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Colon Adenoma and Cancer Cells Aberrantly Express the Leukocyte-Associated Sialoglycoprotein CD43

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CD43 (leukosialin) has hitherto been considered as an exclusive leukocyte marker, but now we report the expression of CD43 in the epithelial cells of all studied colorectal adenomas (21/21) and in about 50% (18/34) of adenocarcinomas as analyzed both at the mRNA and protein levels. Direct evidence showing the causal role of CD43 in colon tumorigenesis is lacking, but its involvement in leukocyte activation and impairment of apoptotic response suggests a role for CD43 in colon cancer development.

CD43 (also known as leukosialin, sialophorin, the major sialoglycoprotein of leukocytes) is a highly sialylated glycoprotein that is expressed at high density on almost all mature leukocytes, except for a population of B- and dendritic cells (1,2). This molecule has a mucin-type extracellular domain consisting of 235 amino acids that is proposed to be involved in regulating and lowering cell-cell/cell-ligand interactions due to the repulsive effect of its high numbers of *O*-linked negatively charged sialic acid residues (3-5). The intracellular domain (123 amino acids) is highly conserved between species. It is proposed to be involved in signal transduction pathways as it has serine/threonine phosphorylation sites that are phosphorylated in resting T cells and hyperphosphorylated upon T cell activation (6). Moreover, it has been shown that CD43 causes tyrosine phosphorylation of certain other proteins (7). Transgenic CD43 knockout mice are viable, but the CD43-deficient T cells show increased *in vitro* proliferative response to several stimuli, a substantial enhancement in homotypic adhesion and ability to bind different ligands (8). It has also been shown that crosslinking CD43 by MAb MEM-59 induces apoptosis in hematopoetic progenitor cells (9) and MAb J393 induces apoptosis in the T-lymphoblastoid cell line Jurkat (10). On the other hand, MAb MEM-59 induces proliferation of T cells by a mechanism analogous to a classic ligand-receptor interaction (11). These results demonstrate that CD43-mediated responses can differ significantly depending on the cell type.

Only a few reports have proposed the expression of CD43 outside the hematopoetic cell lineage. We have unambiguously demonstrated the expression of CD43 in the human colon carcinoma cell line COLO 205 at the protein and mRNA levels (12). Recently, Santamaria *et al.* reported staining of tumor cells in different nonhematopoetic cancers by CD43-specific MAbs (13), but they found only a few tumors that stained with the two best antibodies selected out of a panel of anti-CD43 MAbs. No colon tumors were included in their study. This indicates that currently available CD43-specific MAbs might not be efficient enough to detect CD43 in nonhematopoetic cells.

In the present study the CD43 specific MAb L10 and DIG[1]-labeled antisense RNA-probes were used for detecting the CD43 protein and mRNA in paraffin and cryostat sections of colorectal adenomas and adenocarcinomas. As we found CD43 to be expressed in all cases of colon adenomas and in about 50% of adenocarcinomas, this may suggest an important role for CD43 in colon tumorigenesis.

METHODS

Antibodies. The anti-CD43 MAbs BS-1 and L10 were obtained from Dr. E. Remold-O'Donnell (14), The Center for Blood Research, Boston, MA. and from Drs. Barbara Schwartz and John Harlan (15), Univ. of Washington, Seattle, WA. MAb L60 was purchased from Becton Dickinson (San Jose, CA, USA).

Electrophoresis and Western blotting. Lysates of COLO 205 (ATCC CCL-222) and K562 (ATCC CCL-243) cells were separated on a 8-15% gradient polyacrylamide gel. After electrophoresis, proteins were electrophoretically transferred onto nitrocellulose membrane and strips were probed with anti-CD43 MAbs (5 μg/ml). Bound anti-

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Abbreviations: MAb, monoclonal antibody; DIG, digoxigenin.

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TABLE 1

Detection of the CD43-Protein and mRNA in Frozen- or Paraffin-Embedded Sections of Colon Adenoma and Adenocarcinoma by MAb L10 and by Non-radioactive *in Situ* Hybridization

Specimen	Frozen L10 +	Paraffin L10 +	Frozen *in situ* +	Paraffin *in situ* +
Colon adenoma	1/1	20/20	1/1	20/20
Colon cancer	4/11	14/23	4/11	10/18
Normal colon tissue[a]	0/1	0/8	0/1	0/8

[a] Normal colon tissue adjacent to colon adenoma or carcinoma.

Note. The carcinoma samples positive by *in situ* hybridization were the same ones that stained by immunohistochemistry.

bodies were visualized using alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Dakopatts, Copenhagen, Denmark) diluted 1:500 followed by the NBT/BCIP substrate.

Immunohistochemistry. Paraffin embedded (or frozen) biopsies of colorectal adenomas or adenocarcinomas were cut at 7 μm. After deparaffinization in xylene and rehydration with TBS the sections were blocked with TBS containing 2% BSA for 30 min. Frozen sections were fixed with cold methanol (−20°C) prior to blocking. After incubation with the different CD43-specific MAbs and isotype matched control MAb, bound antibodies were visualized with alkaline-phoshatase conjugated goat anti-mouse secondary antibodies (Dakopatts) 1:100 dilution, and the New Fuchsin/Naphtol AS-BI phosphate substrate. Levamisole (1 mM) was added to the substrate solution to block endogenous phosphatase activity. Sections were finally counter stained with hematoxylin and mounted. Samples were classified as 'positive' if a significant number (>30%) of adenoma or carcinoma cells were stained.

In situ hybridization. DIG-labeled CD43-specific RNA probe was synthesized using CD43 cDNA (obtained from Dr. B. Seed, Harvard Medical School, Boston, MA) cloned into a NotI/HindIII site of the pBluescript II. To synthesize DIG-labeled 'run-off' transcripts, the plasmid was linearized with HindIII and transcribed with T3-RNA polymerase for the anti-sense probe. For synthesizing the sense probe the plasmid was linearized with NotI and transcribed with T7-RNA polymerase. All chemicals for synthesizing DIG-labeled RNA probes and anti-DIG antibody were obtained from Boehringer Mannheim (Germany). Paraffin-embedded sections (7 μm) were dewaxed, rehydrated and washed in PBS. Sections were digested with proteinase K (50 μg/ml) in Tris-EDTA buffer, pH 7.6) for 15 min at 37°C, washed in PBS and dried. Prehybridization was done at 68°C for 1 h in hybridization buffer with 50% formamide. Hybridization with anti-sense and sense probes was performed overnight at 68°C in the same buffer. Washing was done by 1XSSC containing 50% formamide and 0.1% Tween 20 at 68°C, 3 times, 30 min each. The bound probes were detected by sheep anti-DIG antibody (Fab fragment, alkaline phosphatase conjugated, 1:1000 dilution) and visualized by the NBT/BCIP substrate combination. When frozen sections were used for *in situ* hybridizations, sections were first fixed with 4% paraformaldehyde and washed with PBS.

RESULTS

Our earlier finding of a CD43-expressing cell line derived from a colorectal carcinoma (12) led us to explore the expression of CD43 in colon adenomas and carcinomas. The result was striking - all 21 colon adenomas studied showed positive epithelial cell staining by both MAb L10 and *in situ* hybridization (Table 1). Fig. 1a shows an example of a colon adenoma stained with MAb L10 where foci of strongly stained cells (red color) are mixed with less stained areas. Staining of an adjacent section with the anti-sense CD43 probe (dark blue color) shows staining of most epithelial cells (Fig. 1c) and the 'patchy' appearance is not observed. Staining with an irrelevant MAb (Fig. 1b) and the CD43 sense probe (Fig. 1d) were negative. The intensity of CD43 mRNA staining varied between the samples, but a majority of the adenomatous cells were stained. The MAb L10 staining usually showed less positive cells than staining by *in situ* hybridization. A second anti-CD43 MAb (BS-1) failed to stain adenomatous cells, but both MAb L10 and BS-1 readily detected infiltrated lymphocytes in the stroma and epithelium (Fig. 1f, i and j). Normal colon tissues were never stained by MAb L10 or the anti-sense CD43 probe (Table 1), not even when it was adjacent to adenoma cells (Fig. 1k and l).

The expression of CD43 in colon adenocarcinomas of low and high differentiation was studied on both paraffin and frozen sections. Tumor cells were stained with both the MAb L10 (Fig. 1e) and the CD43 anti-sense probe (Fig. 1g) in about 50% (18/34) of the cases (Table 1). In some tumors the staining was confined to well differentiated (gland-forming areas) (Fig. 1e, g), whereas in others staining was found in poorly differentiated (solid) areas (not shown). No correlation between the CD43 staining and histological grading of tumors was observed.

It is noteworthy that only MAb L10 reacted with the adenomatous or carcinoma cells, whereas the anti-CD43 MAbs BS-1 and L60 did not. To study this further the reactivity of these MAbs was analyzed on Western blots of extracts from the CD43-positive cell lines K562 and COLO 205 (Fig. 2a). All three anti-CD43 antibodies detected CD43 equally well in the hematopoetic cell line K562, whereas only MAb L10 reacted with the cell lysate from the colon carcinoma cell line COLO 205. None of the antibodies reacted with the purified, highly glycosylated CD43 secreted from the COLO 205 cells. This molecule migrates as a broad and diffuse band (16) as revealed by an anti-sialyl Lewis a MAb (Fig. 2a). This could suggest that also the MAb L10 should not react with the mature CD43 glycoforms found in the adenomas and adenocarcinomas. When the MAb L10 staining was analyzed in more detail on tissue sections this was shown to be the case as this MAb gave a diffuse cytoplasmic staining often confined to the supranuclear Golgi region (Fig. 2b). No staining was observed over the cell membrane.

DISCUSSION

In this paper we have shown that all 21 colon adenomas and ca 50% of colon carcinomas studied expressed the leukocyte-associated antigen CD43. The fact that all available CD43-specific MAbs recogniz-

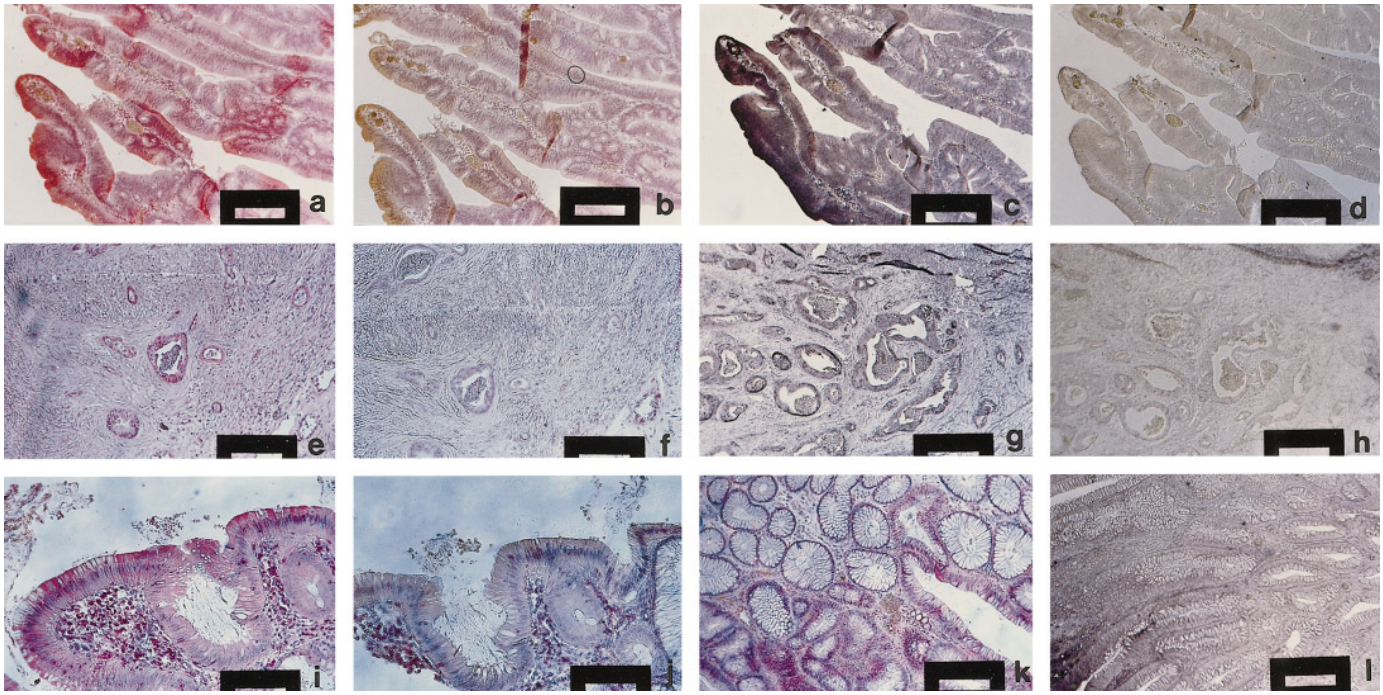

FIG. 1. Immunohistochemical staining and *in situ* hybridization demonstrating the expression of CD43 in colon adenomas and carcinomas. CD43 expression was analyzed in paraffin sections and the CD43-specific MAb L10 and BS-1 were visualized by alkaline phosphatase conjugated secondary antibodies followed by the New Fuchsin/Naphtol AS-BI phosphate substrate, yielding a red color. CD43 mRNA was demonstrated by DIG-labeled RNA probes followed by alkaline phosphatase conjugated Fab-fragments of sheep anti-DIG antibodies and the BCIP/NBT substrate, yielding a dark blue color. (a–d) Four adjacent sections of a colon adenoma stained by (a) MAb L10 giving a variable positive staining over dysplastic epithelium, (b) isotype-matched control MAb showing no staining, (c) DIG-labeled anti-sense RNA probe, staining a majority of dysplastic cells, (d) DIG-labeled sense RNA probe showing no staining. (e–h) four adjacent sections of a moderately differentiated colon carcinoma stained by (e) MAb L10, (f) MAb BS-1, (g) anti-sense RNA probe, (h) sense RNA probe. (i and j) show another colon adenoma stained by MAb L10 and BS-1, respectively. Only MAb L10 recognized the CD43 in epithelial cells, whereas both antibodies stained CD43 in lymphocytes. (k and l) show a third sample of colon adenoma with adjacent normal tissue stained by MAb L10 and by anti-sense RNA probe, respectively. Normal colon crypts are nonstained upper left and right for k and right for l, whereas adenomatous tissue shows expression of the CD43 protein (k, low) or CD43 mRNA (l, lower left). Scale bar in pictures a–h, k–l 100 μm and in i–j 50 μm.

ing CD43 in hematopoetic cells did not regularly detect CD43 in adenoma and carcinoma may be due to different glycoforms of CD43. We have previously shown that CD43 secreted from the COLO 205 cells has sugar chains with an average of 17 sugar residues per chain (12,16), while predominant glycans of leukocyte CD43 has been described as tetra-or hexasaccharides, depending on cell type (2,17). The Western blot analysis (Fig. 2) showed that MAb L10 does not react with the secreted form of CD43 from the colon carcinoma cell line COLO 205. The MAb L10 probably reacts with not fully glycosylated, precursor CD43 molecules giving several bands in Western blot analysis of COLO 205 cell extract. This interpretation is supported by the finding that MAb L10 gave a diffuse cytoplasmic rather than membrane staining in adenomas and carcinomas (Fig. 2).

Direct evidence supporting a causal role for CD43 in colon carcinogenesis is lacking. However, the CD43 expression in 100% of adenomas proposes a role in colon neoplastic development. Induction of CD43 expression might be an early step in adenoma development, because its expression was observed also in small polyps (2 mm). The fact that the CD43 expression in adenocarcinomas was not consistent may indicate that its expression is not critical in later stages of tumor development. This is also supported by the finding that out of four colon carcinoma cell lines only COLO 205 expressed detectable levels of CD43 mRNA (12). Earlier studies on leukocytes may propose how CD43 could be involved in tumor development. Crosslinking of CD43 on leukocytes leads to activation and proliferation of cells and release of CD43 from the cell membrane by a proteolytic cleavage (18,19). In the CD43 expressing adenocarcinoma cell line COLO 205 CD43 has been shown to be quickly phosphorylated and cleaved from its membrane anchor (20), proposing that these phenomena could occur continuously without external activation. Dragone *et al.* (21) have shown that an increased and continuos expression of CD43 in mature B-lymphocytes (where it is normally absent) of transgenic mice caused a marked splenomegaly and prolonged B-

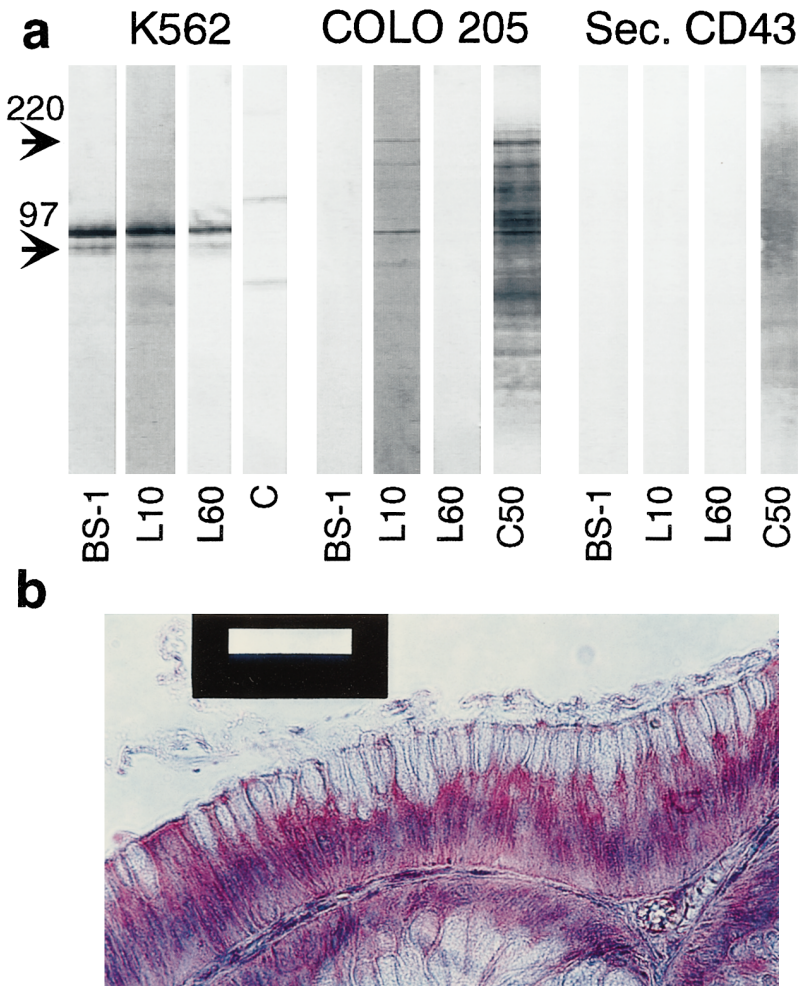

FIG. 2. Reactivity of anti-CD43 MAbs with different glycoforms of CD43. (a) Western blot analysis of K562 cell lysate, COLO 205 lysate and purified secreted CD43 from COLO 205 spent cell culture supernatant (sec. CD43). Cell lysates and purified CD43 were separated by SDS-polyacrylamide gel electrophoresis (8–15% gradient gel), transferred onto nitrocellulose membranes and probed with CD43-specific MAbs BS-1, L10 and L60 or nonrelevant control MAb (C). MAb C50 reacts with the sialyl-Lewis a epitope which is abundantly expressed on several proteins, including secreted CD43 from COLO 205 cells (12). Due to the high glycosylation of CD43 from COLO 205 cells it always appears as a smear on SDS-polyacrylamide gel electrophoresis. The CD43 protein stained by the MAb L10 in a colon adenoma (b) shows diffuse intracellular rather than membrane-bound staining. The staining in the adenomatous cells is confined to the supranuclear Golgi region. Scale bar 25 μm.

cell survival *in vitro* due to a decreased susceptibility to apoptosis. These observations suggest that aberrant CD43 expression during colon tumorigenesis could impair apoptosis and by this being involved in colon cancer development.

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