Decreased expression of Mac-2 (carbohydrate binding protein 35) and loss of its nuclear localization are associated with the neoplastic progression of colon carcinoma

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ABSTRACT The Mac-2 lectin (carbohydrate binding protein 35) is a soluble, 32- to 35-kDa phosphoprotein that binds galactose-containing glycoconjugates. We report here that the colon epithelium is a major site of Mac-2 expression in vivo based on immunohistochemistry of human tissue specimens. In this epithelium, proliferating cells at the base of the crypts do not express Mac-2 but its expression increases with differentiation along the crypt-to-surface axis. Mac-2 expression is concentrated in the nuclei of these differentiated epithelial cells. The progression from normal mucosa to adenoma to carcinoma is associated with significant changes in Mac-2 nuclear localization and expression. In all adenomas (9/9) and carcinomas (13/13) examined, Mac-2 was not present in the nucleus but was localized in the cytoplasm. Sequencing of Mac-2 cDNAs from normal mucosa and carcinoma revealed no specific mutations that could account for this loss of nuclear localization. We also observed a 5- to 10-fold decrease in Mac-2 mRNA levels in cancer compared to normal mucosa as well as a significant reduction in the amount of Mac-2 protein expressed. These observations suggest that Mac-2 exclusion from the nucleus and its decreased expression may be related to the neoplastic progression of colon cancer.

The Mac-2 lectin is a soluble, 32- to 35-kDa phosphoprotein that binds galactose-containing glycoconjugates (1–5). Molecular cloning studies revealed that Mac-2 is identical to carbohydrate binding protein 35 (CBP 35), a lectin characterized initially in 3T3 fibroblasts (3, 4, 6). It is also identical to IgE binding protein (7) and a 32-kDa tumor-associated lectin (8, 9). The COOH-terminal domain of Mac-2, which contains a conserved carbohydrate binding motif, is homologous to a related family of 14-kDa galactose-specific lectins (reviewed in ref. 5). Collectively, Mac-2 and the 14-kDa lectins are referred to as S-lectins because their carbohydrate binding function was thought to be thiol-dependent (10), an assumption that has recently been questioned (2).

The biological functions of Mac-2 remain elusive. Its putative role in cell adhesion has not been substantiated (11). Mac-2 may be associated with cell growth and differentiation because it is found in the nuclei of 3T3 fibroblasts and its nuclear localization may be related to the proliferative state of these cells (5). Also, the expression of Mac-2 occurs as a function of macrophage differentiation (12). These reports are consistent with the recent finding that a homologue of the 14-kDa S-lectin is cytostatic for embryonic fibroblasts (13). Other in vitro studies have suggested that the expression of Mac-2 increases in oncogenically transformed and metastatic cells (14–16).

Progress in elucidating the function of Mac-2 has been hampered, in part, by a lack of data on its expression in vivo.

In the present study, we found that Mac-2 is largely an epithelial-specific lectin with high expression seen in the colonic epithelium. The progression from normal colonic mucosa to adenoma to carcinoma is characterized by a marked reduction in Mac-2 mRNA and protein levels and a loss of its nuclear localization. These observations suggest that Mac-2 exclusion from the nucleus and its decreased expression may be related to the neoplastic progression of colon cancer.

MATERIALS AND METHODS

Tissue Specimens. Specimens of normal colonic mucosa, colonic polyps, and colonic adenocarcinomas were obtained from patients at the time of surgery. Pathological examination was performed on all specimens to assess the presence of preneoplastic and neoplastic disease. Tissue procurement was in accordance with institutional review board standards and procedure.

Immunohistochemistry. Specimens of colonic tissue were formalin-fixed and paraffin embedded. Sections (3 μm) were incubated with the Mac-2-specific monoclonal antibody (mAb) M3/38 (12) at a concentration of 7 μg/ml. Bound antibody was detected by the peroxidase-antiperoxidase technique. The sections were counterstained with hematoxylin.

RNA Blots. RNA was extracted from normal mucosa and tumors using guanidium isothiocyanate. The RNA was transferred onto nylon membranes (Biotrans; ICN) and hybridized with a cDNA probe for human Mac-2 that was obtained by RNA PCR as described below. A cDNA probe for 18S rRNA provided by Dylan Edwards (University of Calgary) was also used.

Protein Blots. Frozen specimens of normal mucosa and tumor were resuspended by probe sonication in Laemmli sample buffer. Samples (25 μg of protein) were resolved by SDS/PAGE (12%) and then transferred to nitrocellulose. The immunoreaction was performed by incubating the nitrocellulose with M3/38 (5 μg/ml) in Tris-buffered saline with 0.05% Tween 20. Bound antibody was detected using a peroxidase-conjugated goat anti-rat IgG.

RNA PCR Cloning. RNA PCR (Perkin-Elmer/Cetus) was performed according to the manufacturer’s instructions. Oligonucleotide primers were chosen that flanked the 5’ and 3’ ends of the Mac-2 coding sequence (3, 17). The primers used were GGAGCCAGCCAAGGCGGAAAATG (upstream primer) and GCCCTTTCAGATTATATCATGTG (downstream primer). First-strand cDNA synthesis was generated by incubating total RNA (2 μg) with the downstream primer and reverse transcriptase at 42°C for 15 min and 99°C for 5

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Abbreviations: mAb, monoclonal antibody; CBP 35, carbohydrate binding protein 35.

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min and then cooling to 5°C. PCR was performed as follows: The mixture was heated to 95°C for 3 min and then cooled to 80°C. At this point, Vent polymerase (New England Biolabs) was added and 30 cycles were completed under the following conditions: 1 min at 60°C, 1 min at 75°C, and 1 min at 95°C. Finally, the mixture was incubated at 60°C for 7 min and cooled to 4°C.

One microliter of the PCR product was then used for ligation in the TA cloning system (Invitrogen, San Diego), and subcloning was performed as recommended by the manufacturer. Positive colonies were analyzed by dyeoxy DNA sequencing (Sequenase; United States Biochemical) of their inserts.

RESULTS

Immunohistochemical Analysis of Mac-2 Expression. The expression of Mac-2 in specimens of human colonic tissue was examined by immunohistochemistry using the M3/38 mAb. The degree of immunostaining was graded according to the percentage of reactive cells as follows: <1% = 0, 1–25% = 1+, 26–75% = 2+, >75% = 3+. In the 15 sections of normal colonic mucosa that were examined, Mac-2 staining was confined to epithelial cells (Fig. 1A). There was no evidence of stromal cell staining with the exception of scattered macrophages in the lamina propria. The distribution of reactivity was distinct because a gradient of staining was noted along the crypt-to-surface axis. All specimens demonstrated 3+ reactivity in the upper third of the crypt and surface cells. In most specimens, cells at the base of the crypt were nonreactive. This pattern of Mac-2 staining indicated that the proliferating cells at the base of the crypts expressed relatively little Mac-2 in comparison to the more differentiated cells along the crypt-to-surface axis.

A striking feature of the Mac-2 immunostaining of normal colon was that it was largely nuclear (Fig. 1 A–C). Intense nuclear staining was evident in all specimens of normal mucosa examined (Fig. 1B and C). This nuclear staining was particularly apparent in the middle third of the crypts (Fig. 1A and B). In surface epithelial cells in the upper third of the crypts, basal and focally basolateral cytoplasmic staining was seen along with intense nuclear staining (Fig. 1B and C).

The distinct localization of Mac-2 staining in normal colonic mucosa prompted us to examine its localization in colon carcinoma. The Mac-2 immunostaining pattern of 13 primary colonic adenocarcinomas was markedly distinct from that of normal mucosa. The most salient feature of this staining pattern was a loss of Mac-2 nuclear localization (Fig. 1D). Reactivity of Mac-2 in carcinomas was predominantly cytoplasmic with only rare isolated staining of nuclei seen. In addition to this shift in Mac-2 localization, we also noted a significant decrease in the intensity of Mac-2 staining compared to normal mucosa (compare Fig. 1 A–C with D). The Mac-2 staining of adenocarcinomas was heterogeneous because not all areas of tumor exhibited staining (Fig. 1D). Specifically, only 54% of the carcinoma specimens exhibited 3+ staining.

Fig. 1. Immunohistochemical analysis of Mac-2 expression in normal colonic mucosa and colon carcinoma. Paraffin-embedded sections (3 μm) of colonic tissue were immunostained with M3/38, a Mac-2 specific mAb (12). Bound antibody was detected by the peroxidase-antiperoxidase technique. Sections were counterstained with hematoxylin. (A) Normal colonic mucosa. Arrows indicate the base of colonic crypts. (×25.) (B) Normal colonic mucosa. Arrow indicates the direction of the crypt-to-surface axis. (×65.) (C) Normal colonic mucosa. (×260.) (D) Colon adenocarcinoma. (×65.)
The above results prompted us to study possible changes in Mac-2 staining that may occur during the progression from normal mucosa to carcinoma. It is widely accepted that most colon carcinomas arise from adenomatous polyps (18). In nine adenomas, Mac-2 staining was considerably less intense than that seen in normal mucosa. However, the percentage of reactive cells was high, with 78% of the adenomas exhibiting 3+ staining. These differences are evident in Fig. 2A in which a region of normal mucosa is adjacent to adenomatous tissue. The immunostaining of adenomas was much more uniform than that of carcinomas because most of the adenomatous cells stained with Mac-2. However, the absence of nuclear staining in carcinomas was also apparent in adenomas. In the high-power photomicrograph shown in Fig. 2B, none of the nuclei exhibited Mac-2 staining. The Mac-2 staining seen is exclusively cytoplasmic. Thus, the neoplastic progression from normal mucosa to adenoma to carcinoma is associated with a loss of Mac-2 nuclear localization. One test of this correlation would be to examine Mac-2 staining in colonic polyps that are not neoplastic. Hyperplastic polyps are small colonic polyps that do not normally progress to cancer (18). The Mac-2 staining pattern of these polyps was strikingly different than that of adenomatous polyps (Fig. 2C). Examination of eight hyperplastic polyps revealed intense nuclear staining in all of the specimens with focal areas of basolateral cytoplasmic staining as seen in normal mucosa.

Expression of Mac-2 Protein and mRNA. The immunostaining results indicated that the progression from normal colonic mucosa to adenocarcinoma was associated with a decrease in Mac-2 expression. Additional evidence to support this possibility was obtained by examining the relative amounts of Mac-2 protein in normal mucosa and tumor. Mac-2 expression was detected by immunoblotting tissue extracts of colon adenocarcinoma and distal normal colonic mucosa obtained from the same patient. As shown in Fig. 3, only a 35-kDa protein, which corresponds to the molecular mass of Mac-2, was detected on the immunoblots using the M3/38 mAb. This observation confirms that this mAb reacted only with Mac-2 in the immunostaining studies described above. Analysis of the immunoblot shown in Fig. 3 indicates a significant decrease in the intensity of the 35-kDa band in cancer relative to normal mucosa in tissue pairs obtained from six different patients. These data corroborate the decrease in the intensity of Mac-2 immunostaining of cancer specimens compared to normal mucosa that was apparent in Fig. 1.

The possibility that the observed decrease in Mac-2 expression resulted from a decrease in the expression of its mRNA was examined by Northern blotting. RNA was extracted from pairs of normal mucosa and cancer obtained from five different patients and hybridized with a Mac-2 cDNA probe obtained from normal colonic mucosa (Fig. 4). A single 1.3-kb transcript was observed in each sample. The intensity of this 1.3-kb band was significantly reduced in the cancer specimens compared to normal mucosa. Densitometric scanning of these blots indicated a 5- to 10-fold decrease in the level of Mac-2 in cancer relative to the amount of 18S RNA in each sample.

cDNA Cloning and Sequencing of Mac-2. We thought it important to clone and sequence Mac-2 from normal colonic mucosa and tumor for several reasons. Foremost, the difference in nuclear and cytoplasmic localization could result from mutations in the Mac-2 coding sequence. Such muta-

![Fig. 2. Immunohistochemical analysis of Mac-2 expression in colonic polyps.](image)

![Fig. 3. Immunoblot analysis of Mac-2 expression in normal colonic mucosa (N) and colon tumors (T).](image)
Fig. 4. Northern blot analysis of Mac-2 mRNA expression in normal colonic mucosa (N) and colon tumors (T). (A) RNA (20 μg) extracted from normal mucosa and tumor obtained from five different patients was resolved by agarose electrophoresis and hybridized with a 32P-labeled Mac-2 cDNA probe. A 1.3-kb RNA transcript was detected using this probe. (B) The relative amount of RNA in each sample was assessed by hybridization with a cDNA probe specific for 18S rRNA (1.8 kb).

sequences have been shown to affect the nuclear localization of other proteins (19). In addition, the four published sequences of human Mac-2 are all derived from tumor cell cDNA libraries (3, 9, 17, 20). No sequence has been reported from a nontumorigenic cell line or normal tissue.

We generated Mac-2 cDNA by RNA PCR of mRNA obtained from normal colonic mucosa, adenocarcinoma, and clone A, a colon carcinoma cell line (21). The nucleotide sequences of all three cDNAs were identical (Fig. 5). However, we did observe allelic variation at codons 191 and 292 upon sequencing several clones of each cDNA (Fig. 5). These sequence variations cannot be considered tumor specific because they were evident in the normal and tumor sequences. Our sequence of human Mac-2 is identical to those published by Robertson et al. (20) and Oda et al. (17) with the exception of the allelic variations that we noted.

DISCUSSION

The data presented here provide new insight into the behavior and possible biological function of Mac-2. We found that the colonic epithelium is a major site of Mac-2 expression in vivo. Expression in the colonic epithelium, as well as other epithelia (unpublished data), is associated with terminally differentiated cells and not with proliferating crypt cells, an observation that had been foreshadowed in an earlier study (22). Perhaps the most interesting observation we make is that Mac-2 expression is concentrated in the nuclei of differentiated, colonic epithelial cells. Nuclear localization of Mac-2 (CBP 35) in cultured 3T3 fibroblasts has been established (23, 24). This report extends these observations by documenting that Mac-2 expression occurs in normal colon epithelial cells. A key difference, however, between our data and previous in vitro studies is that the nuclear localization of Mac-2 (CBP 35) in 3T3 cells is associated with proliferating cells (25, 26). The in vivo data reported here indicate that the nuclear localization of Mac-2 is associated with cells in the upper two-thirds of colonic crypts. These cells, for the most part, are not dividing, as evidenced by the fact that they do not incorporate [3H]thymidine, and they are assumed to be differentiated (27). These observations imply that Mac-2 nuclear localization correlates with either cessation of cell division or differentiation in the intestinal epithelium.
normal colon, from colon carcinoma, and from a colon carcinoma cell line. No mutations were found that would distinguish normal mucosa from neoplastic tissue. One conclusion from these results is that Mac-2 may belong to a
category of nuclear proteins that depend on other proteins for their transport into the nucleus (19). This possibility is also evidenced by the fact that Mac-2 does not contain a well-defined nuclear localization sequence similar to T antigen (Fig. 5), though it is possible that its nuclear localization involves a much larger protein sequence (31). These observations on Mac-2 are similar to recent findings on the nuclear localization of the p53 tumor suppressor protein in breast carcinomas (32). A significant fraction of breast cancers examined (37%) excluded p53 from the nucleus but most of these cancers contained wild-type p53 alleles. These data provide a compelling reason for identifying proteins with which Mac-2 associates and for elucidating its mode of nucleo-transport.

The decreased expression of Mac-2 in colon carcinomas was indicated initially by the immunostaining data and corroborated by analysis of its RNA and protein levels. The down-regulation of gene expression in cancer can result from deletion, mutation, or rearrangement of genomic DNA or from aberrations in either transcriptional or translational regulation (33). In a Southern analysis of three paired specimens of normal colonic mucosa and carcinoma, we found no evidence for gross deletion or rearrangement of the Mac-2 gene (unpublished data). This preliminary result, along with the observation that Mac-2 mRNA levels are reduced in tumors, suggests that decreased Mac-2 expression is caused by altered expression of an intact Mac-2 gene. Additional studies are required to verify this possibility and to define the nature of such control mechanisms.

The results presented in this paper justify further work to investigate the possibility that Mac-2 is a tumor suppressor protein. At present, this justification is based on its decreased expression and loss of nuclear localization in tumors. The key test of this possibility will be to determine if increasing the levels of nuclear Mac-2 in colon carcinoma cells alters their transformed phenotype. Experiments of this type have been done with known tumor suppressor proteins including p53 and RB (34–38). In both cases, expression of the wild-type protein inhibited tumor cell growth in vitro. Several lines of evidence suggest that Mac-2 expression also may affect cell growth or differentiation. Its expression in vivo is associated with terminally differentiated epithelial cells. In addition, its nuclear localization and state of phosphorylation have been correlated with the proliferative state of 3T3 fibroblasts (24–26). Lastly, a 14-kDa galactose-specific lectin that is homologous to the COOH-terminal domain of Mac-2 inhibits the growth of embryonic fibroblasts (13). Collectively, the available data provide reason for investigating the role of Mac-2 in the growth and differentiation of normal and malignant colonic epithelial cells.

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