Overexpression of the Human Erythrocyte Glucose Transporter Occurs as a Late Event in Human Colorectal Carcinogenesis and Is Associated with an Increased Incidence of Lymph Node Metastases

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ABSTRACT
Energy metabolism of human colon cancer in vivo relies predominantly on glucose. Although studies have revealed increased expression of Glut1 mRNA in colon cancer, Glut1 protein (Glut1) expression in the large intestine and its significance are still unknown. The objective of this work was to determine whether Glut1 is present in human colorectal neoplasms and whether that presence is of biological significance. Formalin-fixed, paraffin-embedded tissue sections of 53 colorectal adenocarcinomas, 82 adenomas, 46 hyperplastic polyps, and 38 normal colon samples were immunostained with the anti-Glut1 antibody MYM. The localization was carried out using the avidin-biotin immunoperoxidase technique. No Glut1 immunoreactivity was present in normal colonic mucosa or in hyperplastic polyps, whereas 8 (10%) of 82 adenomas showed such immunoreactivity. The frequency of Glut1 expression in adenomas increased with villous morphology and with the size of the adenoma. Forty-four (83%) of 53 colorectal adenocarcinomas expressed Glut1, and, of these, tumors in which >50% of the cancer cells expressed Glut1 had a significantly higher incidence of metastasis to the lymph nodes (P = 0.0001). It is concluded that (a) Glut1 is expressed as a late event in the carcinogenesis process in human colorectal cancer, and (b) expression of Glut1 in a high proportion of cancer cells is associated with a high incidence of lymph node metastases.

INTRODUCTION
Malignant cells show an increased glucose uptake in vitro and in vivo (1–3). This process is thought to be mediated by Gluts, the expression and activity of which is regulated by oncogenes and growth factors (4–8).

Glut1, the human erythrocyte glucose transporter, is a member of an expanding family of transmembrane proteins known as the facilitative glucose transporters which currently has six members (9). Glut1 mRNA and protein have been found in rat brain, endothelia of the human blood-brain barrier and liver, human erythrocytes, HepG2 hepatic carcinoma cell line, rat kidney, rat mammary gland, and placenta, including fetal membranes (10–16).

Earlier studies have demonstrated the presence of mRNA from different Gluts in human tumors (10, 17–19) and a significant increase in the amount of mRNA for Glut1 in cancers of the esophagus, colon, and pancreas (20). The recent report of increased glucose uptake by human colorectal cancer in vivo (3) suggests an important role for Gluts in the biology of colon cancer. Although Glut1 mRNA was found to be overexpressed in colon carcinoma (20), the expression of Glut1 protein (Glut1) in normal colonic epithelium as well as in benign and malignant tumors is currently unknown. In a recent study, we found Glut1 to be widely expressed in a variety of human neoplasms, including colorectal adenocarcinoma (21). The aim of this study was to determine using immunohistochemistry: (a) whether Glut1 is a late or an early event in human colorectal carcinogenesis and (b) whether such expression has any biological significance.

MATERIALS AND METHODS

Antibody. MYM is a rabbit polyclonal antibody (serum) that we have generated to a 12-amino acid synthetic peptide corresponding to the COOH terminus of human Glut1. This antibody interacted with a Mr 55,000 protein corresponding to the Glut1 glucose transporter in human RBC membranes (21).

Tissues. The study material consisted of formalin-fixed and paraffin-embedded tissues from 53 colorectal adenocarcinomas, 82 adenomas, and 46 hyperplastic polyps. The 38 normal colon controls consisted of sections of nonneoplastic colons as well as tissue adjacent to colon carcinomas or polyps. In all cases, these tissues had no microscopic evidence of inflammation, dysplasia, or other pathology. All cases of adenocarcinoma were staged according to the Astler and Coller staging scheme (22).

Immunohistochemistry. Sections were cut, mounted on Fisher Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), and heated at 58°C for 4 h. Sections were deparaffinized in xylene, rehydrated through decreasing concentrations of alcohol ending in PBS, and microwaved in 10 mm citrate buffer (pH 6.0) for 15 min. Then the sections were incubated with 2% normal goat serum in 1% BSA/PBS for 30 min at room temperature, washed in PBS, and incubated with MYM antibody diluted 1:3000 in 0.1% BSA/PBS for 2 h at room temperature. Finally, sections were washed in PBS, and the bound antibody was

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3 The gene symbol for Glut1 is SLC2A1.
detected using a Vectastain Elite ABC rabbit kit (Vector) with 3,3'-diaminobenzidine as chromogen. Sections were counterstained with hematoxylin, dehydrated, and mounted. Negative controls were sections immunostained as above, but instead of incubation with MYM, the sections were incubated with 1:3000 dilution of the preimmune serum or with the same concentration of MYM preincubated overnight at 4°C with 0.1 mg/ml immunizing peptide. RBCs present in each section served as internal positive control.

Glut1 immunostaining was evaluated by one of us (M. Y.) who had no knowledge of the tumor stage or lymph node status at the time of evaluation. Glut1 immunoreactivity was scored for each tumor based on the percentage of cancer cells that expressed Glut1 in each tumor on a subjective semiquantitative scale: 1, 0%; 2, <10%; 3, 10-25%; 4, 25-50%; 5, >75%.

Statistical Analysis. Statistical analysis was performed with the $\chi^2$ method. The analysis was done using the software StatView for the Macintosh, Version 4.5.

RESULTS

RBCs and perineurium were always positive. None of the 61 normal colons and none of the 49 hyperplastic polyps expressed Glut1, whereas 8 (10%) of the 82 adenomas and 44 (83%) of 53 carcinomas expressed Glut1 ($P = 0.0001$) (Fig. 1).

In adenomas, Glut1 immunoreactivity was always focal, present in areas of high-grade dysplasia, and in no case was >10% of the cells positive. In carcinomas, Glut1 expression was undetectable in 9 cases (17%) and detectable in <10% of the tumor cells in 13 cases (25%), in 10-25% of the tumor cells in 16 cases (30%), in 25-50% of the tumor cells in 5 cases (9%), in 50-75% of the tumor cells in 7 cases (13%), and in >75% of the tumor cells in 3 cases (6%).

Although, as mentioned earlier, some adenomas with high-grade dysplasia expressed focal Glut1 immunoreactivity, there were occasional cases in which Glut1-positive carcinomas were seen arising from Glut1-negative adenomas. Two examples of Glut1 immunostaining of carcinoma arising in adenoma are illustrated in Figs. 2 and 3. Fig. 2 shows a Glut1-positive adenocarcinoma arising in an adenoma, with adjacent adenomatous glands being negative for Glut1. Foci of high-grade dysplasia were present in other areas of the adenoma, some of which did express Glut1. A different case is illustrated in Fig. 3, which shows a Glut1-positive carcinoma arising from the base of an adenoma. The only area of this adenoma positive for Glut1 is the few cells in close contact with the carcinoma. The rest of the adenoma is completely negative for Glut1. The intense membranous Glut1 staining is evident in both Figs. 2 and 3.

When adenomas were divided according to morphology, 3 (21%) of 14 serrated adenomas, 1 (2%) of 44 tubular adenomas, 0 (0%) of 12 tubulovillous adenomas, and 4 (33%) of 12 villous adenomas expressed Glut1 (Fig. 4). The difference in the frequency of Glut1 expression between tubular and villous adenomas was found to be statistically significant ($P = 0.0059$). When the adenomas were divided according to size, we found that only 1 (2%) of the 53 adenomas measuring 0.1-0.5 cm in the largest diameter was positive for Glut1, whereas 7 (24%) of 29 adenomas measuring over 0.5 cm were Glut1 positive ($P = 0.0024$).

Glut1 expression in colorectal adenocarcinomas was associated with increased incidence of lymph node metastases. This incidence increased with the percentage of Glut1-positive cancer cells in nonmucinous carcinomas. Lymph node metastases were present in 0 (0%) of 4 Glut1-negative carcinomas, 3 (30%) of 10 carcinomas with <10% of the cells positive for Glut1, 6 (55%) of 13 carcinomas with 10-25% Glut1-positive cells, 2 (63%) of 3 carcinomas with 25-50% Glut1-positive cells, 3 (75%) of 4 carcinomas with 50-75% Glut1-positive cells, and in 2 (100%) of 2 carcinomas with >75% Glut1-positive cells (Fig. 5). Glut1 expression in >10% of the cancer cells was associated with a higher incidence of lymph node metastases than Glut1 expression in <10% of the cancer cells (59% versus...
DISCUSSION

Several studies have documented that malignant human neoplasms, including colorectal adenocarcinomas, show a significantly increased glucose uptake and utilization, and concluded that glucose is the main source of energy in these tumors (1-3). In a recent study of Glut1 expression in normal and malignant human tissues, using immunohistochemical detection, we found that normal epithelia were negative for Glut1, with rare exceptions (21). Although a significant number of malignant tumors expressed Glut1, other tumors did not show such expression. Furthermore, Glut1 expression varied in the positive tumors from a few cells to the majority of the tumor cells (21). This difference between normal tissues and their malignant counterparts and the heterogeneity of Glut1 expression in the different tumors suggested that such expression may be of biological significance.

Our findings show that Glut1 is expressed as a late event in the adenoma-carcinoma sequence in the human large intestine. This is supported by the following findings: (a) increased fre-

Fig. 3 Glut1 immunostaining of Glut1-positive invasive adenocarcinoma arising from the bottom of a Glut1-negative adenoma (A), and a higher power magnification of the interface between adenoma and carcinoma (B). Immunoperoxidase with hematoxylin counterstaining. A, ×42; B, ×105.

Fig. 4 Glut1 immunoreactivity in colonic adenomas.

Fig. 5 Correlation between the percentage of Glut1-positive cells in adenocarcinomas and the percentage of lymph node metastasis.
frequency of Glut1 expression parallels an increase in the size and villous morphology of adenomas, which in turn correlate with increased risk of high-grade dysplasia and carcinoma (23); (b) only a small percentage of adenomas express Glut1, always in <10% of the cells and in association with high-grade dysplasia; and (c) Glut1-positive carcinomas can arise from Glut1-negative adenomas. Despite the fact that none of the normal colonic tissue samples or hyperplastic polyps expressed Glut1, we cannot completely exclude the possibility that Glut1 may be expressed in these tissues at levels that are too low to be detected by our technique.

Although 14% of the serrated adenomas showed Glut1 immunoreactivity, the meaning of such expression is unknown because the incidence of high-grade dysplasia and carcinoma in this relatively newly described morphological type (24) has not been studied yet.

A highly significant finding in this study is the correlation between Glut1 expression in colon cancer and the frequency of lymph node metastases. Such finding indicates that Glut1, a highly efficient glucose transporter, is important for maintaining the high-energy requirements of aggressive carcinomas. We speculate that immunohistochemical detection of Glut1 in biopsies of colorectal carcinoma may be useful as a marker of aggressive biological behavior. This marker, therefore, may be used to identify a group of patients who are likely to have advanced cancers, i.e., carcinomas with lymph node metastases. These patients may then be offered an optimized preoperative management, based on their predicted risk.

Our results show that Glut1 is expressed late in the adenoma-carcinoma sequence during carcinogenesis in the human colon, and that expression of Glut1 in >10% of the cancer cells is significantly associated with a high incidence of lymph node metastases.

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