Elevated Levels of 2′,5′-linked Oligoadenylate-dependent Ribonuclease L Occur as an Early Event in Colorectal Tumorigenesis

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ABSTRACT

RNA decay in IFN-treated cells is controlled by 2′,5′-linked oligoadenylate (2-5A)-dependent RNase (RNase L), a uniquely regulated endonuclease that requires short 5′-phosphorylated, 2′-5A for its activity. Because RNase L is also implicated in the regulation of cell proliferation, we monitored its expression in colorectal adenocarcinomas and noncancerous polyps from familial adenomatous polyposis patients. Elevated levels of RNase L mRNA and activity were found in 17 of 20 tumors compared with corresponding normal mucosa. An mAb against RNase L revealed elevated amounts of this RNase in sections of the tumors, largely in the base of the villi. The occurrence of elevated levels of RNase L seems to be an early event in colorectal tumorigenesis, suggesting that control of RNA turnover is an important step in tumor progression. These data also indicate that regulating RNase L activity may be a useful strategy in treating colorectal carcinomas.

INTRODUCTION

Control of gene expression requires an intricate balance between RNA synthesis and decay (reviewed in Ref. 1). The 2′-5A system (2) is an RNA degradation pathway present in higher vertebrates that is implicated in some of the antiviral and antiproliferative activities of IFNs (3–5). IFN treatment of cells activates genes encoding several synthetases that produce short 5′-triphosphorylated, 2′:5′-linked oligoadenylates known as 2′-5A from ATP in response to double-stranded RNA (6). Because double-stranded RNA is frequently produced during viral infection, 2′-5A accumulation is observed in some IFN-treated and virus-infected cells (7). Effects of 2′-5A in cells are transient due to the combined activity of 2′,5′-phosphodiesterase and 5′-phosphatase activities that degrade 2′-5A (8). 2′-5A functions by activating the IFN-inducible endonuclease, RNase L (also known as 2′-5A-dependent RNase) (9–11), resulting in the cleavage of single-stranded RNA predominantly after UpUp and UpAp sequences (12, 13). Although cellular levels of RNase L increase during IFN treatment, basal levels of the enzyme are found in most mammalian cells. Human RNase L is an Mr 83,539 protein of 741 amino acid residues that is converted to its catalytically active, homodimeric form by binding to 2′-5A (11, 14, 15, and references therein). The structure of RNase L includes nine ankyrin protein-protein interaction domains also found in all members of the IkB family (inhibitors of transcription factor NF-κB), and in some cell cycle control proteins (5 and references therein). The gene for RNase L maps to chromosome 1q25, a locus that is deleted or rearranged in many breast cancer cell lines, as well as in some gastric adenocarcinomas and oral squamous cell carcinomas (16). Aberrant regulation of RNA stability in tumor cells is suggested by some studies on the 2′-5A system. Elevated endonuclease activity against tRNA has been reported in mononuclear cells from patients with chronic myelogenous leukemia (17). Previously we reported that the antiproliferative activity of IFN (α plus β) was suppressed in murine cells expressing a dominant-negative form of RNase L, suggesting a possible role for the 2′-5A system in cell growth control (5).

Cancer statistics indicate that cases of colorectal cancer in men and women are increasing steadily in the United States as well as worldwide, at least in part because of improvements in diagnosis (18). Cancer mortality due to colorectal cancer ranks second among men and third among women (18). Although the precise etiology of this disease is still unknown, exposure to environmental factors, in particular mutagens and carcinogens in the diet, are contributors. In addition, patterns of genetic changes associated with human colorectal cancer are emerging (19–22). Here we report high amounts of RNase L in a range of colorectal tumor types and in polyps. Results suggest that control of RNA turnover by RNase L is an early event in tumorigenesis. Furthermore, based on these findings, we suggest a therapeutic approach designed to activate RNase L preferentially in tumors as compared with normal intestinal mucosa.

MATERIALS AND METHODS

Isolation of Tumors and Normal Mucosa and Preparation of Cell Extracts. Adenocarcinomas and benign polyps were surgically removed and immediately frozen and stored in liquid nitrogen. The normal colorectal mucosa of the same patients, distal to the tumors and free of metastatic lymph nodes,
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Table 1  RNase L mRNA and activity in colorectal tumors

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>Tumor site</th>
<th>Distal metastasis</th>
<th>Increased levels RNase mRNA</th>
<th>Fold increase in levels of RNase</th>
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<tr>
<td>adc</td>
<td>Sigmoid</td>
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<td>1</td>
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<td>Distal and transverse</td>
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<td>−</td>
<td>1</td>
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<tr>
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<td>Rectum</td>
<td>None</td>
<td>−</td>
<td>1</td>
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<td>Liver, lymph nodes</td>
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<tr>
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<td>Sigmoid</td>
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<td>+</td>
<td>2</td>
</tr>
<tr>
<td>adc</td>
<td>Proximal</td>
<td>Liver, ovaries, lymph nodes</td>
<td>+</td>
<td>2.5</td>
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<tr>
<td>adc</td>
<td>Transverse</td>
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<td>+</td>
<td>2.5</td>
</tr>
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<td>2.5</td>
</tr>
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<td>Liver, lymph nodes</td>
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<td>4</td>
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<tr>
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<td>++</td>
<td>4</td>
</tr>
<tr>
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<td>Distal</td>
<td>Lymph nodes</td>
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<td>4</td>
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<td>+</td>
<td>4.5</td>
</tr>
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<td>FAP P</td>
<td>Desmoid</td>
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<td>+</td>
<td>6+d</td>
</tr>
<tr>
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<td>Liver, ovaries</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>adc</td>
<td>Rectosigmoid</td>
<td>None</td>
<td>++</td>
<td>6</td>
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<td>Sigmoid</td>
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a Approximate relative levels of mRNA encoding RNase L were determined by RT-PCR. −, no change; +, 1.5- to 3-fold; ++, 3- to 5-fold; ++ +, >5-fold increase of the cellular RNase mRNA compared with normal mucosa.

b Numbers indicate the fold increase of levels of 2-5A binding to RNase L in extracts of tumors compared with extracts of corresponding normal mucosa as determined by the 2—5A binding assay (see "Materials and Methods").

c adc, adenoacinaroma; FAP P, FAP polyps.

d Compared with the average level of normal mucosa.

was also collected and stored. The upper central parts of the tumors were used to ensure that the majority of cell populations were cancer cells. None of the patients were treated with IFN or other cytokines, radiation therapy, or chemotherapy before surgery. The frozen tumor or mucosa was grounded to a fine powder in liquid nitrogen, suspended in NP40 lysis buffer supplemented with the protease inhibitor leupeptin (23), and disrupted by vortex mixing. The total cell lysates were centrifuged at 10,000 × g for 10 min at 4°C; the supernatant was collected; and the protein concentration was determined according to the Bradford method (Bio-Rad).

Assay for 2–5A Binding Activity.  Covalent binding of a radiolabeled and bromine substitute 2–5A probe, p(A2'p)2A3'–[32P]Cp, to RNase L was performed as described previously (24). Briefly, a cell extract containing 200 µg protein from either tumor, polyp, or normal mucosa was incubated on ice with the 2-5A probe (50,000 cpm) for 1 h. Covalent cross-linking was achieved under a UV lamp (308 nm) on ice for 1 h; then the proteins were separated on SDS-polyacrylamide (10%) gels. The gels were dried and exposed to X-ray film overnight. Quantitation was performed with a PhosphorImager (Molecular Dynamics). The denaturation-renaturation protocol was performed by a modification (11) of the method of Singh et al. (25). Briefly, after electrophoresis of 300 µg cell protein/lane on SDS-polyacrylamide (10%) gels, the proteins were transferred to nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked with 5% nonfat milk in renaturation buffer [20 mM Tris-HCl (pH 7.5), 0.2 mM magnesium acetate, 80 mM β-mercaptoethanol, 90 mM KCl, and 10 µg/ml leupeptin] containing 0.2 mM poly(U)-[32P]Cp prepared as described (26) and incubated at 30°C for 1 h. Reactions were terminated with 15 µl stop buffer, boiled 3 min, and centrifuged briefly, and 4 µl were loaded to 6% polyacrylamide/urea gels. After electrophoresis, X-ray film was exposed to the gels.

Analysis of RNase L on Western Blots Probed with a mAb.  Extracts of tumors, polyps, and normal mucosa, 100 µg protein each, were subjected to electrophoresis in SDS-polyacrylamide gels, and the proteins were then transferred to nitrocellulose filters. Blots were probed with a 1:5000 dilution of an ascites fluid containing mAb to recombinant human RNase L (15). Detection was with goat antimouse IgG-peroxidase (GIBCO) and enhanced chemiluminescence (Amersham).
**Detection of RNase L mRNA.** Total RNA was prepared from tumor or normal tissue as described (20). Total RNA, 0.1 μg, was used for cDNA synthesis by RT as described by the supplier (GIBCO-Bethesda Research Laboratories). One-tenth of the amount of the first strand of cDNA synthesized was used for 28 rounds of PCR amplifying either RNase L or β-actin mRNA sequences. The PCR cycles included 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1.5 min extension at 72°C, using a GeneAmp PCR Reagent Kit (Perkin Elmer/Cetus). A pair of primers: sense, GCCTTCATGGAAGCCGC, and antisense, CTTGAGTTTGGCAATCATAG, based on the human RNase L cDNA sequence (11), was used to amplify mRNA sequences from nucleotides 382 to 1086, resulting in 705-bp PCR products. Another pair of primers: sense, TCTGGCACAACCTTCTAC, and antisense, AAGGCTGGAAGAGTGCTCA (covering exons 2 and 3 of the β-actin gene) was used to amplify a partial β-actin mRNA sequence (27), resulting in 530-bp products. PCR products were separated on 1.2% agarose gels and analyzed. The RNase L PCR fragments were transferred to a Nytran membrane (Schleicher & Schuell), and hybridized with 10^6 cpm/ml 32P-labeled RNase L cDNA (nucleotides 63–637) labeled by random priming as described (28). Amounts of cDNA were measured with a PhosphorImager. As a control for DNA contamination, no amplified product was detected in the absence of RT in PCR reactions with total cellular RNA.

**Immunoperoxidase Staining.** A total of four frozen samples [FAP (sample 51), tumor (sample 52), and corresponding normal tissues] were cut into 5-μm cryostat sections and...
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RESULTS

RNase L Levels in Human Colorectal Tumors and Polyps. The collection of colorectal tumor samples used in this study included 18 adenocarcinomas isolated from different sites, 8 of which were from patients with distal metastasis (Table 1). In addition, polyps from two patients with FAP were analyzed. The tissues represented a range of different stages and types of colorectal tumors. For comparison, normal colorectal mucosa, distal to the tumors, from the same patients was collected.

To measure RNase L levels in the tumors, we performed covalent cross-linking of a bromine-substituted and 32P-labeled 2-5A probe to the RNase under UV light (24). Based on this highly specific assay, 17 of 20 tumors and polyps showed elevated levels of RNase L compared with normal mucosa removed from the same patients (Fig. 1 and Table 1). Nine tumors contained 4- to 10-fold elevated amounts, and six showed 1.5- to 2.5-fold increased amounts, whereas two benign polyps from FAP patients showed 6-fold elevated amounts of RNase L. An autoradiogram showing the labeling of RNase L by the 2-5A probe from 10 sets of tumor and normal samples are shown in Fig. 1. There was no apparent correlation between the site of the tumor and the levels of RNase L. Indeed, even the benign polyps contained enhanced amounts of the protein. Therefore, the increase in levels of RNase L is a very early event in tumorigenesis.

To determine whether endogenous 2-5A was present in sufficient amounts to reduce binding of the 2-5A probe to the RNase L, denaturing gel electrophoresis was performed, followed by a Southwestern blotting procedure (Fig. 2; Ref. 11). This method, which removes 2-5A that may be prebound to RNase L, also showed that the relative levels of RNase L were significantly higher in the tumor and polyp extracts than in the corresponding normal mucosa (Fig. 2). Therefore, the elevated amounts of 2-5A binding activity in the tumor and polyp extracts were due to increased levels of RNase L itself.

![Fig. 3](image-url) RNase L amounts as determined in a Western blot probed with a mAb. Lanes 1-10, extracts (50 µg protein/lane) of a polyp (sample 51), tumors (samples 41, 52, 40, and 53; both labeled T), and normal mucosa (N), and purified recombinant human RNase L (Lanes 11-14, 10, 3, 1, and 0.3 ng, respectively) were subjected to SDS-PAGE, Western blotting, and probing with a murine mAb against human RNase L (15).
RNase activities of RNase L isolated from tumors and normal mucosa. The RNase L, isolated on an activating affinity matrix, 2–5A-cellulose, was used to cleave radiolabeled poly(U) (see "Materials and Methods"). Lane 1, the input poly(U) alone; Lanes 2–7, tumor and normal mucosa samples as indicated; Lane 8, recombinant human RNase L. An autoradiogram of a sequencing gel is shown.

**Fig. 4** RNase activities of RNase L isolated from tumors and normal mucosa. The RNase L, isolated on an activating affinity matrix, 2–5A-cellulose, was used to cleave radiolabeled poly(U) (see "Materials and Methods"). Lane 1, the input poly(U) alone; Lanes 2–7, tumor and normal mucosa samples as indicated; Lane 8, recombinant human RNase L. An autoradiogram of a sequencing gel is shown.

**Fig. 5** Relative levels of RNase L mRNA and β-actin mRNA from colorectal tumors (T) and corresponding normal mucosa (N) as determined by RT-PCR. Arrows, RT-PCR products of RNase L mRNA (705 bp) and β-actin mRNA (530 bp).

Amounts of RNase L Were Elevated in the Tumors as Determined with a mAb. Because activity and absolute amounts of enzymes are not always directly proportional, for instance, as a result of posttranslational controls, we also measured RNase L amounts on Western blots with a mAb (15). Levels of RNase L as measured with the antibody were significantly elevated in four of five sets of tumors compared with matched sets of normal tissue (Fig. 3). In general, these results were consistent with the 2–5A binding data, although in tumor 53, the amount of RNase in the tumor appeared to be greater as determined by the 2–5A binding assays than in the immunoblot (compare Figs. 1 and 3). The purified, recombinant RNase L was included in the same Western blot for comparison (Fig. 3, Lanes 11–14). These findings show that the amounts of RNase L protein were elevated in the tumors.

The RNase L in the Tumor Extracts Was Catalytically Active in the Presence of 2–5A. Because the 2–5A binding assay may not always discriminate between wild-type and some mutant forms of RNase L (5), RNase assays were performed with RNase L, which was isolated from the tumor and normal mucosa extracts on the activating affinity matrix, 2–5A-cellulose (26). Previously, we showed that poly(U)-3′[32P]pppCp was degraded to discrete oligomers of uridylylate by the human recombinant RNase L (Fig. 4, lane 8; Ref. 14). A similar result was seen with RNase L isolated from three separate tumors and activated with 2–5A-cellulose (Fig. 4). There was significantly less RNase L activity from the corresponding normal tissues. Therefore, both the 2–5A binding and the RNase activities of the RNase L were elevated in the tumors.

**RNase L mRNA Levels Are Elevated in the Tumors.** To determine whether the increased amounts of RNase L were due to an accumulation of its mRNA, RT-coupled PCRs were performed (Table 1 and Fig. 5). For comparison, we also amplified cDNA from β-actin mRNA. Enhanced amounts of RNase L mRNA were observed in the same tumors that showed elevated amounts of RNase L activity (Table 1 and Fig. 5). Therefore, we conclude that in these tumors either increased transcription of the RNase L gene or enhanced stability of the RNase L mRNA were responsible for the increased amounts of RNase L in the tumors. Preliminary analysis of the RNase L gene by Southern blotting did not show any measurable alterations, suggesting that rearrangement of the gene was probably not involved in the overexpression observed in the tumors (data not shown).

**Immunolocalization of RNase L in Colorectal Tumors and Normal Mucosa.** Samples of polyp, tumor, and normal mucosa were sectioned and subsequently probed with the mAb (Fig. 6). Adjacent normal tissue had minimal staining confined to the cytoplasm, mostly in the areas of the base of the crypts of several villi (Fig. 6, A and C). This result seems to be consistent with the basal levels of endogenous RNase L activity in normal tissues. The polyp and tumor specimens (Fig. 6, B and D, respectively) revealed significantly more pronounced staining in the base of the villi as well as other glands in the section, yet inconsistent staining was apparent in the tumor cells themselves. The tumor stained was histopathologically identified to be a well-differentiated neoplasm, and the polyp was a FAP.

As a control for specificity, the staining was prevented by preincubating the antibody with an extract of insect cells expressing recombinant human RNase L (Ref. 14; Fig. 6F). In contrast, an extract of control insect cells lacking recombinant...
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RNase L did not inhibit reactivity with the antibody (Fig. 6, compare E and G). Addition of normal horse serum in place of the mAb (negative control) showed no evidence of staining (Fig. 6H).

DISCUSSION

Here, we have measured elevated amounts of RNase L mRNA and protein and activity in colorectal tumors developed in various regions of the colon with and without metastasis at distal organs. Tumors were from both men and women between the ages of 35 and 80 years. There was no clear correlation between the amounts of RNase L and tumor site. Because high amounts of RNase L were also observed in two benign polyps, the elevation in RNase L levels may be important in tumorigenesis and may be a relatively early event. The implication is that there may be a requirement for enhanced RNA turnover in the rapidly growing cells that develop into cancer cells. The role of the 2-5A-system in controlling RNA turnover in colorectal tumors is unknown. Perhaps RNA turnover rates are limiting for cell growth in normal colonic epithelial cells. For instance, enhanced breakdown of RNA could lead to a rapid replenishment in pools of nucleotides for the synthesis of mRNA. Alternatively, perhaps the RNase L is responsible for selectively enhancing the turnover of mRNA encoding tumor suppressors and other growth-restricting proteins.

Regardless of the physiological significance, the high level of RNase L led us to suggest a novel therapeutic approach for polyps and colorectal tumors. Stable 2-5A analogues with the ability to be both taken up by cells and to activate RNase L could cause the regression of polyps and tumors, which contain high levels of the enzyme. In contrast, normal colonic mucosa, which contain only low levels of RNase L, may be spared or only transiently affected. For example, it was shown that facilitated uptake of 2-5A by human cells has a potent antigrowth affect (29). Anticellular properties of 2-5A are presumably due to the induction of general, rapid RNA decay, including that of rRNA. Alternatively, 2-5A-antisense chimeras could be used, which selectively direct RNase L to mRNA-encoding oncogenic proteins or growth factors, which promote the growth of colorectal carcinomas (30). Previously, we have shown that the 2-5A-antisense strategy selectively ablates target RNA in intact cells (31). Because polyps and colorectal tumor cells have high levels of RNase L, these cells may be especially sensitive to this approach.

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REFERENCES

Elevated levels of 2',5'-linked oligoadenylate-dependent ribonuclease L occur as an early event in colorectal tumorigenesis.
