High Expression of Methionine Aminopeptidase 2 in Human Colorectal Adenocarcinomas

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ABSTRACT

Purpose: Several viral and eukaryotic proteins required for signal transduction and regulatory functions undergo lipoprotein modification by the enzyme N-myristoyltransferase. Previously we reported that N-myristoyltransferase activity is higher in colon and gallbladder carcinoma than in the corresponding normal tissues. Methionine aminopeptidase 2 (MetAP2) is a bifunctional protein that plays a critical role in the regulation of post-translational processing and protein synthesis. To investigate whether MetAP2 contributes to the pathogenesis of colon carcinoma, we investigated the expression of MetAP2 in both normal and invasive tumor components of human samples.

Experimental Design: We evaluated 50 cases of colon carcinoma for this study. In this report we analyzed 15 cases for MetAP2 activity and 13 cases for the expression of MetAP2 by Western blot in both the normal and in invasive tumor components of human samples. In addition, immunohistochemistry analysis was also carried out on samples from all patients.

Results: MetAP activity was elevated in all cancerous tissues compared with normal tissues. Western blot analysis also showed the higher expression of MetAP2 in all cases of cancerous tissues. In addition, immunohistochemistry analysis revealed that all cases of colorectal adenocarcinoma showed moderate to strong cytoplasmic positivity for MetAP2 with increased intensity in the invasive component.

Conclusions: Elevated MetAP protein expression is associated with metastatic tumor progression and appears to be a strong molecular marker for clinical prognosis. MetAP2 inhibition may represent a potential target for therapeutic intervention in colorectal carcinoma.

INTRODUCTION

The translational process on ribosomes is known to start with methionine, and the NH2-terminal methionine is usually removed before the newly synthesized protein is transported to its proper intracellular locus. Methionine aminopeptidase (MetAP) is the enzyme responsible for the removal of NH2-terminal methionine (1) and exists in two different isoforms: MetAP1 and MetAP2 (2). The most significant structural difference between the two is a large helical domain inserted on the surface of the type 2 isozymes (3). Previously, MetAP2 was identified as an eukaryotic initiation factor-2-associated 67-kDa protein (p67; Ref. 4) that regulated protein synthesis by protecting the α-subunit of eukaryotic initiation factor-2 from phosphorylation (5). MetAP2 expression correlates with cell growth, and nondividing cells do not show immunodetectable levels of this enzyme (6). Moreover, MetAP2 is greatly induced by phorbol myristate acetate (5).

Protein myristoylation occurs after the removal of methionine by MetAP. The enzyme catalyzing this cotranslational process is N-myristoyltransferase (NMT). NMT is a ubiquitously distributed eukaryotic cytosolic enzyme that has been purified and characterized from various sources (7, 8). The known myristoylated proteins include the catalytic subunit of cAMP-dependent protein kinase (9), various tyrosine kinases (pp60src, pp60c-src, pp56lck, pp56lck, and cAbl; Refs. 10–12), the β-subunit of calmodulin-dependent protein phosphatase (calcineurin; Ref. 13), the myristoylated alanine-rich C-kinase substrate (14), the α-subunits of several G proteins (15, 16), and several adenosine diphosphate ribosylation factor proteins involved in ADP ribosylation (17). C-Src is frequently observed to be activated or overexpressed in several human cancers, particularly those of colon and breast cells (18–20). The tyrosine kinase activities of N-myristoylated pp60c-src and pp62c-src protein kinases are significantly elevated in primary colorectal adenocarcinoma as well as in their corresponding cell lines relative to those of normal cells (18, 19, 21). It is possible that increased synthesis of pp60c-src in colon cancer requires increased levels of N-myristoyltransferase. The N-myristoyltransferase is responsible for the activation of N-myristoyl-dependent targeting of new synthesized pp60c-src to the cytoskeleton. Previously we reported that NMT activity is higher in colonic epithelial neoplasms than in normal appearing colonic tissue and that increases in NMT activity appear at an early stage in colonic carcinogenesis (22). We also observed that the NMT expression is elevated in colon (23) and gallbladder carcinoma (24). In addition, we have demonstrated low levels of NMT inhibitor protein (NIP2) in high-expressing c-Src cell
lines and high levels of NIP$_{23}$ in low-expressing c-Src cell lines (25). Recently, the importance of NMT in cancer has been reviewed (26).

Angiogenesis is considered a relevant pathogenic event in cancer development. Recently, MetAP2 has been identified as the molecular target of the angiostatic agents fumagillin and ovalicin. These compounds selectively and covalently bind MetAP2 and block its aminopeptidase activity (6, 27). MetAP2 might play a relevant role in the development of various types of cancer. Because we observed higher expression of NMT in various tumors, we investigated the role of MetAP in colorectal adenocarcinoma. Here we report the higher expression of MetAP2 in patients with colorectal adenocarcinoma.

**MATERIALS AND METHODS**

**Materials.** Nitrocellulose membranes were purchased from Bio-Rad Laboratories. Benzamidine, phenylmethylsulfonyl fluoride, leupeptin, and methionine-$p$-nitroanilide were obtained from Sigma-Aldrich Canada. Other reagents were of analytical grade and were purchased from BDH or Sigma-Aldrich Canada. Anti-MetAP2 was obtained from Zymed Laboratories Inc. (San Francisco, CA).

**Human Colorectal Specimens.** Fifty colorectal cancer patients were selected for evaluation of MetAP expression. The human colorectal tissues were collected from the Royal University Hospital, University of Saskatchewan, Saskatoon, SK, Canada. Surgical pathology specimens from 50 patients who had undergone resection for colorectal adenocarcinoma were collected directly from the surgical operating room in the fresh state, i.e., before being immersed in tissue fixative. After gross inspection, samples of tumors were dissected and immediately frozen at $-80^\circ\text{C}$. Normal-appearing colonic mucosa far removed from the cancer was similarly dissected and frozen. Most of the remaining tissue was fixed in neutral-buffered formaldehyde and processed for histological and immunohistochemical evaluation.

**Preparation of Tissue Extracts.** All procedures were carried out at $4^\circ\text{C}$ unless otherwise stated. Tissues were homogenized in 100 mM Tris-HCl (pH 7.4), containing 1 mM EGTA, 1 mM EDTA, 10 mM 2-mercaptoethanol, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 $\mu$g/ml leupeptin. The crude homogenate was centrifuged for 30 min at 10,000 × g, and the supernatant was filtered through glass wool. The supernatant obtained was used for MetAP activity and Western blot analysis.

**MetAP Assay.** MetAP activity was determined by hydrolysis of methionine-$p$-nitroanilide as described elsewhere (28). The assay mixture (total volume, 500 µl) for MetAP activity consisted of 50 mM Tris-HCl (pH 7.5), 0.25 mM methionine-$p$-nitroanilide (substrate), and an appropriate concentration of the enzyme solution. The reaction mixtures were incubated at 37°C for 30 min and left on ice for 15 min. Nitroanilide concentrations were then measured spectrophotometrically at 405 nm. One unit of the aminopeptidase activity was defined as the amount that released 1 $\mu$M/min $p$-nitroanilide under the assay conditions.

**Immunohistochemical Method.** The study material came from 50 patients with colorectal adenocarcinoma. Two tissues were selected from each case: one from the tumor and the other from normal-appearing mucosa far removed from the cancer. Approximately 5-mm-thick sections were cut and subjected to the avidin–biotin complex method as described previously (23) with the anti-MetAP antibody being used as the primary antibody at a concentration of 1–2 $\mu$g/ml. The extent of staining was graded on a five-point scale from 0 to 4 as described previously (29), and the intensity of staining was noted as mild, moderate, or marked.

**Fig. 1 A**, Western blot analysis of extracts from normal tissue (N), polyps (P), and colorectal tumor tissue (C) with anti-MetAP2 antibody. Twenty-five $\mu$g of protein were loaded in each lane of an SDS-polyacrylamide gel and immunoblotted with purified MetAP2 as described in “Materials and Methods.”

**B**, quantitative analysis of the MetAP2 band in normal (□), polyp (■), and colorectal tumor (●) tissue detected by Western blotting was carried out with imaging software from NIH (http://rsb.info.nih.gov/nih-image/download.html). The data presented as the representative mean (SE, bars) of at least three separate experiments. *, significant difference ($P < 0.05$).
**Table 1** Methionine aminopeptidase activity in normal and colorectal cancer tissues from the same patient

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Number of cases</th>
<th>Methionine aminopeptidase activitya</th>
<th>U/mg protein</th>
<th>U/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mucosa</td>
<td>15</td>
<td>41.48 ± 0.029</td>
<td>5.02 ± 0.039</td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>15</td>
<td>61.93 ± 0.094c</td>
<td>11.31 ± 0.543b</td>
<td></td>
</tr>
<tr>
<td>Polyps</td>
<td>3</td>
<td>55.12 ± 0.092</td>
<td>2.96 ± 0.232</td>
<td></td>
</tr>
</tbody>
</table>

a Mean ± SE. Fifty μg of proteins from normal, polyp, and colorectal tumor tissues were analyzed for methionine aminopeptidase activity as described in “Materials and Methods.” The data presented are representative of at least three separate experiments.

b Significant difference from normal mucosa (P < 0.05).

**SDS-PAGE and Western Blot Analysis.** Proteins isolated from human normal and cancerous colon tissues were separated on 10% SDS-polyacrylamide gels according to the procedure described by Laemmli (30). MetAP protein expression in colon cancer tissues was determined by the immunoblotting method of Towbin et al. (31) and was probed with anti-MetAP2 antibody at a concentration of 0.5 μg/ml. The immunoreactive proteins were visualized by chemiluminescence reagent (NEN Life Science Products, USA) and exposure to Kodak autoradiograph film.

**Other Methods.** Protein concentrations were measured by the method of Bradford (32) with BSA as the standard.

**Statistical Analysis.** The data are expressed as the mean ± SE. The significance of the difference between normal human colorectal tissue and cancerous tissue was analyzed with a two-sided Student’s t test. The critical level of significance was set at P < 0.05.

**RESULTS AND DISCUSSION**

We evaluated 50 colorectal cancer patients for the expression of MetAP. To analyze the alteration of MetAP in colorectal mucosa, equivalent amounts of protein from matched pairs of normal human colorectal mucosal tissue and adenocarcinomas from 12 patients were subjected to immunoblotting and probed with anti-MetAP2 (Fig. 1). The results demonstrated that the immunoreactive band of MetAP2 with an apparent molecular mass of 67 kDa was highly expressed in colorectal tumor tissues (Fig. 1A, Lanes C) compared with the mucosal sections taken distant from the tumors (Fig. 1A, Lanes N). Quantitative analysis of the 67-kDa immunoreactive band demonstrated a significant increase in MetAP2 expression in colorectal cancer tissues (Fig. 1B, columns C) compared with the normal tissue (Fig. 1B, columns N). Furthermore, results for MetAP activity in all 15 patients revealed that MetAP activity was higher in adenocarcinomas than in normal human colorectal mucosal tissue (Table 1). MetAP activity was 2-fold higher in almost all patients, and these results are in agreement with the protein expression results obtained by immunoblotting (Fig. 1). Immunohistochemical analysis performed in all cases of colorectal cancer showed weak expression of MetAP2 in normal mucosa (Fig. 2A). The intensity of staining was strong in colorectal tumor tissue (Fig. 2C) and moderate in polyps (Fig. 2B). All samples of colorectal adenocarcinoma stained with anti-MetAP2 demonstrated moderate to strong cytoplasmic positivity with increased intensity in the invasive component (Fig. 2C). The staining appeared to be cytoplasmic rather than nuclear (Fig. 2C).

MetAPs are responsible for removal of the initiator methionine from newly synthesized proteins if the penultimate residue is small and uncharged. MetAP2 is also the molecular target of the angiogenesis inhibitor TNP470 (33). In addition, it has recently been suggested that MetAP2 has an antiapoptotic function in mesothelioma (34). A high level of MetAP2 expression was observed in germinal center B cells of malignant lymphomas of various subtypes (35). MetAP2 plays a critical role in the proliferation of endothelial cells and certain tumor cells; thus it serves as a promising target for antiangiogenesis and anticancer drugs (35). These results demonstrated that MetAP2 may regulate tumor growth also by a direct effect on cancer cell survival. In our study, the moderate staining of MetAP2 in polyps revealed that this gene is up-regulated as a part of molecular events that take place during the malignant formation of colon.

![Fig. 2](Immunohistochemical analysis of normal human colorectal tissue (A), colorectal polyps (B), and colorectal tumor tissue (C). A, section from the normal mucosa far removed from the cancer shows a mild degree of focal staining (see arrows; stained with immunoperoxidase; original magnification, ×120). B, section from polyps shows a weak degree of antibody reactivity (stained with immunoperoxidase; original magnification, ×120). C, section from colorectal adenocarcinoma shows a marked degree of antibody reactivity in the majority of the tumor cells (stained with immunoperoxidase; original magnification, ×120).)
tissues, whereas the activity and protein expression of MetAP2 in polyps was not as high as in cancerous tissues, but was higher than in normal tissues. For the first time we demonstrate the high expression of MetAP2 in colorectal adenocarcinoma patients. Our previous findings demonstrated elevated NMT activity in rat and human colonic tumors (22) as well as elevated NMT activity in human colorectal (23) and gallbladder carcinomas (24). The present results highlight the involvement of MetAP in the elevation of NMT activity in cancerous tissues. It appears that higher expression of MetAP is required for the overexpression of NMT in colon carcinogenesis. The myristoylation process may be controlled by MetAP through the co- and/or post-translational process. Further studies will be required to demonstrate the mechanism(s) of regulation of myristoylation by MetAP.

Catalano et al. (34) demonstrated the high expression of MetAP2 in human mesothelioma tissue, and its expression is associated with antiapoptotic function in those neoplastic cells. These authors showed that inhibition of MetAP2 expression in mesothelioma cells leads to cell death and that such apoptosis is avoided in cases in which there is overexpression of Bcl-2. The up-regulation of Bcl-2 in colorectal cancer has been well established by various investigators (36–38). One of the other mechanisms correlating with MetAP2 and apoptosis is through caspase (34). A recent observation suggested that mesalazine induced apoptosis in colon cancer cells, possibly through activation of caspase-3 (39). Chen et al. (40) reported a reduction in protein levels of caspase-3, -7, and -9 in human colon cancer specimens. Future detailed studies related to MetAP2 and apoptosis could shed light on the involvement of this enzyme in the regulation of various apoptotic factors.

In conclusion, we have demonstrated that there is increased expression of MetAP in human colorectal adenocarcinoma. These findings represent the first description of increased MetAP expression in colorectal tumors. Similarly, the moderate staining of MetAP2 in polyps demonstrated the potential use of MetAP2 as a marker for the early detection of colorectal adenocarcinoma in immunohistochemical analyses. Further studies of this work could be useful to reveal the mechanism of action of MetAP in colon carcinoma.

REFERENCES
