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CMAP: A Novel Cystatin-like Gene Involved in Liver Metastasis

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ABSTRACT

A novel metastasis-associated gene was identified with a differential display system in murine carcinoma cells showing a high rate of metastasis to the liver. A human homologue was also identified using a PCR-based strategy. The protein coded by this gene was named cystatin-like metastasis-associated protein (CMAP) and showed 22.1–28.1% homology to human family 2 cystatins. CMAP mRNA was selectively overexpressed in all murine liver metastatic tumors but not in any pulmonary metastatic tumors examined. Transfection of CMAP antisense DNA into highly metastatic liver cells greatly decreased their metastatic potential and CMAP expression, indicating that CMAP is involved in liver metastatic ability after intravasation of malignant cells. The human homologue of CMAP was found to be expressed in various human cancer cell lines established from malignant tumors. Our discovery of this novel liver metastasis-related gene indicates a new approach to the diagnosis and/or prevention of liver metastasis of human cancer.

INTRODUCTION

To establish metastases, cells must complete the following sequential steps: neovascularization, migration with invasive destruction of the extracellular matrix components at the site of the primary lesion, intravasation, transportation through the channels with survival against host immune defenses, arrest in the capillary bed of a distant organs, extravasation, and growth with angiogenesis in the secondary lesion (1–3).

For cancer therapy, the regulation and control of malignant phenotypes are matters of great concern. In attempts to identify metastasis-related genes, down-regulated genes in highly metastatic tumors, e.g., NM23 (4), Kiss-1 (5), cystatin M (6), CC3 (7), and up-regulated genes, e.g., mts1 (8), stromelysin-3 (9), and thymosin β15 (10), have been identified by differential/subtractive hybridization or mRNA differential display.

For further studies on metastasis, animal models are required. Several models, such as experimental metastasis by i.v. injection, intrasplenic implantation, spontaneous metastasis by s.c. implantation, intrafootpad implantation, and orthotopic transplantation, have been developed (11). The IMC-HM murine carcinoma cell line showing high metastasis to the liver was isolated from nonmetastatic IMC-LM cells in our laboratory (12). IMC-HM cells metastasized spontaneously from a s.c. site mainly to the liver and then caused lethal multiple metastases in mice. In liver slices of the dead mice, diffuse infiltration of metastatic pleomorphic tumor cells was observed with focal necrosis histologically (12). The metastases were seen from an early stage of tumor growth at the primary site. Surgical removal of the primary lesion with the inguinal plexus 3 days after implantation (day 3) resulted in almost no life prolongation because latent micrometastases in target organs had already occurred (12). "Occult" tumor cells were seen microscopically in the metastatic lesions from day 10. Gross and histological examinations showed that IMC-HM cells injected i.v. into mice exhibited similar metastatic properties to those implanted s.c., suggesting that spontaneous metastases of the cells occurs via the blood circulation, not by dissemination or the lymph system in vivo. Although it involves ectopic transplantation, this liver metastasis system is a useful model of micrometastasis in the liver, differing from the predominantly pulmonary metastases of Lewis lung carcinoma (13).

Besides the lungs, the liver is a common site of hematogenous metastasis of various types of carcinomas. Because the metastatic behavior of these tumors, including extensive multiple metastases, resembles that of IMC-HM cells, we tried to identify a metastasis-associated factor(s) in this model. For this, we compared the transcriptional differences between IMC-HM and IMC-LM cells by mRNA differential display, because of the close genetic backgrounds of these two cell lines. Here, we report the identification and cloning of a novel cDNA for CMAP, which is transcriptionally up-regulated in IMC-HM cells as well as other liver metastatic tumor cells, and its close correlation with the liver metastatic potential of the cells in vivo.

MATERIALS AND METHODS

Mice. Female CDF1 (BALB/c × DBA/2) mice were purchased from Charles River Japan (Kanagawa, Japan). All mice were used at 5–8 weeks old.

Cell Lines. IMC murine carcinoma cells were kindly provided by Dr. M. Ishizuka of the Institute of Microbial Chemistry (Shizouka, Japan). IMC-HM cells were isolated in our laboratory. After the isolation of IMC-HM cells, the original IMC cells were named IMC-LM cells to distinguish them from the highly metastatic variant. The cells were passaged weekly in the peritoneal cavity of female CDF1 mice and also maintained in culture in RPMI 1640 supplemented with 10% FCS and 20 μM 2-mercaptoethanol. Clonal lines of IMC-HM cells and IMC-LM cells, named IMC-HA1 and IMC-LE5 cells, respectively, were obtained by limited dilution, followed by random selection. These cell lines did not show any notable differences from the cells before cloning in their in vivo metastatic properties, morphology, growth rate, or CMAP expression (data not shown). IMC-HA1 cells were entrusted for maintenance and provision to the Agency of Industrial Science and Technology (Tsukuba, Japan). The ascites form murine tumors, Ehrlich, Meth A, Sarcoma 180, MH134, M-5076, L5178Y, P388, and L1210 cells, were maintained in the abdominal cavity of mice. The cells were transferred to in vitro culture at least 2 weeks before isolation of total RNA. B-16-BL6 melanoma and colon 26 carcinoma cells were cultured in vitro under the same conditions as described above. LX-1 and KP-1N cells were kindly provided from Dr. M. Inaba of Cancer Institute, the Japanese Foundation for Cancer Research (Tokyo, Japan), and Dr. A. Kono of the National Kyushu Cancer Center (Fukuoka, Japan), respectively. Lu-135, SEKI, and PSN-1 cells were gifts from Drs. T. Terasaki and T. Sekiya, respectively, of the National Cancer Center Research Institute (Tokyo, Japan). PC-13 and MKN-45 cells were purchased from Immuno-Biological Laboratories Co. (Gunma, Japan). KU812 and T24 cells were obtained from the Riken Cell Bank (Ibaraki, Japan). All other human tumors were purchased from the American Type Culture Collection (Manassas, VA).

mRNA Differential Display. mRNA differential display was performed using a Delta RNA Fingerprinting Kit (Clontech). Total RNA was prepared from IMC-HM and IMC-LM cells using ISOGEN (Nippongene). After DNase I treatment with the MessageClean Kit (GenHunter), total RNA (2 μg) was reverse-transcribed with 200 units of Superscript II (Life Technologies, Inc.) in 151

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The abbreviations used are: CMAP, cystatin-like metastasis-associated protein; mCMAP, murine CMAP; hCMAP, human CMAP; RT-PCR, reverse transcription-PCR; EST, expressed sequence tag.
the presence of 0.1 μM oligo(dT) anchor primer and then amplified by PCR using different arbitrary primer sets (Clontech) with 50 units/ml of LATaq (Takara). The cycling conditions were 5 min at 94°C, 5 min at 40°C, 5 min at 68°C (1 cycle), 2 min at 94°C, 5 min at 40°C, 5 min at 68°C (2 cycles), 1 min at 94°C, 1 min at 60°C, and 2 min at 68°C (25 cycles). Two independent reaction products from both IMC-HM and IMC-LM cells were separated in 6% polyacrylamide gel with glyceraldehyde-3-phosphate running buffer (United States Biochemical). Differentially expressed cDNA bands were directly excised from the gel and reamplified with the same primer sets in high-stringency conditions. After subcloning into pcR II vector (Invitrogen), the proper cDNAs were sequenced by a Dye Primer Cycle Sequencing Ready Reaction (PerkinElmer Corp.).

**Northern Blot Analysis.** Poly(A) RNA was isolated from exponentially growing cells using a Messenger RNA Isolation Kit (Stratagene) or separated from total RNA using a mRNA Separator Kit (Clontech). Samples of 2 μg of poly(A) RNA were size-fractionated in denaturing formaldehyde-agarose gel (1.0%) and transferred to a Hybond-N membrane (Amersham) according to the manufacturer’s recommended protocol. Filters were prehybridized for 1 h at 42°C and then hybridized by addition of denatured probe labeled with [α-32P]dCTP (Amersham), using Megaprime DNA-labeling systems (Amersham). Blotted filters were washed to a final stringency of 0.2 × SSC-0.1% SDS at 55°C. Image analysis was performed with BAS2000 (Fuji).

**Southern Blot Analysis.** Genomic DNA extracted from cells with a DNA Extraction Kit (Stratagene) was digested by EcoRI (Toyobo) and loaded onto 1.0% agarose gel for electrophoretic separation. After transfer to a Hybond-N+ membrane, hybridization was performed under the same conditions as for Northern analysis. Filters were washed to a final stringency of 0.1 × SSC-0.1% SDS at 68°C.

**cDNA Library Screening.** An oligo(dT)-primed cDNA library was constructed using the ZAP Express Vector (Stratagene) with poly(A) RNA obtained from IMC-H1 clonal line. The library was screened using a 32P-labeled cDNA fragment, as a probe, pBK-CMV phagemid vector with a positive insert clone was excised from the ZAP Express Vector in vivo in the presence of ExAssist helper phage. Nucleotide sequences were determined with the primers hCMAP-1, AATTTTAGAAGCAAATGGATAC; hCMAP-2, AATTTTAGAAGCAAATGGATAC; hCMAP-3, ATCTACCAAAAGGCTTCGCAC; and hCMAP-4, ATCTACCAAAAGGCTTCGCAC, which do not show obvious homology to the reference gene for hCMAP.

**Sequence Determination of cDNA Library.** Total RNA was reverse-transcribed and amplified using the cDNA expression system (Clontech). The cDNA library was constructed using a 32P-labeled cDNA fragment, as a probe, pBK-CMV phagemid vector with a positive insert clone was excised from the ZAP Express Vector in vivo in the presence of ExAssist helper phage. Nucleotide sequences were determined with the primers hCMAP-1, AATTTTAGAAGCAAATGGATAC; hCMAP-2, AATTTTAGAAGCAAATGGATAC; hCMAP-3, ATCTACCAAAAGGCTTCGCAC; and hCMAP-4, ATCTACCAAAAGGCTTCGCAC, which do not show obvious homology to the reference gene for hCMAP.

**Vector Construction of Antisense Nucleotide of mCMAP and Preparation of Transfectants.** mCMAP mRNA was amplified by RT-PCR under stringent conditions with the specific primer set retaining a NheI or KpnI recognition site, mCMAP-F1 (5′-AATTCGCTACCAGCTGAGCTAC- CCCACCATGCCC-3′) and mCMAP-R1 (5′-AGTCGCTAGCAGAGGAGAACAGGCACTCCTAAAAC-3′), to obtain a cDNA including the open reading frame of mCMAP with the short 5′-untranslated segment. The pBK-CMV vector was cut with NheI and KpnI restriction enzymes (Toyobo) to delete the lac promoter region and the spare multicloning sites. The PCR product was also excised with the same restriction enzymes and ligated into the linear vector using the ligation kit version II (Takara) in inverse orientation. After cloning using One Shot INVesD cells (Invitrogen), the vector with mCMAP cDNA in an antisense orientation was extracted using a QIAfilter (Qiagen), cleaved by ApalI treatment, and introduced into IMC-HA1 cells by electroporation using a Gene Pulser II (Bio-Rad). Transfectants stably expressing the induced vector were selected by continuous neomycin treatment at 0.3 mg/ml for 2 weeks. The neomycin-resistant cells were cloned by the limited dilution technique with increase in neomycin concentration to 0.8 mg/ml. After ~2 weeks, 35 clones were obtained by random selection. Suitable clones were selected by analysis of reduction in the amount of their mCMAP mRNA by RT-PCR.

**Analysis of Metastatic Activity in Vivo.** Exponentially growing cells in culture or in freshly prepared ascites were harvested, washed, and resuspended in PBS. The cells were inoculated into the flanks of female CDF1 mice at 5 × 10^7 cells/mouse for studies on spontaneous metastasis or into a tail vein at 1 × 10⁸ cells/mouse for studies on experimental metastasis. In studies on spontaneous metastasis, the local site tumor and a lymph node were excised 3 days later. The survival times of mice were recorded, or the mice were sacrificed on day 13–15 and their liver and spleen were removed and weighed. The livers or spleens that showed a medial mass were photographed immediately or after fixation with neutralized formalin. Tumor masses at implantation sites were also examined to determine generation times in vivo. Volumes of tumors were calculated by the following formula, V = (L × W²)/2, where L = length and W = width. Gross examinations, mainly of the liver, were performed on mice with tumors that died and on surviving mice that were sacrificed.

**RESULTS**

**Identification and Cloning of cDNA.** We compared the gene expression patterns of the nonmetastatic IMC-LM and liver-metastatic IMC-HM murine carcinoma cell lines. In the mRNA fingerprint patterns amplified with each primer set of 25- or 26-mer arbitrary primer (P primer) and 30-mer oligo(dT) anchor primer (T primer), 50–200 cDNA fragments were usually distinguishable on the gel. Several candidates have been obtained with 30 different primer sets so far. One of them is a cDNA fragment, 23-1, derived from IMC-HM cells (Fig. 1A). Its band was extracted from the gel and reamplified with the same primer set. On subcloning, we obtained the clone 23-1#2 (656 bp).

A gene transcript hybridizing with 32P-labeled 23-1#2 was confirmed by Northern analysis (Fig. 1B). A major 1.1-kb poly(A) RNA was detected in IMC-HA1 cells, a clonal line of IMC-HM cells, but not in IMC-LE5 cells, a clonal line of IMC-LM cells, although there was no difference in the reference gene for G3PDH in the two cell lines. Similar results were obtained with IMC-HM cells and IMC-LM cells (data not shown). We named the gene mCMAP because of its structural similarity with cstatin, described below. On Southern blot analysis, two EcoRI-digested genomic DNAs of ~5.4 kb and 3.5 kb from the two cell lines hybridized with the same probe on Northern blot analysis (Fig. 1C).

To elucidate the total sequence of mCMAP, we screened a cDNA library derived from IMC-HA1 cells, which was constructed with the ZAP Expression Vector by applying 32P-labeled 23-1#2. In this way, a full-length cDNA clone, pBK-CMV#9.31334, was isolated (GenBank accession no. AB015224). Search of the GenBank and EMBL nucleotide sequence data bases with FastA using GCG software (Genetics Computer Group Inc., University of Wisconsin, Madison, WI), indicated that mCMAP does not show obvious homology to...
Correlation between mCMAP Expression and Metastatic Activity in Experimental Metastases. To determine whether mCMAP is actually involved in metastasis in vivo, we prepared cells with stably reduced mCMAP expression by transfection of the antisense DNA into IMC-HA1 cells. Several clones, 57C4, 53A9, and 53E9, showed reduced mCMAP mRNA expressions of ~49, 32, and 16%, respectively, of that of control IMC-HA1 cells (Fig. 3A). These transfectants showed no gross alterations except in mCMAP expression and neomycin sensitivity during in vitro cultivation.

All these transfectants, including a vector control, clone 18B5, showed almost the same tumorigenicity at an inguinal site (Fig. 3B). When IMC-HA1 cells were inoculated i.v. into mice at 1 × 10^6 cells/mouse, they caused liver metastases, and the mice died about 2 weeks after cell inoculation (data not shown). Then, we examined the metastatic activities of the transfectants. On day 13 after i.v. injection of the transfectants, the mice were sacrificed, and their livers and spleens were weighed (14). Clone 18B5 caused no apparent difference

Fig. 1. Identification and cloning of mCMAP selectively overexpressed in IMC-HA1, a clonal line of IMC-HM cells. A, signal pattern in an mRNA differential display made by amplification with a pair of primers, P2 (5'-ATTAAACCCCTCACTAAATGCTGCAGG-3') and T3 (5'-CATTATGCTAGTGATATCTTTTTTTTTTAG-3'). Arrow, DNA selectively amplified in IMC-HM cells, 23-1. B, Northern blot analysis with 2 μg of poly(A) RNA from both IMC-HA1 and IMC-LES cells probed with 32P-labeled 23-1#2, a clone of 23-1. The expression of mCMAP was confirmed as a 1.1-kb single band. The membrane was rehybridized with a G3PDH probe as a control. C, genomic Southern blot analysis with 10 μg of EcoRI-digested genomic DNA from both cell lines probed with 32P-labeled 23-1#2.

previously identified genes without several ESTs. Namely, AA089339 (517 bp), AA089317 (428 bp), AA423624 (328 bp), and AA537189 (178 bp) derived from murine cDNA libraries showed homologies of 99.6, 99.5, 99.4, and 96.6% over the full-stretch sequences, respectively.

Distribution of mCMAP mRNA in Various Murine Tumors and Normal Tissues. To elucidate the extents of mCMAP transcription in various murine tumors, we designed a primer set for its specific detection by the RT-PCR. When the transcribed cDNA was serially quartered, the PCR products of mCMAP and G3PDH in IMC-HA1 cells showed linearity at under 1:4 and 1:16 dilution, respectively (data not shown). The dilution of the initial template was fixed at 1:10 for mCMAP and 1:40 for G3PDH amplification.

Total RNAs extracted from various murine tumors were reverse-transcribed and then subjected to shuttle-PCR under fixed semiquantitative conditions for IMC-HA1 cells. As shown in Fig. 2A, mCMAP mRNA was detected only in M-5076 reticulum cell sarcoma, L5178Y lymphoma, and P388 and L1210 leukemia. All these tumor cells are known to cause multiple metastases mainly in the liver on i.v. injection into mice at 1 × 10^6 cells/mouse. In contrast, IMC-LES, Ehrlich, Meth A, Sarcoma 180, and MH134 in which mCMAP mRNA is not expressed, did not metastasize on i.v. inoculation, although they showed strong tumorigenicity on s.c. or i.p. implantation. Although B-16-BL6 murine melanoma and colon 26 carcinoma tumors are well known to cause pulmonary metastases, they did not cause liver metastases detectable on gross examination.

mCMAP mRNA expression in various normal tissues from female CDF1 mice was also analyzed. As shown in Fig. 2B, the expression of mCMAP was only detected in “lymphoid organs,” such as thymus, spleen, and lymph nodes, although its expression was less than in IMC-HA1 cells. In other tissues, no mCMAP mRNA was detected. This unique distribution of mCMAP in lymphoid organs suggests its physiological association with the immune system.

Fig. 2. Expression pattern of CMAP-specific mRNA. A, RT-PCR detection of mCMAP in various murine tumor cell lines. Primers (9-4F2, 5'-CTGAAAGCTACCCCACCATGC- CCTGG-3' and 23-1#2F2, 5'-GACTGTATCTGCTTTGCTCTGCAC-3') were used for specified amplification of mCMAP. Control amplification was performed with a G3PDH-specific primer set. B, distribution of mCMAP in various normal mouse tissues. Fifteen normal murine tissues were excised from two to three normal female CDF1 mice (5 weeks old) and homogenized in 10 volumes of ISOGEN in a polytron, then total RNA was extracted. RT-PCR was performed under semiquantitative conditions for IMC-HA1.
from IMC-HA1 in the mean weights or appearances of the liver or spleen (Fig. 3, C–E). In contrast, clones 57C4, 53A9, and 53E9, showed significantly lower metastatic activity than IMC-HA1 or 18B5 cells. The liver and spleen weights of mice were almost the same as that of mice with the nonmetastatic clone, IMC-LE5 cells ($P < 0.0001$ for each). Under similar conditions, the survival period of mice with the transfectants were significantly longer than that of mice with IMC-HA1 or vector control cells. In particular, one of five
mice injected with clone 53E9 survived until day 60 (Table 1). There was a good inverse correlation between the extent of mCMAP expression and the survival period of mice (Table 1). Similar results were obtained in both spontaneous metastasis studies with and without tylectomy of the primary lesion (data not shown).

**Sequence Determination of hCMAP.** The human homologue of CMAP (hCMAP) was identified by a PCR-based strategy. Two human ESTs, N56875 (414 bp) and N47763 (428 bp), which showed 72.0 and 75.7% homology to mCMAP, respectively, were identified in the nucleotide sequence database of a human EST project of Merck Research Laboratories. These ESTs possessed 34 bp of overlapping region, indicating that they are of the same origin (Fig. 4A). A hybrid cDNA based on these ESTs showed high homology to mCMAP, especially around the open reading frames, indicating that this hybrid gene is a human homologue of CMAP (Fig. 4B).

To refine the incomplete sequence of the hybrid, we designed several primers using the regions of both termini and amplified them by RT-PCR with human spleen total RNA as a template source. A 800-bp product was directly sequenced by dye terminator cycle sequencing. In this way, we determined the sequence of hCMAP (Genbank accession no. AB015225) encoding 167 amino acids. The homology between murine and human CMAP was 54.0% for the sequences of nucleotide bases and 62.3% for their complete amino acid sequences.

**Protein Structure Alignment of CMAP with Human Family 2 Cystatins.** The structure of CMAP resembles that of human family 2 cystatins classified in the cystatin superfamily, which are endogenous inhibitors of papain-like thiol proteinases, such as cathepsins (15). Human CMAP shows low homology with family 1 cystatins, stefin A or B: 15.3% on full-stretch comparison with both (data not shown). Human CMAP shows homologies with cystatin M (also called cystatin E; Ref. 16), C, D, SN, SA, and S, and chicken cystatin in the full sequences of 22.1, 28.1, 22.5, 25.5, 25.5, 26.2, and 25.9%, respectively. hCMAP and mCMAP were aligned with human family 2 cystatins and chicken cystatin (Fig. 5). hCMAP and mCMAP showed hydrophobicity from Arg25 to Thr40 or from Leu15 to Thr36 on Kite-Doolittle analysis, respectively (data not shown), although all family 2 cystatins are practically or theoretically cleaved of the hydrophobic signal segment between 20 and 29 residues (Fig. 5). hCMAP or mCMAP have second Met at position 23 or 24 (Fig. 5, underlined sequence), respectively. The entire sequence of CMAP may be produced as a membrane-associated protein. In contrast, if the translation is started from the second Met, the product may be a secretory protein with a putative cleavage site same as family 2 cystatins (Fig. 5). This important issue remains to be elucidated. Chicken cystatin has been extensively analyzed and shows 41.7% homology with human cystatin C. Data from x-ray diffraction and NMR spectroscopy of chicken cystatin (17, 18) reveal that the evolutionarily conserved four Cys residues at positions 121, 132, 146, and 166 in CMAP may form two disulfide bridges. In addition, three underlined segments indicated in Fig. 5, the NH2-terminal region including Gly59, Gln103-X-Gly107 and Val153-Phe154, Trp155, which are also present in CMAP, may be essential for interaction with the substrate (19). These sequences are highly conserved in family 2 cystatins (Fig. 5). Obvious differences between CMAP and cystatins are the Lys57 and Lys106 residues in the underlined segments, Cys residues without the consensus cystin bridges (Fig. 5, boxed sequence), and Asn residues (Fig. 5, boldface italicized sequence). No family 2 cystatin contains any basic amino acid residues in the three underlined segments, suggesting that the binding specificity of CMAP to its unknown target is different from that of cystatins. Human family 2 cystatins contain no Cys residue except signal sequences and the four consensys Cys residues near the C-OOH terminus (Cys18 in cystatin M and Cys9 in cystatin S and SA). In contrast, mCMAP has Cys residues at positions 7, 9, 19, 33, 34, 48, and 85 and hCMAP has Cys residues at positions 33, 34, 48 and 85, although it is unclear whether these Cys residues form an additional

<table>
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<th>Cell line</th>
<th>mCMAP mRNA (%)</th>
<th>Mean ± SD</th>
<th>Md</th>
<th>St/C</th>
<th>Mt/T</th>
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<tr>
<td>IMC-HA1</td>
<td>100</td>
<td>14.0 ± 0.7</td>
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<td>0/5</td>
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<tr>
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<td>19.2 ± 1.5</td>
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<td>21.4 ± 2.6</td>
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<td>153</td>
<td>0/5</td>
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<tr>
<td>53E9</td>
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<tr>
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<td>&gt;60.0 ± 5.0</td>
<td>&lt;0.0001</td>
<td>&gt;429</td>
<td>5/5</td>
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* Values were calculated as radio intensities of mCMAP to that of G3PDH on Northern analysis.
* Statistical analysis was performed by the Fisher’s protected least significant difference test.
* Values are indicated as percentages of the mean survival days of mice bearing IMC-HA1 cells.
* No. of survivors on day 60/No. of mice tested.
* No. of mice with liver metastasis/No. of mice tested.
* Significant difference, P < 0.001.

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**Table 1 Extension of survival of mice bearing clonal lines expressing reduced levels of mCMAP mRNA in vivo**

Cells were inoculated i.v. into female CDF1 mice at 1 × 103 cells/mouse on day 0.

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**Fig. 4.** Sequence determination of hCMAP. A, identification of hCMAP using the sequence similarities of two human ESTs, N47763 and N56875. Open reading frames are indicated [●]. For details see text. B, comparison of sequences of hCMAP and mCMAP cDNA. The sequence relationship between hCMAP and mCMAP cDNAs was calculated by the Compare program in GCG software.
disulfide bridges. This unique NH2-terminal region might result in a different specificity of the physiological role from that of cystatins. The Asn residues at positions 84 and 124 in human and at position 84 in murine CMAP are putative glycosylation sites according to analysis with GCG software, although cystatins do not contain any putative glycosylation site except Asn137 in cystatin M.

**hCMAP Expression in Tissue Culture Cell Lines.** To evaluate the expression of hCMAP, over 50 human tissue culture cell lines were subjected to RT-PCR. We found that not all tumors but some malignant cell lines, such as HL-60 acute promyelocytic leukemia; COLO 201 colorectal adenocarcinoma; P388 leukemia; and L929 fibroblasts, showed much stronger expression than normal spleen (Fig. 6). Other tumors expressed little or no hCMAP except two of three osteosarcoma cell lines, Saos-2 and U-2 OS, which showed moderate expression. Unexpectedly, few leukemias or lymphomas were high producers of hCMAP. The mammary carcinomas established from brain metastasis or pleural effusion showed almost no expression. In contrast, a high proportion of malignant melanomas were hCMAP positive. Important information was obtained from two melanoma cell lines, WM-115 and WM-266-4, derived from the primary tumor and metastatic site of a malignant melanoma of the same patient. WM-266-4 showed a much stronger signal than WM-115. Although exact metastatic lesions are not shown in the literature, hCMAP expression is up-regulated in metastatic sites rather than primary lesions in the same patient. In addition, LX-1 was
also established from metastatic site. Moreover, the donor of PSN-1 was deceased by extreme liver metastasis. Pancreatic or lung cancer also established from metastatic site. Moreover, the donor of PSN-1 was deceased by extreme liver metastasis. Pancreatic or lung cancer also established from metastatic site.

DISCUSSION

Here, we showed that a novel gene named CMAP is involved in liver-selective metastasis in a murine system. CMAP is not one of the metastasis-related genes previously isolated and has homology with cystatins, inhibitors of cysteine proteases, despite their ant metastatic profiles (6, 20, 21). Because proteinases are, in general, upregulated and/or abnormally activated in metastatic tumor cells (9, 22, 23), our observation is not consistent with the current concept of the mechanism of metastasis. Thus, our findings seem to indicate a new aspect of the molecular mechanism of metastasis.

The expression of hCMAP was also found in some malignant human cancer cells, although studies were performed with tumor cell lines (Fig. 6). For more exact information on the relation of hCMAP expression and malignant progression including metastasis, analysis of surgical or biopsy specimens may be needed. However, preliminary studies on human cancer cell lines suggested that CMAP may be also related to malignancy of some human cancers. Therefore, further investigations on the molecular mechanisms on the involvement of CMAP in liver metastasis are important. Such studies may lead to a new approach for diagnosis and prevention of liver metastasis in humans. The following are important aspects for future studies on CMAP.

CMAP: A Conceivable Mediator of Liver Metastasis. We analyzed the expressions of >20 metastasis-related genes in IMC-HA1 and IMC-LE5 cells by RT-PCR. Most of them, e.g., NM23 (4), calcyclin (24), and the CD44 splicing variant (25), showed almost no transcriptional differences. In contrast, mts1 (8) and fibronectin (26) were reasonably up- (−10-fold) and down- (−100-fold) regulated in IMC-HA1 cells. These results suggest that multiple gene alterations are required to achieve linked sequential steps in metastasis. The population of mCMAP mRNA showed the most extreme up-regulation (>100-fold) in IMC-HA1 cells compared with that in IMC-LE5 cells thus examined. All the murine tumors expressing mCMAP mRNA metastasized to the liver by i.v. inoculation and induced multiple metastases and death (data not shown). In contrast, mCMAP-negative tumors, including pulmonary metastatic carcinomas, did not metastasize to the liver. Unlike mCMAP expression, mts1 expression does not seem to be correlated with liver metastasis (data not shown). A previous report supports the observation that mts1 was poorly expressed in a spontaneous liver metastatic tumor, despite its high expression in lung or lymphonodus metastatic carcinomas (8). These results suggest that mCMAP is a possible mediator of liver metastases.

CMAP: Possible Mechanisms in Hematogenous Liver Metastasis. The data shown in Fig. 3 and Table 1 suggest that CMAP is mainly involved in hematogenous liver metastasis after intravasation of the tumor cells. Several possible roles of CMAP in metastasis are conceivable. First, CMAP may protect producer cells themselves from excess proteolysis or from the attack of exogenous proteinases. In contrast, CMAP may act as a positive modulator of proteinases during extravasation and infiltration into target organs by antagonizing to cystatins using the structural resemblance. Second, CMAP may act in adapting the microenvironment in metastatic lesions. There are several reports that endogenous proteinase inhibitors exert various biological activities other than as antiproteolyc functions, such as mitogenic activity (27–30), chemotactic activity (31), or differentiation-inducing activity (32). Another possibility is as follows: there are several reports suggesting that factors involved in programmed cell death, such as p53 (33), CC3 (7), and DAP kinase (34), may be metastasis suppressors of several tumor cell lines. Possibly, CMAP may be involved in promoting survival. There is a report that the cysteine proteinase cathepsin B may be involved in apoptosis in mammary tissue (35). Furthermore, the aspartic protease, cathepsin D (36), and several ICE-like cysteine peptidases (37) are considered to play roles in programmed cell death, although cathepsins and caspases are categorized as different types (15). The actual target of CMAP in liver metastasis remains to be elucidated.

CMAP: Possible Target for Antimetastatic Strategy. Recent discoveries of several metastasis suppressors were important not only for specifying endogenous metastatic suppressors but also for indi-
cating diversity of metastasis. There are also positive regulators of metastasis, which might be a good target for actual therapy for prevention of metastasis. The data shown in Fig. 3 and Table 1 indicate that mCMAP expression is involved in hematogenous liver metastasis. Therefore, suppression of mCMAP transcription or inhibition of biological property of protein coding would be new approaches to antiliver metastatic therapy. Thus, biochemical characterization of CMAP and the molecular mechanisms underlying transcriptional activation of CMAP are important issues to be elucidated. We are now working on preparation of the recombinant protein, its biochemical characterization and the structural analysis of the genomic DNA.

After preparation of this manuscript, we learned that Halfon et al. (38) reported isolation of the same gene from human dendritic cells and mouse T helper 2 cells. Their sequences (leukocystatin) were found to be identical to our hCMAP and mCMAP, although their mouse gene cDNA sequence is not complete. They also reported that the expression is selective to hematopoietic cells (38).

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