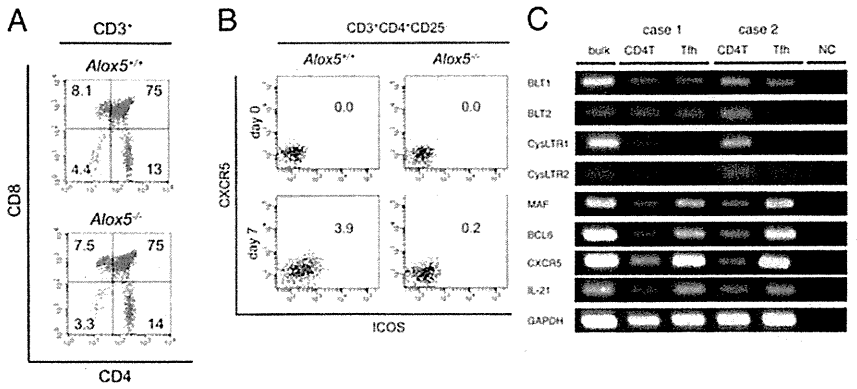

Figure 5. Developmental defects of Tfh cells in Alox5-deficient mice. **A:** FACS analysis of thymocytes in $Alox5^{+/+}$ and $Alox5^{-/-}$ mice. The population recognized by the expression of CD4 and CD8 are shown. **B:** FACS analysis of spleen cells of $Alox5^{+/+}$ and $Alox5^{-/-}$ mice before (day 0) and after (day 7) immunization with SRBCs. After immunization, the population of Tfh cells ($CD3^+CD4^+CD25^-ICOS^+CXCR5^+$) in $Alox5^{+/+}$ mice increased from 0.0% to 3.9% of the total $CD3^+CD4^+$ cells, and that of $Alox5^{-/-}$ mice increased from 0.0% to 0.2%. **C:** Expression levels of transcripts of leukotriene receptors in Tfh cells sorted from human tonsils. Results of two cases assessed by means of RT-PCR (28 cycles) are shown. Glyceraldehyde-3-phosphate dehydrogenase was used as the positive control. Bulk, total lymphocytes; NC, no template control. The results presented in (**A**) and (**B**) are representative of three to four independent experiments. In each experiment, three to six mice per group were tested.

agent for MCL, resulted in dramatic reduction of the growth activities of MINO cells. Therefore, Alox5 may be associated with the cellular integrities of not only primary B cells of the mantle zone, from which they originate, but also of MCL cells.

Discussion

In this study, we followed comprehensive immunoprecipitation and proteomics methods to identify an L22 Ag as Alox5. As implicated by the expression of Alox5 in mantle zone B cells ($CD23^+$ and $CD23^-$) around germinal centers, Alox5 plays a pivotal role in specific immunity as a regulator of cell fate and responsiveness to IL-21 of naive follicular B cells.[37,38,44] Alox5 defects also impinge on the generation of memory B cells, suggesting that it would completely support the preservation of B-cell repertories maintained by naive and memory B cells. The Alox5 pathway is essentially associated with a variety of inflammatory diseases, including asthma, atherosclerosis, rheumatoid arthritis, liver cirrhosis, and cancer, which are caused by an underlying anomaly of acquired immunity.[15,31] Therefore, primary B cells may act as a modulator in these pathologic situations. As a major source of leukotrienes in lymphoid tissues, primary B cells may contribute to establishment of the histologic features, such as lymphadenitis lesions.

Infection of mice with RNA or DNA viruses induces an antiviral antibody response, which is largely restricted to IgG2a. This also provides the functional importance of Alox5 as a coordinator of host defense. The regulation of IgG2a, which is an IL-4–independent Ig isotype, and the mechanism of interferon γ derived from type I helper T cells remain investigative priorities. Although we could not fully elucidate the precise mechanism of IgG2a production by Alox5, it has been previously reported that Alox5 deficiency may lead to an imbalance of type I and II helper T cells.[45] Thus far, we considered the possible involvement of Alox5 in the development of helper T-cell subpopulations. $CD4^+$ T cells possess leukotriene receptors, which can instructively work for the mobilization of effector T cells to inflammatory foci; however, little is known about the role of leukotrienes in the development of Tfh cells.[14,40–42,46] Leukotriene receptors encoded in germlines form a receptor spanning seven membranes coupled with G-proteins in the cytoplasm, similar to the chemokine receptors of Tfh cells, such as CXCR5, CCR6, and CXCR3. Leukotriene receptors seem to be down-regulated (or at least not up-regulated) in Tfh cells; therefore, such lipid mediators might act on the initial process of differentiation of naive helper T cells. The prerequisite for the differentiation of naive helper T cells is the interaction of the T-cell receptor on naive $CD4^+$ helper cells with major histocompatibility complex class II molecules on professional APCs. Therefore, professional APCs, which express Alox5, profoundly affect the initial steps of the differentiation of naive helper T cells. *Rag1*^{Alox5} mice exhibited defects of specific antibody responses to foreign antigens, although Tfh cells could be recognized in response to the administration of foreign antigens in these

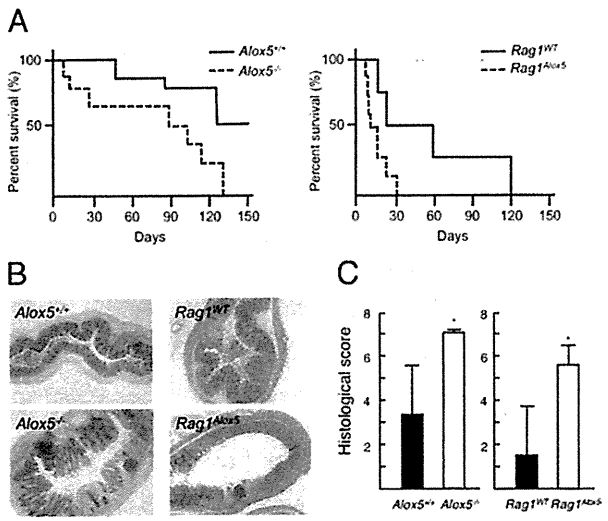

Figure 6. Alox5 defects attenuate intestinal tissue damage, with high mortality in experimentally induced chronic enterocolitis models. **A:** Survival curves of DSS-induced colitis models. Mice were maintained in a conventional facility and were allowed to freely drink water containing 1.5% DSS. The **left** and **right panels** illustrate the percentage survivals of $Alox5^{+/+}$ and $Alox5^{-/-}$ mice and $Rag1^{WT}$ and $Rag1^{Alox5}$ mice, respectively. $n = 7$ to 8 per group. **B:** Histologic findings of mice intestines after DSS administration on day 7. The **left** and **right panels** demonstrate the intestines of $Alox5^{+/+}$ or $Alox5^{-/-}$ mice and $Rag1^{WT}$ or $Rag1^{Alox5}$ mice, respectively. The intestines of $Alox5^{-/-}$ and $Rag1^{Alox5}$ mice manifested severe enterocolitis. Original magnification, ×100. **C:** The total histologic score is presented as mean with SEM ($n = 3$ to 4 mice per group). $^*P < 0.05$ compared with the wild-type control.

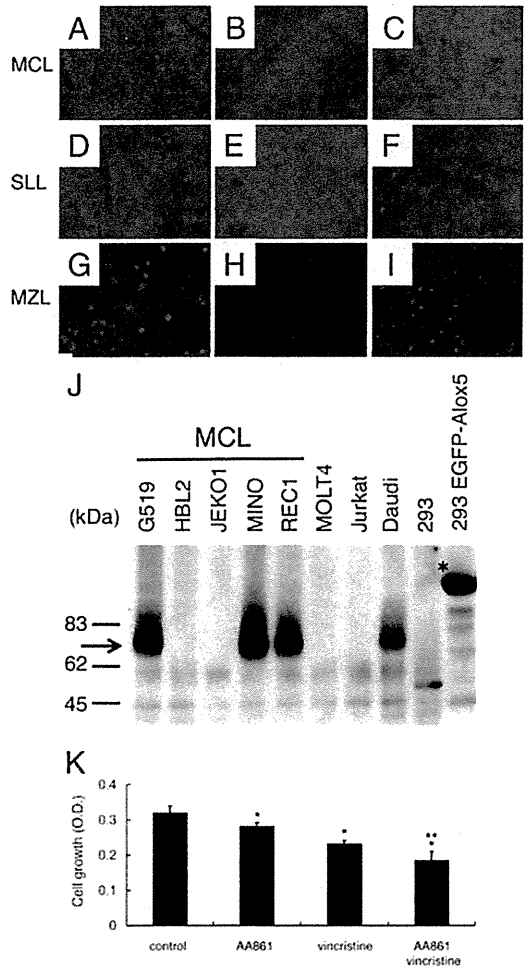

Figure 7. Alox5 defines cell growth of mantle cell–derived B-cell malignancies. **A–I:** Alox5 expression in B-cell lymphomas composed of small lymphoid cells. Immunohistochemical analysis using 1.22 mAbs was performed on frozen sections of independent tumor tissues: MCL (**A–C**), SLL (**D–F**), and marginal zone lymphoma (MZL) (**G–I**). Original magnification, ×200. **J:** Alox5 expression in cells derived from MCL at the protein level (**arrow**). Immunoblot analysis with anti-Alox5 pAbs was performed on human cell lines. HEK 293 cells with or without EGFP-Alox5 (**asterisk**) were used as the control. **K:** Cell growth of MINO under 10 μmol/L AA861 or 50 μg/ml of vincristine assessed by using WST1. A 96-well plate containing 1×10^5 cells per well was incubated with reagents for 24 hours. Results are representative of four independent experiments. $^*P < 0.01$ compared with the control; $^{**}P < 0.05$ compared with vincristine alone.

mice (data not shown). Thus, the lipid mediators derived from macrophages or dendritic cells but not from B cells would affect the process of differentiation of Tfh cells.

In this study, we could not fully elucidate a mechanism of the production of leukotrienes from primary B cells. Macrophages or dendritic cells produce Alox5-related leukotrienes by activating cytosolic phospholipase A2. It may be supposed that B-cell receptor signaling leads to the mobilization of calcium ions through inositol phosphate, leading to the activation of cytosolic phospholipase A2 and the subsequent liberation of arachidonate from membrane glycerolipids as a substrate of Alox5. We observed that *Alox5*$^{-/-}$ mice can produce B1 B cells ($B220^+CD5^+IgM^+$), related to producing natural antibodies, in the spleen and peritoneal cavity (data not shown). In this context, leukotrienes of primary B2 B cells may regulate the adaptive humoral immune response.[47] Indeed, we found transcripts of Alox5 in mouse follicular B cells, and we are trying to detect Alox5 in these cells at protein levels.

Maintenance of the B-cell repertoire is one of the most important elements in achieving adaptive humoral responses and protecting the host from pathogens. Perhaps primary naive and memory B cells express Alox5 and preserve their integrity to maintain the B-cell repertoire. According to this story, it was of interest to note that MCL, one of the most refractory tumors against conventional therapies, might depend at least in part on the function of Alox5 in terms of cellular growth. The evidence that primary B cells and MCL, which originates from primary B cells, rely on Alox5 for their cellular integrity can provide insights for understanding the unique tumor biology of MCLs. Further investigations should elucidate the mechanism of the additive effects of Alox5 inhibitors as chemotherapeutic reagents to treat MCL.[48,49]

In summary, we demonstrated the fundamental role of Alox5 in establishing specific antibody responses. Alox5 regulates not only primary resting B cells of the naive and memory B-cell phenotypes but also Tfh cell generation, thereby preserving specific antibody production. It has not yet been determined whether Alox5-related lipid mediators take part in the plasticity of the generation of helper T cells. However, studies of the expression profiles of leukotriene receptors on helper T-cell species would enable us to recognize the biological significance of lipid mediators in the differentiation of helper T cells. Lipid metabolism of arachidonic acid, affected by amounts from the oral intake or function of related enzymes, may affect the primary B-cell response by altering IL-21– and Tfh-mediated stimulation.

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Stem Cells, Tissue Engineering, and Hematopoetic Elements

Cytotoxic T Lymphocytes Efficiently Recognize Human Colon Cancer Stem-Like Cells

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Cancer stem-like cells (CSCs) and tumor-initiating cells (TICs) are a small population of cancer cells that share three properties: tumor initiating ability, self-renewal, and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence, and distant metastasis. Here, we show that cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. Using Hoechst 33342 dye staining, we isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and showed resistance to chemotherapeutic agents such as irinotecan or etoposide.To evaluate the susceptibility of SP cells to CTLs, we used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Cep55/c10orf3_193 (10) (VYVKGLLAKI). The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*. These observations suggest that Cep55/c10orf3_193(10) peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs. *(Am J Pathol 2011, 178:1805–1813; DOI: 10.1016/j.ajpath.2011.01.004)*

Colon cancer is one of the most common malignancies worldwide. With recent progress in treatment, the prognosis has improved to some extent. In advanced disease, however, the prognosis remains unfavorable, because of recurrence, distant metastasis, and resistance to treatment. Thus, novel treatment modalities are needed.

Cancers contain morphologically heterogeneous populations. This fact has led to the cancer stem cell theory,[1] the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer.[2–6] In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice.[3] Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs. Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis.[7] These reports[3–6] support the hypothesis that malignant cancers comprise heterogeneous populations that organize in a hierarchical differentiation model. The CSCs/TICs are located at the top of this hierarchy, and targeting CSCs/TICs is essential to achieve efficient effects for treatment of malignant diseases. Recently, some trials targeting CSCs/TICs have been reported for hema-

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topoietic malignancies.[8] Hedgehog signaling is essential for maintenance of myeloid leukemia stem cells, and inhibition of hedgehog signaling by cyclopamine is effective for imatinib-resistant myeloid leukemia.[9] To date, however, no such CSC/TIC targeting approach has been reported for colon cancer.

In the present study, we evaluated the efficiency of CTL-based immunotherapy targeting colon CSCs/TICs. Using Hoechst 33342 dye, we isolated colon CSCs/TICs as side population (SP) cells from six colon cancer cell lines. The SP cells derived from SW480, HT29, and HCT15 showed higher tumorigenicity than did main population (MP) cells. On the other hand, SP cells from KM12LM, Lovo, and Colo320 did not show any increase in tumorigenicity, compared with MP cells. This suggests that SW480, HT29, and HCT15 SP cells (but not KM12LM, Lovo, and Colo320 SP cells) were enriched with CSCs/TICs. In RT-PCR analysis the SW480, HT29, and HCT15 SP cells showed a stem cell-like gene expression signature, including SOX2, POU5F1, LGR5, and ALDH1A1. Furthermore, these SP cells also showed resistance to chemotherapeutic agents, including irinotecan and etoposide. These observations support the idea that these SP cells had stem cell-like features. To assess the immunogenicity of SP cells, we evaluated the expression of HLA class I and of CEP55, which is a tumor-rejection antigen of breast and colon cancer.[10,11] The SP cells expressed HLA class I (and also HLA-A24) at the same level as MP cells. The SP cells also expressed CEP55 messenger RNA (mRNA) at the same level as MP cells in RT-PCR. To confirm the susceptibility of SP cells to cytotoxic T lymphocytes (CTLs), we used CTL clone 41, which recognizes CEP55 in an HLA-A24-restricted manner.[10] CTL clone 41 killed SW480, HT29, and HCT15 SP cells at the same level as it killed MP cells and presorted cells. These observations suggest that colon CSCs/TICs are also sensitive to CTLs, as non-CSC/TIC populations are. Furthermore, adoptive transfer of CTL clone 41 inhibited the tumor growth of SW480 SP cells in immunodeficient mice. These observations suggest that CTL-based colon cancer immunotherapy is efficient for colon CSCs/TICs. To our knowledge, the present study provides the first direct evidence that colon CSCs/TICs are susceptible to CTLs and thus opens possibilities for future applications in immunotherapy using CSC/TIC-specific vaccines.

Materials and Methods

Cell Lines

Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402), HCT15 (HLA-A*0201/2402), HT29 (HLA-A1/24), Lovo, and Colo320 were kind gifts of Dr. K. Imai (Sapporo, Japan), and KM12LM was a kind gift of Dr. K. Itoh (Kurume, Japan). All cell lines except K562 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). K562 was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum. HCT15-B2M, a stable transfectant of HCT15 cells with B2M (2 microglobulin) cDNA, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 g/mL puromycin (Sigma-Aldrich).[11]

Side Population Analysis

Side population analysis was performed as described previously, with some modifications.[12] Trypsinized cultured cells were washed with PBS and were resuspended at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. After 10 minutes preincubation, the cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD) for 90 minutes at concentrations of 3.75 g/mL for Colo320, 5 g/mL for SW480 and Lovo, 7.5 g/mL for HT29 and KM12LM, and 10 g/mL for HCT15, with or without verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 50 mol/L for SW480, HCT15, and Colo320, 75 mol/L for Lovo, and 100 mol/L for HT29. Cells were counterstained with 1 g/mL propidium iodide to label dead cells. Next, 1 10^6 viable cells were analyzed and sorted using a BD FACSAria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The Hoechst dye was excited at 355 nm, and its fluorescence was measured at two wavelengths using optical filters 405 DF20 [450/20 nm bandpass filter O (Hoechst Blue)] and 635LP [635 nm longpass edge filter (Hoechst Red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

Xenograft Model

The SP cells, MP cells, and presorted cells from colon cancer cell lines were mixed 1:1 by volume with Matrigel (BD Biosciences) and were injected subcutaneously into the backs of female 4- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size in cubic millimeters was assessed weekly with calipers and was calculated as Tumor Size (Longest Diameter Shortest Diameter^2)/2.

RT-PCR Analysis of SP and MP Cells

RT-PCR analysis was performed as described previously.[10] Total RNAs were isolated from both SP cells and MP cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 g of total RNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen). The PCR amplification was performed in 20 L of PCR mixture containing 1 L of cDNA mixture, 0.5 L of Taq DNA polymerase (Qiagen) and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5=-CATGATG-

GAGACGGAGCTGA-3= and 5=-ACCCCGCTCGCCATGCTATT-3= for SOX2, with an expected PCR product size of 410 bp; 5=-TGGAGAAGGAGAAGCTGGAGCAAAA-3= and 5=-GGCAGATGGTCGTTTGGCTGAATA-3= for POU5F1, with an expected PCR product size of 163 bp; 5=-CTCTTCCTCAAACCGTCTGC-3= and 5=-GATCGGAGGCTAAGCAACTG-3= for LGR5, with an expected PCR product size of 181 bp; 5=-TGTTAGCTGATGCCGACTTG-3= and 5=-TTCTTAGCCCGCTCAACACT-3= for ALDH1A1, with an expected PCR product size of 154 bp; 5=-TGAGTTTGCCATCACAGAGC-3= and 5=-TTGCTTGCTGGTGCATTAAC-3= for CEP55, with an expected PCR product size of 521 bp; and 5=-ACCACAGTCCATGCCATCAC-3= and 5=-TCCACCACCCTGTTGCTGTA-3= for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with an expected product size of 452 bp. GAPDH was used as an internal control.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Applied Biosystems). Thermal cycling was performed using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, with normalization to the *GAPDH* gene as an internal control.

Flow Cytometric Analysis and Monoclonal Antibodies

Cells were incubated with mouse monoclonal antibodies at saturation concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for 30 minutes. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma, a kind gift from Dr. P.G. Coulie, Brussels, Belgium) were prepared from hybridomas.

Survival Studies for Etoposide and Irinotecan

We isolated SP and MP cells of SW480 and HCT15 and seeded them into 96-well culture plates at 1 10^4 cells per well for each population of cells. The cells in both populations were treated with etoposide (1 and 5 g/mL) or irinotecan (40 and 400 g/mL for SW480, 10 and 100 g/mL for HCT15). After 72 hours of exposure to the chemotherapeutic agents, viability of the cells was determined using the SOD assay kit WST-1, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan; Rockville, MD).

Cytotoxicity Assay for SP Cells with CTL Clone 41

We had previously established CTL clone 41, which recognizes an HLA-A24 restricted antigenic peptide (VYVKGLLAKI) termed Cep55/c10orf3_193(10), from an HLA-A24-positive breast cancer patient's peripheral blood mononuclear cells.[8] The lytic activity of CTL clone 41 for SP cells, MP cells, and presorted cells was evaluated by ^{51}Cr release assay. Briefly, SP cells, MP cells and presorted cells were labeled with 100 Ci of ^{51}Cr for 1 hour at 37°C, washed four times with PBS, and resuspended in AIM-V medium (Invitrogen). The ^{51}Cr-labeled target cells (2000 cells/well) were then incubated with various numbers of effector cells for 6 hours at 37°C in 96-well culture plates. Radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % Specific Lysis (Experimental Release Spontaneous Release) 100/ (Maximum Release Spontaneous Release). Target cells were treated with 100 units/mL interferon- for 48 hours before the assay.

Winn Assay

SW480 SP cells were mixed with CTL clone 41 at a ratio of 1 SP cell to 10 CTL cells. The resulting mixture (200 L with 1 10^6 CTL clone 41 and 1 10^5 SP cells) was injected subcutaneously into the backs of NOD/SCID mice. A control group of five mice was injected with SP cells alone. Tumor size was assessed weekly.

CTL Adoptive Transfer

NOD/SCID mice were inoculated subcutaneously on the back with 1 10^3 SW480 SP cells. Three weeks later, when the tumor started to be palpable, 5 10^4 Cep55/c10orf3_193(10)-specific CTL clone cells or PBS was injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical Analysis

In the xenograft model, survival studies using chemotherapeutic agents, cytotoxicity assay, Winn assay, and adoptive transfer model, the data were analyzed using the Mann-Whitney *U*-test, with P 0.05 conferring statistical significance.

Results

Isolation of Colon CSCs/TICs as SP Cells

Several methods to isolate colon cancer CSCs/TICs has been reported, including cell surface markers such as CD44 or PROM1 (CD133), SP cells, and the Aldefluor assay.[3–6,13] In the present study, we isolated colon CSCs/TICs using SP cell analysis. Several colon cancer cell lines were dyed with Hoechst 33342 and then analyzed with a BD FACSAria II flow cytometer as

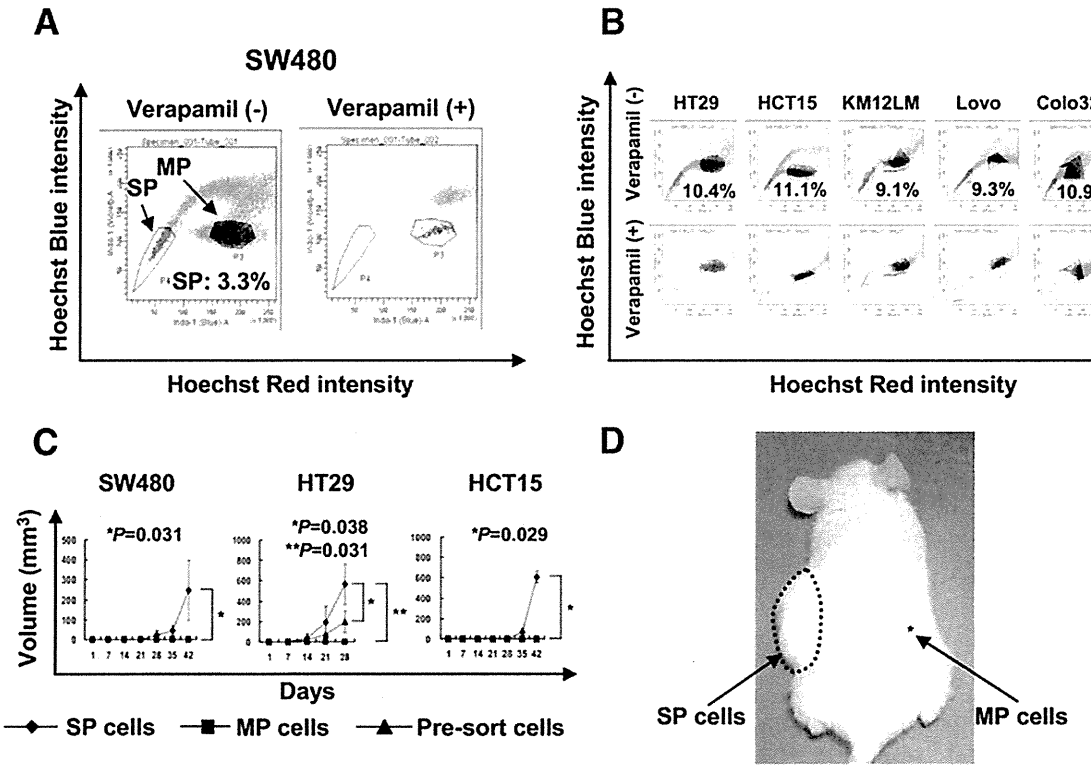

Figure 1. Isolation of colon CSCs/TICs from colon cancer cell lines and tumor growth of the SP cells. A: Colon cancer cell line SW480 was stained with Hoechst 33342 dye as described under *Materials and Methods*, with or without verapamil. Stained cells were analyzed using a BD FACSAria II fluorescence-activated cell sorting system. Frequency of SP cells was 3.3%. B: Colon cancer cell lines (HT29, HCT15, KM12LM, Lovo, and Colo320) were stained with Hoechst 33342 dye with or without verapamil. Stained cells were analyzed using a BD FACSAria II system. Frequencies of SP cells ranged from 9.1% for KM12LM cells to 11.1% for HCT15 cells. C: SP cells, MP cells, and presorted cells of colon cancer cell lines SW480, HT29, and HCT15 were inoculated subcutaneously into the backs of NOD/SCID mice (1 10^3 cells injected). Data are reported as means SD. *P* values indicate differences between cell types according to a Mann-Whitney *U*-test. D: Representative tumor growth in NOD/SCID mice at the SP cell injection site (1 10^3 cells injected). SP cells and MP cells were inoculated subcutaneously into the left and right side of the back, respectively.

described under *Materials and Methods*. Side population cells could be detected in all six colon cancer cell lines analyzed (ie, SW480, HT29, HCT15, Colo320, Lovo, and KM12LM) (Figure 1, A and B). The frequency of SP cells ranged from 3.3% for SW480 to 11.1% for HCT15 cells. All these SP cells were specifically inhibited by verapamil, as has been shown previously,[14] suggesting that these SP cells were specific for ABC transporter expression. Because previous studies showed that some colon cancer SP cells were not enriched with a CSC/TIC population,[15] it was essential to confirm the presence of CSCs/TICs in SP cells for further analysis. We inoculated these SP cells subcutaneously into the back of immunodeficient NOD/SCID mice using serial dilution. The SP cells derived from SW480, HCT15, and HT29 showed higher tumor initiating ability, compared with MP cells (Table 1). Furthermore, SW480, HT29, and HCT15 SP cells showed faster tumor growth, compared with MP cells (Figure 1, C and D), suggesting the presence of CSCs/TICs in these SP cells. In contrast, the SP cells derived from Colo320, Lovo, and KM12LM did not show any difference in tumorigenicity or tumor growth, compared with MP cells. We therefore restricted further analysis to the SW480, HT29, and HCT15 SP cells as colon cancer CSCs/TICs.

RT-PCR Analysis of Colon Cancer SP Cells

To examine the molecular properties of SP cells, we performed RT-PCR analysis. SOX2 and POU5F1 are representative markers for embryonal stem cells and CSCs/TICs.[16] The SP cells derived from SW480, HT29, and HCT15 showed higher expression of both SOX2 and POU5F1, compared with MP cells (Figure 2A). ALDH1A1, a colon CSC/TIC marker,[6] was expressed at a higher level in SP cells of HCT15 than in MP cells, but SP cells of SW480 and HT29 did not show any difference in comparison with MP cells. SW480 and HT29 SP cells also showed higher expression of LGR5, which is known as a normal colon stem cell marker.[17] To confirm the expression of stem cell markers, we also performed real-time PCR. The SW480 SP cells expressed 90 times higher SOX2, 7 times higher POU5F1, 153 times higher LGR5, and 6.1 times higher ALDH1A1, compared with MP cells (Figure 2B). These findings indicate that these SP cells had molecular properties similar to those of embryonal stem cells.

Resistance to Chemotherapeutic Reagents

Although SP cells derived from liver cancer cell line HuH7 have showed resistance to chemotherapy,[13] we know of no conclusive previous studies of such resistance in co-

Table 1. Tumor Initiating Ability of Colon Cancer SP Cells

Cell line (% SP cells)	Tumor initiating ability* 1 10^{4}†	1 10^{3}†	1 10^{2}†
SW480 (3.3)			
SP cells	4/4	4/6	4/4
MP cells	2/4	3/5	0/4
HT29 (10.4)			
SP cells	3/3	2/3	3/3
MP cells	3/3	0/3	0/3
HCT15 (11.1)			
SP cells	3/3	3/4	3/3
MP cells	1/3	1/4	0/3
Colo320 (10.9)			
SP cells	2/2	1/2	1/2
MP cells	2/2	2/2	1/2
Lovo (9.3)			
SP cells	0/1	1/1	0/1
MP cells	1/1	0/1	0/1
KM12LM (9.1)			
SP cells	1/2	2/2	1/1
MP cells	1/2	2/2	1/1

MP, main population; SP, side population.
*Tumor initiating ability is expressed as the ratio of tumor-initiation to injection.
†The tumor initiation abilities were evaluated at day 42 after injection of the indicated number of cells.

lon SP cells. We performed a cell survival study of colon cancer SP cells using the chemotherapeutic agents irinotecan and etoposide. The SW480 and HCT15 SP cells were more resistant to both irinotecan and etoposide than were MP cells (Figure 3, A and B). This finding is consistent with findings for CSCs/TICs derived from other organs.[22,24]

Expression of HLA and Tumor-Associated Antigens in SP Cells

Because CTLs recognize tumor-associated antigen (TAA)-derived antigenic peptides presented by HLA class I molecules, expression of HLA class I molecules is essential for activation of CTLs. Several types of malignancies have been reported to lose the expression of HLA class I molecules through various mechanisms and so escape CTL attack.[18] We therefore evaluated the expression of HLA class I molecules and TAA. We assessed the differences of HLA class I and HLA-A24 expression between SP cells and MP cells by flow cytometry. Because ELISA study has revealed that HCT15 cells lack B2M because of gene mutations of *B2M*,[19] we transduced wild-type B2M cDNA into HCT15 cells and so established HCT15-B2M cells. The SW480, HT29, and HCT15-B2M SP cells showed HLA class I and HLA-A24 expression at the same level as MP cells (Figure 4, A and B). Furthermore, we assessed the expression of one of the colon cancer TAAs, CEP55, by both RT-PCR and real-time PCR (Figure 2, A and B). Both SP cells and MP cells derived from SW480, HT29, and HCT15-B2M expressed CEP55 mRNA at the same level. These data raised the possibility that SP cells are also sensitive to CTLs specific for the CEP55-derived antigenic peptide. Because both SP cells and MP cells expressed CEP55 mRNA at the same level, this appeared to be an ideal target for comparing the susceptibilities of SP cells and MP cells to CTLs.

Susceptibility of CSCs/TICs to CTLs, in Vitro *and* in Vivo

We had previously established CTL clone 41, which is specific for the cancer-related, antigen-derived, HLA-A24-restricted peptide Cep55/c10orf3_193(10).[10] CTL clone 41 recognized CEP55-positive and HLA-A24-positive cancer cells, as described previously.[10,11] In the present study, we used a ^{51}Cr release assay to examine whether CTL clone 41 can recognize SP cells. All SP cells derived from SW480, HT29, and HCT15-B2M showed susceptibility to CTL clone 41 at the same level as the MP cells and the presorted bulk cell lines (Figure 4C). This indicates that the colon CSCs/TICs were sensitive to CTLs.

To analyze the cytotoxic activity of the CTL clone *in vivo*, we performed a Winn assay. SW480 SP cells with or without CTL clone 41 were injected into the backs of NOD/SCID mice subcutaneously. CTL clone 41 significantly inhibited the tumorigenicity of SW480 SP cells (Figure 4D). Because CTLs were injected at the same time and place as the SP cells in the Winn assay, we could not rule out the possibility that SP cells were killed *in vitro*. We

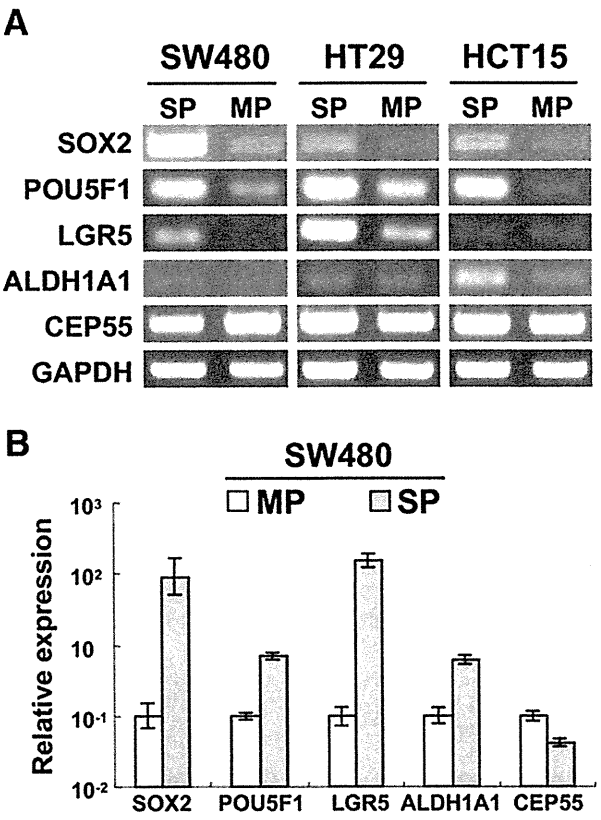

Figure 2. Expression of stem cell markers in SP and MP cells. A: mRNAs purified from SW480, HT29, and HCT15 SP and MP cells were analyzed by RT-PCR. B: mRNA purified from SW480 SP cells and MP cells were analyzed with real-time PCR. mRNA expression level is relative to MP cells. Data are reported as means SD.

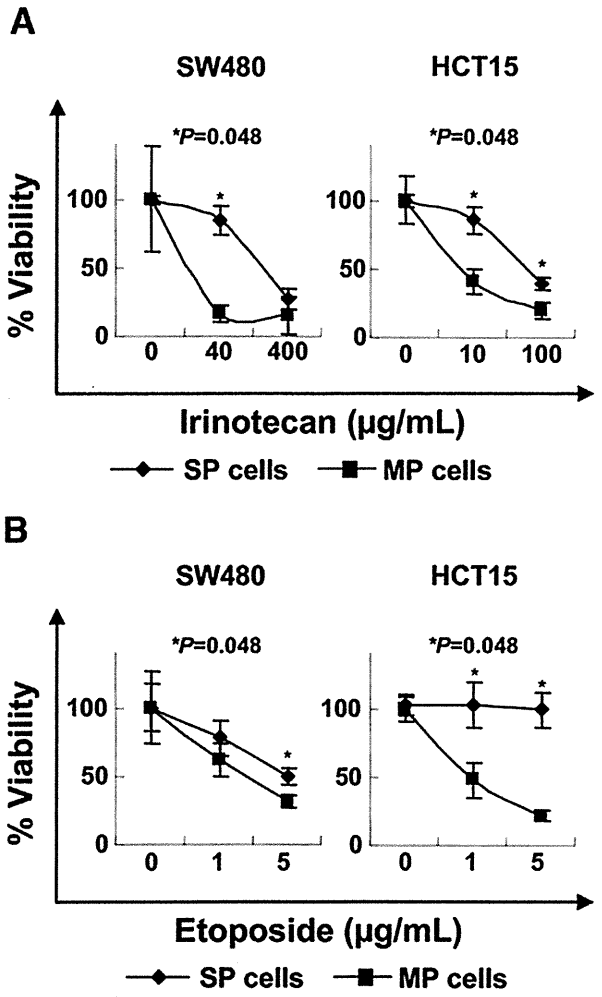

Figure 3. Sensitivity to chemotherapeutic agents. SP cells and MP cells derived from SW480 and HCT15 were incubated in the presence of irinotecan (CPT-11) (A) or etoposide (VP-16) (B) for 3 days. After incubation, the cell viabilities were measured by WST-1 assay. Data are reported as means SD. Differences between SP cells and MP cells were examined for statistical significance using the Mann-Whitney *U*-test.

therefore used an adoptive transfer model, as described under *Materials and Methods*. SW480 SP cells were inoculated into the back of NOD/SCID mice subcutaneously. Three weeks later, after confirmation of palpable tumors, CTLs were injected intravenously. Tumors of CTL-injected mice were significantly inhibited in growth, compared with tumors of control mice (Figure 4E). These data indicate that CTLs could recognize CSCs/TICs both *in vitro* and *in vivo*.

Discussion

In the present study, we successfully isolated colon cancer CSCs/TICs as SP cells, using Hoechst 33342 staining. Side population cells were first described by Goodell et al,[12] and CSCs/TICs of several types of malignancies were successfully isolated as SP cells in subsequent studies.[14,20–24] Haraguchi et al[13] isolated SP cells from gastrointestinal cancer cell lines; they reported the gene expression profiles and resistance to chemotherapeutic agents of SP cells derived from liver cancer cell line Huh7, but did not determine their tumorigenicity. Burkert et al[15] found that SP cells derived from gastrointestinal cancers cell lines HT29, HGT101, Caco2, and HRA19a1.1 were not enriched with a CSC/TIC population. In the present study, we were able to isolate SP cells from all six colon cancer cell lines studied (SW480, HT29, HCT15, KM12LM, Lovo, and Colo320). However, in only three of the six cell lines did the SP cells show higher tumorigenicity than MP cells, suggesting that these SP cells were enriched with CSC/TIC populations. Thus, SP cells might not be the definitive phenotype of CSCs/TICs, and confirmation of tumorigenicity in immunodeficient mice is essential for validation of SP cells as a source of CSCs/TICs. In the present study, the SP cells derived from SW480, HCT15, and HT29 cells were confirmed to be enriched with CSCs/TICs. Furthermore, these SP cells expressed stem cell markers, including SOX2, POU5F1 and LGR5, at higher levels than MP cells, suggesting correspondence with CSCs/TICs. Thus, these SP cells would be a useful tool for analysis of colon CSCs/TICs.

In the present study, we evaluated the immunogenicity of colon CSCs/TICs. Colon cancer CSCs/TICs expressed HLA class I molecules, and also CEP55, which is one of the TAAs. Furthermore, colon CSCs/TICs expressed several other TAA-encoding genes (data not shown), including BIRC5 (encoding apoptosis inhibitor survivin), BIRC7 (encoding livin), WT1, CTAG1B (alias *NY-ESO-1*), and MAGEA4. As a novel finding, colon cancer CSCs/TICs were sensitive to CTLs both *in vitro* and *in vivo*. Recently, Todaro et al[25] showed that colon CSCs/TICs were sensitive to T cells. Because both CTLs and T cells kill target cells through secretion of perforin (encoded by the *PRF1* gene) and granzyme B (encoded by *GZMB*), these observations strongly suggest that CSCs/TICs are sensitive to PRF1- and GZMB-dependent apoptosis. Todaro et al[26] had earlier reported that PROM1-positive (CD133) colon cancer CSCs/TICs secrete IL-4 in an autocrine manner and upregulate the antiapoptotic proteins CFLAR (c-FLIP), BCL2L1 (Bcl-xL), and PEA15 (PED), thereby gaining resistance to chemotherapeutic agents. Saigusa et al[27] reported that distant recurrence of rectal cancer after chemotherapy was related to the expression of CSC/TIC markers such as PROM1 (CD133), POU5F1 (Oct3/4), and SOX2. These reports support the idea that colon CSCs/TICs are resistant to apoptotic cell death. The fact that immunocytes induce apoptosis in their target cells raises the question of whether colon CSCs/TICs are also sensitive to immunotherapy.

In the present study, and in that of Todaro et al,[25] colon CSCs/TICs were sensitive to perforin- and granzyme B-dependent apoptosis. Thus, both CTLs and T cells can be useful tools for colon CSC/TIC targeting therapy. However, because T cells do not recognize target cells in an antigen-specific manner, immunotherapy using T cells should also recognize the non-CSC/TIC population. Because the number of T cells is restricted *in vivo*, it may be in doubt whether T cell can recognize colon

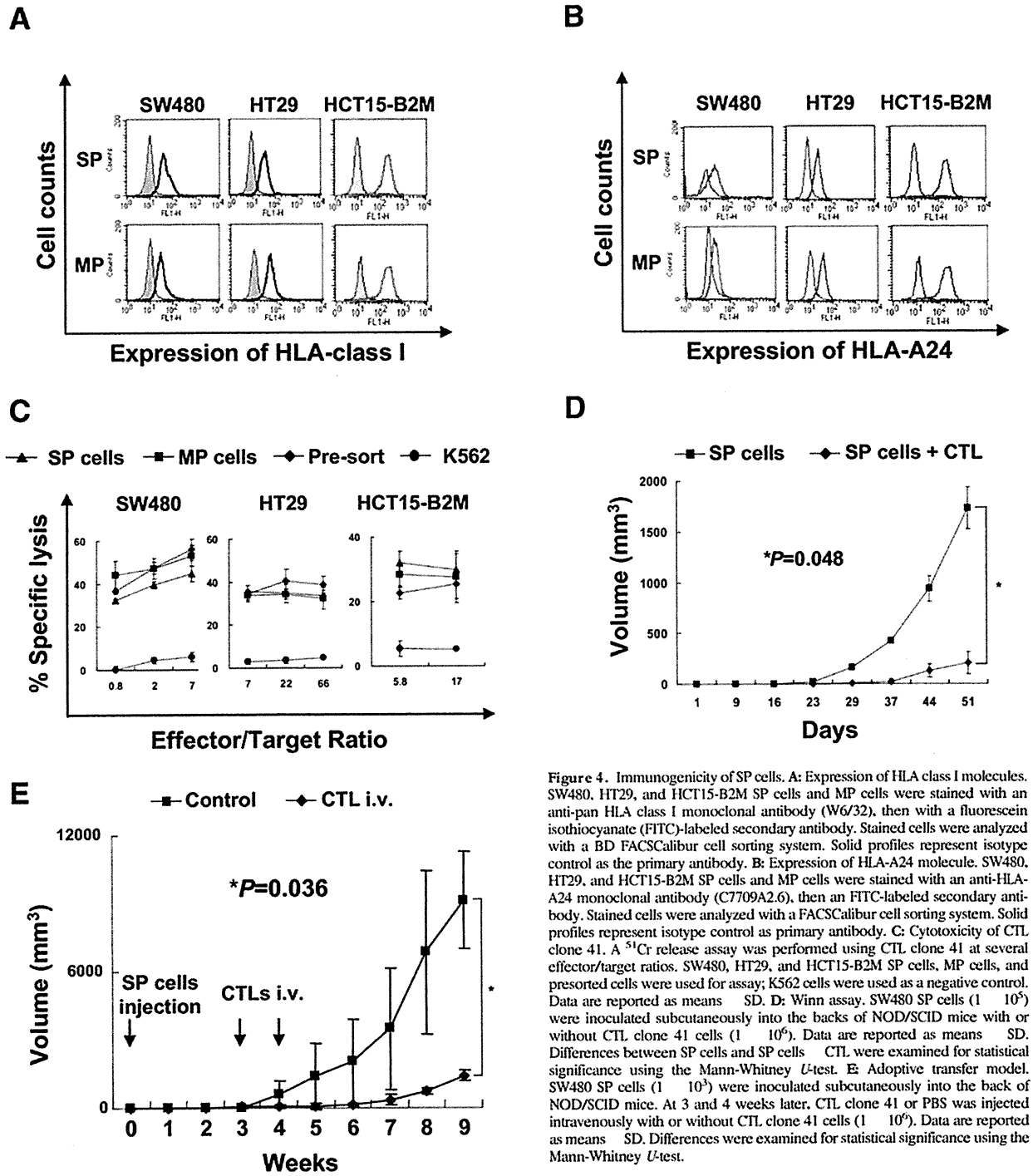

Figure 4. Immunogenicity of SP cells. A: Expression of HLA class I molecules. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-pan HLA class I monoclonal antibody (W6/32), then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Stained cells were analyzed with a BD FACSCalibur cell sorting system. Solid profiles represent isotype control as the primary antibody. B: Expression of HLA-A24 molecule. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-HLA-A24 monoclonal antibody (C7709A2.6), then an FITC-labeled secondary antibody. Stained cells were analyzed with a FACSCalibur cell sorting system. Solid profiles represent isotype control as primary antibody. C: Cytotoxicity of CTL clone 41. A ^{51}Cr release assay was performed using CTL clone 41 at several effector/target ratios. SW480, HT29, and HCT15-B2M SP cells, MP cells, and presorted cells were used for assay; K562 cells were used as a negative control. Data are reported as means SD. D: Winn assay. SW480 SP cells (1 10^5) were inoculated subcutaneously into the backs of NOD/SCID mice with or without CTL clone 41 cells (1 10^6). Data are reported as means SD. Differences between SP cells and SP cells CTL were examined for statistical significance using the Mann-Whitney *U*-test. E: Adoptive transfer model. SW480 SP cells (1 10^3) were inoculated subcutaneously into the back of NOD/SCID mice. At 3 and 4 weeks later, CTL clone 41 or PBS was injected intravenously with or without CTL clone 41 cells (1 10^6). Data are reported as means SD. Differences were examined for statistical significance using the Mann-Whitney *U*-test.

cancer CSCs/TICs *in vivo* efficiently. Recently, based on a large cohort study, Ogino et al[28] reported that lymphocytic reaction to tumor was associated with longer survival of colorectal cancer patients. They did not analyze the subtypes of infiltrating lymphocytes; however, the findings from this large-scale study strongly support the notion that immune reaction to tumor cells is important for control of the disease.

Wei et al[29] reported recently that glioma-derived CSCs/TICs suppressed T-cell proliferation and activation, and induced T-cell apoptosis through expression of co-stimulatory inhibitory molecule CD274 (B7-H1) and soluble LGALS3 (galectin-3); glioma CSCs/TICs enhance the induction of regulatory T cells. We also observed that SW480 SP cells express higher mRNA of the immunosuppressive cytokine IL-10 than MP cells (data not

shown). Thus, colon CSCs/TICs may have immunosuppressive potential and so inhibit CTL induction. However, colon CSCs/TICs are efficiently killed by CTLs, and colon CSCs/TICs have no influence on the effector phase of CTLs. Thus, adoptive cell transfer of CSC/TIC-specific CTL clones, T-cell-receptor–induced T cells, or peptide vaccination accompanied by an anti-IL-10 monoclonal antibody might be an effective approach for eliminating colon CSCs/TICs.

In the present study, we observed that both colon CSCs/TICs and non-CSCs/TICs were sensitive to CEP55-specific CTLs at the same level. This finding seems reasonable, given that CSCs/TICs express CEP55 mRNA at the same level. Huge numbers of TAAs have already been reported,[30,31] and the next challenge is to identify which TAAs would be the most suitable targets for cancer immunotherapy. According to the manner of expression in CSCs/TICs and non-CSCs/TICs, TAAs can be classified into three categories: i) CSC/TIC-specific antigens, such as SOX2 and ALDH1A1; ii) non-CSC/TIC-specific antigens; and iii) shared antigens, such as CEP55.[32] The frequencies of colon CSCs/TICs are 1% to 10%, and in the present study these cells had 10- to 100-fold higher tumorigenicity than non-CSCs/TICs. It is likely, therefore, that 1% to 10% of colon CSC/TIC populations have almost the same tumorigenic potential as 90% to 99% of the non-CSC/TIC population. To achieve a complete cure of the disease, shared antigens seem to a be reasonable candidate strategy. *In vivo*, however, CTL numbers are limited. Given that 1 L of peripheral blood contains approximately 5 10^9 lymphocytes, there are approximately 5 10^8 CD8 T cells in 1 L of peripheral blood and approximately 3 10^9 CD8 cells in the total volume of peripheral blood in a human adult. If the CTL precursor frequency reaches 0.1% of CD8 T cells in a patient receiving peptide vaccination therapy, then the total peptide-specific CTLs can be calculated as 3 10^6 cells in whole blood. This is not an inconsiderable number. Visible tumors as large as 1 cm diameter contain 1 10^9 tumor cells, and the estimated effector/target ratio (E/T) *in vivo* is 0.003. This ratio may be too low to expect an anti-tumor effect *in vivo*. However, if we focus only on CSCs/TICs, then the effector/target ratio will be improved. For targeting CSCs/TICs with 1% frequency, the effector/target ratio is correspondingly improved (E/T 0.3). Thus, focusing only on the CSC/TIC population with CSC/TIC-specific antigens seems to be a better approach for advanced cancer cases. For prevention of disease recurrence after treatment, the target cells are likely to be limited, so shared antigens might be a reasonable choice for cancer immunotherapy.

Recently, some research groups have reported that monoclonal antibodies for insulin-like growth factor-1 receptor (IGF-1R), -like 4 ligand (DLL4), and CD47 efficiently eliminate colon cancer and leukemia CSCs/TICs.[33–36] These approaches are also fascinating, and a reasonable option for elimination of CSCs/TICs. An antibody is a relatively stable protein, but the half-life in peripheral blood is approximately 2 to 3 weeks, and therefore serial administration is needed to maintain the effects of the antibody. On the other hand, antigenic peptide vaccination can induce specific CTLs as memory cells *in vivo*, such that the specific immunity will last for several years. Thus, peptide vaccination therapy may also be useful for prevention of post-treatment cancer recurrence.

In conclusion, we report here the novel finding that colon cancer CSCs/TICs are as sensitive to CTLs as are non-CSCs/TICs, and that CEP55, a tumor-associated antigen, is a suitable antigen for targeting colon cancer CSCs/TICs.

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Inhibition of Osteopontin Reduces Liver Metastasis of Human Pancreatic Cancer Xenografts Injected into the Spleen in a Mouse Model

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Abstract

Purpose. Pancreatic cancer is associated with the poorest prognosis of any digestive cancer due to the high incidence of liver metastasis. This study evaluated the possibility that osteopontin (OPN) RNA interference (RNAi) and anti-OPN antibody (Ab) could have anti-metastatic effects.

Methods. The differential gene expression was measured in a parental cell line, HPC-3, and an established highly liver metastatic cell line, HPC-3H4. This study investigated the effect of OPN RNAi and anti-OPN Ab on the metastatic ability of HPC-3H4 to the liver. An OPN RNAi-expressing vector was introduced into HPC-3H4 cells (HPC-3H4/miOPN), in which OPN production was reduced to the level of the parental HPC-3 cells. Finally, the ability of anti-OPN Ab to suppress liver metastasis was investigated.

Results. Osteopontin was upregulated 11.1-fold in HPC-3H4 in comparison to HPC-3. The metastatic rate of HPC-3H4/miOPN was significantly reduced to 25% in comparison to the 100% metastatic rate of HPC-3H4 and control HPC-3H4/miNeg cells ($P < 0.01$). The metastatic rate of the group given anti-OPN Ab was 50%.

Conclusion. OPN RNAi and anti-OPN Ab had remarkable inhibitory effects against liver metastasis by the pancreatic cancer cell line.

Key words Osteopontin · RNA interference · Pancreatic cancer · Liver metastasis

Introduction

Pancreatic cancer patients have an extremely poor prognosis because liver metastasis is commonly observed even after extensive curative surgery.[1] Some chemotherapeutic regimens, such as gemcitabine, prolong survival of patients with resectable pancreatic cancer,[2] but in almost all cases of pancreatic cancer with liver metastasis, chemotherapy, radiation, and extensive surgery are unable to achieve long-term survival. It is critically important for oncologists to introduce a novel therapeutic means to achieve better survival for patients suffering from pancreatic cancer.

A highly liver metastatic HPC-3H4 cell line was derived from the human pancreatic cancer cell, HPC-3, which shows a low metastatic potential. These lines were used to investigate the molecular mechanisms involved in the liver metastasis of pancreatic cancer. A cDNA microarray was used to analyze the genes differentially expressed in these two cell lines. The microarray analysis revealed a variety of differentially expressed genes. This revealed that osteopontin (OPN) was highly expressed in metastatic HPC-3H4 cells.

Osteopontin is a secreted glycophosphoprotein with cell-adhesive and migratory properties, which can bind to extracellular matrix proteins and signal transducing receptors.[3–5] The expression of OPN is correlated with the metastasis of some malignant neoplasms.[6,7]

The current study evaluated the possibility that OPN might play an important role in the development of pancreatic cancer metastasis, and that OPN RNAi and anti-OPN Ab could have an anti-metastatic effect.

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Materials and Methods

Cell Lines

The human pancreatic cancer line, HPC-3 (parental cell line), its established highly metastatic variant, HPC-3H4 (liver metastatic line), and established sublines (HPC-3H1, HPC-3H2, and HPC-3H3) were used in this study. The procedure for establishing such variants has been described previously.[8–14] HPC-3 was inoculated into the spleens (5×10^6 cells) of nude mice. The mice developed cachexia after several weeks. The mice were then killed and a few metastatic nodules were harvested aseptically. The cells were expanded in culture and designated HPC-3H1. Thereafter, each selection cycle was repeated four times to yield HPC-3H4. These cell lines were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37°C in RPMI 1640 medium (Asahi Techno Glass, Funabashi, Japan) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 8 mg/l tylosin tartrate (Sigma-Aldrich, St. Louis, MO, USA), 50 units/ml penicillin, and 50 mg/ml streptomycin (Gibco). The cells were passaged and expanded by trypsinization of the cell monolayer and replating every 4–5 days. The culture medium was changed every 2–3 days.

Animals

Athymic female BALB/c nu/nu mice, 5–7 weeks old, weighing 20–22 g, and originating from the Central Institute for Experimental Animals (Kawasaki, Japan), were purchased from Clea Japan (Tokyo, Japan). The mice were maintained in a laminar airflow cabinet under specific pathogen-free conditions and also were provided with sterile food and water. All experiments were performed according to the institutional ethical guidelines on animal care.

cDNA Microarray Analysis

The differential gene expression was determined in the HPC-3 and HPC-3H4 cell lines. Poly(A)+ mRNA was extracted from cultured cell pellets using a FastTrack mRNA Isolation Kit (Invitrogen, Leek, the Netherlands) according to the manufacturer's instructions, and quantified spectrophotometrically. Double-stranded cDNA was synthesized from the purified poly(A)+ mRNA using the T7-(dt) 24 primer (5′-GGC CAG TGA ATT GTA AGT AAT ACG ACT CAC TAT AGG GAG GCG G-(dt) 24–3′) and a Superscript kit (Invitrogen, Carlsbad, CA, USA). Double-stranded cDNA was purified by phenol/chloroform extraction with phase-lock gels. Biotin-labeled antisense cRNA (target) in an in vitro transcription reaction (IVT) was produced using the ENZO BioArray RNA Transcript Kit (Affymetrix, Santa Clara, CA, USA). Thereafter, the IVT product (cRNA) was cleaned up using Qiagen RNeasy Columns (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cRNA was subsequently fragmented for target preparation. A hybridization cocktail was prepared using the Gene Chip Eukaryotic Hybridization Control Kit (Affymetrix). This cocktail was then hybridized on the Gene Chip Human Cancer G110 Array (Affymetrix) during a 16-h incubation in a 45°C oven. Immediately following hybridization, the hybridized probe array was subjected to an automated washing and staining protocol on the fluid station according to the Gene Chip Fluidics Station 400 User's Manual. Each probe array was scanned twice. The computer workstation automatically overlaid the twice-scanned images and averaged the intensities of each probe cell for the greatest assay sensitivity.

The data were analyzed using the Microarray Suite 4.0 software package (Affymetrix). The software calculated the average intensity of each probe array signal and then applied the selected array algorithm to determine the expression level for each gene. The average expression level of each gene in each cell line was calculated, as well as the ratio of the average expression level between the two cell lines.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from each cultured cell pellet using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 1 µg of total RNA using the Super Script Choice System for cDNA Synthesis (Invitrogen) in 19 µl of a reaction mixture according to the manufacturer's instructions. One microliter of synthesized first-strand cDNA was amplified by PCR in a final volume of 50 µl containing 5 µl of a 10× PCR buffer, 4 µl of 2.5 mM deoxynucleotide triphosphates (dNTP), 2 µl each of 10 µM forward and reverse primers, 0.5 µl of ExTaq polymerase (TaKaRa, Shiga, Japan) and 35.5 µl of water. The sequences of the primers for OPN and GAPDH used were 5′-TCC AAG TAA GTC CAA CGA AAG C-3′ (forward), 5′-GAC CTC AGT CCA TAA ACC ACA C-3′ (reverse) and 5′-GAG TCA ACG GAT TTG GTC GT-3′ (forward), 5′-TTA TTT TGG AGG GAT CTC G-3′ (reverse), respectively. PCR amplification of the cDNA was performed under the following conditions: 30 cycles, 15 s at 98°C, 2 s at 52°C, 30 s at 74°C. Before the first cycle, a denaturation step for 2 min at 98°C was included, and after 30 cycles the extension was prolonged for 7 min at 72°C.

Enzyme-Linked Immunosorbent Assay (ELISA) of OPN

The cells were cultured in 96-well plates at a cell density of 1×10^4 cells/well in an RPMI 1640 medium supplemented with 10% FCS for 24h, and, the next day, the culture medium was changed to serum-free RPMI 1640. After 2 days, the supernatants were collected, and the concentrations of OPN were measured using ELISA kits according to the manufacturer's instructions (IBL, Gunma, Japan). The data were normalized to cell number in the wells at the time of collection of conditioned medium with 1×10^4 cells.

Histological Treatment

To evaluate differences in histological features, 5×10^6 cultured cells were inoculated subcutaneously, and tumor specimens were fixed by the perfusion of 10% formaldehyde for 7–10 days. They were sectioned serially into 3-μm slices and stained with hematoxylin–eosin.

Immunohistochemical Study

Immunohistochemical staining was carried out using 4-μm-thick sections from formalin-fixed, paraffin-embedded tissues mounted on silane-coated glass slides. The tissues were deparaffinized in xylene and dehydrated through a graded alcohol series. The slides were heated in a 10mM citrate buffer by autoclaving for 10min at 120°C. Then, a monoclonal mouse antibody (1:100) against OPN (Anti-Human Osteopontin [10A16] Mouse IgG MoAb, IBL, Gunma, Japan) was applied overnight in a humid chamber at 4°C.

OPN-Expressing Vector Construction and Transfection

Human OPN cDNA was obtained by RT-PCR using total RNA from HPC-3H4 cells. The PCR oligonucleotides were 5′-CGG GAT CCA TGA GAA TTG CAG TGA TTT G-3′ (sense) and 5′-GGG AAG CTT GAC CTC AGA AGA TGC AC-3″ (antisense). The PCR product was digested with *Bam*HI and *Hin*dIII and cloned into pcDNA3.1/myc-His(–) A (Invitrogen) mammalian expression vector. The absence of undesired mutations in the construct was verified by sequencing.

Then, 2×10^4 HPC-3 cells were seeded at a 10-cm^2 surface area/plate with 2ml of an RPMI 1640 medium containing 10% FCS 24h before transfection. The transfection cocktail was made with 5μg of mixing plasmids (pcDNA3.1/OPN) and 15μl of Lipofectamine 2000 (Invitrogen) in 500μl of OptiMEM for 20min. The transfection was performed by adding the transfection cocktail to the culture medium. After 12h, the transfection cocktail was replaced with an RPMI 1640 medium containing 10% FCS. After 3 weeks of selection with 500μg/ml of G418, G418-resistant HPC-3 cells were cloned as HPC-3opn. The stable transfectant was maintained in a medium containing 100μg/ml of G418. HPC-3 cells transfected with an empty vector were cloned as HPC-3neg for a negative control.

OPN RNAi Design, Vector Construction, and Transfection

Nineteen-nucleotide (nt) DNA sequences targeting OPN were designed using the BLOCK-iT RNAi Designer at http://www.invitrogen.com/rnaidesigner. The sequences (5′-TGG CTA AAC CCT GAC CCA TCT-3′) corresponded to the sequence of human osteopontin mRNA (GenBank accession number: NM-000582). A 19-nt oligonucleotide 5′-GTC TCC ACG CAG TAC ATT T-3′, which had no significant homology to any known human mRNA in the databases, was used as a negative control. Synthetic sense and antisense oligonucleotides were used to create the template for generating RNA composed of two identical 19-nt sequence motifs in an inverted orientation separated by a 9-base-pair (bp) spacer to form a double-stranded hairpin of small interfering RNA. Two micrograms of each oligonucleotide was annealed by heating for 4min at 95°C and cooling for 10min to room temperature, and then ligated into 2μl of pcDNA 6.2-GW/EmGFP-miR, a linearized plasmid vector (containing the spectinomycin and Blasticidin resistance gene and the *Pol*II promoter; Invitrogen). These constructs were cloned in TOP10 chemically competent *Escherichia coli* cells according to the manufacturer's instructions (Invitrogen). The sequence of each insert was confirmed by automated sequencing. The two constructs were named pcDNA/miOPN as an OPN RNAi-expressing vector and pcDNA/miNeg as a negative control vector.

Next, 2×10^4 HPC-3H4 cells were seeded at 10-cm^2 surface area plates with 2ml of an RPMI 1640 medium containing 10% FCS 24h before transfection. The transfection cocktail was made by mixing 5μg of plasmids (pcDNA/miOPN or pcDNA/miNeg) and 15μl of Lipofectamine 2000 (Invitrogen) in 500μl of OptiMEM for 20min. The transfection was performed by adding the transfection cocktail to the culture medium. After 12h, the transfection cocktail was replaced with an RPMI 1640 medium containing 10% FCS. After 3 weeks of selection with 5μg/ml of Blasticidin, Blasticidin-resistant HPC-3H4 cells were cloned as HPC-3H4/miOPN and HPC-3H4/miNeg. The stable transformants

were maintained in a medium containing 5 μg/ml of Blasticidin.

Evaluation of Liver Metastatic Potential of Cell Lines

Cells (5×10^6) of each intended cell line were injected into the spleens of nude mice using a 27-gauge needle. The mice were killed approximately 4 weeks after the injection, and autopsies were performed thereafter. The ability of cells to produce liver metastasis in nude mice was evaluated microscopically.

Blocking Assay of Liver Metastasis in Vivo Using Anti-OPN Ab

First, 5×10^6 of HPC-3H4 cells suspended in 0.1 ml of PBS were injected into the spleens of nude mice. The mice were then randomly divided into two groups of ten animals each and given the following treatments: the OPN Ab group was injected intraperitoneally with an anti-OPN (M5) Ab,[15] and the control group was injected intraperitoneally with rabbit γ-globulin. Both treatments were administered on the day before and the day after inoculation at a dose of 450 μg dissolved in 1 ml of PBS. The mice were killed 4 weeks later, and the liver metastasis rate was calculated.

Statistical Analysis

Statistical differences were evaluated using the Mann–Whitney *U*-test and Fisher's exact test. *P* values of less than 0.05 were considered to be significant.

Results

Differential Gene Expression in a Liver Metastasis Cell Line

Table 1 shows the list of genes differentially expressed in HPC-3H4 in comparison to HPC-3. The ratio represents the expression value in a metastatic cell line, HPC-3H4, in comparison to that in the parental cell line, HPC-3. Nine upregulated genes and six downregulated genes were recognized to have over 5-fold expression differences in the ratio. Among differentially expressed genes, OPN was the most highly upregulated gene in HPC-3H4 in comparison to the HPC-3 line. The OPN mRNA expression level in HPC-3H4 was 11.1-fold that in HPC-3.

Relation of the Metastatic Rate and OPN Expression Among Cell Lines

The metastatic rates of the cell lines are presented in Fig. 1A. Four metastatic cell lines were established from the minimally/nonmetastatic HPC-3 parental cell line; HPC-3H4 with 100% metastatic ability, followed by HPC-3H3 with 90%, HPC-3H2 with 75%, and HPC-3H1 with less than 30%.

The expression of OPN transcripts was examined by nonquantitative RT-PCR (Fig. 1B) to investigate the role of OPN as a metastasis-related protein. The amplification of OPN in the highly metastatic cell lines seemed to be stepwise higher than that of the less metastatic cell lines. Amplified OPN fragments of the expected size (285 bp) were detected more strongly in HPC-3H4 than

Table 1. Differential gene expression in HPC-3H4 in comparison to that in HPC-3

Accession no.	Gene name	Intensity in HPC-3	Intensity in HPC-3H4	Ratio
Upregulated gene				
M28130	interleukin 8 (il8)	40.9	1284.3	31.0
M62402	insulin-like growth factor binding protein 6 (igfbp6)	−1.1	715.1	24.9
J04765	osteopontin	320.9	3111.6	11.1
U09937	urokinase-type plasminogen receptor	30.3	222.2	9.1
U04313	maspin	−9.4	241.3	9.0
M62403	insulin-like growth factor binding protein 4 (igfbp4)	42.9	326.4	8.4
U67988	guanylate kinase associated protein (gkap)	−11.2	145.4	6.4
M60314	bone morphogenetic protein 5 (bmp-5)	−0.5	153.2	6.4
L31584	G protein-coupled receptor (EBI 1)	9.0	159.1	6.0
Downregulated gene				
M22490	bone morphogenetic protein-2B (BMP-2B)	377.3	91.8	8.4
M14752	c-abl	133.5	4.5	7.2
U93867	RNA polymerase III subunit (RPC62)	65.7	−98.4	7.0
M35011	integrin beta-5	1156.6	169.7	6.8
U59423	Smad1	754.3	140.0	5.9
M90657	tumor antigen (L6)	3187.2	566.6	5.1

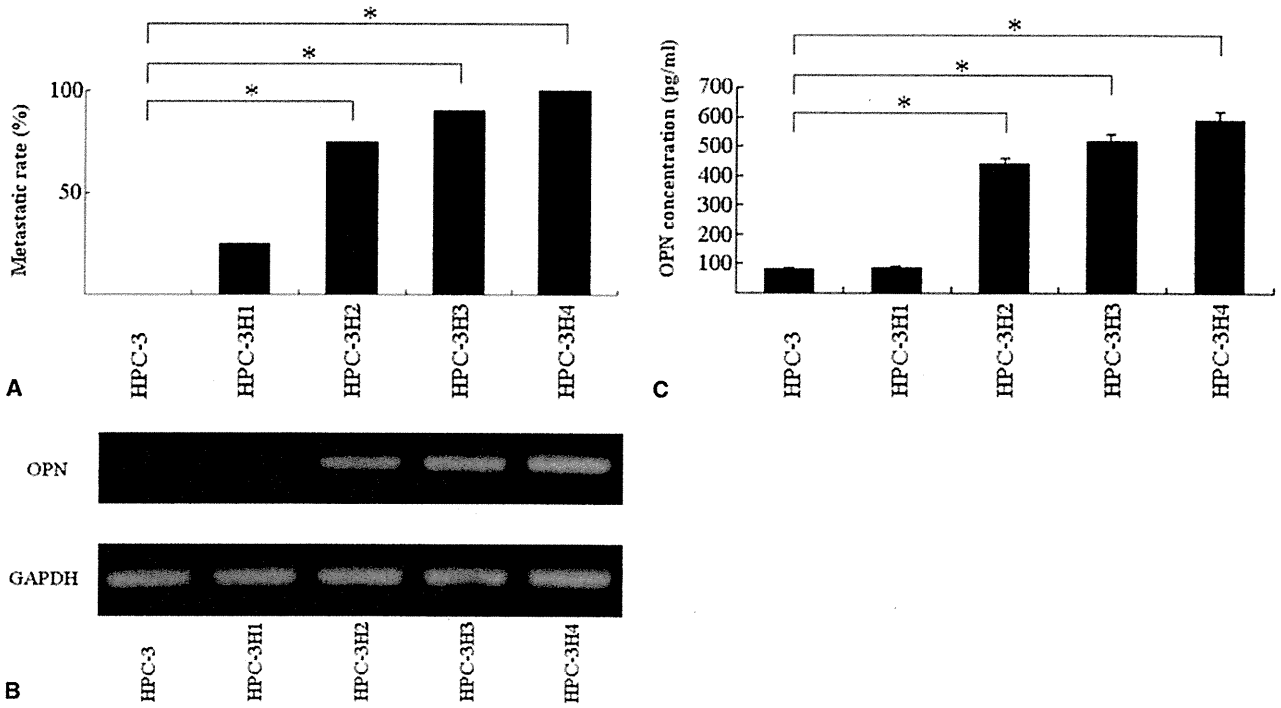

Fig. 1. A Metastatic rate of the established cell lines. The metastatic rate was higher in each metastatic subline than it was in each parental cell line. The numbers are the percentage values. $*P < 0.01$(Fisher's exact probability test) vs HPC-3. **B** Reverse transcription–polymerase chain reaction for the detection of osteopontin (*OPN*) mRNA. The amplification of OPN by the more metastatic cell line was stepwise higher than that of a lower metastatic cell line. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is shown as a quantitative control. **C** Enzyme-linked immunosorbent assay of OPN production. OPN production by highly liver metastatic cell lines was significantly higher than that by the parental cell line. The results are expressed as mean ± SD. $*P < 0.01$, significantly different from HPC-3

in HPC-3. The identity of the amplified fragments was verified by sequencing.

The level of OPN protein expression by various cell lines was determined using an ELISA to correlate the degree of OPN expression with the metastatic potential. Figure 1C shows that the production of OPN by the highly metastatic cell lines was stepwise higher than that of less metastatic cell lines. The OPN levels of HPC-3, HPC-3H1, HPC-3H2, HPC-3H3, and HPC-3H4 were 85.3 ± 0.66, 87.0 ± 0.63, 439.7 ± 2.95, 517.6 ± 6.41, and 586.9 ± 9.89 pg/ml, respectively (mean ± SE). Osteopontin production by HPC-3H2, HPC-3H3, and HPC-3H4 were significantly higher than that by HPC-3 ($P < 0.01$).

Two representative cell lines, HPC-3 and HPC-3H4, were used in following experiments to further dissect the molecular role of OPN in liver metastasis. The histological features were initially examined. Two cell lines were injected into mice, and the tumor tissues that developed in vivo were stained with hematoxylin–eosin and immunohistochemically with the OPN antibody. There was no remarkable difference in the morphology between HPC-3 and HPC-3H4. The pathological findings corresponded to poorly differentiated adenocarcinoma, and the two cell lines had essentially the same appearance (Fig. 2). The immunohistochemical analysis showed a similar amount and pattern of OPN staining between the two cell lines (Fig. 2): OPN was mostly present in the cytoplasm of tumor cells.

Effect of Forced Alteration of the OPN Production Ability on Cell Lines

The metastatic ability was further analyzed by manipulating OPN expression by silencing the endogenous OPN gene or introducing an exogenous OPN gene. First, an HPC-3opn cell line was established, in which the exogenous OPN gene was introduced, and its negative control was the HPC-3neg cell line. Figure 3A shows that OPN production by HPC-3opn was 587.2 ± 2.80 pg/ml, which was similar to the level produced by the highly metastatic HPC-3H4 cells. In sharp contrast, both HPC-3neg and HPC-3 produced significantly lower amounts of OPN in comparison to HPC-3H4 cells. Consistent with the ELISA results, the amplifica-

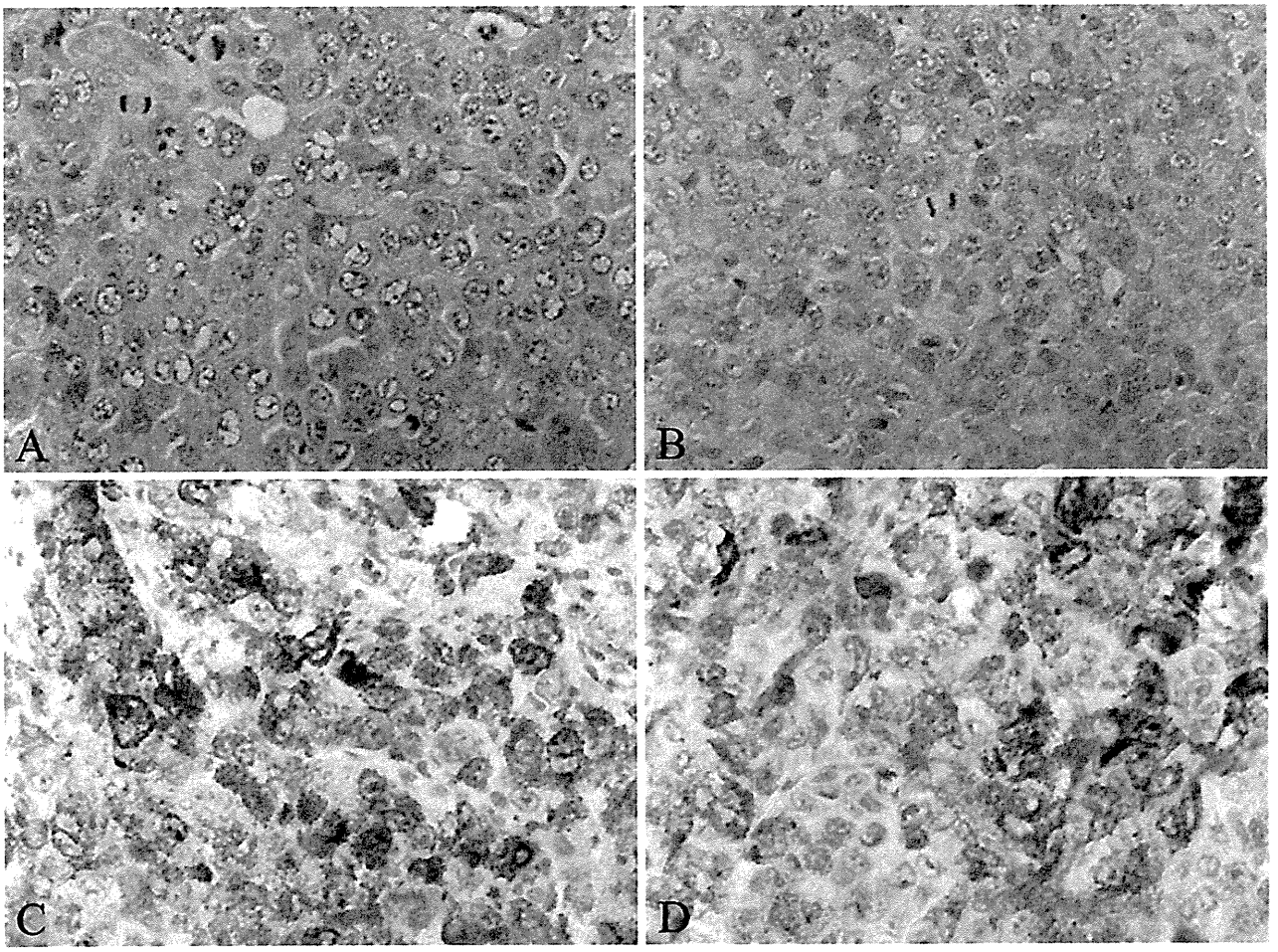

Fig. 2. Hematoxylin–eosin and immunohistochemical staining of tumors formed by cell lines. Histopathology of tumors showing poorly differentiated adenocarcinoma, with the same morphology of cancer growth. **A** HPC-3: parental tumor growing in a subcutaneous lesion, **B** HPC-3H4: a metastatic lesion growing in the liver. Immunohistochemical staining for OPN, with the same level of immunostaining intensity. OPN expression is localized in the cytoplasm of tumor cells and the extracellular matrix. **C** HPC-3: a parental tumor growing in a subcutaneous lesion. **D** HPC-3H4: metastatic lesion growing in the liver

tion of mRNA of OPN by HPC-3opn was at the same level as that by HPC-3H4, and was significantly stronger than that by HPC-3 and HPC-3neg (Fig. 3B).

Subsequently, the metastatic rate was evaluated using these cell lines. Unexpectedly, the metastatic rate of the HPC-3opn was 0% (0/10), which was the same as those of HPC-3 and HPC-3neg. Therefore, the introduction of exogenous OPN gene alone into low metastatic HPC-3 cells did not promote liver metastasis.

The following experiments attempted to inhibit the liver metastasis of highly metastatic HPC-3H4. An HPC-3H4/miOPN cell line was established, in which endogenous OPN expression was silenced by OPN RNAi and its negative control, HPC-3H4/miNeg cell line. Stable blocking of OPN by the RNAi-expressing vector was confirmed by verifying that the OPN production was at the same level in a transformant (HPC-3H4/miOPN) cultured for 1 week, a transformant (HPC-3H4/miOPN) cultured for 4 weeks, and a parental cell line (HPC-3) using an ELISA. Figure 3A shows that the OPN level in HPC-3H4/miOPN was 96.6 ± 0.68 pg/ml, which was significantly lower than that of HPC-3H4 and HPC-3H4/miNeg ($P < 0.01$) in ELISA. In addition, the amplification of OPN mRNA by HPC-3H4/miOPN was at the same level as that of low metastatic HPC-3 (Fig. 3B).

The metastatic rates of these established cell lines are presented in Fig. 3C. The metastatic rate of the HPC-3H4/miOPN was 25% (2/8), a significantly lower rate than those of HPC-3H4 and HPC-3H4/miNeg. Therefore, the silencing of the endogenous OPN expres-

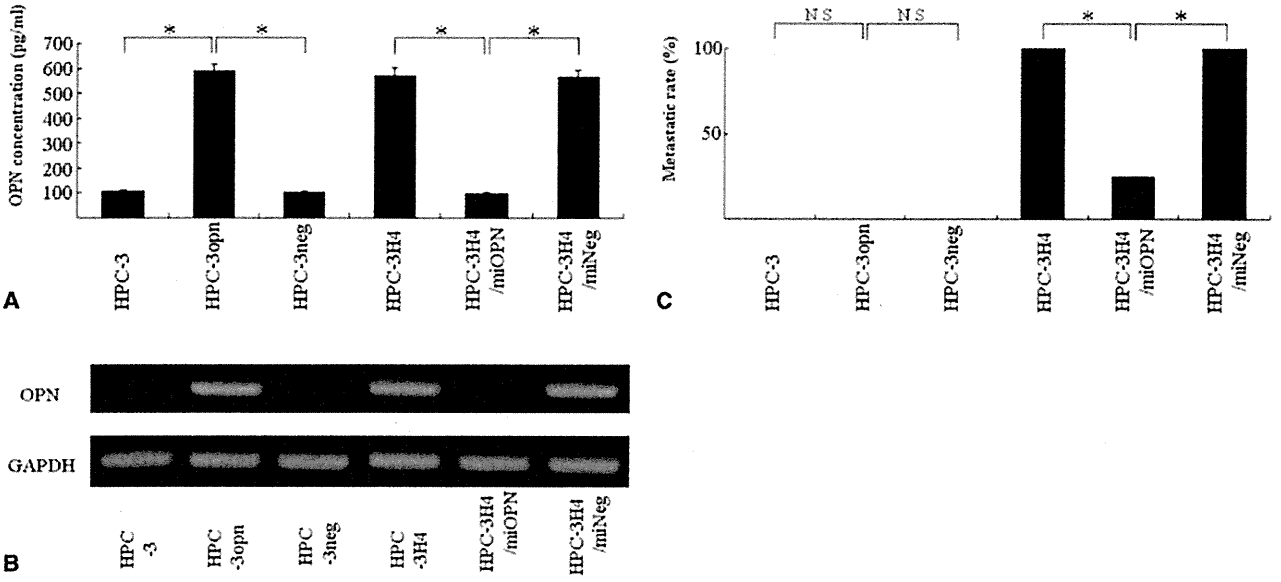

Fig. 3. **A** Enzyme-linked immunosorbent assay of OPN production. OPN production by HPC-3H4, HPC-3opn, and HPC-3H4/miR-neg was significantly higher than that by HPC-3, HPC-3neg, and HPC-3H4/miOPN. OPN production by HPC-3H4/miOPN was at the same level as that by HPC-3. The results are expressed as mean ± SD. $*P < 0.01$, significantly different from HPC-3opn and HPC-3H4/miOPN. **B** Reverse transcription–polymerase chain reaction for detection of OPN mRNA. An amplified fragment of the expected size (285 bp) was detected to a greater extent in HPC-3H4, HPC-3opn, and HPC-3H4/miNeg than in HPC-3, HPC-3neg, and HPC-3H4/miOPN. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) is shown as a quantitative control. **C** Metastatic rate of the cell lines. The metastatic rate of the OPN-forced expression cell line, HPC-3opn, is 0% (0/10), the same level as that of HPC-3. The metastatic rate of the OPN-suppressed expression subline, HPC-3H4/miOPN, is 25 (2/8). The OPN RNAi-expressing vector reduced the metastasis to the liver. The numbers are percentage values. $*P < 0.01$ (Fisher's exact probability test) vs HPC-3H4/miOPN

sion in HPC-3H4 cells significantly reduced the liver metastasis ($P < 0.01$).

Effect of Anti-OPN Ab on the Liver Metastasis of HPC-3H4 Cells

Finally, anti-OPN Ab or control Ab was administered into the peritoneal cavity of mice at 1 day before and after intrasplenic HPC-3H4 cell injection. The metastatic rate of the control group was 100% (10/10), while that of the metastatic rate of OPN Ab group was 50% (5/10; Fig. 4). All mice in the control group had multiple and bilateral liver metastatic nodules, while most of the mice in the OPN Ab group had only a single or several unilateral metastatic nodules. These results demonstrated that the anti-OPN Ab administration significantly reduced the liver metastatic rate of HPC-3H4, which is a highly metastatic cell line ($P < 0.01$).

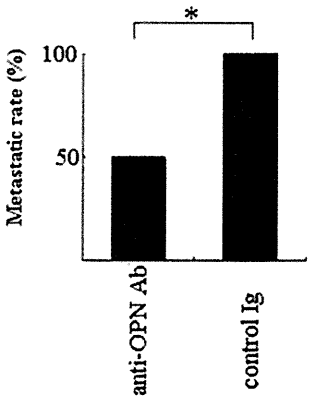

Fig. 4. Effect of anti-OPN antibody (*Ab*) on the liver metastasis of the HPC-3H4 cells in vivo. The metastatic rate of the control group (rabbit γ-globulin) was 100% (10/10), and all mice developed liver metastasis. On the other hand, the metastatic rate of the anti-OPN Ab group was 50% (5/10), and half of the mice developed liver metastasis. These results demonstrated that anti-OPN Ab administration reduced the liver metastatic rate of HPC-3H4. The numbers are percentage values. $*P < 0.01$ (Fisher's exact probability test) vs control

Discussion

Molecular-targeted agents, such as trastuzumab, which is effective against HER-2 overexpressed breast cancer,