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A novel apoptosis-inducing monoclonal antibody (anti-LHK) against a cell surface antigen on colon cancer cells

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Background. Apoptosis is a crucial element in the behavior of mammalian cells in many different situations. We here report the establishment of a novel monoclonal antibody (anti-LHK mAb) that has apoptosis-inducing activity against colon cancer Colo205 cells. ***Methods.*** The mechanism of anti-LHK mAb-induced cell death was assessed by microscopic morphology, Annexin V/Hoechst 33528 staining, and detection of DNA fragmentation. The molecular weight of LHK antigen was determined by Western blotting. Growth inhibition of Colo205 cells induced by anti-LHK mAb was determined by in vitro and in vivo studies. ***Results.*** Anti-LHK reacted with a 70-kDa antigen and completely blocked the proliferation of Colo205 cells bearing LHK in vitro in a manner characteristic of apoptosis. Strikingly, anti-LHK mAb suppressed tumor growth in a murine peritoneal dissemination model. ***Conclusions.*** LHK antigen, which is restricted to epithelial cells, may be a novel death receptor that plays a critical role in controlling the growth, invasion, and metastasis of human colon cancer cells.

Key words: anti-LHK mAb, apoptosis, Fas, colon cancer

Introduction

Monoclonal antibodies (mAbs) are accepted as ideal adjuvant therapeutic agents for some diseases. In 1989, two groups independently isolated the same mouse-derived antibody, designated anti-Fas[1] and anti-APO-1 mAb,[2] respectively, which had a cytotoxic effect on various human cell lines.[1,2] Fas belongs to the tumor necrosis factor receptor (TNF-R) family.[3–6] The mechanism of cell death induced by Fas signaling is apoptosis. Furthermore, Fas is broadly expressed in a variety of normal organs and tissues, including hematopoietic cells and various normal and cancer epithelial cells.[7] It is well known that colon cancer cells express relatively lower levels of Fas and are more resistant to the Fas system than normal colonic epithelial cells.[6] In fact, Moller and colleagues[8] reported that Fas-negative subpopulations, which occur rarely in colon adenomas and normal cells, are common in colon cancers. In the immune response to neoplasms, cytotoxic T lymphocytes (CTLs) are activated by tumor cells and thereafter express Fasligand(L) on their own surfaces. FasL-expressing CTLs then bind to Fas-expressing target cells, and the binding induces the intrinsic apoptotic pathway in tumor cells. In contrast, it has been reported that colon cancer cells also express functional FasL on the surface and kill Fas-expressing Jurkat cells in a Fas-mediated manner as "Fas counterattack".[9] This converse phenomenon suggests that colon cancer cells can kill CTLs infiltrating around them through the Fas system and escape from immunological rejection.

Although it appears that an anti-Fas strategy is not feasible for the treatment of patients with colon cancers because of Fas resistance and its fatal side-effects, apoptotic cell death is still potentially of importance for clinical application. Here we report our establishment and analysis of a novel antibody (anti-LHK) that had a cytotoxic effect on Fas-resistant Colo205 colon cancer cells.

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Methods

Reagents and antibodies

Recombinant human (rh) interferon (IFN)-γ, with a specificity of 4×10^6 units/mg, was kindly provided by Shionogi Pharmaceutical (Osaka, Japan); rh TNF-α (1.7×10^7units/ml) and the murine mAb anti-TNF-R IgG2a were purchased from GenZyme (Cambridge, MA, USA). Ethidium bromide, propidium iodide (PI), cycloheximide (CHX), Hoechst 33528, and dithiothreitol were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). The murine mAb anti-Fas IgM (CH11) was purchased from MBL (Nagoya, Japan). Fluorescein iso thiocyanate (FITC)-conjugated goat polyclonal anti-mouse IgM or IgG was purchased from Tago (Burlingame, CA, USA). [^{35}S]-methionine was purchased from ICN Pharmaceuticals (Irvine, CA, USA). Dispase was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN, USA). Block ace was purchased from Dainihon Pharmaceuticals (Osaka, Japan). Streptavidin-enzyme conjugates (Vectastatin ABC kit) were purchased from Vector Laboratories (Burlingame, CA, USA).

Mice

BALB/c and C.B.17 severe combined immunodeficiency (SCID) mice (6–8 weeks old, male) were purchased from Japan Clea (Tokyo, Japan). The mice were maintained in the Animal Care Facility of Keio University. The review board of the University has approved our experimental animal studies.

Cell lines and human tissues

The human colon cancer cell lines (Colo205, WiDr, HT-29, and Caco2) and the human gastric cancer cell line (KATO-III) were purchased from ATCC (Rockville, MD, USA). The mouse myeloma cell line Sp2/0 was kindly given by Dr. S. Aiso (Keio University). The human hepatoma cell line derived from hepatitis B virus (HBV)-infected patients (Alexander) and the cervical epidermoid carcinoma cell line (HeLa) were kindly given by Dr. H. Saito (Keio University). The human T-cell leukemia cell line Jurkat was kindly given by Dr. Kayagaki (Juntendo University, Tokyo). The cell culture and all assays in this study were performed in RPMI 1640 or DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 1% penicillin/streptomycin, and 2 mercapto ethanol (ME) (5×10^{-5} M). All normal human tissues and tumor tissues were obtained from biopsied or surgical specimens. The diagnostic criteria for the histological examinations were based on the Japanese classification of colorectal carcinoma (6th edition) of the Japanese Society for Cancer of the Colon and Rectum. Written informed consent was obtained from all patients prior to the study and all experiments were approved by the Committee on Human Subjects of Keio University Hospital.

Preparation of mAb

Before immunization, 1×10^7 Colo205 cells were stimulated with 100 U/ml human rhIFN-γ for 48 h. Then, 6-week-old BALB/c mice were immunized intraperitoneally with IFN-γ-stimulated Colo205 cells (4.0×10^7) four times, every 3 weeks. Four days after the last injection, spleen cells of immunized mice were fused with Sp2/0 cells by the standard hybridization technique. A hybridoma cell (termed B3D11-H12) producing an mAb with cytotoxicity to Colo205 cells was cloned twice by limiting dilution at a concentration of 0.3 cells per well. After the injection of B3D11-H12 cells into the abdominal cavity of BALB/c mice, ascites were purified. The mAb was termed anti-LHK mAb, and its isotype was determined by enzyme-linked immunosorbent assay (ELISA; Biomeda, Foster City, CA, USA).

Immunoprecipitation

Prior to the immunoprecipitation assay, cell extracts were prepared from 5×10^6 Colo205 cells in methionine-free minimal essential medium, which were first labeled with [^{35}S]-methionine (500 μCi; ICN Pharmaceuticals) overnight. Cells were then harvested, washed with TBS (10 mM Tris-Cl, 150 mM NaCl, pH 7.5), resuspended in lysis buffer (50 mM Tris-buffered saline, pH 7.6, 150 mM NaCl, 0.5% Triton X-100, 1 mM phenyl methylsulfonyl fluoride [PMSF], 10 mM iodoacetamine, 10 μg/ml leupeptin, 10 μg/ml pepstatin, and 10 μg/ml aprotinin), and sonicated three times for 40 s each time with a Branson sonifer (Branson Ultrasonics, Danbury, CT). The supernatants were centrifuged at 15 000 rpm for 10 min to remove soluble debris and used as sources of antigen. An immunoprecipitation assay was performed as described before.[10] Ten micrograms of anti-LHK mAb or isomatched anti-mouse IgM was incubated with 2 μg of protein A-Sepharose beads or protein A-Agarose beads (Sigma-Aldrich) pre-swollen in 500 μl of IPP buffer (500 mM NaCl, 0.1% Nonidet P-40 [Wako Chemicals, Tokyo, Japan], 10 mM Tris-Cl, pH 8.0) overnight at 4°C in a microfuge tube with end-to-end rotation. The antibody-coated beads were washed three times with 500 μl of IPP buffer, and then 400 μl of IPP buffer was added and the antibody-coated beads were incubated for 2 h at 4°C with 200 μl of [^{35}S]-labeled cell extract, which was equivalent to 5×10^5 Colo205 cells. After three washes, the beads were heated at 95°C

in sodium dodecyl sulfate (SDS) sample buffer (62.5 mM Tris-Cl, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue, pH 6.8) for 5 min to extract the bound proteins. The proteins were loaded onto 10% polyacrylamide-SDS gel and subjected to electrophoresis. The gel was then soaked for 30 min in 0.5 M sodium salicylate, dried, and exposed to X-ray film (XAR-5 film; Eastman Kodak, Rochester, NY, USA) with an intensifying screen at –70°C.

Immunohistochemistry

Serial sections (6 μm) from the fresh frozen tissues were air-dried for 2–3 h, fixed in acetone for 10 min at room temperature, and immunostained immediately. After the blocking of nonspecific binding sites with Block ace (Dainippon Pharmaceutical) for 10 min at 37°C, the sections were incubated with purified mouse anti-LHK IgM mAb (1 μg/ml) or isomatched mouse IgM (1 μg/ml, as a negative control) for 30 min at 37°C, then with biotinylated goat anti-mouse IgM for 30 min at 37°C, and finally with streptoavidin-enzyme conjugates (Vectastatin ABC kit). The localization of LHK antigen was visualized by incubation with diaminobenzidine solution, and faint counterstaining was performed with Mayer's hematoxylin.

Flow cytometry

An immunofluorescence technique[11] was used to estimate the quantity of the LHK antigen, Fas, or TNF-R expression by flow cytometry. Cultured cells were washed in phosphate-buffered saline (PBS) and suspended in 50 μl of staining buffer (PBS containing 2% FCS and 0.1% NaN_3), then 5 μg/ml of each primary antibody (anti-LHK, anti-Fas, anti-TNF-R) or isotype-matched control antibody (polyclonal mouse IgM or IgG) was added, and the samples were placed on ice for 20 min. FITC-conjugated goat anti-mouse IgM or goat anti-rat IgG was stained for 20 min. Samples were rewashed in staining buffer and suspended at 1×10^6 cells/ml in 50 μl of PBS. Fluorescence intensity on the surface was then analyzed using a FACScan (BD Sciences, San Jose, CA, USA). Changes in LHK antigen, Fas, and TNF-R expression after pretreatment with 100 U/ml IFN-γ for 48 h were also assessed. In addition, early apoptotic cells were detected by flow cytometric analysis, using FITC-labeled Annexin V, a protein with high affinity for phosphatidylserine exposed upon the cell membrane in the early apoptotic phase.[12]

Morphological analysis

Approximately 5×10^4 cells were seeded in 25-cm^2 culture flasks and incubated with isotype-matched control IgM (1 μg/ml), anti-LHK mAb (1 μg/ml), anti-Fas IgM (1 μg/ml), or TNF-α (50 ng/ml) in complete medium for 12 h, then washed twice with PBS. After exposure, the cells were trypsinized and harvested. Cell viability was counted by trypan blue staining. Morphological changes caused by these mAbs were observed by phase-contrast microscopy. In addition, apoptotic cells were assessed morphologically by staining with Hoechst 33258 (Sigma-Aldrich).

Demonstration of DNA laddering

DNA fragmentation was visualized by the method of Sellins and Cohen,[13] with some modification. Briefly, cells (5×10^6) cultured for 3 h with anti-LHK mAb (1 μg/ml) or isotype-matched control IgM (1 μg/ml) were lysed in 1 ml of DNA extraction solution containing 20 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 5 mM ethylenediamire tetraacetic acid [EDTA], and 0.5% SDS. The lysates were incubated with 100 μg/ml proteinase K at 37°C for 16 h. After incubation, the enzyme-digested cell lysates were carefully mixed with 1 ml of phenol/chloroform (1:1) and centrifuged at 15 000 *g* for 20 min. DNA in the aqueous phase was incubated with 5 μg/ml DNase-free RNase A at 37°C for 1 h and again extracted with phenol/chloroform. DNA was collected by precipitation with 1 ml isopropanol and 0.1 ml NaCl at –20°C overnight. After centrifugation, the resulting DNA was dissolved in 10 mM Trs-HCl and 1 mM EDTA, and its concentration was determined at 260 nm by spectrophotometry. DNA electrophoresis was carried out in 1.2% agarose gels containing 1 μg/ml ethidium bromide, and DNA banding was visualized by exposing the gels to UV light.

Inhibition of Colo205 cell proliferation by anti-LHK IgM

Colo205 cells and Fas-sensitive Jurkat cells (5×10^4 cells/well) were incubated with the isotype-matched control IgM, anti-LHK mAb, anti-Fas IgM, or rhTNF-α at various concentrations in 100 μl of complete medium in a 96-well round culture plate for 24 h at 37°C in a 5% CO_2 incubator. Then cells were labeled with [^{3}H] thymidine by the addition of 50 μl of complete medium containing 0.5 μCi of [^{3}H]-thymidine for 4 h. The radioactivity incorporated was counted in a liquid scintillation counter to quantify the level of DNA synthesis.

Preparation of isolated epithelial cells from the intestinal mucosa

Human colonic epithelial cells were isolated from surgical specimens as previously described.[14] Briefly, resected samples of colonic mucosa were dissected from

the underlying musculature and washed in calcium- and magnesium-free Hanks' balanced salt solution (CMF-HBSS). Tissues were then treated with 1 mM dithiothreitol (Sigma-Aldrich) in CMF-HBSS for 15 min at 22°C. After three washes with CMF-HBSS, the tissue pieces were incubated twice in CMF-HBSS containing 1 mg/ml dispase (Boehringer Mannheim Biochemicals, Indianapolis, IN, USA) for 30 min at 37°C. During the treatment, epithelial cells and intraepithelial lymphocytes were released from the tissues. The cell suspensions resulting from dispase treatment were washed twice, pelleted, and resuspended in 3 ml of 100% Percoll (Pharmacia Biotech, Piscataway, NJ, USA). Three-ml layers of 60%, 40%, and 30% Percoll were layered successively on top prior to centrifugation at 1500 rpm for 30 min at 4°C. Cells at the top 0/30% layer interface contained more than 95% pure epithelial cells. Cells were washed three times in RPMI, and viability was determined by trypan blue exclusion.

Xenograft model of Colo205 cells

A xenograft model of human colon cancer was made as previously reported.[15] Briefly, cultured Colo205 cells were harvested and the pellet of cells obtained by centrifugation was suspended in RPMI 1640 with 10% FCS to a concentration of 1×10^7 cells/ml. Aliquots of 5×10^6 cells were injected into the peritoneal cavities of 8-week-old BALB/c SCID mice. Twenty-four hours after the injection, 500 µg of anti-LHK IgM or control mouse IgM mAb in 0.5 ml of RPMI 1640 was injected intraperitoneally into Colo205-transplanted mice, and the injection was repeated weekly. The dosage was selected based on the preliminarily observed effects of several doses. Eight weeks after the first injection, mice were killed by cervical dislocation. The peritoneal tumors were dissected out and the total volumes were measured. Tumor volumes were calculated by using the formula ($A \times B^2/2$), where A and B are tumor caliper measurements expressed in millimeters, and $A > B$. The volume of tiny nodules in the mesentery was negligible, because they were less than 0.2 mm in diameter.

Statistical analysis

The values for results were expressed as means ± SD. Groups of data were compared by the Mann-Whitney U-test for correlations between the frequency of LHK^+ cells and histology of colon cancer, and tumor numbers and tumor volume examined in the xenograft model, and by the χ^2 test for the frequencies of abdominal masses and peritoneal dissemination in the xenograft model. Differences were considered to be statistically significant when P was less than 0.05.

Results

Anti-LHK mAb induces apoptosis

To establish novel mAbs, with cytotoxicity against colon cancer cells, BALB/c mice were immunized with IFN-γ-stimulated Colo205 colon cancer cells. Among more than 10000 hybridoma cells, one clone produced an mAb that had cytotoxicity against Colo205 cells. The isotype of the mAb was proven to be IgM, and the mAb was designated anti-LHK antibody. Anti-LHK mAb was purified to homogeneity, and the purified anti-LHK mAb killed Colo205 cells as well as crude culture supernatant or ascites of mice undergoing intraperitoneal injection of hybridomas. Anti-LHK mAb was added to Colo205 cells cultured in complete media at the concentration of 1 µg/ml, and cells were observed sequentially by phase-contrast microscopy. Budding, which is a characteristic of the apoptotic process,[16] was observed 3 h after the start of culture (Fig. 1A, upper right) and cell death was induced 12 h after the culture (Fig. 1A, lower right). In contrast, cell death was not induced in Colo205 cells cultured in the presence of control IgM (Fig. 1A, upper and lower left). Next, suppression of proliferation of Colo205 cells treated with anti-LHK mAb was assessed using [^{3}H]-thymidine. The proliferation of Colo205 cells was markedly suppressed by anti-LHK mAb in a dose-dependent manner (Fig. 1B).

To confirm the mechanism of cell death induced by anti-LHK mAb, three types of assays for apoptosis were performed. Hoechst 33528 staining of DNA revealed morphological features characteristic of apoptosis in Colo205 cells: fragmented and condensed nuclei were observed after 12 h of treatment with anti-LHK mAb (Fig. 2A). In the electrophoresis of DNA extracted from Colo205 cells treated with anti-LHK mAb, DNA fragmentation was visualized as DNA ladders (Fig. 2B). In the double-staining of Annexin V/propidium iodide (PI), Colo205 cells treated with anti-LHK mAb for 12 h were mainly Annexin V^-/PI^+ cells and Annexin V^+/PI^+ cells, as were many of those treated with CHX (Fig. 2C). These results suggest that apoptosis was critically involved in the mechanism of anti-LHK-induced cell death.

The antigen for anti-LHK mAb

Because Fas and TNF receptor (TNF-R) are known to be cell death receptors that induce apoptotic cell death, we next assessed whether the mechanism of anti-LHK mAb-induced apoptosis of Colo205 cells was identical to those of anti-Fas or TNFα. Phase-contrast microscopic time-course observation revealed that anti-LHK mAb and TNFα, but not anti-Fas mAb (CH11), had strong cytotoxicity for Colo205 cells (Fig. 3A, left). In

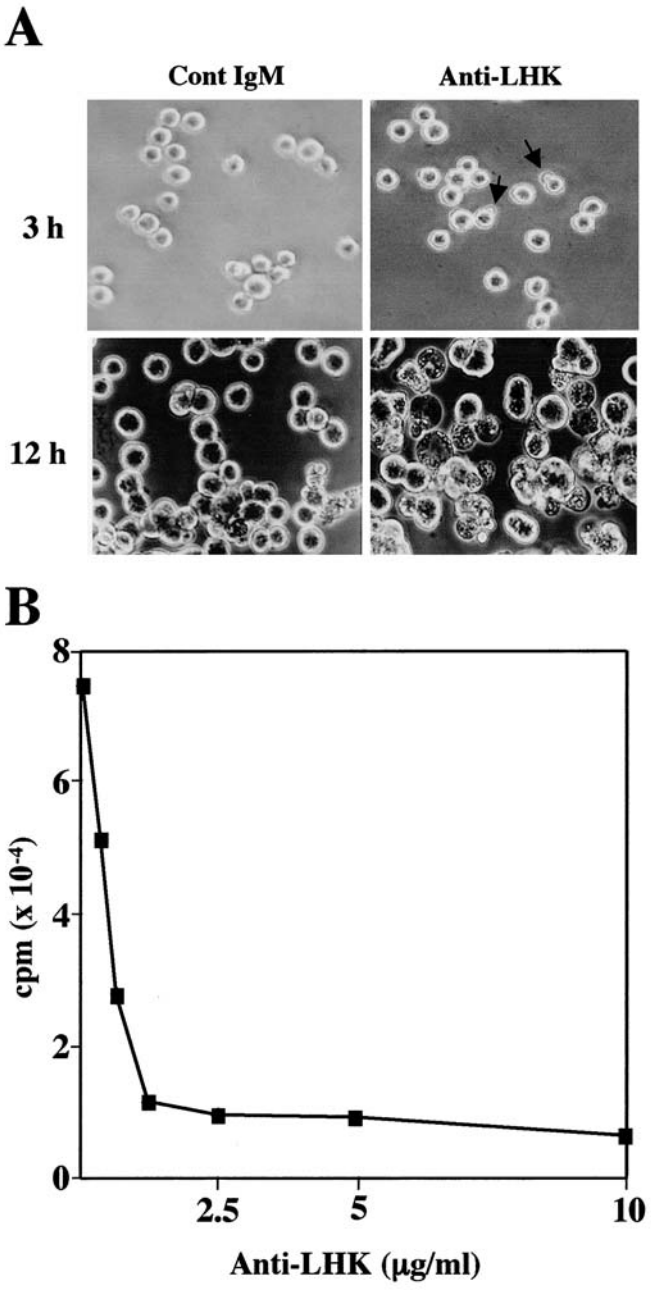

Fig. 1. **A** Morphological changes and proliferative responses of Colo205 cells induced by anti-LHK monoclonal antibody (mAb). Dying Colo205 cells observed under a phase-contrast microscope. Three h (*upper left*) and 12-h (*lower left*) with control IgM (1 μg/ml) and 3 h (*upper right*) and 12 h (*lower right*) with anti-LHK mAb (1 μg/ml). ×120. *Arrows* indicate budding phenomena. **B** Suppression of proliferative responses of Colo205 cells induced by anti-LHK mAb at various concentrations

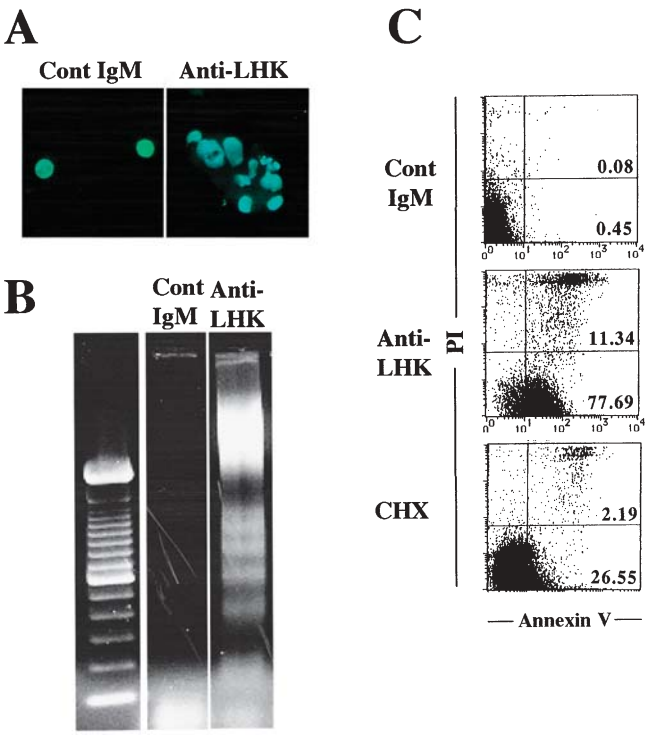

Fig. 2A–C. Anti-LHK-induced apoptosis. **A** Hoechst 33528 staining revealed morphological changes characteristic of apoptosis in Colo205 cells after treatment for 12 h with anti-LHK mAb (1 μg/ml; *left*). Not observed after treatment for 12 h with control IgM (1 μg/ml; *right*). ×120. **B** DNA ladders produced in Colo205 cells after culture with anti-LHK mAb (1 μg/ml; *right*) for 12 h. They were not observed after culture control (*cont*) IgM (1 μg/ml; *middle*). *Left*, size markers. **C** Double-staining of Colo205 cells with Annexin V and propidium iodide (PI). Colo205 cells incubated for 12 h with control (*cont*) IgM (1 μg/ml; *upper*), anti-LHK mAb (1 μg/ml; *middle*), or cycloheximide (CHX; 100 ng/ml; *lower*) were stained with Annexin V and PI

sharp contrast, anti-Fas mAb and TNFα, but not anti-LHK mAb, showed cytotoxicity toward to Jurkat cells, a human T-cell leukemia cell line (Fig. 3A, right). In the analysis of proliferation using [^{3}H]-thymidine, anti-LHK mAb, but not anti-Fas and TNFα, blocked the proliferation of Colo205 cells in a dose-dependent manner (Fig. 3B, upper). With Jurkat cells, the result corresponded with that of the microscopic observation (Fig. 3B, lower). Because Fas expression is reported to be upregulated by IFN-γ treatment,[17] we further investigated the modification of LHK antigen and Fas by IFN-γ treatment. As shown in Fig. 4, Fas expression on Colo205 cells was significantly upregulated by exogenously added IFN-γ, whereas LHK antigen was not affected at all. To further compare LHK, Fas, and TNF-R, we examined the expression profiles of LHK antigen, Fas, and TNF-R on various human cancer cell lines by flow cytometry. Although LHK antigen was markedly expressed on Colo205 cells, it was also expressed on other colon cancer cell lines, such as WiDr, HT29, and Caco2, albeit to a lesser extent (Fig. 5). Furthermore, LHK was moderately to highly positive on the gastric cancer cell line Kato-III, marginal on Jurkat cells, and negative on hepatocellular carcinoma Alexander cells

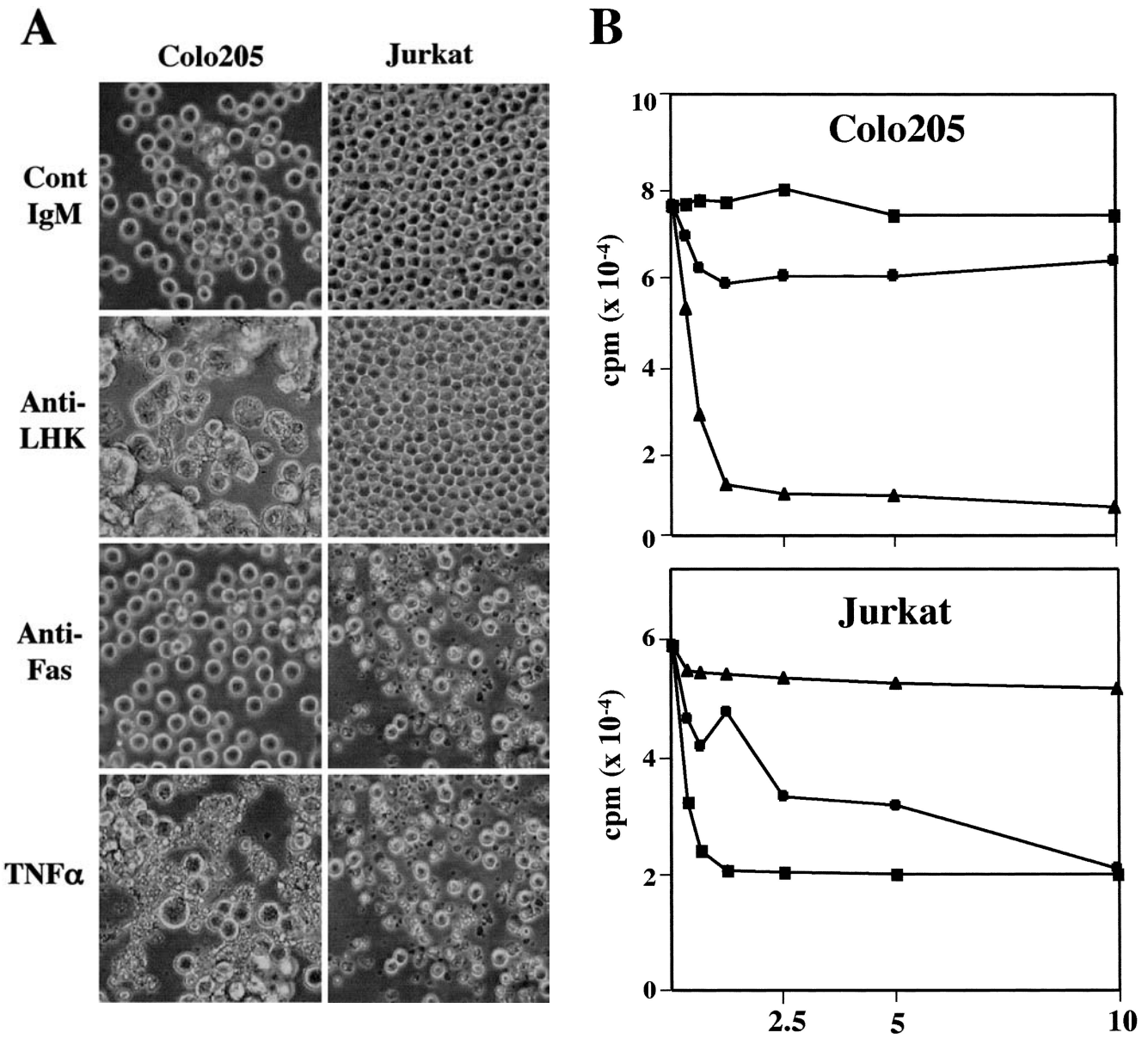

Fig. 3A,B. Distinct sensitivities of Colo205 and Jurkat cells to cell death induced by anti-LHK mAb, anti-Fas mAb, and tumor necrosis factor-α (*TNFα*). **A** Morphological changes of Colo205 cells and Jurkat cells induced by anti-LHK mAb (1 μg/ml), anti-Fas mAb (CH11; 1 μg/ml), and TNFα (50 ng/ml) after culture for 12 h. ×120 **B** Proliferative responses of Colo205 cells and Jurkat cells to control IgM, anti-LHK mAb (*triangles*; μg/ml), anti-Fas mAb (*large squares*; μg/ml), and TNFα (*small squares*; ng/ml). Cells were cultured in the presence of control IgM, anti-LHK mAb, anti-Fas mAb, or TNFα at various concentrations in triplicate for 48 h, and then labeled with [^{3}H]-thymidine for a further 4 h

and uterine cervical cancer HeLa cells. Based on these expression profiles, LHK was clearly distinguishable from Fas and TNF-R.

The molecular mass of LHK antigen was next assessed by immunoprecipitation assay. LHK antigen from Colo205 cells was specifically immunoprecipitated under reducing conditions and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE), using protein A-Sepharose beads (Fig. 6; lane 2, isomatched anti-mouse IgM; lane 3, anti-LHK mAb) and protein A-Agarose beads (lane 4, isomatched anti-mouse IgM; lane 5, anti-LHK mAb), where it appeared as a main band of 70 kDa (Fig. 6). Although several other bands were also observed, the band of 70 kDa was found only in lanes of anti-LHK, and not in lanes of control IgM by two different methods. Collectively, the results suggest that LHK antigen is a novel molecule that is distinct from Fas and TNF-R.

We next analyzed LHK antigen expression on human colon cancer cells and normal colonic epithelia by im-

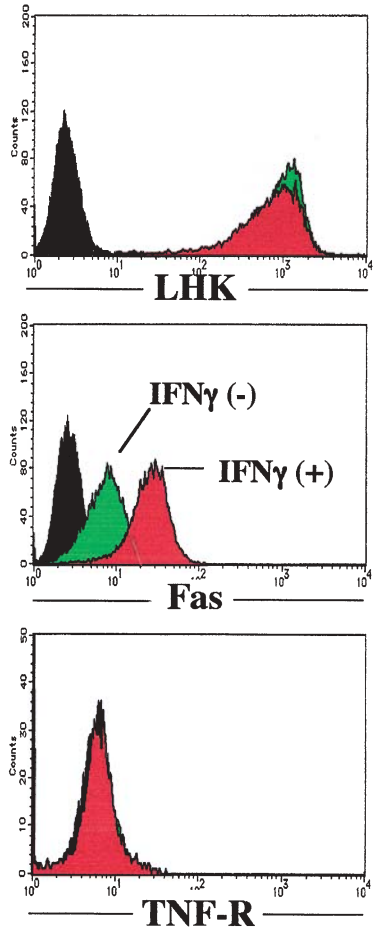

Fig. 4. Modulated expression of LHK, Fas, and TNF-receptor (*R*) by exogenously added interferon-γ (IFN-γ). The expression of LHK antigen (*upper*), Fas (*middle*), and TNF-R (*lower*) on Colo205 cells was determined after 48 h of culture with INF-γ (100 U/ml)

munohistochemical and flow cytometric analysis. Interestingly, immunohistochemistry revealed that LHK was expressed on epitheia in normal colonic epithelial cells (Fig. 7A, upper) and other epithelial cells such as tongue, esophagus, stomach, small intestine, pancreas, and skin, whereas it was never expressed in liver, spleen, tonsil, thymus, lung, heart, and kidney (data not shown). In assessing the expression of LHK antigen on human colon cancer tissues, we found that some colon cancer tissues expressed less or no LHK antigen (Fig. 7A, lower). This tendency was confirmed by flow cytometric analysis, using freshly isolated cancer and normal epithelial cells (Fig. 7B). To assess this in detail, we stained many colon cancer tissues and normal epithelial cells and found that the reactivity of anti-LHK mAb decreased significantly in parallel with the dedifferentiation of colon cancer (normal epithelial cells vs poorly differentiated adenocarcinoma; $P < 0.001$; Table 1), indicating a relationship between LHK antigen and the malignant potential of colon cancer.

Table 1. Immunohistochemical reactivity of anti-LHK mAb decreased in proportion with dedifferentiation of colon cancer

Histology	% Positive LHK
Normal colonic epithelial cells	100% (61/61[a])
Tubular adenoma with severe dysplasia	100% (10/10)
Well-differentiated adenocarcinoma	74% (28/38)
Moderately differentiated adenocarcinoma	49% (19/39)
Poorly differentiated adenocarcinoma	40% (8/20)

[a] Number of mice with positive staining/number of mice examined

Therapeutic effect of anti-LHK mAb in a xenograft model of human colon cancer

Finally, we examined the therapeutic effect of anti-LHK mAb, using a xenograft model of Colo205 cells. BALB/c SCID mice that had received injection of 1×10^6 Colo205 cells intraperitoneally and had been treated with 1 mg of anti-LHK mAb weekly were kilted 8 weeks after the cell injection. We then examined the frequency of appearance of abdominal masses and tiny nodules in the mesentery (peritoneal dissemination) and compared the mean volumes of the tumors. An abdominal mass was observed in 7 of 17 (41%) mice treated with anti-LHK mAb, but in 16 of 18 (89%) mice treated with control IgM ($P < 0.01$). The risk ratio was 0.46 (95% confidence interval, 0.26–0.84; $P < 0.05$). Peritoneal dissemination (Fig. 8) was observed in 2 of 17 (12%) mice treated with anti-LHK mAb and in 8 of 18 (44%) mice treated with control IgM ($P < 0.01$). The risk ratio was 0.26 (95% confidence interval, 0.07–1.07; not significant). Mean tumor numbers were 1.0 ± 1.41 and 3.3 ± 2.91, respectively ($P < 0.01$). Mean total tumor volumes were 188.5 ± 88.6 and $514.8 \pm 152.5\,mm^2$, respectively ($P < 0.05$). These results indicate that anti-LHK mAb also exerts tumorcidal activity in vivo.

Discussion

The goal of the present study was to establish a novel mAb capable of killing colon cancer cells that are resistant to Fas-induced apoptosis. We established an anti-LHK mAb produced by a hybridoma from mice immunized with Colo205 colon cancer cells. The mechanism of anti-LHK mAb-induced cell death was demonstrated to be apoptosis. The LHK antigen was a novel protein of 70 kDa that is distinct from Fas and TNF-R. Interestingly, the expression of LHK in colon cancer tissues decreased significantly in parallel with their grade of dedifferentiation. Furthermore, we demonstrated that the anti-LHK mAb suppressed tumor growth in a murine peritoneal dissemination model.

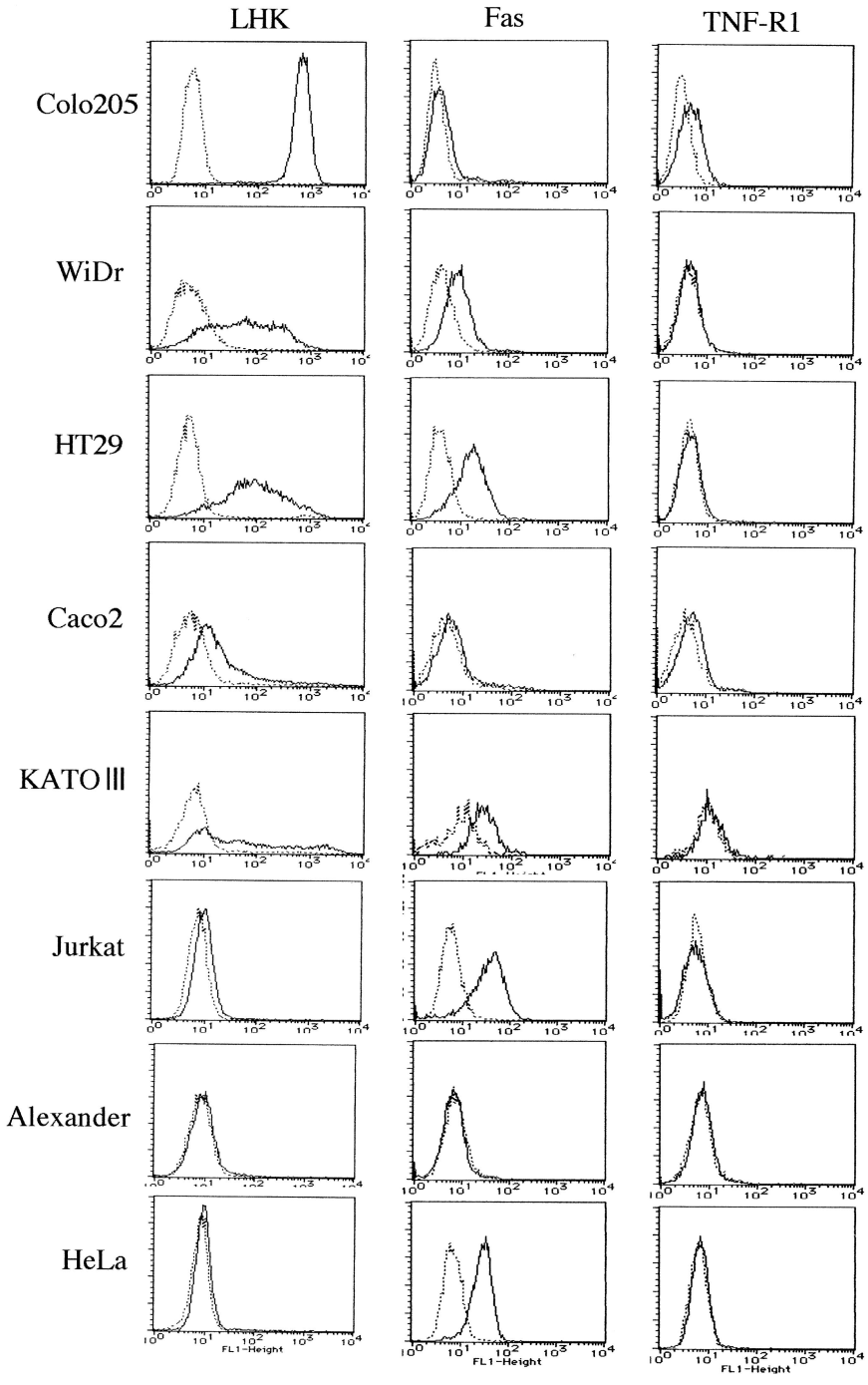

Fig. 5. Expression of LHK, Fas, and TNF-R on various human cancer cell lines. Aliquots of 1×10^6 cells were incubated on ice with control IgM and IgG, anti-LHK, anti-Fas, or anti-TNF-R mAb. Then the cells were washed, stained with fluorescein isothiocyanate (FITC)-coupled goat anti-mouse IgM or IgG, and analyzed by flow cytometry

Apoptotic cell death is morphologically characterized by membrane blebbing, shrinkage involving nuclear margination, and chromatin condensation. Moreover, the nuclear apoptotic events known as DNA ladder formation; that is, degeneration of chromatin induced by endogenous nuclease activation, have become a biochemical hallmark of apoptosis.[16] These characteristics were also observed in Colo205 cells treated with anti-LHK mAb, indicating that the mechanism of anti-LHK-induced death in the Colo205 cells was apoptosis. This was supported by two further assays for apoptosis; namely, Hoechst 33528 and Annexin V/PI staining.

Fas is functionally expressed on the basolateral surface of colonic epithelial cells regardless of their position along the crypt axis. In contrast to the normal epithelium, colon cancer cell lines are mostly resistant to Fas-induced apoptosis. In the normal situation, senescent epithelial cells in the gut mucosa are eliminated by apoptosis, but it is not yet clear how apoptosis is induced in these cells. Thus, death receptors including

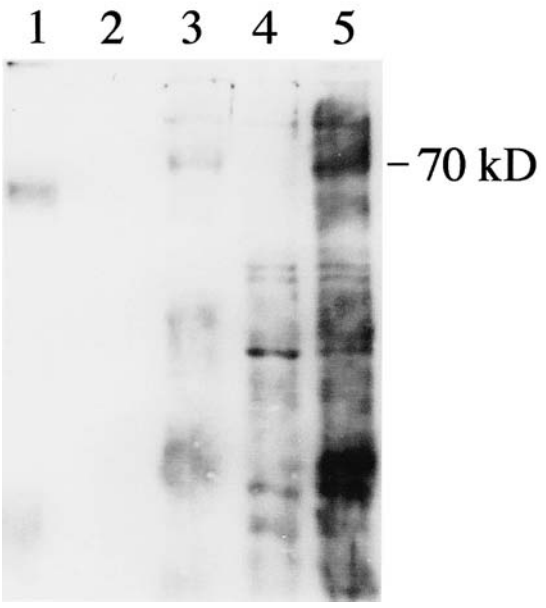

Fig. 6. Immunoprecipitation of LHK antigen on Colo205 cells. *Lane 1*, marker; *lane 2*, isotype-matched control IgM-conjugated protein A-Sepharose beads; *lane 3*, anti-LHK IgM-conjugated protein A-Sepharose beads; *lane 4*, isotype-matched control IgM-conjugated protein A-agarose beads; *lane 5*, anti-LHK mAb-conjugated protein A-Agarose beads

Fas may be involved in this elimination system. This may also be the case with the LHK antigen, because the expression of LHK in colon cancer and normal tissues decreased significantly in parallel with their grade of dedifferentiation. Indeed, these observations indicate that this mAb may not be ideal for cancer therapy, but, rather, that LHK antigen may be useful as a marker of malignancy for patients with colon cancer, because its down-regulation may enable colon cancer cells to escape from apoptosis. Of note, the expression of LHK antigen on colon cancer cells in patients with liver metastasis was marked, suggesting a useful predictor for their prognosis.

In the analysis of the LHK antigen, we first aimed to clarify whether LHK antigen was distinguishable from Fas and TNF-R. We concluded that LHK antigen is a novel death receptor, for the following reasons. First, the molecular mass of LHK is 70 kDa, whereas those of Fas, TNF-R1, and TNF-R2 are 48, 55, and 75 kDa, respectively. Second, the expression profiles from immunohistochemistry and flow cytometry revealed that Fas and TNF-R were broadly expressed on various cells, including hematopoietic cells and fibroblasts, whereas LHK was restricted to epithelial cells in the gastrointestinal tract and skin. Regarding the difference between LHK and Fas, it is noteworthy that Fas, but not LHK, was significantly upregulated on Colo205 cells by IFN-γ, and that Colo205 and Jurkat cells differed in their sensitivity to cell death induced by anti-LHK and anti-Fas (CH11) mAbs. Furthermore, because the TNF-R family is a still-growing group of homologous transmembrane proteins, it was important to assess whether or not LHK is identical to other recently-described molecules, such as TNF-related apoptosis-inducing ligand (TRAIL) receptors, DR3, DR4, and DR5. Like LHK, but unlike Fas ligand, TRAIL appears to preferentially induce apoptosis in tumor cells over normal cells.[18] Five distinct receptors for TRAIL have been identified (TRAIL-R1, R2, R3, R4, and osteoprotegrin [OPG]). Only TRAIL-R1 and R2, which bear a cytoplasmic death domain, can induce apoptosis. The latter three are thought to be nonsignaling decoy receptors and to be related to the regulation of apoptotic cell death via TRAIL systems.[19–22] Unlike LHK, TRAIL-R1 and R2 are expressed in most tissues, such as spleen, thymus, peripheral blood lymphocytes, activated T cells, and small intestine. The molecular masses of TRAIL-R1 and R2 were 46 and 52 kDa, respectively, indicating that LHK is also distinguishable from TRAIL receptors. Further study involving the direct cloning of the LHK molecule will be needed to resolve this issue.

Finally, we assessed the therapeutic effect of anti-LHK mAb in a murine peritoneal dissemination model. Although targeting specific death receptors in cancer therapy may have therapeutic effects on tumors, the clinical use of both TNF and FasL (or anti-FAS mAbs) has been prevented by their toxic side effects. The systemic administration of certain doses of TNF causes a severe inflammatory response syndrome that resembles septic shock, and injection of agonistic antibody to Fas in tumor-bearing mice can be lethal, apparently because of the induction of apoptosis in hepatocytes. Although TRAIL showed promising efficacy in a preclinical study and was suggested to be a safer agent than TNF and FasL,[23,24] a report indicated that human primary hepatocytes were also efficiently killed by TRAIL signaling.[25] In our murine model, anti-LHK mAb treatment had a potent tumoricidal effect. Importantly, no mice died or showed apparent adverse effects, such as severe inflammatory response syndrome, fulminant hepatic failure, or damage of normal colonic epithelial cells (data not shown). Of course, we cannot conclude that anti-LHK mAb is an effective and safe agent for the treatment of patients with colon cancer. Further investigation will be needed to clarify the mechanism of action of anti-LHK mAb before its clinical trials.

In conclusion, LHK antigen, which is uniquely restricted to epithelial cells in the gastrointestinal tract and skin, may be a novel death receptor that is critically involved in controlling the growth, invasion, and metastasis of human colon cancer cells. Therefore, this study might provide a basis for the practical application

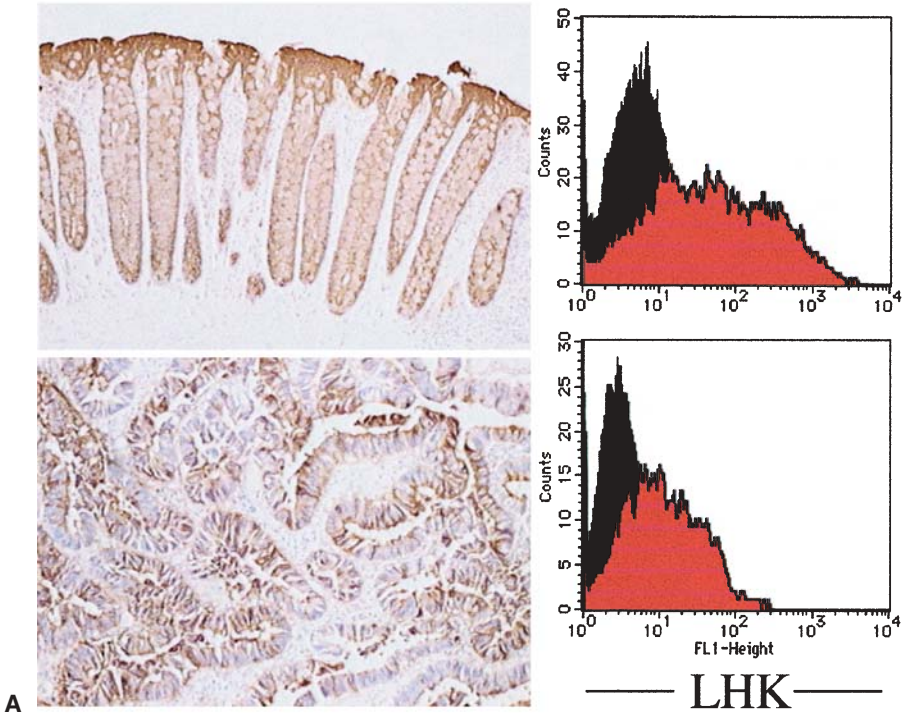

Fig. 7A,B. LHK expression in normal human colonic epithelial cells and colon cancer cells. **A** LHK antigen in normal human colonic epithelial cells (*upper*) and colon cancer cells (*lower*) was visualized by immunohistochemistry. ×100. **B** Flow cytometric analysis of freshly isolated human normal colonic epithelial cells (*upper*) and colon cancer cells (*lower*) is demonstrated

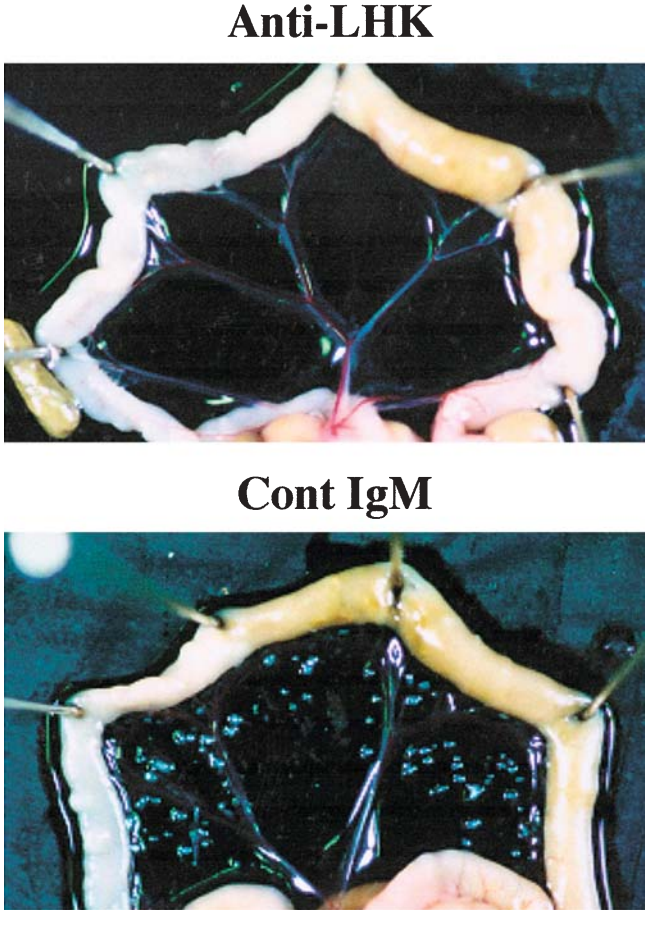

Fig. 8. Reduction of peritoneal dissemination in a murine xenograft model by anti-LHK mAb treatment. Colo205 cells (5×10^6) were injected into the peritoneal cavities of 8-week-old BALB/c severe combined immunodeficiency (SCID)mice. Twenty-four hours after the injection, 500 μg of anti-LHK IgM or control (*cont*) mouse IgM mAb was injected intraperitoneally into Colo205-transplanted mice. The injection was repeated weekly for 8 weeks then mice were killed by cervical dislocation. Tiny nodules in the mesentery were observed in mice treated with control IgM, but not in mice treated with anti-LHK mAb

of therapy using anti-LHK mAb for the treatment of patients with colon cancer.

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