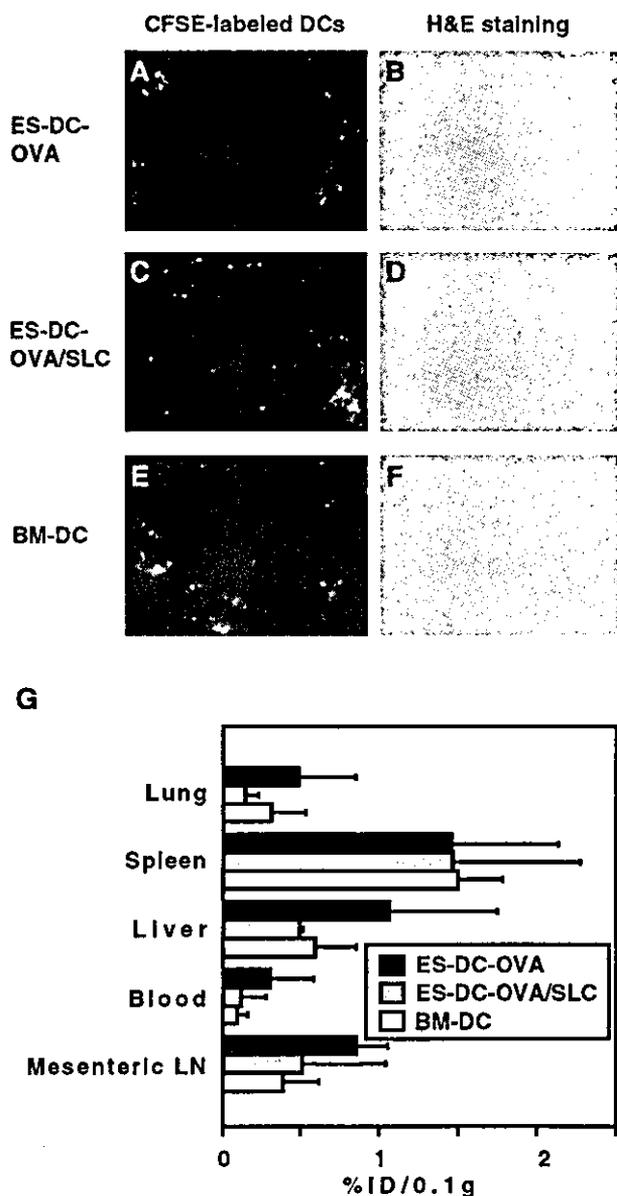
The migration capacity of ES-DCs in vivo

To test the migration capacity of ES-DCs in vivo, we histologically examined the migration of transferred ES-DCs to the spleen. In addition, we tested whether or not the expression of SLC, the chemokine with DC-attracting property, by ES-DCs would affect their in vivo migration. As shown in Fig. 4, A–F, CFSE-labeled ES-DC-OVA, ES-DC-OVA/SLC, and BM-DCs migrated to the spleen to the same extent, mostly localizing in the white pulp and the marginal zone (Fig. 4, B, D, and F).

A

B

ES-DC-OVA/SLC
ES-DC-OVA
ES-OVA/SLC
ES-OVA

SLC produced (ng/1x10⁴ cells/48 h)

C

ES-DC-OVA/Lptn
ES-DC-OVA
ES-OVA/Lptn
ES-OVA

Lptn produced (ng/1x10⁴ cells/48 h)

D

ES-DC-OVA/Mig
ES-DC-OVA
ES-OVA/Mig
ES-OVA

Mig produced (ng/1x10⁴ cells/48 h)

FIGURE 3. Stimulation of OVA-specific T cell hybridoma and chemokine production by genetically modified ES-DCs. A, Stimulation of K[b]-restricted OVA-specific T cell hybridoma, RF33.70, with ES-DC-OVA (■), ES-DC-OVA/mock (□), ES-DC-OVA/SLC (▲), ES-DC-OVA/Lptn (△), ES-DC-OVA/Mig (○), or negative control, ES-DC-TT2 (●) without OVA-expression, was analyzed. Stimulators and RF33.70 were cocultured for 24 h, and IL-2 produced by RF33.70 was quantified by measuring proliferation of CTLL-20 cells. Results were expressed as mean cpm of triplicate cultures ± SD. Data are representative of three independent and reproducible experiments. B–D, The 48-h culture supernatants of the 1 × 10⁶ ES-DC-OVA expressing chemokine or ES-DC-OVA in petri dishes and that of 1 × 10⁶ ES-OVA expressing chemokine or ES-OVA on layers of primary embryonic fibroblasts were harvested. The concentrations of chemokine in the supernatants were measured using ELISA. Production of chemokine SLC (B), Lptn (C), and Mig (D) by respective transfectants was quantified. Results are expressed as mean amounts of chemokine per 1 × 10⁴ cells of triplicate cultures ± SD. Data are representative of two independent and reproducible experiments.

We also investigated the distribution of ¹¹¹In-labeled DCs in lymphoid organs after i.p. transfer. The distribution of ES-DCs shown in Fig. 4G indicated that ES-DCs and BM-DCs similarly accumulated in the spleen and mesenteric LN 40 h after the transfer, and that expression of SLC by ES-DCs made no significant difference in the migration pattern.

Collectively, the migratory capacity toward lymphoid tissues of ES-DCs is almost comparable to that of BM-DCs, and the SLC produced by ES-DC-OVA/SLC did not prevent them from migrating toward lymphoid tissues.

FIGURE 4. The migration capacity of ES-DCs in vivo. *A–F*, DCs (2 × 10⁶) were labeled with CFSE and injected i.p. into mice. At 40 h later, frozen sections of spleens were prepared. Injected DCs were ES-DC-OVA (*A* and *B*), ES-DC-OVA/SLC (*C* and *D*), and BM-DCs (*E* and *F*). *A*, *C*, and *E* are fluorescense images of the sections serial to H&E-stained sections shown in *B*, *D*, and *F*, respectively. *G*, ¹¹¹In-labeled DCs (1 × 10⁶) were injected i.p. into mice, and radioactivity of indicated organs was measured 40 h later. The measured radioactivity in tissues was expressed as percentage of injection dose per 0.1 g tissue (%ID/0.1 g) as described in *Materials and Methods*. Results were expressed as mean %ID/0.1 g + SD (*n* = 3 per group).

Priming of Ag-specific CTLs with genetically modified ES-DCs in vitro and in vivo

We analyzed the capacity of ES-DC-OVA to prime OVA-specific T cells in vitro. ES-DC-TT2, ES-DC-OVA, heat-killed ES-DC-OVA, or BM-DCs prepulsed with OVA peptide were cocultured with splenic T cells derived from unprimed CBF₁ mice. After 5 days, cells were recovered and OVA-specific CTL activity was analyzed. The results shown in Fig. 5*A* indicate that OVA-specific CTLs were primed in vitro by intact ES-DC-OVA but not by ES-DC-TT2, BM-DCs prepulsed with OVA₂₅₇₋₂₆₄ peptide, or heat-
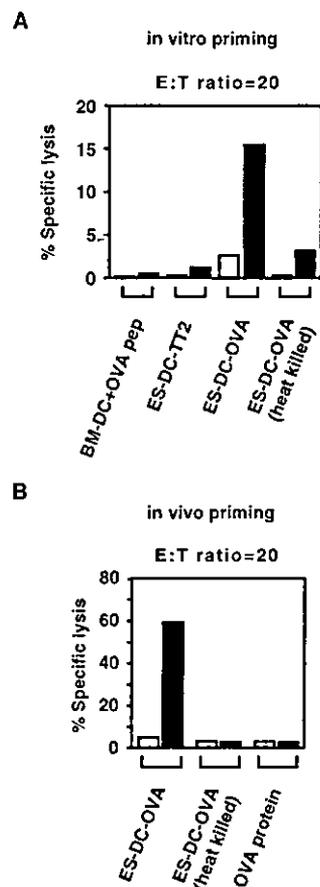
FIGURE 5. Priming of OVA-specific CTLs with genetically modified ES-DCs. *A*, BM-DCs prepulsed with OVA peptide (10 μM), ES-DC-TT2, ES-DC-OVA, or heat-killed ES-DC-OVA were cocultured with splenic T cells of unprimed CBF₁ mice. After 5 days, the resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide (■) or left unpulsed (□) at an E:T ratio of 20. *B*, Mice were transferred i.p. twice with ES-DC-OVA (2 × 10⁴), alive or heat killed, or OVA protein (50 μg) on days −14 and −7. Spleen cells were harvested from the mice on day 0, pooled for each group (four mice per group), and cultured in the presence of OVA₂₅₇₋₂₆₄ (0.1 μM) for 5 days. The resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide (■) or left unpulsed (□) at an E:T ratio of 20. Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of two independent and reproducible experiments.

killed ES-DC-OVA. BM-DCs prepulsed with OVA peptide could prime OVA-specific CTLs in vitro only in the presence of exogenous IL-2, whereas ES-DC-OVA could prime OVA-specific CTLs, regardless of whether or not IL-2 had been added (our unpublished observations).

Furthermore, the capacity of ES-DC-OVA to prime OVA-specific T cells in vivo was analyzed. ES-DC-OVA (2 × 10⁴), heat-killed ES-DC-OVA (2 × 10⁴), or OVA protein (50 μg) were injected i.p. into CBF₁ mice twice within a 7-day interval. Spleen cells were isolated 7 days after the second injection and then cultured in vitro in the presence of OVA₂₅₇₋₂₆₄ peptide. After 5 days, cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2ᵇ) prepulsed with the OVA peptide. The results shown in Fig. 5*B* indicate that CTLs specific to the OVA epitope were primed in vivo with ES-DC-OVA but not with heat-killed ES-DC-OVA or soluble OVA protein.
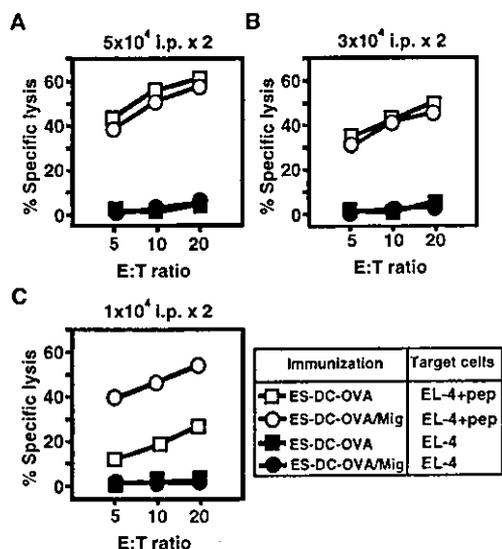
These results demonstrated that live ES-DCs genetically modified to express an antigenic protein have the capacity to prime

Ag-specific CTLs both in vitro and in vivo. There is little possibility that endogenous host DCs, which phagocytosed ES-DCs expressing OVA or OVA protein, played a major role in priming CTLs, based on the result that CTLs were not primed either by injection with heat-killed ES-DC-OVA or by OVA protein.
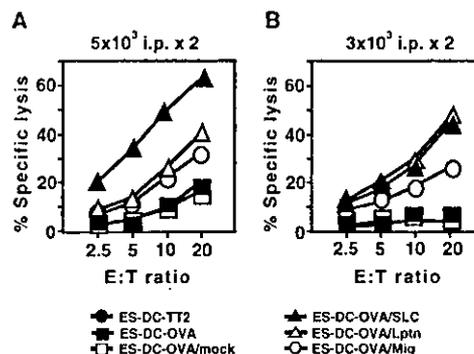
Efficient priming of OVA-specific CTLs by DCs producing chemokine along with OVA

We analyzed the capacity of genetically modified ES-DCs expressing Mig along with OVA to prime OVA-specific T cells in vivo. Graded numbers of ES-DC-OVA or ES-DC-OVA/Mig were transferred i.p. to mice twice at a 7-day interval. Spleen cells were isolated 7 days after the second transfer then cultured in vitro in the presence of OVA$_{257-264}$ peptide. After 5 days, cells were recovered and assayed for their capacity to kill EL-4 thymoma cells (H-2^b) prepulsed with the OVA peptide (Fig. 6). When 5×10^4 or 3×10^4 DCs were transferred twice, a comparable level of OVA-specific CTL activity was primed by ES-DC-OVA and ES-DC-OVA/Mig. In contrast, when 1×10^4 DCs were transferred twice, ES-DC-OVA/Mig primed CTL activity to a greater extent than seen with ES-DC-OVA. As we reported, OVA-specific CTLs were not primed by transfer of ES-DC-TT2, even when 5×10^5 DCs were transferred twice (16).

We next analyzed effects of expression of the three chemokines on in vivo CTL-priming using the same experimental procedure as previously described except that smaller numbers of DCs were transferred into the mice (Fig. 7). When mice were given 5×10^3 ES-DCs twice, all OVA-expressing DCs stimulated OVA-specific CTLs, and the T cell-priming capacity of DCs coexpressing either of the three chemokines was significantly stronger than those expressing OVA alone. Even when only 3×10^3 DCs were transferred twice, OVA-specific CTLs were primed by the three kinds of ES-DC-OVA chemokine. Conversely, priming of CTLs by ES-DCs expressing OVA alone was not detected under this condition.

FIGURE 7. Enhanced priming of OVA-specific CTLs in vivo by immunization with ES-DCs expressing chemokine along with OVA. Mice were transferred i.p. twice on days −14 and −7 with 5 × 10³ ES-DCs (A) or 3 × 10³ ES-DCs (B). ES-DCs expressing chemokine along with OVA were ES-DC-OVA/SLC (▲), ES-DC-OVA/Lptn (△), ES-DC-OVA/Mig (○). ES-DCs expressing OVA alone as controls were ES-DC-OVA/mock (□) and ES-DC-OVA (■). DCs differentiated from the parental OVA gene single-transfectant ES cell clone. Spleen cells of the mice were isolated on day 0, pooled for each group (three to seven mice per group), and assayed for the CTL activity using the same procedure as in Fig. 5. For all effectors, specific lysis was <2% when target EL-4 cells were not prepulsed with OVA peptide. Results were expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of three independent and reproducible experiments.
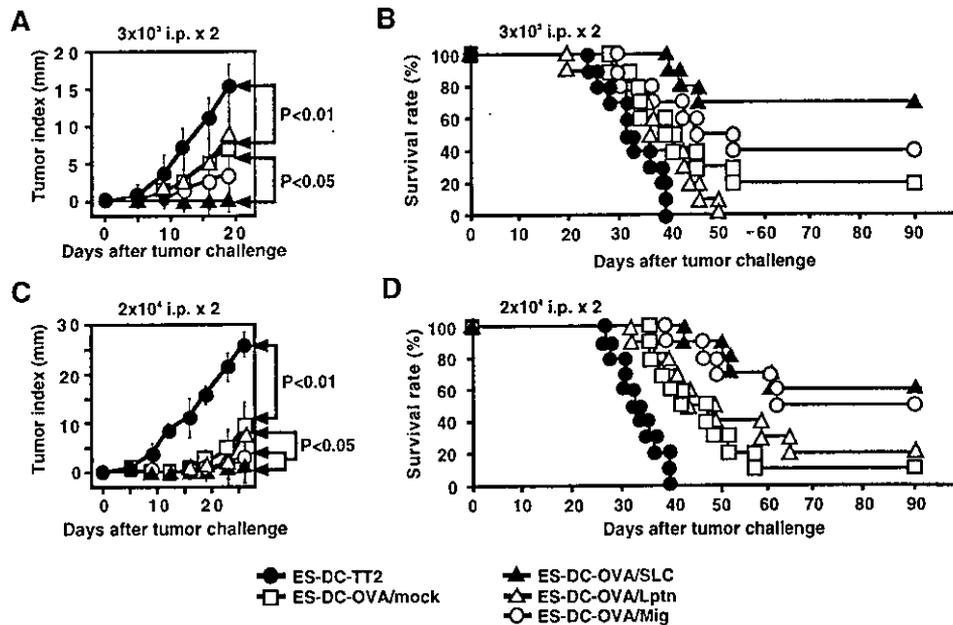
These results clearly demonstrate that coexpression of the chemokines along with Ag in DCs enhances their capacity to prime the Ag-specific CTLs in vivo. The results shown in Fig. 7 also indicate that coexpression of SLC or Lptn in DCs is more effective than that of Mig in the priming of CTLs in vivo.

Protective effects of immunization with chemokine gene-modified DCs against tumor cell challenge

We next asked whether coexpression of chemokine with OVA in DCs would enhance their capacity to induce protective immunity against tumor cells expressing OVA. We immunized mice by twice i.p. transfers of DCs at 7-day intervals, and 7 days after the second transfer, the mice were challenged s.c. with 3×10^5 MO4 cells, OVA-expressing melanoma cells derived from B16. In case of two transfers of 3×10^3 ES-DCs, as shown in Fig. 8A, immunization with ES-DCs expressing OVA alone (ES-DC-OVA/mock) provided significant protection against the MO4 challenge, in comparison with ES-DC-TT2 ($p < 0.01$). Conversely, transfer of ES-DC-TT2 gave no significant protection, compared with no DC transfer (data not shown). Immunization with ES-DC-OVA/SLC provided greater protection than did immunization with ES-DC-OVA/mock ($p < 0.05$). In contrast, protection given by immunization with ES-DC-OVA/Mig or ES-DC-OVA/Lptn was at a comparable level to that provided by ES-DC-OVA/mock. As shown in Fig. 8B, immunization with ES-DC-OVA/mock showed a significant prolongation of survival, compared with immunization with ES-DC-TT2 ($p < 0.05$). Immunization with ES-DC-OVA/SLC resulted in a further prolongation of survival. However, coexpression of Lptn or Mig had no significant additive effect on survival.

In case of twice transfers of 2×10^4 ES-DCs, as shown in Fig. 8C, immunization with ES-DC-OVA/mock provided significant protection against MO4 challenge, compared with ES-DC-TT2 ($p < 0.01$). Under this condition, immunization with ES-DC-OVA/SLC and ES-DC-OVA/Mig provided greater protection than that seen with ES-DC-OVA/mock ($p < 0.05$). In contrast, effect of immunization with ES-DC-OVA/Lptn was comparable to that of

FIGURE 6. Priming of OVA-specific CTLs in vivo by immunization with ES-DC-OVA/Mig. Mice were transferred i.p. twice with ES-DC-OVA or ES-DC-OVA/Mig on days −14 and −7 with 5 × 10⁴ (A), 3 × 10⁴ (B), or 1 × 10⁴ (C) ES-DCs. Spleen cells from transferred mice were harvested on day 0, pooled for each group (three mice per group), and cultured in the presence of OVA$_{257-264}$ (0.1 μM) for 5 days. The resultant cells were assayed for the capacity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide or left unpulsed. Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of three independent and reproducible experiments.

FIGURE 8. Suppression of tumor growth and prolongation of survival by immunization with ES-DCs expressing chemokine along with OVA. Mice were transferred i.p. twice on day -14 and -7 with 3×10^3 (A and B) or 2×10^4 ES-DCs (C and D). The mice were challenged s.c. with 3×10^5 MO4 tumor cells expressing OVA on day 0. Tumor index (A and C) and survival rate (B and D) were monitored. The differences in tumor index between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ($p < 0.01$ and $p < 0.05$, respectively) (A). The differences in survival rates between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ($p < 0.05$) (B). The difference in tumor index between ES-DC-TT2 and ES-DC-OVA/mock is statistically significant ($p < 0.01$) (C). The differences in tumor index between ES-DC-OVA/mock and ES-DC-OVA/Mig as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are also statistically significant ($p < 0.05$) (C). The differences in survival rate between ES-DC-TT2 and ES-DC-OVA/mock as well as between ES-DC-OVA/mock and ES-DC-OVA/SLC are statistically significant ($p < 0.01$) (D). The difference in survival rate between ES-DC-OVA/mock and ES-DC-OVA/Mig is also statistically significant ($p < 0.05$) (D). A and C, Results are expressed as mean tumor index $\pm$ SD ($n = 10$ per group). B and D, Kaplan-Meier plot depicts the survival rate ($n = 10$ per group).

ES-DC-OVA/mock. As shown in Fig. 8D, immunization with ES-DC-OVA/SLC resulted in a longer survival time than that seen with ES-DC-OVA/mock ($p < 0.01$). In addition, ES-DC-OVA/Mig was more effective than ES-DC-OVA/mock ($p < 0.05$), but less effective than ES-DC-OVA/SLC. Immunization with ES-DC-OVA/Lptn again resulted in survival at the same level as seen with ES-DC-OVA/mock. When mice were twice transferred with 2×10^4 ES-DCs and challenged with 3×10^6 MO4 tumor cells, among the three chemokine-expressing ES-DCs, only immunization with ES-DC-OVA/SLC was more effective than ES-DC-OVA/mock (data not shown).

Collectively, ES-DC-OVA/SLC was always more effective than ES-DC-OVA/mock. Expression of Mig in ES-DC increased survival time under some experimental conditions. In contrast, ES-DC-OVA/Lptn did not elicit more protection than did ES-DC-OVA/mock under the conditions we tested. These results suggest that expression of SLC along with antigenic protein is the most effective among the three chemokines for induction of protective immunity against tumor cells expressing the Ag.

No effect of SLC simultaneously injected with ES-DCs

As described, coexpression of SLC along with OVA in ES-DCs enhanced their capacity to induce protective immunity against tumor cells expressing OVA (Fig. 8). To examine the effect of SLC upon simultaneous injection with ES-DCs expressing OVA, we compared immunization with 2×10^4 ES-DC-OVA/SLC to immunization with 2×10^4 ES-DC-OVA/mock accompanying i.p. or systemic (i.v.) injection of recombinant mouse SLC (3 μg). The amount of injected recombinant mouse SLC was much higher than that expected to be produced by injected ES-DC-OVA/SLC after the transfer (Fig. 3B). Transfer of ES-DCs and tumor cell challenge with

3×10^5 MO4 cells were done using the same schedule as previously described. The tumor index in millimeters 30 days after MO4 challenge is shown in Fig. 9. In case of cotransfer of recombinant mouse SLC i.p. or i.v. with ES-DC-OVA/mock, tumor indexes were similar
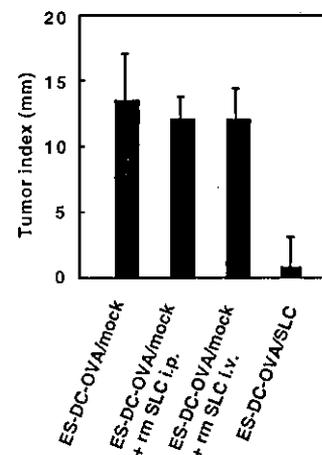
FIGURE 9. No effect of simultaneous injection of recombinant mouse SLC together with ES-DC-OVA. Mice were immunized with ES-DC-OVA/mock (2×10^4/mouse) with or without simultaneous injection of recombinant mouse SLC (3 μg, i.v. or i.p.). Other mice were immunized with ES-DC-OVA/SLC (2×10^4/mouse). Transfers of ES-DCs plus SLC were done twice at a 7-day interval, and 7 days after the second transfer, mice were challenged with 3×10^5 MO4 cells. The tumor index (in millimeters) 30 days after the MO4 challenge was shown. In mice immunized with ES-DC-OVA/SLC, the tumor index was significantly smaller than the others ($p < 0.05$). Results are expressed as mean tumor index $+$ SD ($n = 4$–6 per group).
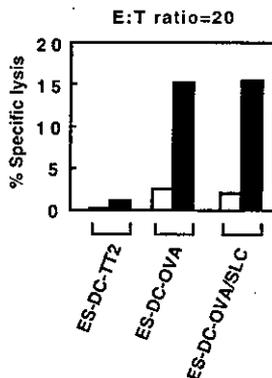
to those in case of immunization with 2×10^4 ES-DC-OVA/mock, indicating that coinjection of recombinant mouse SLC was without effect. In contrast, in case of immunization with ES-DC-OVA/SLC, the tumor index was significantly smaller than those in other conditions ($p < 0.05$), such being consistent with the data shown in Fig. 8.

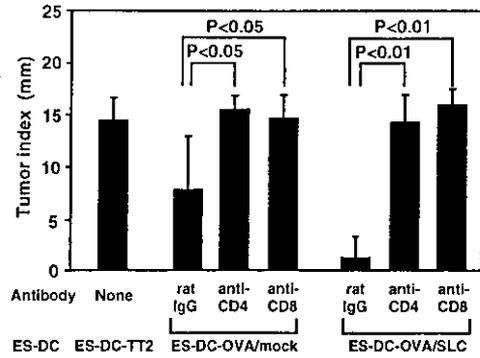### No effect of SLC on survival of ES-DCs and on CTL priming activity of ES-DC in vitro

We tested to see whether the SLC would have any effect on the survival of DCs in vitro. ES-DC-OVA/mock and ES-DC-OVA/SLC were cultured for 7 days. Other ES-DC-OVA/mock were cultured in the presence of recombinant mouse SLC (300 ng/ml). Numbers of recovered ES-DCs after the culture were 77.8%, 88.3%, and 77.7% of the starting cells in case of ES-DC-OVA/mock, ES-DC-OVA/SLC, and ES-DC-OVA/mock plus recombinant mouse SLC, respectively. Dead cells were fewer than 1% of the recovered cells under any conditions. These results indicate that the SLC have no significant effect on the survival of DCs in vitro. In addition, there was no difference in the in vitro CTL-priming capacity between ES-DC-OVA and ES-DC-OVA/SLC (Fig. 10). These results suggest that the enhanced CTL-priming by ES-DC-OVA/SLC observed in case of in vivo injection is not due to the direct effect of SLC on ES-DCs.

Involvement of both CD4+ and CD8+ T cells in protection against MO4 induced by ES-DCs expressing OVA

To determine the role of CD4+ and CD8+ T cells in protection against tumor cells induced by genetically modified ES-DCs, we depleted mice of CD4+ or CD8+ T lymphocytes by treatment with anti-CD4 or anti-CD8 mAb in vivo, respectively. By this treatment, >90% of CD4+ and CD8+ T cells were depleted (data not shown). During this procedure, mice were immunized with ES-DC-OVA/SLC or ES-DC-OVA/mock and challenged with MO4 cells. As shown in Fig. 11, depletion of either CD4+ or CD8+ T cells totally abrogated the protective immunity induced by ES-DC-OVA/SLC or ES-DC-OVA/mock. Although some populations of physiological DCs have been reported to express CD4 or CD8 molecules, the number of CD11c+ splenic DCs did not change with this treatment (data not shown), indicating that the abrogation

FIGURE 11. Involvement of both CD4+ and CD8+ T cells in antitumor immunity induced by ES-DCs. CD4+ or CD8+ T cells were depleted in vivo by inoculation of anti-CD4+ or anti-CD8+ mAbs during immunization with ES-DC-OVA/mock or ES-DC-OVA/SLC. As control, other mice were given i.p. by transfer of ES-DC-TT2. The mice were challenged s.c. with 3×10^6 MO4 tumor cells, and tumor measurements were made 15 days after the tumor cell challenge. In case of immunization with ES-DC-OVA/mock, the differences in tumor index between mice inoculated with rat IgG and those with anti-CD4 mAb as well as between mice inoculated with rat IgG and those with anti-CD8 mAb are statistically significant ($p < 0.05$). In case of immunization with ES-DC-OVA/SLC, the differences in tumor index between mice inoculated with rat IgG and those with anti-CD4 mAb as well as between mice inoculated with rat IgG and those with anti-CD8 mAb are statistically significant ($p < 0.01$). Results were expressed as mean tumor index + SD ($n = 8$ per group).
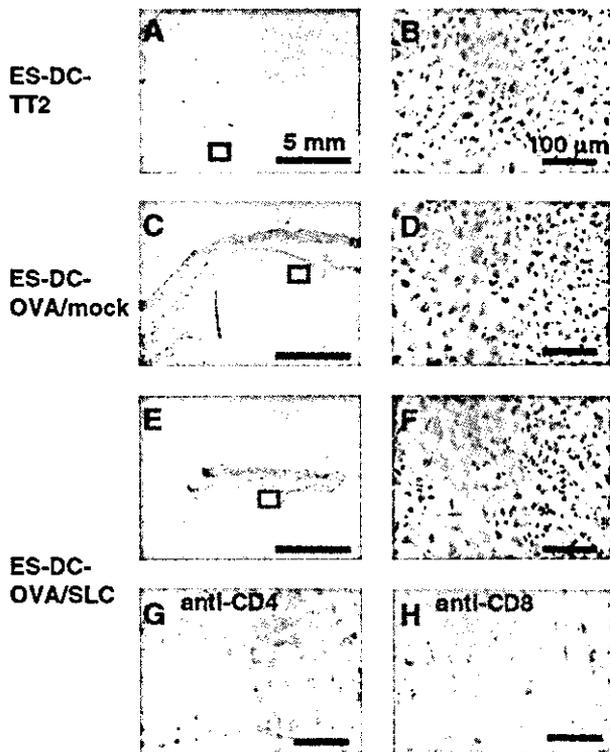
of protective immunity by Ab treatment is due to the depletion of T cells and not due to the effect on endogenous host DCs. These results suggest that both CD4+ and CD8+ T cells play critical roles in antitumor immunity induced by OVA-expressing DCs, regardless of whether or not they coexpress SLC.

We histologically investigated the tumor tissues to search for infiltration of lymphocytes. As shown in Fig. 12, A–F, the size of the tumor in mice immunized with ES-DC-OVA/SLC was much smaller than that of mice immunized with ES-DC-OVA/mock or ES-DC without OVA (ES-DC-TT2). There was a large number of inflammatory cells infiltrating into tumor tissues of mice immunized with ES-DCs expressing OVA, particularly in mice immunized with ES-DC-OVA/SLC. The infiltrating cells consisted of both CD4+ and CD8+ T cells (Fig. 12, G and H). These results also suggest that the antitumor effect induced by ES-DC expressing SLC along with OVA is mediated by both CD4+ and CD8+ T cells.

Discussion

In the present study, we attempted to improve the capacity of in vivo transferred DCs to prime T cells by genetic modification to express a chemokine with a T cell-attracting property. Among the chemokines, we comparatively evaluated the effects of three chemokines, SLC, Mig, and Lptn, not produced by DCs under physiological conditions. For the genetic modification of DCs, we used a method to generate DCs from mouse ES cells. By sequential transfection of ES cells with expression vectors for OVA Ag and for chemokines and by subsequent induction of differentiation to DCs, we generated DCs expressing a chemokine along with OVA.

ES-DCs have a migratory capacity toward lymphoid tissues (Fig. 4) and the capacity is almost comparable to that of BM-DCs. ES-DCs expressing OVA could induce the Ag-specific priming of CTLs both in vivo and in vitro (Fig. 5). ES-DCs expressing OVA could prime OVA-specific CTLs in the absence of IL-2 in vitro, whereas stimulation with CD40 ligand (30) or presence of exogenous IL-2 (our unpublished observations) is essential for BM-DCs to prime Ag-specific CTLs in vitro. Therefore, the capacity of

FIGURE 10. Similar capacity of ES-DC-OVA and ES-DC-OVA/SLC to prime OVA-specific CTLs in vitro. ES-DC-TT2, ES-DC-OVA, and ES-DC-OVA/SLC (4×10^5/well) were cocultured with nylon wool-purified splenic T cells (2.5×10^6/well) of unprimed CBF_1 mice in 24-well culture plates. After 5 days, the cells were harvested and assayed for the capacity to kill EL-4 tumor cells either pulsed with 10 μM OVA peptide (■) or left unpulsed (□). Results are expressed as mean specific lysis of triplicate assays, and SDs of triplicates were <2%. Data are representative of two independent and reproducible experiments.

FIGURE 12. Infiltration of both CD4$^+$ and CD8$^+$ T cells into tumor tissues. Mice were transferred twice with ES-DC-TT2 (A and B), ES-DC-OVA/mock (C and D), or ES-DC-OVA/SLC (E–H). Seven days after the second transfer, mice were challenged with 3 × 10^6 MO4 tumor cells. Twelve days after the tumor cell challenge, frozen sections of tumor tissues were made and stained with H&E (A–F) or immunostained with anti-CD4 (G) or anti-CD8 (H) mAb. F–H, Serial sections are shown. B, D, and F, Enlarged views of the portion indicated in the square of A, C, and E, respectively. Note that size of the tumor in mice immunized with ES-DC-OVA/SLC (E) was much smaller than that of mice immunized with ES-DC-TT2 (A) and ES-DC-OVA/mock (C). Scale bars are 5 mm (A, C, and E) and 100 μm (B, D, F, G, and H).

Ag-expressing ES-DCs to induce CTLs specific to the Ag is no way inferior to BM-DCs. Recently, several reports suggested transfer of Ag or peptide-MHC complexes from adoptively transferred DCs to endogenous host DCs (8, 31). Therefore, it is possible that intrinsic host DCs played some role also in priming of CTLs in our system. However, based on the finding that transfer of heat-killed ES-DC-OVA did not induce priming of CTLs (Fig. 5B), we consider that the OVA-specific CTL-priming in our system mainly depends on the direct action of injected ES-DCs expressing OVA.

Among the three chemokines, expression of SLC was the most effective in eliciting protection against OVA-expressing tumor cells (Fig. 8). However, simultaneous injection of recombinant mouse SLC i.p. or i.v. together with an i.p. injection of DCs expressing OVA had no significant additive effect on protection against tumor (Fig. 9). In addition, SLC had no significant effect on the survival of DCs and CTL-priming capacity in vitro (Fig. 10). These results suggest that the enhanced immunizing effect of ES-DC-OVA/SLC observed with in vivo transfer is not due to the effect of SLC on ES-DCs but rather due to attraction of T cells to the site of transferred ES-DCs, and emphasize the significance of the production of the chemokine by DCs.

We consider that antitumor effects induced by transfer of ES-DCs expressing OVA are primarily mediated by CD4$^+$ and CD8$^+$ T cells reacting to OVA. This notion is supported by findings that

the antitumor effect was abrogated by depletion of either CD4$^+$ or CD8$^+$ T cells by treatment with specific mAbs in vivo (Fig. 11). In addition, immunohistochemical analyses demonstrated obvious infiltration of both CD8$^+$ and CD4$^+$ T cells into tumor tissues in mice immunized with ES-DC-OVA/SLC (Fig. 12). The total abrogation of antitumor effects upon challenge with B16 tumor cells or derivative cells not only by depletion of CD8$^+$ T cells but also by depletion of CD4$^+$ T cells is consistent with reported data (32–34). In addition to providing aid for activation of CD8$^+$ T cells, CD4$^+$ T cells may directly attack B16 or MO4 cells that express MHC class II molecules upon stimulation with IFN-γ (33).

Expression of Lptn in DCs enhanced CTL priming no less effectively than that of Mig. In contrast, expression of Lptn in DCs did not result in any significant enhancement of protection against tumor challenge. This observation is inconsistent with the report by Cao et al. (35) that showed the effect of expression of Lptn in peptide Ag-pulsed DCs on promoting protective antitumor immunity. The discrepancy between their report and ours may be attributed to retention of OVA-specific activated T cells nearby transferred ES-DC-OVA/Lptn in our experiments. Lptn attracts memory or activated rather than naive T cells (36). We consider that, under our experimental conditions, significant numbers of OVA-specific T cells primed with DCs transferred by the first transfer were particularly attracted toward ES-DCs expressing Lptn transferred by the second transfer, which was given 7 days before the tumor challenge, and the T cells could not efficiently migrate to site of the tumor cell inoculation. Although this speculation has not been experimentally verified, the selective attraction of effector/memory T cells by Lptn could be beneficial when we attempt to down-modulate immune responses by genetically modified ES-DCs, aiming at treatment of autoimmune diseases, and allergy or prevention of transplant rejection.

Although it has been demonstrated that SLC gene-introduced and tumor cell lysate-loaded DCs promoted strong antitumor responses (37), ours is the first study to comparatively evaluate effects of three chemokines. We generated DCs expressing chemokine simultaneously with antigenic protein. For induction of antitumor immunity, gene-based Ag-expression by DC is considered superior to peptide, protein, or cell lysate-loading in DC-based immunization. The expression of genes encoding for entire tumor-specific Ags circumvents the need for identification of specific CTL epitopes within the protein (38). Expression of tumor-specific Ags within DCs provides a continuous and renewable supply of Ags for presentation, as opposed to a single pulse of peptides or tumor cell lysates. In fact, in the current study, transfer of genetically modified ES-DCs (3 × 10^3 crossed two times) elicited significant CTL responses and protection against tumor challenge. Numerous tumor-associated Ags have been identified by investigators including us (39–41). We are planning to test antitumor effects of the newly identified natural tumor Ags in in vivo experiments using genetically modified ES-DCs expressing the Ags.

As for the methods for gene transfer to DCs, electroporation, lipofection, and virus vector-mediated transfection have been developed. Many clinical trials using DCs transfected with virus-based vectors are now in progress. However, there are several problems related to the presently used strategies, i.e., efficiency of gene transfer, stability of gene expression, potential risk accompanying the use of virus vectors, and immunogenicity of virus vectors. Although improvements have been made in these methods (42, 43), development of more efficient and safer means is needed. For ES cells, efficient methods for gene-transfer and for isolation of appropriate recombinant cell clones have been established. In the present study, we introduced ES cells sequentially with two

expression vectors containing puromycin-resistant and neo-R genes. It should be feasible to generate more than triple-gene transfectant ES-DCs by sequential or simultaneous transfection with multiple expression vectors, or by using an exchangeable gene-trap system (16, 44). Although formation of teratomas accompanying the transfer of ES cell-derived cells may be anticipated (45), we observed no apparent abnormality, including teratoma formation in mice transferred with ES-DCs 300 days before. When we tested our in vitro differentiation protocol with ES cell lines other than TT2 cells, we observed that DCs can be generated from all of these lines, which included ES cell lines of 129 and C57BL/6 mice origin. We are now planning to generate DCs expressing immunoregulatory molecules along with antigenic proteins, attempting Ag-specific immunosuppression as well as immunostimulation.

A method was established to generate mouse ES cell lines of an appropriate genetic background by nuclear transfer from allogeneic somatic cells to already established ES cell lines (46, 47). Recently, differentiation of hematopoietic cells from human and monkey ES cells has been reported (48, 49). Generation of DCs from human ES cells should also be feasible. With advances in the ES cell-related technologies, immunomodulation by genetically engineered ES-DCs may be applied to the treatment of autoimmune diseases and allergy, prevention of rejection of transplanted organs, and antitumor immunotherapy.

Acknowledgments

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Glypican-3, overexpressed specifically in human hepatocellular carcinoma, is a novel tumor marker ☆

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Abstract

With the global pandemic of hepatitis B and C infections, the incidence of Hepatocellular carcinoma (HCC) is rapidly increasing world wide. We identified glypican-3 (GPC3), a novel oncofetal gene over-expressed specifically in human HCC, as based on data of cDNA microarrays. As GPC3 is a GPI-anchored membrane protein and could be secreted, we attempted to detect secreted GPC3 protein in sera from HCC patients using Western blotting and ELISA. GPC3 protein was positive in sera of 40.0% (16/40) of HCC patients, and negative in sera from subjects with liver cirrhosis (LC) (0/13), chronic hepatitis (CH) (0/34), and healthy donors (0/60). All subjects were Japanese. Although 12 of 40 HCC patients were negative for both α-fetoprotein (AFP) and PIVKA-II well known tumor markers of HCC, four of these were GPC3-positive in the sera. We also observed vanishing GPC3 protein in the sera of three patients after the surgical treatment for HCC. On the other hand, immunohistochemical analysis revealed that HCC expressed GPC3 protein in all 14 HCC patients tested. In conclusion, GPC3, as defined in this study was shown to be a useful tumor marker for cancer-diagnosis for large numbers of patients with HCC.
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Keywords: Glypican-3; Hepatocellular carcinoma; Tumor marker; cDNA microarray; Oncofetal protein

Primary hepatocellular carcinoma (HCC) is one of the most common malignancies in the world. Because of the global pandemic of hepatitis B and C viral infections, the incidence of HCC is rapidly increasing in Asian and Western countries [1,2], and this trend is expected to continue for the next 50 years because of the long latency between infection and the development of HCC. The prognosis of advanced HCC remains poor,

and novel treatment and diagnosis strategies are urgently needed.

cDNA microarray technology, by which investigators can obtain comprehensive data with respect to gene-expression profiles, is rapidly progressing. Several studies have demonstrated the usefulness of this technique for identification of novel cancer-associated genes and for classification of human cancers at the molecular level [3]. We identified genes of which expression was altered during hepatocarcinogenesis in 20 subjects with primary HCCs through the use of a genome-wide cDNA microarray containing 23,040 genes [4].

In the present work, we identified glypican-3 (GPC3) over-expressed specifically in human hepatocellular carcinoma, as based on cDNA microarray data. We

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☆ Abbreviations: HCC, hepatocellular carcinoma; GPC3, glypican-3; HD, healthy donor; LC, liver cirrhosis; CH, chronic hepatitis; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; AFP, α-fetoprotein; PIVKA-II, protein induced by vitamin K absence or antagonist-II.
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detected soluble GPC3 protein in sera of HCC patients, but not in case of other liver diseases or other cancers. We propose that GPC3 is a novel tumor marker for HCC.

Materials and methods

cDNA microarrays. Profiling of gene expression by cDNA microarrays was done, as reported [4]. Primary HCCs and corresponding non-cancerous liver tissues were obtained with informed consent from 20 Japanese patients who underwent hepatectomy in the Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine. These specimens were used only for cDNA microarray analysis, as reported [4]. Poly(A)$^+$ RNAs isolated from human bone marrow, brain, heart, kidney, liver, lung, mammary gland, pancreas, placenta, prostate, skeletal muscle, small intestine, spleen, stomach, testis, thymus, thyroid, uterus, fetal brain, fetal kidney, fetal liver, fetal lung (Clontech), colon, and ovary (Biochain) were used as probes for the cDNA microarray [5].

HCC tissues, blood samples, and cell lines. After obtaining informed written consent, we independently obtained tissue and blood samples at random from HCC patients and from various donors treated in the Department of Surgery II, and the Third Department of Internal Medicine, Kumamoto University School of Medicine.

We collected patient profiles from medical records to determine the clinical stages, according to the UICC TNM classification (Table 1). Hep G2, Hep 3B, PLC/PRF/5, and HuH-7 were kindly provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer Tohoku University, and SK-Hep-1 was provided by Dr. K. Itoh of Kurume University, Kurume, Japan. Hep G2, Hep 3B, PLC/PRF/5, and HuH-7 were cultured in DMEM supplemented with 10% FCS, and SK-Hep-1 was cultured in RPMI1640 supplemented with 10% FCS.

RT-PCR. RT-PCR was done, as described [6]. We designed GPC3 gene-specific PCR primers to amplify fragments of 939 bp and used RT-PCRs consisting of initial denaturation at 94 °C for 5 min and 30 amplification cycles at an annealing temperature of 58 °C. GPC3 PCR primer sequences were: sense, 5'-GTTACTGCAATGTGGTCATGC-

3' and antisense, 5'-CTGGTGCCCAGCACATGT-3'. β-Actin: sense, 5'-CCTCGCCTTTGCCGATCC-3' and antisense, 5'-GGATCTTC ATGAGGTAGTCAGTC-3'. After normalization by β-actin mRNA, as a control, we compared the expression of GPC3 mRNA in HCC tissues and cell lines.

Western immunoblot analysis. Cell samples were lysed in appropriate amounts of lysing buffer 150 mM NaCl, 50 mM Tris, pH 7.4, 1% Nonidet P-40, 1 mM sodium orthovanadate (Wako, Osaka, Japan), 1 mM EDTA, plus a protease inhibitor tablet (Amersham, Arlington Heights, IL). Supernatant fluids of the lysates were electrophoresed on SDS-PAGE gels and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). After blocking with 5% skimmed milk and 0.2% Tween 20 in Tris-buffered saline, the membrane was incubated with the anti-GPC3, rabbit polyclonal antibody raised against a recombinant protein corresponding to human GPC3 303-464 (Santa Cruz, California), washed extensively with PBS, and subjected to chemiluminescence detection using peroxidase-conjugated anti-rabbit Ig, horseradish peroxidase linked F(ab')$_2$ fragment (from donkey) (Amersham), using an ECL kit (Amersham). We purchased GPC3 303-464 produced in *Escherichia coli* as a 45 kDa tagged fusion protein (Santa Cruz, CA) and this protein was added in SDS-PAGE loading buffer to serve as a positive control.

Immunohistochemical examination and ELISA. Immunohistochemical examinations were done, as described [5]. We stained 4-μm-thick sections of formalin-fixed and paraffin-embedded tissue samples with anti-GPC3 Ab at a dilution of 1:200. To set up ELISA detection of GPC3 in sera from patients and healthy donors, we purchased FluoReporter Mini-Biotin-XX Protein Labeling Kits (F-6347) (Molecular Probes, Eugene) for biotinylating anti-glypican-3, rabbit polyclonal antibody. The 96-well ELISA plates (Nunc, Denmark) were coated overnight at 4 °C with 0.1 μg/well anti-human GPC3 303-464 (Santa Cruz) in PBS, pH 7.4. Then, the plates were blocked with Block Ace (Dainippon pharmaceutical, Osaka) for 1 h at room temperature. Serum samples from patients and healthy donors were diluted at 1:200 with 10× Block Ace to serve as samples for ELISA. We added standard samples of positive control and culture supernatants with biotinylated anti-GPC3 Ab, followed by incubation for 2 h at room temperature. After washing three times with PBS, HRP-Conjugated Streptavidin (ENDOGEN, Woburn) was added to each well. After 30 min of incubation, the plates were washed three times with PBS and TMB Substrate Solution (ENDOGEN) was added. We then used an

Table 1
Profiles of serum donors and detection of GPC3 using ELISA

Disease	Mean age (years)	Sex		Virus-positive donor[a]			UICC stage[b]				GPC3 positive rate
		M	F	HCV	HBV	Non-B–non-C	I	II	III	IV	
HCC	66	36	4	27	8	6	1	15	14	10	·16/40 (40%)
Liver cirrhosis	65	6	7	8	4	1					0/13 (0%)
Chronic hepatitis	60	15	19	31	3	0					0/34 (0%)
Autoimmune hepatitis	65	0	2								0/2 (0%)
Primary biliary cirrhosis	79	0	1								0/1 (0%)
Healthy donors	40	25	35								0/60 (0%)
Cancers											
Colon	66	16	- 5				1	6	5	9	0/21 (0%)
Gastric	71	9	5				7	3	4	0	0/14 (0%)
Pancreatic.	58	6	5				0	0	0	11	0/11 (0%)
Biliary	70	2	4				0	3	1	2	0/6 (0%)
Esophageal	59	6	0				1	0	2	3	0/6 (0%)
Lung	64	7	0				3	0	0	4	0/7 (0%)
Breast	50	0	10				4	2	2	2	0/10 (0%)

[a] HCV was detected using RT-PCR. HBsAg was examined using radioimmunoassays.
[b] International Union Against Cancer Classification; TNM Classification of malignant tumors.

ELISA reader (model 550, Bio-Rad) at 450 nm for measurement of optical density (OD).

Results

Identification of the GPC3 gene over-expressed specifically in HCC

We obtained data comparing expression profiles between 20 HCCs (10 cases were hepatitis B virus (HBV)-positive and 10 were hepatitis C virus (HCV)-positive) and their corresponding non-cancerous liver tissues [4] and in various normal human tissues [6], using cDNA microarrays. We then searched for genes over-expressed specifically in HCC using these data and we identified GPC3. In 16 cases of the 20 HCCs, the expression of GPC3 mRNA in the cancer tissue was 5 or more times higher than that in non-cancerous tissues (Fig. 1A). GPC3 is an over-expressed gene in most HCCs and is not related to HBV or to HCV viral infection. GPC3 mRNA is highly expressed in the placenta, fetal liver, fetal lung, and fetal kidney and is low in most adult normal tissues (Fig. 1A). Data on GPC3 have been published by other investigators, as based on Northern blotting studies [7,8]. Thus, like AFP, GPC3 is a novel onco-fetal antigen in HCC.

Expression of GPC3 mRNA in human HCC

We examined GPC3 mRNA expression using RT-PCR. HCC tumor of 6 patients, 37, 35, 34, 38, 42, and 43 among 7 patients tested (85.7%), showed a much stronger expression than did non-cancerous liver tissue in these patients. The HCC tumor of patient 41 showed no such expression (Fig. 1B). Hep G2, Hep 3B, and HuH-7 HCC cell lines showed a stronger expression of GPC3 mRNA than PLC/PRF/5 while, SK-Hep-1 showed no such expression (Fig. 1C).

The presence of soluble GPC3 protein in culture supernatants of HCC cell lines and sera from HCC patients

As GPC3 is a GPI-anchored membrane protein and could be secreted, we next attempted to detect secreted GPC3 protein. We used Western blotting techniques for Hep G2 cell lysates and the culture supernatant harvested after the indicated culture period to gain support for the existence of soluble GPC3 protein in culture supernatants of Hep G2. Hep G2 cell lysates prepared from 1×10^5 cells after cultivation for 6, 12, 24, and 48 h (lanes 1, 3, 5, and 7 in Fig. 2A) in serum-free medium showed similar amounts of 60 kDa GPC3 protein. On the other hand, Hep G2 culture supernatants (20 μl of 1 ml/well) after cultivation for 6, 12, 24, and 48 h (lane 2, 4, 6, and 8) showed a gradual increase in 60 kDa GPC3
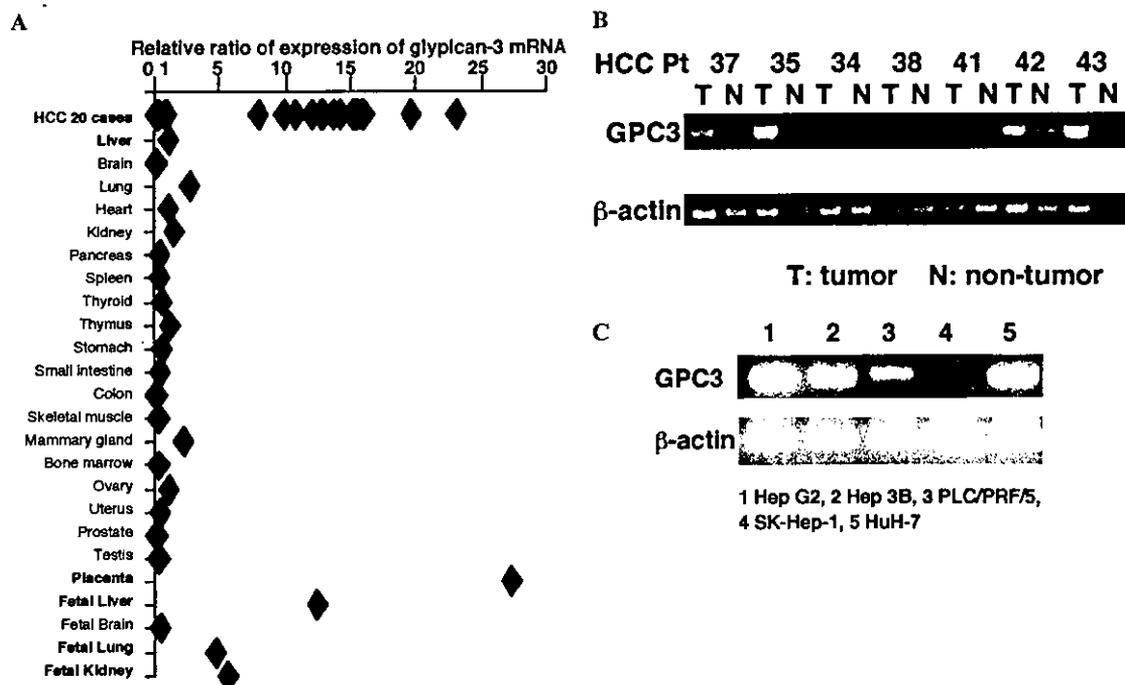
Fig. 1. HCC-specific expression of GPC3 mRNA. (A) The relative ratio (RR) of expression of human GPC3 mRNA in 20 HCC patients and in disease-free tissues. RR in HCC show cancerous tissue versus adjacent non-cancerous liver tissue intensity ratio in each case. RR in disease-free tissues show each disease-free tissue versus disease-free liver intensity ratio. (B) Expression of GPC3 mRNA detected using RT-PCR in human HCC tissues. (C) Expression of GPC3 mRNA detected using RT-PCR in human HCC cell lines.

A

culture time

| 6h | 12h | 24h | 48h |

1 2 3 4 5 6 7 8 9

60kDa ━━━━━━━━━━━━━━━━━━━━ GPC3

cell sup. cell sup. cell sup. cell sup. med.

B

M: Marker 220, 97, 66, and 45kD,
Sera of 1: Pt 4, 2: Pt 5, 3: Pt 7, 4:HD 54, 5: HD 55, 6: HD 56,
7: HD 57,
8: Positive control (GPC3 303-464),
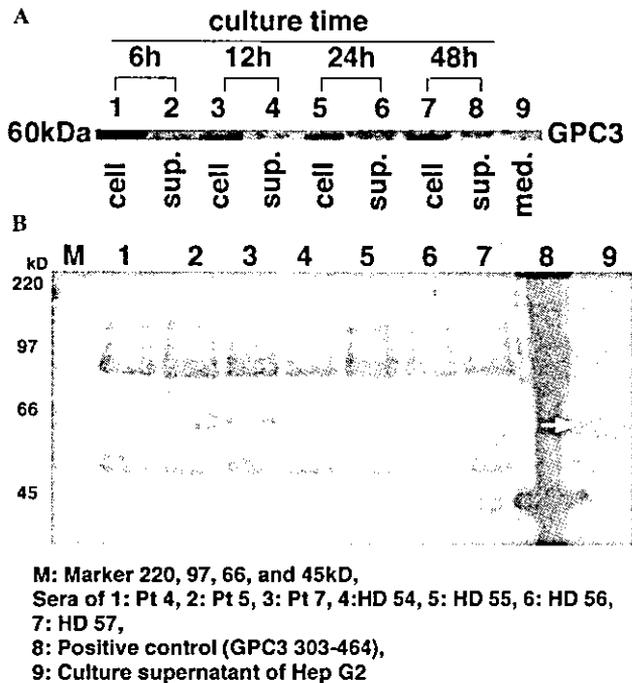9: Culture supernatant of Hep G2

Fig. 2. Evidence for secretion of GPC3 from human HCC. (A) Evidence for the presence of GPC3 protein in the culture supernatant of Hep G2 as based on Western blots. Lanes 1, 3, 5, and 7: lysate of 1×10^5 Hep G2 cells after cultivation for 6, 12, 24, and 48 h. Lanes 2, 4, 6, and 8: 20 µl of Hep G2 culture supernatant after cultivation for 6, 12, 24, and 48 h. (B) Evidence for soluble GPC3 protein in sera from HCC patients. Arrows indicate bands of GPC3 protein. Lane 8: positive control, 45 kDa GPC3 303-464. Lane 3: 20 µl of sera of Pt. 7. Lane 9: culture supernatant of Hep G2.

protein, that is, GPC3 protein was indeed secreted from Hep G2 into the culture supernatant.

We then searched for soluble GPC3 protein by Western blotting of sera of three HCC patients and four healthy donors (HDs). We detected the band of positive control, 45 kDa GPC3 303-464 (lane 8 in Fig. 2B), and detected the band of 60 kDa GPC3 protein in 20 µl of sera from Pt 7 (lane 3 in Fig. 2B) and in culture supernatant of Hep G2 (lane 9 in Fig. 2B), but not in sera from two other HCC patients (patients 4 and 5) or from four healthy donors (HDs 54–57).

We next detected soluble GPC3 using ELISA. We defined the concentration of GPC3 protein in the 1ml of the culture supernatant of 1×10^5 Hep G2 cells after cultivation for 24 h as 1 U/ml. The amount of GPC3 protein in the culture supernatant of the Hep G2, PLC/PRF/5, and HuH-7 was much larger than that of the Hep 3B, and that of the SK-Hep-1 was not detected (Fig. 3A), although the amount of GPC3 mRNA of the Hep 3B was much larger than that of the PLC/PRF/5 (Fig. 1C). Thus, there was some discrepancy between the expression levels of GPC3 mRNA in HCC cells and the amount of GPC3 protein secreted into the culture supernatant.

The quantification by ELISA of GPC3 protein in sera of 40 HCC, 13 LC, 34 CH, and other patients and of 60 HDs is indicated in Figs. 3B and C, Tables 1 and 2. As we did not have recombinant GPC3 protein useful for a positive control for this ELISA system, serially diluted culture supernatant of Hep G2 was used to estimate the
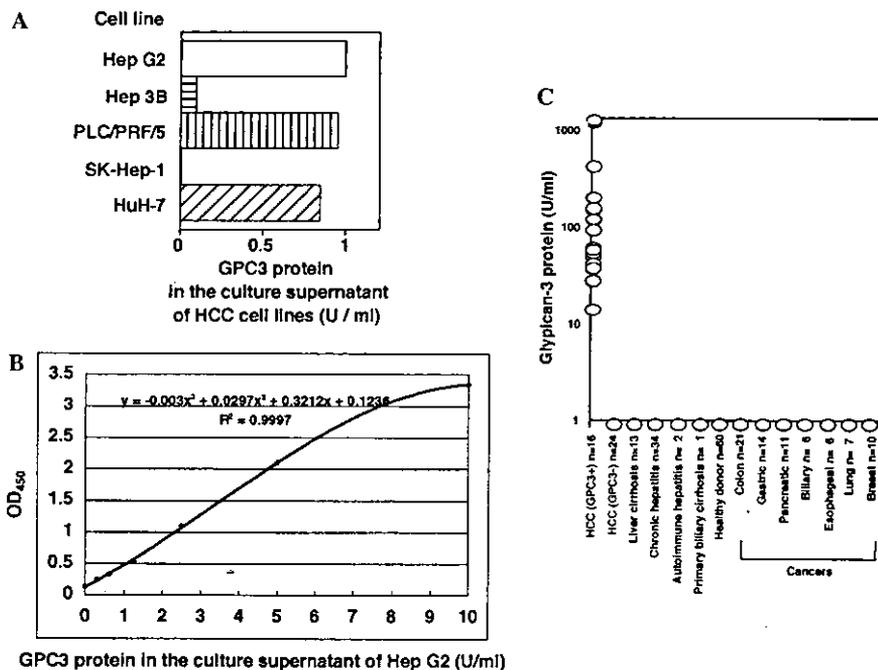
Fig. 3. Quantification of GPC3 protein using ELISA. (A) Quantification of GPC3 protein secreted in the culture supernatant of HCC cell lines. We defined the concentration of GPC3 protein in 1 ml of the culture supernatant of 1×10^5 Hep G2 cells after cultivation for 24 h to be 1 U/ml. (B) Standard curve to quantify the GPC3 protein based on OD data. Serially diluted culture supernatant of Hep G2 was used to estimate the standard curve. (C) Quantification of GPC3 protein in sera from 40 HCC patients and other patients with liver diseases or other cancers and healthy donors.

Table 2
Profiles of 40 Japanese patients with HCC and quantification of AFP, PIVKA-II, and GPC3 in sera of these patients

Pt ID	Age (years), sex	Virus[a]	UICC stage	AFP (ng/ml)[b] (<20)[c]	PIVKA-II (mAU/ml)[d] (<40)	GPC3 (U/ml) (<10)
3	56, M	HBV	IVA	54[c]	957	51
5	71, M	HCV	IIIB	8900	31,577	15
11	69, M	Non-B, non-C	IVA	9400	319	215
32	71, M	Non-B, non-C	II	30	508	30
1	64, M	HCV	IIIA	50	15	448
2	53, M	HCV	II	45	38	162
8	69, M	HCV	III	21	21	99
39	78, M	HCV	IIIA	94	25	45
9	73, M	HCV	IIIA	25	242	–
12	61, M	HCV	IVA	349	169	–
17	70, M	HBV	IVA	56	133	–
18	71, F	HBV	II	930	994	–
19	77, M	HCV	II	163	96	–
24	50, M	HCV	II	29	41	–
26	63, M	HBV	IVA	5280	1549	–
38	60, M	HCV	II	16,200	3556	–
4	69, M	HCV	IIIA	178	28	–
15	67, M	HCV	II	100	32	–
22	72, M	HCV	IIIA	25	12	–
33	60, M	HCV	IIIA	2030	14	–
7	62, M	HCV	IVA	10	239	1301
16	72, M	HCV	II	<1	840	130
30	75, M	HCV	II	5	102	166
37	59, M	Non-B, non-C	II	3	707	65
14	62, M	HCV	II	3	69	–
20	75, F	Non-B, non-C	IIIA	<1	63	–
27	71, M	HCV	IVA	10	19,288	–
35	52, M	HBV	II	13	431	–
6	72, F	HCV	II	9	20	58
25	63, M	HCV	IVB	<1	<10	1349
29	56, M	Non-B, non-C	II	<1	22	62
40	58, M	HBV	II	3	11	40
10	58, M	Non-B, non-C	IIIA	3	18	–
13	69, M	HCV	IVB	6	<10	–
21	71, M	HBV, HCV	IVA	2	26	–
23	59, M	HBV	IIIA	3	18	–
28	69, M	HCV	IIIA	<1	28	–
31	69, M	HCV	IIIA	17	13	–
34	63, F	HCV	I	4	14	–
36	61, M	HCV	IIIB	13	29	–

[a] HCV was detected using RT-PCR. HBsAg was examined using radioimmunoassay.
[b] AFP was quantified using radioimmunoassay.
[c] Values in parentheses represent cut-off value.
[d] PIVKA-II was quantified using enzyme immunoassays.
[e] Positive values are underlined.

standard curve to quantify the GPC3 protein based on OD data (Fig. 3B). We detected and quantified GPC3 protein in the sera of 16 of 40 HCC patients, but not in sera of patients with liver cirrhosis (LC), chronic hepatitis (CH), autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), HD, and another kinds of cancers. Fig. 3B shows the standard curve for ELISA-detection of GPC3 that guarantees the quality of this ELISA. Ac-cording to these data, we were convinced that the lowest limit for detection of serum GPC3 was 10 U/ml by using 200× diluted serum samples and we determined more than 10 U/ml to be positive. The evidence that our ELISA system detected soluble GPC3 in culture super-natant of NIH3T3 transfected with mouse GPC3 gene but not in that of wild type NIH3T3 cells also supports the accuracy of ELISA (data not shown).

The prevalence of GPC3 protein in the sera of HCC patients was significantly higher than that in other donors ($P < 0.0001$). The GPC3 evaluated by ELISA for patients 4, 5, and 7 is given in Fig. 2B and was 0, 15, and 1301 U/ml, respectively (Table 2). Namely, 1301 U/ml of

GPC3 protein was detectable and 15 U/ml of GPC3 was not detectable using Western blotting techniques. Although as shown in Fig. 1B, HCC cells of Pt. 35 showed much stronger expression of GPC3 mRNA than did those of Pts. 37, 34, and 38, serum GPC3 was detected

Table 3
Lack of correlation between serum GPC3 levels and injury of hepatocytes or liver function in 40 Japanese patients with HCC

Pt ID	GPC3 (U/ml)	Injury of hepatocytes		Liver function				
		AST[a] (IU/l) (<40)[b]	ALT[c] (IU/l) (<40)	Ascites	T-Bil[d] (mg/dl) (<1.0)	Alb[e] (g/dl) (>4.0)	ICGR$_{15}$ (%)[f] (<15)	PT[g] (%) (>70)
25	1349[h]	38	36	−	1.0	3.7	37.0	68
7	1301	104	53	+	1.2	3.7	43.0	65
1	448	138	166	−	1.0	3.5	31.3	90
11	215	22	14	−	0.6	3.7	12.7	97
30	166	60	59	−	0.7	3.6	15.1	85
2	162	47	68	−	1.5	4.6	6.8	82
16	130	18	9	−	0.4	3.8	9.8	91
8	99	96	59	−	1.0	4.2	23.9	99
37	65	26	34	−	0.6	4.3	9.2	94
29	62	23	9	−	0.8	4.6	8.6	110
6	58	55	60	−	1.1	3.7	18.4	92
3	51	341	157	−	1.1	4.0	33.9	96
39	45	63	50	−	1.4	3.3	45.4	82
40	40	23	25	−	0.7	4.1	11.8	91
32	30	44	47	+	1.1	3.8	20.4	83
5	15	65	46	−	0.9	3.2	22.5	81
	Positive rate	10/16 (62.5%)	10/16 (62.5%)	2/16 (12.5%)	6/16 (37.5%)	10/16 (62.5%)	10/16 (62.5%)	2/16 (12.5%)
4	—	36	20	−	1.2	3.0	40.6	77
9	—	101	70	−	0.8	2.6	34.3	85
10	—	37	57	−	1.0	4.2	12.9	104
12	—	62	49	−	1.1	3.7	28.7	77
13	—	163	72	−	0.4	2.1	33.6	90
14	—	55	21	−	1.2	3.3	50.5	69
15	—	39	37	−	1.3	3.1	39.0	59
17	—	62	74	−	0.6	3.7	24.9	77
18	—	29	22	−	0.6	3.6	27.9	86
19	—	23	10	−	1.6	2.8	13.9	66
20	—	55	29	+	1.1	3.5	47.0	66
21	—	47	37	−	1.0	3.2	36.4	85
22	—	78	56	+	1.0	2.9	54.0	77
23	—	37	57	−	1.0	4.2	12.9	104
24	—	49	41	−	0.8	3.9	16.0	97
26	—	19	20	−	0.7	3.7	12.4	87
27	—	38	36	−	1.2	3.0	48.1	84
28	—	54	52	−	1.0	3.7	14.5	85
31	—	56	50	−	1.5	3.1	21.7	85
33	—	52	55	+	1.1	3.0	41.4	77
34	—	29	32	−	1.6	4.6	10.9	90
35	—	32	51	−	0.5	4.1	14.5	89
36	—	67	70	−	0.4	4.0	22.0	87
38	—	64	89	−	1.0	4.3	17.2	96
	Positive rate	14/24 (58.3%)	14/24 (58.3%)	3/24 (12.5%)	10/24 (41.7%)	17/24 (70.8%)	17/24 (70.8%)	4/24 (16.7%)

[a] Serum levels of aspartate transaminase.
[b] Values in parentheses represent cut-off value.
[c] Serum levels of alanine transaminase.
[d] Serum levels of total bilirubin.
[e] Serum levels of albumin.
[f] Indocyanine green retention at 15 min level.
[g] Prothrombin test.
[h] Positive values are underlined.

only in Pt. 37 suggesting that there was no correlation between serum GPC3 concentrations and mRNA expressions of GPC3 in the cancer tissues. We propose that GPC3 may be a novel tumor marker for HCC. There was no correlation in the positive state of tumor markers among the three markers, α-fetoprotein (AFP), protein induced by vitamin K absence or antagonist-II (PIVKA-II), and GPC3 (Table 2). Although 12 patients were negative for both AFP and PIVKA-II, four patients (6, 25, 29, and 40) of 12 were GPC3-positive, and HCC Pts 6, 29, and 40 were classified as a relatively early UICC Stage II (Table 2).

Lack of correlation between serum concentrations of GPC3 and the activity of hepatitis or liver function

As shown in Fig. 1A, GPC3 mRNA expression was found in fetal liver as well as in placenta, suggesting that regenerating hepatocytes in injured liver may express GPC3. To investigate this possibility, relationship between serum concentrations of GPC3 and injury to hepatocytes or liver function were assessed in patients with HCC (Table 3). Serum levels of aspartate transaminase (AST) and alanine transaminase (ALT) are good indexes for injury to hepatocytes. The presence of ascites, serum levels of total bilirubin (T-Bil) and albumin (Alb), indocyanine green retention at 15 min level (ICGR$_{15}$ (%)), and prothrombin test (PT (%)) were chosen as indexes of liver function. There was no significant difference in positive rates of abnormalities in these factors between 16 serum GPC3-positive patients and 24 negative patients. Furthermore, GPC3 was not detected in the sera of any patient with active CH, and serum concentrations of GPC3 were not increased when regeneration of liver occurred after surgical resection of HCC. These data clearly show no correlation between serum concentrations of GPC3 and the activity of hepatitis or liver function.

GPC3 protein in the sera of HCC patients disappeared after surgical treatments

Changes in serum levels of three tumor markers, AFP, PIVKA-II, and GPC3, and tumor masses detected by computed tomography (CT) before and after surgical treatments for HCC in three patients (Pts. 30, 40, and 37) are shown in Fig. 4. GPC3 protein was detectable in these three patients prior to surgery, but GPC3 was not detectable after the surgical treatments for patients with HCC. It should be noted that GPC3 was the only useful tumor marker for Pt. 40.

Expression of GPC3 protein in human HCC tissues

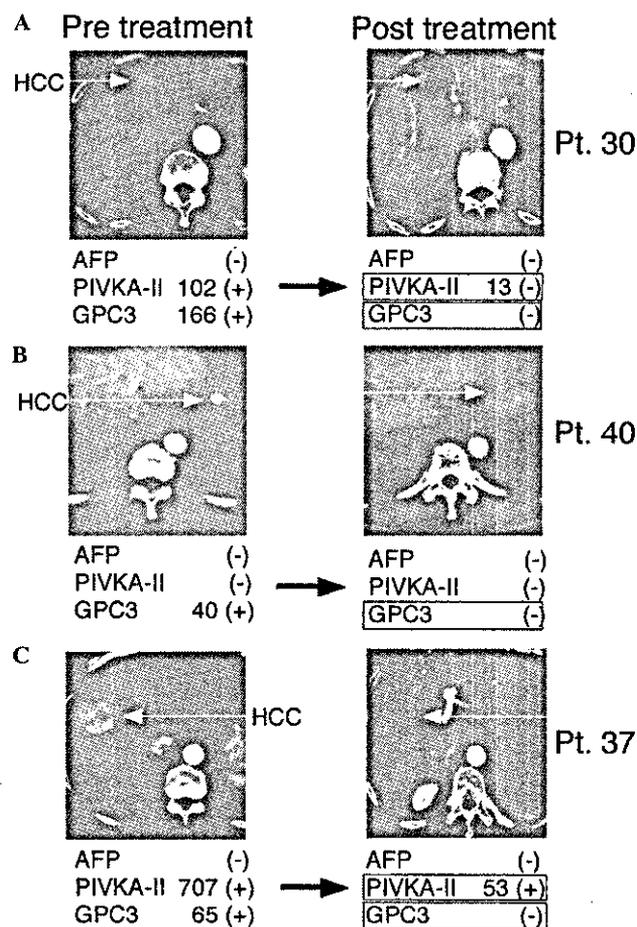We made an immunohistochemical analysis of GPC3 in HCC and non-cancerous liver tissue surrounding

Fig. 4. Disappearance of soluble GPC3 in patients' sera after the surgical treatments of HCC. Computed tomography (CT) of HCC lesion and serum levels of three kinds of tumor markers, AFP, PIVKA-II, and GPC3 before and after surgical treatments are indicated for patients 30 (A), 40 (B), and 37 (C).

HCC excised from 14 patients with HCC. Seven patients (Pts. 2, 6, 7, 8, 11, 29, and 37) were positive for serum GPC3 (secreting type) and other seven were negative (non-secreting type; Pts. 10, 12, 17, 23, 26, 34, and 35). The expression of GPC3 protein in seven tumors derived from secreting type patients was divided into two patterns. Secreting type-1 pattern (Pts. 2, 7, and 11) showed a much stronger expression of GPC3 protein in HCC cells than in non-cancerous liver cells (Fig. 5). Secreting type-2 pattern (Pts. 6, 8, 29, and 37) showed weak expression of GPC3 protein in HCC cells and showed some expression in non-cancerous liver tissue (Fig. 5). Because GPC3 mRNA isolated from tumor of Pt. 37 showed a higher expression than did that of non-cancerous liver tissue, it was thought that the majority of GPC3 protein in this type of HCC cells was almost secreted away. On the other hand, all seven non-secreting type tumors showed moderate expression of GPC3 protein with a speckled pattern in HCC cells (Fig. 5).
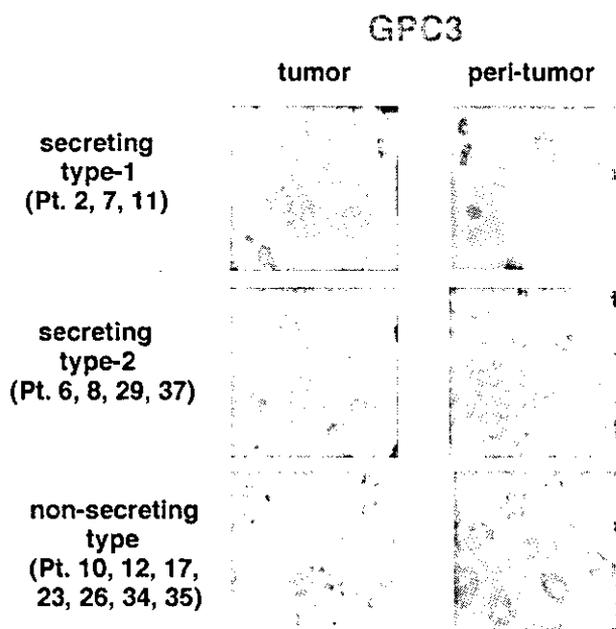
GPC3

Fig. 5. Immunohistochemical analysis of expression pattern of GPC3 protein in HCC cells. GPC3 immunostaining (colored brown) of sections of tumor (left) and of peri-tumor (right) was indicated at objective magnifications; 400×.

Discussion

In 1996, Pilia et al. reported that *GPC3*, which encodes one member of the glypican family, is mutated in patients with Simpson–Golabi–Behmel syndrome (SGBS) [9]. SGBS is an X-linked disorder characterized by pre- and postnatal overgrowth, and a broad spectrum of clinical manifestations which vary from a very mild phenotype in carrier females to infantile lethal forms in some males [10]. The list of clinical manifestations of SGBS includes a distinct facial appearance, cleft palate, syndactyly, polydactyly, supernumerary nipples, cystic and dysplastic kidneys, congenital heart defects, and so on [11–14]. Most GPC3 mutations are point mutations or small deletions encompassing a varying number of exons [15,16]. Given the lack of correlation between patient phenotype and location of the mutations, it has been proposed that SGBS is caused by the lack of a functional GPC3 protein, with additional genetic factors being responsible for the intra- and interfamilial phenotypic variation [15]. The development of GPC3-deficient mice added a strong support for this hypothesis [17], these mice have several abnormalities found in SGBS patients, including overgrowth, and cystic and dysplastic kidneys.

Because GPC3 is an inhibitor of cell proliferation and can induce apoptosis in certain types of tumor cells [18], reports indicating that GPC3 expression is down-regulated in tumors of different origin were not surprising. Lin et al. [19] showed that, although GPC3 is expressed in the normal ovary, the expression is undetectable in any significant proportion of ovarian cancer cell lines. In all cases where GPC3 expression was lost, the GPC3 promoter was hypermethylated, and mutations were nil in the coding region. GPC3 expression was restored by treatment with a demethylating agent. In addition, the authors demonstrated that ectopic expression of GPC3 inhibits colony-forming activity in several ovarian cancer cell lines. Other data associating GPC3 with cancer were derived from a differential mRNA display study on normal rat mesothelial cells and mesothelioma cell lines [20]. In this study GPC3 was consistently down-regulated in tumor cell lines and a similar down-regulation was noted in primary rat mesotheliomas and in cell lines derived from human mesotheliomas. Similar to cases of ovarian cancer, mutations in the GPC3 coding sequence have not been found, but most cell lines had an aberrant methylation in the GPC3 promoter region. As reported [18], the study showed that ectopic expression of GPC3 in mesothelioma cell lines inhibits colony-forming activity. Xiang et al. [21] reported that GPC3 expression was also silenced in cases of human breast cancer. Collectively, these data suggest that GPC3 can act as a negative regulator of growth in these cancers. Insomuch as the expression of GPC3 is reduced during tumor progression in cancers originating from tissues that are GPC3-positive in adults and this reduction seems to play a role in generation of the malignant phenotype.

On the contrary, in the case of HCC, tumors originating from tissues that express GPC3 only in the embryo, GPC3 expression tends to reappear with malignant transformation. In this study, more than 80% of 27 HCC tumors showed a much stronger expression of GPC3 mRNA than did non-cancerous liver tissue, and immunohistochemical analysis revealed that HCC expressed GPC3 protein in all 14 HCC patients tested. On the other hand, GPC3 protein was positive in sera of 40.0% (16/40) of HCC patients. There was a discrepancy between GPC3 expression and GPC3 secretion. We could classify three GPC3 protein expression patterns in HCC (secreting type1, 2, and non-secreting type). Further investigations are needed to determine why serum GPC3 was detected in only 40% of our HCC patients. Furthermore, whether GPC3 re-expression plays a role in progression of these tumors is unknown. During the last few years it has been clearly established that cell-surface heparan sulfate proteoglycans (HSPGs) are required for the optimal activity of heparin-binding growth factors, such as fibroblast growth factors (FGFs) and Wnts [22,23]. Glypicans are a family of GPI-anchored cell surface HSPGs. We speculate that tissue-specific differences in the relationship between oncogenesis and the expression level of GPC3 are due to the fact that GPC3 may regulate growth and survival factors differently in each tissue.

GPC3 seems to behave in these organs, at least as an oncofetal protein. In general, oncofetal proteins do not seem to play a critical role in tumor progression but have been used as tumor markers or as targets for immunotherapy [24,25]. Whether or not the oncofetal behavior of GPC3 can be tested clinically and whether re-expression of this glypican plays a role in the progression of HCC are under investigation.

AFP and PIVKA-II [26] are well known major tumor markers for HCC. Generally, AFP shows high sensitivity but also high false-positivity. Serum AFP levels are often increased in patients with benign liver diseases, such as CH and LC, when AFP is detected using a more sensitive method. *Lens culinaris* agglutinin-reactive fraction of α-fetoprotein (AFP-L3%) is a recently described marker of HCC. AFP-L3% shows a much higher specificity than AFP, but a lower sensitivity. On the other hand, PIVKA-II shows a lower false-positivity, but is not always sensitive enough to detect small HCCs. In our study, the sensitivity of AFP, AFP-L3%, PIVKA-II, and GPC3 was 20/40 (50%), 10/36 (27.7%), 20/40 (50%), and 16/40 (40%), respectively. We could not diagnose 12 of 40 (30%) HCC patients using AFP and PIVKA-II. Although, we could diagnose an additional four patients as cases of HCC among 12 patients, three were classified as being in a relatively early UICC Stage II, hence GPC3 may be useful for diagnosis of early stage HCC. We could diagnose 80% of our patients with HCC using AFP, PIVKA-II, and the novel tumor marker, GPC3. Furthermore, GPC3 protein in the sera was detectable only in HCC patients and not in patients with other liver diseases or other kinds of cancers and healthy donors, thereby indicating that the specificity is 100%. Furthermore, we confirmed that GPC3 protein had disappeared from the sera of three patients after surgical treatments for HCC. Taken together, these results indicate that GPC3, as defined in our study, may prove to be an appropriate candidate for use in cancer-diagnosis for large numbers of patients with HCC.

Acknowledgments

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