Increased Expression of \textit{S100A4}, a Metastasis-associated Gene, in Human Colorectal Adenocarcinomas$^1$

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\textbf{ABSTRACT}

The \textit{S100A4} gene (also known as \textit{pEL98/mtsl/p9KaI/18A2/42A/calvasculin/FSP1/CAPL}) encoding an \textit{S100}-related calcium-binding protein is implied to be involved in the invasion and metastasis of murine tumor cells. In the present study, the expression of \textit{S100A4} in human colorectal adenocarcinoma cell lines (SW837, LoVo, DLD-1, HT-29, SW480, SW620, WiDr, and Ccolo201) and surgically resected neoplastic tissues was examined to investigate whether \textit{S100A4} plays a role in the invasion and metastasis of human tumor cells. Northern blot analysis using total RNA isolated from the adenocarcinoma cell lines revealed that five of the eight cell lines expressed substantial amounts of \textit{S100A4} mRNA. Normal colon fibroblasts (CCD-18Co) expressed little of the RNA. Using surgically resected specimens, it seemed that the amount of \textit{S100A4} mRNA in adenomas was nearly equal to that in normal colonic mucosa, whereas adenocarcinomas expressed a significantly higher amount of the RNA than did the adjacent normal colonic mucosa. Immunohistochemical analysis using formalin-fixed paraffin-embedded surgical specimens and monoclonal anti-\textit{S100A4} antibody demonstrated that none of 12 adenoma specimens were immunopositive, whereas 8 of 18 (44\%) focal carcinomas in carcinoma in adenoma specimens and 50 of 53 (94\%) adenocarcinoma specimens were immunopositive. Interestingly, the incidence of immunopositive cells increased according to the depth of invasion, and nearly all of the carcinoma cells in 14 metastases in the liver were positive. These results suggest that \textit{S100A4} may be involved in the progression and the metastatic process of human colorectal neoplastic cells.

\textbf{INTRODUCTION}

A number of \textit{S100}-related small calcium-binding proteins have been identified in mammalian cells (1). Although their functions are still obscure, they are thought to mediate calcium signals in normal and transformed cells and play important roles in many biological events. One such protein is \textit{S100A4}. The human \textit{S100A4} gene, which is located on 1q21 (2), encodes an acidic 101-amino acid protein containing two EF-hand motifs. The gene product has been implied to be involved in cell immortalization (3), cell growth (4), differentiation of mammary epithelial stem cells to myoepithelial-like cells (5), and fibrogenesis (6). In addition, \textit{S100A4} has been reported to be specifically expressed in metastatic tumor cells (7). We also demonstrated that the expression level of \textit{S100A4} is positively correlated with the invasive abilities of various clones established from Lewis lung carcinoma (8). Introduction and enforced expression of the \textit{S100A4} gene induce the metastatic phenotype in a benign rat mammary epithelial cell line (9) and in B16 melanoma cells (10), and the expression of antisense \textit{S100A4} RNA suppresses the metastatic potential of mammary adenocarcinoma cells (11) and Lewis lung carcinoma cells (12). Furthermore, two groups have recently demonstrated a high incidence of lung metastasis of mammary carcinomas in hybrid mice transgenic for the \textit{S100A4} gene (13, 14).

The \textit{S100A4} gene product has been shown to physically interact with cytoskeletal proteins such as nonmuscle tropomyosins (15), myosin (16, 17), and actin (18), implying that \textit{S100A4} is involved in cytoskeletal dynamics and cell motility. In fact, embryonic trophoblast cells, lymphoid cells, and macrophages, all of which are highly motile, are found to express a relatively high amount of \textit{S100A4} (4, 7, 19, 20). Furthermore, we and others have recently demonstrated the enhanced cell motility found in \textit{S100A4} cDNA-transfected tumor cells (8, 21).

Presently, little is known about the expression of \textit{S100A4} in human tissues and tumors and the role of \textit{S100A4} in the process of invasion and metastasis of human tumor cells. To this end, we examined the expression of \textit{S100A4} in human colorectal carcinoma cell lines and surgically resected normal colonic mucosa, adenomatous polyps, carcinoma in adenoma specimens, adenocarcinomas, and metastatic nodules in the liver. The results suggest that \textit{S100A4} may play a role in the progression and in the invasive and metastatic process of colorectal carcinoma cells.

\textbf{MATERIALS AND METHODS}

\textbf{Cells and Cell Culture.} Human colon fibroblasts (CCD-18Co) and colorectal adenocarcinoma cell lines (SW837, ...
Expression of S100A4 in human colon fibroblasts and colorectal adenocarcinoma cell lines.

**Fig. 1** Expression of S100A4 in human colon fibroblasts and colorectal adenocarcinoma cell lines. A, Northern blot analysis of S100A4 mRNA expression. Twenty μg of total RNA were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to nylon filters, and hybridized with a 32P-labeled human S100A4 probe. Ethidium bromide staining of the gels is also shown. B, Western blot analysis of S100A4 protein expression. Cell extracts (25 μg of total protein) were electrophoresed on acrylamide gels under reducing conditions, and the resolved proteins were transferred to nitrocellulose membrane. The sequence of the PCR product was identical with the reported S100A4 sequence (25). Filters were finally washed at 50°C in 30 mM NaCl, 3 mM sodium citrate, and 0.1% SDS.

**Preparation of Cell Extracts, SDS-PAGE, and Immunoblot Analysis.** Subconfluent cultures of colorectal adenocarcinoma cells were lysed in extraction buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride) (15, 19). Cell lysates were centrifuged at 10,000 × g for 10 min, and the supernatant was used for immunoblot analysis. SDS-PAGE was carried out under reducing conditions (26). Immunoblot analysis was performed as described previously (27) using anti-S100A4 polyclonal antibody (15) and an enhanced chemiluminescence Western blotting detection kit (Amersham). Protein concentration was determined by the method of Bradford (28) using BSA as a standard.

**Immunohistochemical Staining.** The 4-μm sections from formalin-fixed paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. They were then air-dried and deparaffinized. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 20 min. The sections were rehydrated and washed with DPBS. After blocking nonspecific binding sites with 10% normal sheep serum or goat serum in DPBS for 10 min at room temperature, the sections were incubated with 1 μg/ml rat anti-S100A4 mAb (mAb 4A2; Ref. 19) or mouse anti-SLea mAb (clone KM-93; Seikagaku Corp., Tokyo, Japan) in DPBS containing 0.1% normal sheep serum overnight at 4°C. After rinsing with DPBS,
Expression of S100A4 in Human Colorectal Adenocarcinoma Cell Lines. To examine whether S100A4 is expressed in colorectal adenocarcinoma, we first examined the expression of S100A4 mRNA in human colorectal adenocarcinoma cell lines (SW837, LoVo, DLD-1, HT-29, SW480, SW620, WiDr, and Colo201) by Northern blot analysis. As shown in Fig. 1A, HT-29, SW480, SW620, WiDr, and Colo201 cells were found to express S100A4 mRNA, whereas SW837, LoVo, and DLD-1 cells expressed little of the mRNA. We also examined the expression of S100A4 mRNA in human colon CCD-18Co fibroblasts and found that they did not express it. Western blot analysis using anti-S100A4 antisera demonstrated the presence of the S100A4 protein in HT-29, SW480, SW620,
Expression in Human Colorectal Carcinomas

mAb 4A2 positive, and others were defined as mAb 4A2 negative. Note that the infiltrating lymphocytes and macrophages (examples are indicated by arrows) are positively stained, whereas epithelial cells are negative. B, adenoma specimen stained with mAb 4A2. Note that the infiltrating lymphocytes and macrophages are positively stained, whereas adenoma cells are negative. C, carcinoma in adenoma specimen stained with mAb 4A2 (positive case). a, adenoma region; c, carcinoma region. Note that immunopositive cells reside in the carcinoma region. D, adenocarcinoma specimen stained with mAb 4A2. Adenocarcinoma cells are positively stained.

Table 1  mAb 4A2 reactivity in human colorectal neoplastic epithelial cells examined immunohistochemically

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<th>mAb 4A2 reactivity</th>
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<tr>
<td></td>
<td>Positive/total</td>
<td>%</td>
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<tr>
<td>Adenoma</td>
<td>0/12</td>
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<td>Carcinoma in adenoma*</td>
<td>1/18</td>
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<td>Adenoma</td>
<td>8/18</td>
<td>44</td>
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<tr>
<td>Carcinoma</td>
<td>50/53</td>
<td>94</td>
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<td>Liver metastasis</td>
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*Only focal carcinoma cells were positive, except one case in which approximately 10% of adenoma cells were positive.

WiDr, and Colo201 cells (Fig. 1B). Thus, five of the eight adenocarcinoma cell lines tested expressed a substantial amount of S100A4.

Expression of S100A4 mRNA in Human Colorectal Adenomas, Adenocarcinomas, and Metastases. Total RNA was isolated from surgically resected colorectal adenomas, adenocarcinomas, and adjacent normal colonic mucosa and subjected to Northern blot analysis. Fig. 2 shows several representatives of the expression level of S100A4 mRNA. A low amount of the RNA was detected in normal mucosa. The amounts of the RNA in adenomas were nearly equal to those in normal mucosa (Fig. 2A). However, relatively higher amounts of the RNA, although in varying degrees, were expressed in adenocarcinomas than in normal mucosa (Fig. 2B). After semiquantitating the level of S100A4 mRNA in normal mucosa (18 cases), adenomas (5 cases), and adenocarcinomas (25 cases) by video densitometry, and after determining the ratio of S100A4 mRNA expression in each case to the positive Colo201 control sample on each blot, the relative expression levels of S100A4 mRNA in all cases were determined. These are presented in a scattergram plot shown in Fig. 3. Clearly, S100A4 mRNA was highly expressed in adenocarcinoma specimens compared to normal mucosa (P < 0.05) and adenoma specimens. We also examined the expression of S100A4 mRNA in metastatic nodules in the liver. Although no detectable level of the RNA was expressed in normal liver, a significant amount of RNA was detected in the metastatic nodules (Fig. 2C). There was no apparent correlation between the S100A4 mRNA level and histological differentia-

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The content is a combination of immunohistochemical staining, table data, and discussion on the expression of the S100A4 protein in human colorectal tissues, detailing the positive findings observed in adenoma and carcinoma regions, as well as the implications of these findings in relation to Northern blot analysis and mRNA expression levels.
Immunohistochemical staining of human colorectal adenocarcinomas and hepatic metastases. A, adenocarcinoma specimen stained with mAb 4A2. Note that the deeply invaded adenocarcinoma cells are more intensely stained than those in mucosal region. B, adenocarcinoma specimen stained with anti-sLe^a antibody. An adjacent serial section used in A was stained. C, adenocarcinoma cells in veins (v) and lymphatic vessels (ly) stained with mAb 4A2, n, necrotic region. D, metastatic nodules in the liver stained with mAb 4A2.

Immunohistochemical Analysis of S100A4 Expression in Normal Colonic Mucosa, Colorectal Adenomas, Carcinoma in Adenoma Specimens, Adenocarcinomas, and Liver Metastases. To examine the expression of S100A4 at the cellular level, we performed immunohistochemical analyses using histological preparations of formalin-fixed paraffin-embedded surgical specimens and anti-S100A4 mAb (mAb 4A2). The antibody specifically immunoprecipitated the S100A4 protein from cell lysates and reacted with the S100A4 protein on a Western blot (19). However, it did not react with the closely related calcium-binding proteins calcyclin, S-100a subunit, and S-100b subunit (data not shown).

In normal colon tissues, a wide range of cell types seemed to be stained with mAb 4A2. There was a high level of staining of the smooth muscle, of the smooth muscle in the walls of vessels, and of some but not all lymphocytes in the lymph nodes. A weak but definitive staining was observed in the adipose tissue (data not shown). The infiltrating lymphocytes and macrophages in the stroma were intensely stained with mAb 4A2 (Fig. 4A), consistent with the previous reports (7, 19, 20). The staining was abolished when tissue sections were incubated with mAb 4A2 that had been previously absorbed with recombinant S100A4 protein (data not shown). In contrast, there was little staining of fibroblasts and of the mucus-secreting goblet cells (Fig. 4A).

Next we immunohistochemically stained colorectal adenomatous polyps, carcinoma in adenoma specimens, adenocarcinomas, and metastatic nodules in the liver and evaluated the mAb 4A2-immunopositive neoplastic cells in these tissues. The results presented in Table 1 showed that none of 12 adenomas tested was positive for mAb 4A2 staining, whereas the infiltrating lymphocytes and macrophages were positively stained (Fig. 4B). In carcinoma in adenoma specimens, 8 of 18 (44%) cases were positive (Table 1; see Fig. 4C for a positive case). It should be noted that the positive cells resided only in the focal carcinoma region in seven of the eight positive cases, and approximately 10–40% of the carcinoma cells were positive. In one case, besides carcinoma cells, approximately 10% of adenoma cells were also immunopositive. In adenocarcinomas, 50 of 53 (94%) cases were positively stained (Table 1; Fig. 4D). Interestingly, we noticed that most of the adenocarcinoma cells that invaded deeply were much more intensely stained than those in the mucosal region (Fig. 5A). This staining pattern was more prominent than that observed after immunostaining adjacent...
serial sections with anti-sLe" mAb (Fig. 5B). Then the percentage of mAb 4A2-immunoreactive adenocarcinoma cells was determined according to the depth of invasion in 10 adenocarcinoma specimens in which we could clearly evaluate the extent of invasion (Table 2). Except for case 6, the percentage of immunopositive adenocarcinoma cells was higher in the deeply invaded region than it was in the mucosal region (see case 8 for a typical example). In addition, the carcinoma cells that invaded into capillaries and lymphatic vessels were immunopositive (Fig. 5C). Furthermore, almost all of the carcinoma cells that metastasized to the liver were immunoreactive (Fig. 5D, Tables 1 and 2).

**DISCUSSION**

S100A4 has been implied to be involved in the invasion and metastasis of murine tumor cells (7–14). However, information on S100A4 expression in human tumor cells is limited thus far, although high S100A4 expression in breast, ovary, colon, and thyroid carcinoma has been reported (29, 30). In the present study, we examined the expression of S100A4 and evaluated whether it could be correlated with the invasive and metastatic potential of human colorectal adenocarcinoma using cultured cell lines and surgically resected neoplastic tissues.

Among the human adenocarcinoma cell lines we tested, HT-29, SW480, SW620, WiDr, and Colo201 cells but not SW837, LoVo, and DLD-1 cells expressed a substantial amount of S100A4. In the literature, SW837, LoVo, and DLD-1 cells are reported to be noninvasive or quite minimally invasive (31–35), whereas HT-29, SW620, and WiDr cells are shown to be invasive and/or metastatic in nude mice (32, 34–36). To our knowledge, there is no report concerning the invasive and metastatic ability of Colo201 cells, but they were established from ascites fluid of a patient with metastases in the liver (37). Therefore, it seemed likely that there is a correlation, although not a strict one, between the expression of S100A4 and invasive potential among the cell lines tested. However, there is the exception of SW480 cells, which have been shown to be noninvasive (34, 38).

Cell lines are not always comparable with the in vivo situation, and their properties may be altered during in vitro passages. Therefore, we examined the expression of S100A4 in human colorectal neoplastic tissues. For this, total RNA was isolated from surgically removed adenoma and adenocarcinoma specimens. Northern blot analyses using human S100A4 cDNA as a probe revealed that the S100A4 mRNA expression level in adenomas was nearly equal to that in normal mucosa. However, the expression level was higher in adenocarcinomas than it was in normal mucosa. Furthermore, expression of S100A4 mRNA was observed in metastases in the liver. These results imply that expression of S100A4 may be linked to the progression of colorectal neoplastic cells. The role of S100A4 in tumor progression has also been demonstrated in a human breast cancer cell line (39).

Because S100A4 has been reported to be expressed in a variety of cell types such as lymphocytes, macrophages, and smooth muscle (4, 7, 19, 20), it is premature to conclude that the higher expression of S100A4 mRNA in adenocarcinoma specimens indeed reflects the expression in carcinoma cells themselves. To evaluate this point and examine the expression of S100A4 in invasive cells more closely, we performed immunohistochemical analyses using mAb 4A2. In the colon, a variety of cell types were immunopositive for mAb 4A2. In addition to the infiltrating lymphocytes and macrophages, there was an intense staining of the smooth muscle and of the smooth muscle in the walls of the vessels. A weak staining of the adipose tissues was also evident. These results were fairly consistent with the previous report in which the distribution of S100A4 mRNA in adenocarcinoma specimens indeed reflects the expression in carcinoma cells themselves. To evaluate this point and examine the expression of S100A4 in invasive cells more closely, we performed immunohistochemical analyses using mAb 4A2. In the colon, a variety of cell types were immunopositive for mAb 4A2. In addition to the infiltrating lymphocytes and macrophages, there was an intense staining of the smooth muscle and of the smooth muscle in the walls of the vessels. A weak staining of the adipose tissues was also evident. These results were fairly consistent with the previous report in which the distribution of S100A4 mRNA in normal tissues was examined immunohistochemically (40). S100A4 mRNA detected in normal colonic mucosa may be derived from these immunopositive cells. Coinciding with the results of S100A4 expression in CCD-18Co cells, there was little staining of normal colon fibroblasts.

It seemed that colorectal epithelial cells are scarcely stained with mAb 4A2. Little if any staining was observed in adenoma cells. In contrast, the percentage of mAb 4A2-immunopositive carcinoma cells was quite high in adenocarcinoma...
specimens, especially in deeply invaded regions. Although there was a difference in the percentage of S100A4-positive adenocarcinoma specimens when examined at the mRNA level and immunohistochemically, this may reflect the increased sensitivity and resolution of the immunohistochemical techniques relative to Northern blotting. Interestingly, 8 of 18 (44%) carcinoma in adenoma specimens were immunopositive (10–40% of carcinoma cells were positive). These results imply that the expression of S100A4 may be turned on during the so-called adenoma-carcinoma sequence.

The mechanisms regulating S100A4 expression in colorectal epithelial cells are largely unknown. Tulchinsky et al. (41, 42) have reported that DNA methylation of the S100A4 gene plays a critical role in the expression of S100A4; that is, the high expression of S100A4 in metastatic cells may be associated with the demethylation of a CD3 δ enhancer in the first intron of the murine S100A4 gene. Supporting these reports, our preliminary experiment has shown that the S100A4 gene is hypermethylated in CCD-18Co, SW837, LoVo, and DLD-1 cells compared to HT-29, SW480, SW620, WiDr, and Colo201 cells. We are now examining whether DNA methylation of the S100A4 gene could account for the difference in S100A4 expression between colorectal adenoma and adenocarcinoma cells.

S100A4 has been shown to be involved in regulating the cell motility of tumor cells (4, 7, 8, 20, 21). It is widely accepted that the increased motility of tumor cells is one of the determinants in the acquisition of invasive and metastatic capability. Therefore, if S100A4 plays a role in the invasion and metastasis of human colorectal carcinoma cells, the percentage of S100A4-positive carcinoma cells may be high in deeply invaded regions and in metastases in lymph nodes and distant organs. The present results showed that the number of mAb 4A2-immunopositive carcinoma cells increased as carcinoma cells invaded deeply, and more than 90% of the cells in the regions of the deeply invaded carcinoma cells may be high in deeply invaded regions and in metastases in lymph nodes and distant organs. The present results showed that the number of mAb 4A2-immunopositive carcinoma cells increased as carcinoma cells invaded deeply, and more than 90% of the cells in the regions of the deeply invaded carcinoma cells may be high in deeply invaded regions and in metastases in lymph nodes and distant organs. Although our results are consistent with the idea that increased expression of S100A4 correlates with increased invasiveness and metastatic potential, further work is required to provide evidence for a causative role of S100A4 in the invasive potential of human colorectal carcinoma cells. In addition, it should be evaluated whether S100A4 expression could be used as a marker for prognosis in colorectal carcinoma patients.

N. Nakamura and K. Takenaga, unpublished observations.

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