Inverse Association of Cell Adhesion Regulator Messenger RNA Expression with Metastasis in Human Colorectal Cancer

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ABSTRACT

Alterations in several classes of adhesion molecules have been implicated in the progression of colorectal cancer. Cell adhesion regulator (CAR) has been identified as a regulator molecule of integrin-dependent cell adhesion. We have explored a possible involvement of the CAR gene in colorectal cancer. Reverse transcription-PCR revealed that CAR expression was detected in normal colon tissue, whereas it was decreased or undetectable in 13 (46.2%) human colon cancer cell lines. To further study the biological significance of CAR expression in colon cancer cells, a CAR expression vector was introduced into HT-29 cells, in which CAR is not expressed. Adhesion of HT-29 cells to extracellular matrix components was up-regulated by the introduction of CAR. In spite of similar growth properties with the controls, CAR-transfected HT-29 cells showed a significantly reduced spontaneous metastatic potential in nude mice. To determine whether these experimental results are of relevance with respect to actual human tumors, we investigated CAR expression in 30 surgical specimen pairs of human colorectal cancer and adjacent noncancerous tissue using semiquantitative reverse transcription-PCR. In 14 of 30 cases (46.7%), CAR expression in cancer was less than one-tenth of that in matched noncancerous tissue. The tumor: normal ratio of CAR expression was significantly lower in patients with lymph node metastasis than in those without it (P < 0.01) and in patients with distant metastasis than in those without it (P < 0.05). CAR expression was significantly lower in more advanced Dukes' stage tumors (P < 0.05). Our results suggest that down-regulation of CAR expression may play an important role in the progression and metastasis of colorectal cancer.

INTRODUCTION

Metastasis is one of the principle reasons for the high rate of mortality in colorectal cancer (1). Evidence has shown that the process of cancer metastasis requires a complex coordinated set of changes in intercellular and cell to matrix interactions (2–4). Under normal conditions, these interactions play a role in maintaining cancer cells at a primary site (5). Therefore, disturbance of the adhesive interactions seems to favor release of cancer cells from the primary site (6).

Several lines of evidence support this paradigm in colorectal cancer; down-regulation of E-cadherin and several classes of integrin have been shown to be associated with the progression and metastasis of colorectal cancer (7–10). In this regard, it is of considerable interest that CAR (11) has been isolated as a regulator molecule of integrin-dependent cell adhesion (11). This molecule up-regulates cell adhesion to extracellular matrix components without increasing the expression of integrin itself. However, this enhanced adhesion is inhibited by the anti-integrin antibody, indicating that CAR may regulate integrin-dependent cell adhesion by modifying the integrin function (11). The probable contribution of CAR is highlighted by the finding that the metastasis-derived colon cancer cell line SW620, which has lost CAR expression, demonstrated lower binding activity to extracellular matrix components than its counterpart, SW480, derived from the primary tumor of the same patient. Loss of normal adhesive properties is characteristic of invasive and metastatic cancer cells (2–4). Moreover, CAR-transfected SW620 demonstrated adhesiveness comparable to SW480 (11). Therefore, it seems promising to explore a possible involvement of CAR in colorectal cancer.

In this study, we examined CAR mRNA expression in human colon cancer cell lines and the effect of CAR expression on the growth and metastatic potential in a colon cancer cell line. We also investigated CAR expression in 30 surgical specimen pairs of human colorectal cancer and adjacent noncancerous tissue using semiquantitative RT-PCR.

MATERIALS AND METHODS

Cell Culture and Surgical Specimens. All colon cancer cell lines were obtained from the Japanese Cancer Research Resources Bank. Cells were maintained in DMEM containing 10% fetal bovine serum, with the exception of those noted. CHC-Y1, COLO 201, and DLD-1 were maintained in RPMI 1640 containing 10% fetal bovine serum. SW837 and SW948 were maintained in L15 containing 10% fetal bovine serum. Thirty surgical specimen pairs of primary colorectal cancer and adjacent noncancerous tissue were obtained from patients receiving surgical treatment. Informed consent was obtained from each subject. All tissues were frozen in liquid nitrogen and later used for RNA extraction. Specimen sets of colorectal cancer, adenoma, and adjacent normal colon tissue were obtained from five patients who had both colorectal cancer and adenoma.

Detection of CAR mRNA Expression Using RT-PCR. Total RNA was extracted from cell lines and specimens using the acid guanidinium thiocyanate-phenol-chloroform extraction method (12) and treated with DNase I. The cDNA was synthesized from 1 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Perkin Elmer/Cetus, Norwalk, CT) with random hexamers. The cDNA was amplified with the PCR using CAR-specific oligonucleotides (Ref. 11; 5'-TGGTCATCGCCGCTGTTGCA-3' as a sense primer and 5'-TTCCAACTACACAGTTTAT-3' as an antisense primer) or β-actin-specific oligonucleotides (Ref. 13; 5'-CTGTCTGGCGGCCACAC-CAT-3' as a sense primer and 5'-GCCAAGCTATGCTAGTCGCC-3' as an antisense primer). β-actin served as an internal control of the reaction. PCR amplification was carried out in a Perkin Elmer/Cetus thermal cycler under the following conditions: denaturation at 94°C for 1 min; annealing and extension at 60°C for 1 min; 30 cycles for CAR; and 20 cycles for β-actin. Using the same RNA samples, we simultaneously performed RT-PCR without reverse transcriptase.

Southern Blotting of PCR Products. The PCR products were electrophoresed in a 3% Nusieve agarose gel (FMC Bioproducts, Rockland, ME) and transferred to Hybond N plus (Amersham, Arlington Heights, IL) in 0.4 N NaOH. The filters were hybridized with cDNA probes labeled using the random primer method in 50% formamide/5X Denhardt’s solution/3X SSC/100 μg/ml salmon sperm DNA/1% SDS at 42°C. Probes are partial cDNAs obtained using RT-PCR, each of which was respectively revealed to be identical to the original CAR and β-actin cDNA with DNA sequencing.

DNA Transfection. A full-length cDNA encoding CAR was isolated from normal human colon tissues using PCR and cloned into the BamHI site of the eukaryotic expression vector pcDNA neo (Invitrogen, San Diego, CA) which contains the cytomegalovirus promoter/enhancer for expression and a neoycin-resistance gene allowing G418 selection. A transfection of HT-29 cells was performed with LipofectAMINE (Life Technologies, Inc., Grand Island, NY)
following the manufacturer's protocol. After 2–3 weeks of 0.6 mg/ml G418 selection, individual colonies were picked and expanded for further analyses. Transfectants containing the selection plasmid pcDNA neo alone were used as controls and designated HT-mock.

**Cell Adhesion Assay.** A cell adhesion assay was performed as described previously, with some modification (14). Briefly, microwells were coated with type I collagen, type IV collagen, laminin, or fibronectin (5 μg/cm<sup>2</sup>) for 2 h at 37°C and washed with PBS. Suspended cancer cells (5 × 10<sup>5</sup>) in serum-free medium were added to each coated well and incubated for 60 min at 37°C. At the end of the incubation period, nonadherent cells were washed off by streaming PBS over the plate three times, and the remaining adherent cells were counted. Results were expressed as a percentage of the initially added number of cells. To test the inhibition of cell adhesion to these substrates, cells were preincubated with monoclonal antibodies against integrin subunits for 60 min at 4°C before seeding on the wells.

**Growth in Culture.** At the initial density of 5 × 10<sup>5</sup> cells/well, cancer cells were seeded on the culture plates coated with type I collagen, type IV collagen, laminin, or fibronectin (5 μg/cm<sup>2</sup>). Viable cells from triplicate plates were detached with trypsin and counted every 2 days.

**Growth and Metastasis in Vivo.** Cancer cells (1 × 10<sup>6</sup>) were s.c. injected into the hind limb footpad of 4-week-old athymic BALB/c nude mice. Tumor size was measured with calipers every 5 days. Tumor volume was calculated as the product of the longest and shortest diameters (4π/3) of the ellipsoid resulting from amputation with a hemilogarithmic scale.

**Preparation of Standard Curves.** Standard curves for PCR were prepared as described previously (15). Plasmid (1 μg) containing amplified regions of CAR or β-actin was diluted serially and logarithmically. Diluted samples were amplified with PCR using the same intervals and cycles as those used for the cell lines and clinical samples. Southern blotting of amplified products was done with radiolabeled CAR or β-actin cDNA. The resulting band intensities of autoradiograms were measured with a densitometer. The band intensity of each sample was expressed as the peak height (mm) of the curve drawn with the densitometer. The correlation between the quantity of cDNA before PCR and band intensities of CAR and β-actin was analyzed using a hemilogarithmic scale.

**Statistical Analysis.** The Wilcoxon rank sum test was used for statistical comparisons between the two groups. In multiple groups, data were initially analyzed with one-way ANOVA followed by the Bonferroni multiple comparison method. A nonparametric test was used to compare ordered groups across different Dukes’ stages. In all tests, the level of significance was set at P < 0.05.

**RESULTS**

**Expression of CAR mRNA in Colon Cancer Cell Lines.** CAR mRNA expression was examined in 13 human colon cancer cell lines using RT-PCR. When RNA samples had been incubated in RT reactions without reverse transcriptase, no gene products of interest were amplified using PCR (data not shown). This indicates that a trace amount of residual genomic DNA did not show any band even after hybridization with the CAR cDNA probe. Among 13 cell lines, CHC-Y1, COLO 201, and SW837 showed reduced CAR expression as compared to normal human colon tissues (Fig. 1). Moreover, CAR expression was undetectable in HuMLIC-06, LoVo, and HT-29. Altogether, 6 of 13 (46.2%) colon cancer cell lines showed a reduction or loss of CAR expression.

**Transfection with CAR cDNA.** Exogenous CAR mRNA expression was examined in transfecteds. CAR expression was detectable in six CAR transfectants that showed stable integration of CAR, but not in the other CAR transfectants, parental cells, or mock transfectants (Fig. 2). With the exception of HT-CAR-5, levels of exogenous CAR expression in CAR transfectants were within the range colon cancer cell lines endogenously expressed (Figs. 1 and 2). Flow cytometric analysis showed that transfection of the CAR gene caused no alteration in the surface levels of integrin subunits (positive for α<sub>1</sub>, α<sub>2</sub>, α<sub>3</sub>, α<sub>6</sub>, α<sub>v</sub>, β<sub>1</sub>, and β<sub>4</sub>; negative for α<sub>4</sub>, α<sub>5</sub>, β<sub>2</sub>, and β<sub>3</sub>) normally found on HT-29 cells (data not shown). Parental HT-29 cells, three CAR transfectants with different levels of CAR expression, HT-CAR-2 (high), HT-CAR-7 (moderate), HT-CAR-9 (low), and one mock transfectant were selected for further analysis.

**Effect of CAR Expression on Adhesion of HT-29 Cells to Various Extracellular Matrix Components.** Adhesion of HT-29 cells and select transfectants to various extracellular matrix components was examined. Fig. 3 shows the representative data of HT-CAR-7. CAR transfectants demonstrated significantly enhanced adhesion to...
REDUCED CAR EXPRESSION IN COLORECTAL CANCER

Fig. 5. Growth properties of CAR transfectants similar to those of the controls in wells coated with type I collagen (upper left), type IV collagen (upper right), fibronectin (lower left), or laminin (lower right). Cells were plated in wells coated with substrates at the initial density of $5 \times 10^4$ cells/well. At the indicated times, viable cell numbers were counted. Points, means of triplicate wells of three separate experiments; SD of the mean was within 10% for each point.

Fig. 5. Growth of s.c. tumors in nude mice. Tumor size was measured with calipers every 5 days. Tumor volume was calculated according to the formula: tumor volume = maximum length $\times$ maximum width $\times$ 0.5. Points, mean from six mice in each group.

extracellular matrix components as compared with the controls. On the other hand, adhesion to poly-L-lysine was almost equivalent between the CAR transfectants and controls. Enhanced adhesion of CAR transfectants to type I and type IV collagen, laminin, and fibronectin was inhibited by the anti-α1, anti-α6, and anti-αv antibody, respectively.

Growth in Vitro. HT-29 cells and select transfectants were examined for their growth properties on the extracellular matrix components. HT-29 cells, CAR transfectants, and mock transfectants had comparable growth rates when seeded on the culture plates coated with type I collagen, type IV collagen, laminin, or fibronectin (Fig. 4).

Growth and Metastasis of CAR Transfectants in Vivo. HT-29 cells and select transfectants were s.c. injected into the hind limb footpad of nude mice. In all cases, palpable tumors were evident. HT-29 cells, three CAR transfectants, and mock transfectants had a comparable growth rate in nude mice (Fig. 5). Examination of histological sections did not reveal any obvious differences among the tumors. To assess the stability of CAR expression in vivo, we examined its expression in the tumors using RT-PCR. CAR was actually expressed in the primary tumors arising from HT-CAR-2 and HT-CAR-7, but not in those from HT-CAR-9 (Fig. 6A). Despite a comparable growth rate with the controls, HT-CAR-2 and HT-CAR-7 showed significantly lower spontaneous metastatic potential as compared to the controls (Table 1). However, no significant suppression

Fig. 6. Expression of CAR mRNA in s.c. tumors (A) and lung metastatic foci (B) in nude mice. One μg of total RNA extracted from tissues was used for RT-PCR.
REDUCED CAR EXPRESSION IN COLORECTAL CANCER

Table 1 Metastatic potential of HT-29 cells after transfection with a CAR expression vector

<table>
<thead>
<tr>
<th>No. of metastatic foci in lung/mouse (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>s.c. Injection&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>HT-29</td>
</tr>
<tr>
<td>HT-CAR-2</td>
</tr>
<tr>
<td>HT-CAR-7</td>
</tr>
<tr>
<td>HT-CAR-9</td>
</tr>
<tr>
<td>HT-mock</td>
</tr>
</tbody>
</table>

<sup>a</sup> Cells were injected s.c. into the hind limb footpad of nude mice (1 x 10<sup>6</sup>/mouse). Mice bearing tumors of more than 1000 mm<sup>3</sup> were anesthetized, and the hind limb was amputated. Twelve weeks after amputation, mice were killed and examined for metastasis. For each group, n = 6.

<sup>b</sup> Cells were injected into the lateral tail vein of nude mice (1 x 10<sup>6</sup>/mouse). Twelve weeks later, mice were killed and examined for metastasis. For each group, n = 6.

Statistical significance: HT-CAR-2 or HT-CAR-7 to HT-29, HT-CAR-9, and HT-mock (P < 0.001). Initially, individual data for each cell were analyzed using one-way ANOVA, followed by the Bonferroni multiple comparison method.

Fig. 7. Correlation between band intensities and amounts of serially diluted cDNAs. A, standard curves for CAR cDNA. Correlation coefficients (r) = −0.975, P < 0.05; paired t test. Vertical and horizontal axes, band intensity (mm) and the amount of serially diluted cDNA (μg), respectively. B, standard curve for β-actin cDNA. r = −0.954, P < 0.05; paired t test. The linear relationships, determined by the least squares approximation, were: 1 (CAR) = −27.2 log Q + 107.5; 1 (β-actin) = −25.0 log Q + 98.8. The relationships between band intensities and plasmid concentrations were almost linear within the ranges 10<sup>−4</sup> to 10<sup>−7</sup> (μg) of plasmids containing CAR (correlation coefficients, r = −0.975, P < 0.05; paired t test) and 10<sup>−3</sup> to 10<sup>−6</sup> (μg) of β-actin plasmids (r = −0.954, P < 0.05; paired t test). These results indicate that the expression of CAR and β-actin mRNA could be evaluated semiquantitatively within these ranges.

Expression of CAR mRNA in Human Colorectal Cancer and Adenoma Tissues. CAR mRNA was expressed in all 30 noncancerous samples at similar levels; expression was comparable or reduced in matched cancer tissues. Representative cases with different Dukes' stages are shown in Fig. 8. The tumor:normal ratio of CAR expression was corrected for that of β-actin expression. In 14 of 30 cases (46.2%), CAR expression in cancer was less than one-tenth of that in matched noncancerous tissue. The relationship between CAR expression and clinicopathological features is summarized in Table 2. There was no correlation of CAR expression with histological differentiation of tumor or tumor depth. In contrast, CAR expression was significantly lower in patients with lymph node metastasis than in those without (P < 0.01) and in patients with distant metastasis than in those without (P < 0.05). Finally, CAR expression was significantly lower in more advanced Dukes' stage tumors (P < 0.05, Fig. 9). We also investigated CAR expression in five cases who had both colorectal cancer and adenoma. In all five cases, CAR expression was almost equivalent between adenoma and normal tissues, irrespective of its expression in matched cancer tissues (Fig. 10).

Table 2 Relationship between clinicopathological characteristics and tumor:normal ratio of CAR mRNA expression in patients with colorectal cancer

<table>
<thead>
<tr>
<th>Tumor:normal ratio of CAR mRNA expression&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of cases</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Differentiation grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>11</td>
<td>0.58 ± 0.52</td>
</tr>
<tr>
<td>Moderately</td>
<td>16</td>
<td>0.47 ± 0.52</td>
</tr>
<tr>
<td>Poorly</td>
<td>3</td>
<td>0.37 ± 0.60</td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sm</td>
<td>2</td>
<td>0.53 ± 0.73</td>
</tr>
<tr>
<td>pm</td>
<td>11</td>
<td>0.58 ± 0.53</td>
</tr>
<tr>
<td>ss</td>
<td>12</td>
<td>0.45 ± 0.52</td>
</tr>
<tr>
<td>s</td>
<td>5</td>
<td>0.44 ± 0.58</td>
</tr>
<tr>
<td>Node categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N (−)</td>
<td>17</td>
<td>0.80 ± 0.44</td>
</tr>
<tr>
<td>N (+)</td>
<td>13</td>
<td>0.11 ± 0.28</td>
</tr>
<tr>
<td>Metastasis categories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M (−)</td>
<td>26</td>
<td>0.54 ± 0.52</td>
</tr>
<tr>
<td>M (+)</td>
<td>4</td>
<td>0.02 ± 0.02</td>
</tr>
</tbody>
</table>

<sup>a</sup> The tumor:normal ratio of CAR mRNA expression are expressed in averages ± SD.

Fig. 9. Inverse correlation between CAR mRNA expression and Dukes' stage. Lower expression is present in patients with a higher Dukes' stage (P < 0.05). Points, mean; bars, SE.
Our experimental demonstration of a relationship between the CAR expression and metastatic potential of colon cancer cells was corroborated by the follow-up examination of CAR expression in 30 surgical specimen pairs of human colorectal cancer and adjacent noncancerous tissue. In about one-half of the cases, cancer tissues expressed less CAR than their noncancerous counterparts. More important, there was a significant statistical association between reduced CAR expression and evidence of lymph node or distant metastasis. We also found a statistically significant inverse correlation between the expression level and advanced disease in Dukes’ stages. Thus, down-regulation of CAR expression seems to be closely associated with the progression and metastasis of colorectal cancer. On the other hand, CAR expression was almost the same between adenoma and normal tissues, irrespective of its expression in matched cancer tissues; expression seems to be impaired late in colorectal carcinogenesis. The mechanism of reduction or loss of CAR expression has not been found in this study. High levels of allelic loss on 16q in the region of CAR have not been demonstrated in colorectal cancers. Further investigation of the CAR genome in cases with reduced CAR expression might provide more information regarding the mechanism.

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