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The GCTM-5 Epitope Associated with the Mucin-Like Glycoprotein FCGBP Marks Progenitor Cells in Tissues of Endodermal Origin

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Abstract

Monoclonal antibodies against cell surface markers are powerful tools in the study of tissue regeneration, repair, and neoplasia, but there is a paucity of specific reagents to identify stem and progenitor cells in tissues of endodermal origin. The epitope defined by the GCTM-5 monoclonal antibody is a putative marker of hepatic progenitors. We sought to analyze further the distribution of the GCTM-5 antigen in normal tissues and disease states and to characterize the antigen biochemically. The GCTM-5 epitope was specifically expressed on tissues derived from the definitive endoderm, in particular the fetal gut, liver, and pancreas. Antibody reactivity was detected in subpopulations of normal adult biliary and pancreatic duct cells, and GCTM-5-positive cells isolated from the nonparenchymal fraction of adult liver expressed markers of progenitor cells. The GCTM-5-positive cell populations in liver and pancreas expanded greatly in numbers in disease states such as biliary atresia, cirrhosis, and pancreatitis. Neoplasms arising in these tissues also expressed the GCTM-5 antigen, with pancreatic adenocarcinoma in particular showing strong

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

M.F.P. is Director of Biotrophix Pty. Ltd., a company with a commercial interest in the GCTM-5 antibody.

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and consistent reactivity. The GCTM-5 epitope was also strongly displayed on cells undergoing intestinal metaplasia in Barrett's esophagus, a precursor to esophageal carcinoma. Biochemical, mass spectrometry, and immunochemical studies revealed that the GCTM-5 epitope is associated with the mucin-like glycoprotein FCGBP. The GCTM-5 epitope on the mucin-like glycoprotein FCGBP is a cell surface marker for the study of normal differentiation lineages, regeneration, and disease progression in tissues of endodermal origin.

Keywords

Endoderm; Cell surface antigen; Liver progenitor; Barrett's esophagus; FCGBP; Pancreatic cancer

INTRODUCTION

Longstanding evidence suggests that the molecular factors regulating embryonic morphogenesis and lineage determination also govern tissue regeneration and repair [1, 2]. The gastrointestinal tract, liver, and pancreas are formed from the definitive endoderm. These tissues share a close ontogeny and common developmental regulation of many critical transcriptional and growth factors [3–5]. For example, cell lineage tracing experiments in mice show that Sox-9 expression characterizes progenitors in the liver and pancreatic ducts and small intestinal crypts [6]. In these adult tissues, resident stem cells or facultative progenitors may mediate regeneration through recapitulation of the developmental program [7–9]. Recent experiments have demonstrated interconversion of mature pancreatic and hepatic lineages through the introduction of transcriptional regulators or growth factors, demonstrating extraordinary plasticity in hepatopancreatic ontogeny [10–13]. Furthermore, these reprogramming studies suggest a key role for stem cells and progenitors in the pathological processes of metaplasia and neoplasia that occur in these organs.

The liver is capable of regeneration not only through hepatocyte proliferation but also via a biliary ductular reaction, wherein bipotential cells give rise to bile duct and hepatic parenchyma. These facultative bipotential progenitor cells reside in the Canals of Hering [14–17]. Although there is significant heterogeneity in this population of ductular progenitors, EpCAM and NCAM provide useful markers for these cells [9, 17]. The GCTM-5 monoclonal antibody epitope is a candidate marker for this putative progenitor cell, since GCTM-5 reacts with a subpopulation of biliary cells in normal liver and demonstrates colocalization with NCAM in the expanded biliary population found in cirrhotic liver [18].

Regenerative mechanisms in the pancreas are not as well-established as those in the liver. A candidate population of progenitors found in chronic pancreatitis resides in a poorly defined structure termed the tubular complex that has been proposed to originate in various compartments including acinar cells, centroacinar cells, and terminal ductules [19–21]. In both animal and human studies, these structures have been identified as a susceptible population for neoplastic transformation, and they may represent the cell of origin of pancreatic intraepithelial neoplasia (PanIN), the precursor of pancreatic ductal adenocarcinoma [22–26]. An analogous sequence of premalignant changes has been proposed for intrahepatic cholangiocarcinoma, but the distinct cell of origin is largely unknown for this highly malignant tumor [27, 28].

Columnar metaplasia of the esophagus is a preneoplastic lesion that occurs secondary to gastroesophageal reflux disease [29–32]. For many years, the incomplete intestinal metaplasia found in this condition has been thought to originate from the transdifferentiation that occurs during regeneration following reflux-induced injury. However, more recently,

the ductal epithelium of the esophageal submucosal glands has been implicated as the source of a multipotent stem or progenitor cell that might give rise to the pathogenesis of neoplasia [33–38].

There is a paucity of cell surface markers for the identification and prospective isolation of progenitor cell populations in all these tissues. In this study, we sought to define the distribution of the GCTM-5 antigen in regenerative, metaplastic, and neoplastic states in the gastrointestinal tract and hepatopancreatic ducts. Furthermore, we have characterized the population of GCTM-5-expressing epithelia in the biliary duct and identified the protein bearing the epitope biochemically.

MATERIALS AND METHODS

Human Fetal, Pediatric, and Adult Tissue Samples

All human tissue specimens were obtained following appropriate ethical consent or Institutional Review Board approval at the respective institutions. Details on tissue types and acquisitions can be found in Supporting Information.

Immunohistochemistry, Histochemistry, and Histopathological Analysis

Immunohistochemistry was performed using standard protocols. Primary antibodies used in this study were: GCTM-5 (neat hybridoma cell line culture supernatant), IgG1 isotype negative control (DakoCytomation, Carpenteria, CA, www.dako.com), anti-Ki-67 (DakoCytomation), rabbit anti-c-peptide (Millipore Corporation, Billerica, MA, www.millipore.com), mouse anticy-tokeratin 19 (DakoCytomation), mouse anti-Cytokeratin 7 (OV-TL 12/30; DakoCytomation), and EpCAM (Ber Ep4, DakoCyto-mation). Appropriate isotype controls were used for all experiments and were shown to be negative on the relevant tissue.

A total of 21 esophageal biopsy cases were evaluated for foci of GCTM-5 reactivity. The GCTM-5 stained esophageal specimens were evaluated by two expert gastrointestinal surgical pathologists (L.M.P. and P.T.C.).

Isolation of GCTM-5-Positive Cells from Adult Human Liver

Adult human liver cells were provided by CellzDirect (Life Technologies, Grand Island, NY, www.lifetechnologies.com). The supernatant postisolation of hepatocytes was rinsed with ice-cold 0.1% bovine serum albumin/2 mM EDTA in phosphate buffered saline several times and processed further as liver nonparenchymal cells. The liver nonparenchymal cells were then loaded onto a 20%–40% percoll-gradient column which was centrifuged at 1,000*g* for 10 minutes. The cell fraction banding at 20% percoll included the majority of GCTM-5-positive cells, and this fraction was collected and rinsed. Then GCTM-positive cells were isolated by magnetic-positive isolation with purified GCTM-5 antibody and Dynabeads Rat anti-Mouse IgG1 (DYNAL, Life Technologies) according to manufacture's protocol. The GCTM-5-positive cells were directly lysed for RNA purification or seeded on collagen IV-coated plates and then cultured in Kubota's medium supplemented with 2% fetal calf serum, 10 ng/ml HGF, and epidermal growth factor. The cultured cells were gently dissociated with TripLE (Invitrogen, Life Technologies) and passaged every week.

Quantitative PCR Analysis

Total RNA was isolated with Trizol (Life Technologies) and further purified with RNeasy mini kit with DNase I (Qiagen, Valencia, CA, www.qiagen.com). Reverse transcription was performed using the Omniscript kit (Qiagen) and random hexamer primers. Quantitative PCR was performed using gene-specific primer/probe mixtures (TaqMan Gene Expression

Assays, Life Technologies), Taq-Man 2× Master Mix, and the ABI PRISM 7900 Sequence Detection system (Applied Biosystems, Life Technologies) according to the manufacturer's protocols. The PCR data were analyzed by the delta/delta cycle threshold (CT) method and normalized to PPIA expression with RQ Manager software (Applied Biosystems). The fold expression was calculated relative to human embryonic stem cells. Human dermal fibroblast and HepG2 cells were used as negative and positive control, respectively.

Immunostaining

The isolated GCTM-5-positive cells were passaged to remove magnetic beads after 5 days culture and fixed with 4% paraformaldehyde in phosphate buffered saline 2 days after passage. All mouse IgG1 antibodies were labeled with a fluorescent-conjugated $F(ab')_2$ fragment (Zenon Mouse IgG Labeling kit Life Technologies (Invitrogen), and staining was performed according to manufacture's protocol. The other antibodies used were detected indirectly with fluorescent-conjugated secondary antibodies. The purified GCTM-5, anti-NCAM (CD54) (clone HA58, BD Pharmingen, BD Biosciences, San Diego, CA, www.bdbiosciences.com), anti-CD133 (clone EMK08, eBio-science, San Diego, CA), anti-Cytokeratin 8 (C51, Santa Cruz Bio-technology, Santa Cruz, CA, www.scbt.com), anticytokeratin 19 (DakoCytomation), anti-E-cadherin antibody (HECD-1, Invitrogen, Life Technologies), antiepithelial antigen (EpCAM) (clone Ber-EP4, DakoCytomation), anti-ICAM antibody (BD Pharmingen), and anti-albumin (DakoCytomation) were used as primary antibodies, and Alexa Fluor 488- or 594-conjugated antibodies (Molecular Probes, Life Technologies) were used as secondary antibodies.

Immunochemical and Biochemical Characterization of the GCTM-5 Antigen

CFPAC-1 pancreatic adenocarcinoma cells were grown in T175 flasks to 75% confluence in Iscove's Modified Dulbecco's Medium containing 10% fetal calf serum. Serum containing medium was then removed and the cells were washed three times with phosphate buffered saline. Serum-free/antibiotic-free medium was then added to the CFPAC-1 cells that were subsequently cultured in a humidified environment at 37°C, 5% CO_2 for a further 3 days. The conditioned medium was collected, passed through a 0.22 μM filter, and stored at 4°C. CFPAC-1 conditioned medium was added to 2× Laemmli sample buffer and proteins were separated on either 10% or 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE), transferred to nitrocellulose or polyvinylideneflouride membranes, and immunoblotted with GCTM-5 antibody as previously described [18].

Using a stirred ultracentrifuge cell (Millipore) and a 30 kDa molecular weight cut-off filter, 500 ml of CFPAC-1 conditioned medium was concentrated down to ~15 ml under nitrogen gas pressure (~20 psi). Concentrated conditioned medium was then filtered through a 0.22 μM filter. Immunoprecipitation (with Protein A or Protein G beads) or immunoblotting with GCTM-5 or with commercial antibodies against peptides from the C or N terminus of FCGBP (Sigma Aldrich, St. Louis MO, www.sigmaaldrich.com) were carried out on concentrated conditioned medium. Conditioned medium or immunoprecipitates were separated on SDS PAGE on precast 4%–20% gradient polyacrylamide gels or 8% gels in Tris-HEPES-SDS running buffer. Detection was carried out either with anti-mouse or anti-rabbit Ig or with or the corresponding $F(ab')_2$ conjugates.

For carbohydrate analysis, 200 μl of concentrated condition medium was added to 100 μl of 3× Laemmli sample buffer containing dithiothreitol then treated with deglycosylating enzymes according to manufacturers' instructions (GLYKO, Prozyme, Hayward, CA, www.prozyme.com). Denatured concentrated conditioned medium in sample buffer was treated with *N*-Glycanase, *O*-Glycanase, Sialidase A, or all three enzymes, and the samples

were run on SDS PAGE gels and blotted with GCTM-5 antibody as described above to assess the effect of enzyme treatment on antibody reactivity.

GCTM-5 antibody, purified by affinity chromatography on protein G-Sepharose, was covalently coupled to protein G-Sepharose using dimethylpimelimidate in 0.2 M sodium borate buffer. Concentrated conditioned medium from CFPAC-1 cells, prepared as described above, was applied to a column of protein G-Sepharose coupled to GCTM-5. The column was washed with 10 mM sodium phosphate buffer at pH 6.8 and eluted with 100 mM glycine at pH 2.5. Immunoblotting was carried out with GCTM-5 or with commercial antisera to FCGBP as described above. The column eluate was sequenced at the USC Proteomics Core facility on a Thermo linear trap quadropole/electron transfer dissociation (LTQ-ETD) mass spectrometer.

RESULTS

Expression of the Epitope Detected by the GCTM-5 Monoclonal Antibody Is Specific for Endodermal Tissues of the Fetus and Adult and Is Modulated in Disease States

A survey of normal and diseased tissues representative of the three embryonic germ layers indicated that GCTM-5 immunore-activity is highly specific for tissues of endodermal origin (Supporting Information Tables S1 and S2 and Supporting Information Figs. S1--S3). In particular, the GCTM-5 antigen was detected in normal biliary ducts and pancreatic ducts, with expansion of the antigen-positive cell population in diseased states in these tissues and in metaplastic esophagus (Supporting Information Table S1). A description and additional figures of GCTM-5 immunoreactivity in fetal and adult tissues may be found in Supporting Information. In some tissues, GCTM-5 staining was associated with mature cells, such as mucin-secreting cells in the adult colon. However, in many tissues, the antibody stained progenitor compartments (below).

In normal and preneoplastic tissues, GCTM-5 immunore-activity was found at the apical surface of epithelia, in a mucin-like pattern of distribution. In cancerous tissue, where epithelial polarity is disrupted, reactivity was distributed throughout the surface of the cell.

Putative Hepatic Progenitors and a Subset of Cholangiocarcinoma Express the GCTM-5 Antigen

Previous studies indicated that the GCTM-5 monoclonal antibody is a novel cell surface marker of putative biliary progenitors in normal liver that demonstrates colocalization with NCAM and CK19 in cirrhotic tissues [18]. Further immunohistochemical investigation confirmed that GCTM-5 is expressed in a subpopulation of biliary ductal cells in the portal tracts of normal adult liver (Fig. 1A). In regenerating cirrhotic livers, GCTM-5 marked an expanded population of ductal cells with the appearance of biliary ductular reaction (Fig. 1B). We studied coexpression of the GCTM-5 antigen with EpCAM and albumin, markers of hepatic stem cells [15]. GCTM-5 colocalized with EpCAM (Fig. 1C) and demonstrated coexpression with albumin where GCTM-5-positive cells merge at the periphery of regenerative nodules of parenchymal hepatocytes (Fig. 1D). The malignant cells of intrahepatic cholangiocarcinoma reacted variably and heterogeneously with GCTM-5 (Fig. 1E; Supplemental Table 1).

GCTM-5-positive cells were more widely distributed in bile ducts from pediatric liver. Staining of serial sections showed that immunoreactive cells could be found in the distal portions of the duct extending well into the hepatic parenchyma (Fig. 2), consistent with localization in the Canals of Hering. GCTM-5 reacted with a subset of CK19-positive cells in the distal portions of the bile ducts. In livers of patients with biliary atresia, GCTM-5-reactive cells were positive for Ki-67 (Supporting Information Fig. S4). In these specimens,

there was some overlap with NCAM and EpCAM, but as in the adult, the two markers were not always coincident (Supporting Information Fig. S5). An antibody to the transcription factor Sox-9 decorated a subset of GCTM-5-positive cells within the hepatic ducts (Supporting Information Fig. S6).

GCTM-5-Positive Cells in Adult Liver Have a Progenitor Cell Phenotype

To characterize further the GCTM-5 subpopulation in adult liver, we isolated antigen-positive cells from the nonparenchymal fraction of dissociated liver tissue using GCTM-5 coupled to immunomagnetic beads, then analyzed the cells by quantitative reverse transcriptase polymerase chain reaction (QRT-PCR) for expression of liver progenitor and hepatocyte markers (Fig. 3). Relative to whole liver, total nonparenchymal cells, HepG2 hepatocellular carcinoma, or human dermal fibroblasts, the GCTM-5-positive fraction expressed high levels of HNF1beta, HNF6, HHex, GATA-6, and Pdx-1. GCTM-5 cells expressed GATA-4, HNF3a, HNF4, Sox-17, Sox-7, albumin, and alpha1-antitrypsin at similar levels to other hepatic cell types but did not express AFP.

GCTM-5-positive cells from adult liver could be serially cultivated in vitro for several passages in media previously reported to support propagation of human liver progenitor cells [39]. The isolated cells formed small epithelial colonies throughout which most cells retained GCTM-5 expression (Fig. 4). Most cells in the colonies were positive for EpCAM, E-cadherin, and albumin. A subpopulation of cells in growing colonies expressed cytokeratins 8 and 19. Most cells were negative for NCAM and ICAM, and a subset expressed CD133 (not shown).

GCTM-5 Antigen Is Expressed in a Subpopulation of Normal Pancreatic Duct Cells that Undergoes Expansion of Expression in Ductal Metaplasia, PanIN, and Adenocarcinoma

In the adult pancreas, infrequent and scattered GCTM-5 reactivity in small ductules and in larger ducts was observed (Fig. 5A). Expansion of the population of GCTM-5-expressing ductules was observed in areas of tissue affected by mild chronic pancreatitis (Fig. 5B). In cases of severe chronic pancreatitis, a subpopulation of ductules in the tubular complex formation, also known as acinarductal metaplasia, expressed the GCTM-5 antigen (Fig. 5C). Poorly defined structures known as transforming tubular complexes, which show morphological overlap with PanIN but occur in the acinar compartment, variably expressed the GCTM-5 antigen (Fig. 5D) [22, 40]. Both low-grade and high-grade pancreatic epithelial neoplasia lesions showed intense membrane reactivity (Fig. 5E). Primary and metastatic pancreatic ductal adenocarcinoma strongly and consistently expressed the GCTM-5 antigen in a high percentage of tumor cells, irrespective of the degree of differentiation of the tumor (Fig. 5F).

GCTM-5 Antigen Is Expressed in the Reflux to Adenocarcinoma Sequence of Barrett's Esophagus in Proliferating Cells

Further study of the reflux to adenocarcinoma sequence of Barrett's esophagus was performed to evaluate the significance of GCTM-5 antigen expression in this condition. The normal squamous epithelium of the esophagus did not express the GCTM-5 antigen (Fig. 6A). However, GCTM-5 immunoreacted with the majority of cases containing metaplastic epithelia, dysplasia, and adenocarcinoma in the esophagus (Supporting Information Table S1 and Fig. 6B–6F). This indicates that the GCTM-5 antigen is expressed in the metaplasia to adenocarcinoma sequence of Barrett's esophagus. A lesion known as the multilayered epithelium, thought to be a precursor to intestinal metaplasia, was strongly decorated with GCTM-5 (Fig. 6B), as were cardiac mucosa type metaplasia (Fig. 6C) and intestinal metaplasia (Fig. 6D) itself. The majority of dysplastic lesions (Fig. 6E) expressed the

antigen, as did adenocarcinoma (Fig. 6F), although only portions of these tissues were reactive with the antibody.

Biochemical Characterization of the GCTM-5 Antigen

Previously, the GCTM-5 antigen was characterized as an epitope on a ~50 kDa protein present in cell lysates of primary biliary epithelial cells and differentiating populations of human embryonic stem cells [18]. The antigen is also expressed on the cell surface of cultured pancreatic adenocarcinoma cell line CFPAC-1 (live cell staining analyzed by flow cytometry, Supporting Information Fig. S7); immunoblotting of whole cell lysates of CFPAC-1 cells lysed into SDS PAGE gel reducing sample buffer showed a strong band of ~55 kDa and diffuse high molecular weight bands (Fig. 7A). CFPAC-1 cells secreted or shed the antigen into the cell culture medium, where the high molecular weight species represented the predominant form (Fig. 7B, lane 1).

We Sought to Characterize the GCTM-5 Antigen Further

Treatment of CFPAC-1 concentrated conditioned medium with deglycosylating enzymes demonstrated significant sensitivity of the epitope to sialidase A treatment (Fig. 7B lanes 3 and 5) but not to *N*- or *O*-glycosidases (Fig. 7B lanes 2 and 4). The antigen was difficult to extract from CFPAC-1 cells using nonionic detergents. However, the secreted form could be recovered from concentrated conditioned medium by immunoprecipitation (Fig. 7C, lane 1) or affinity chromatography. Affinity purified antigen from CFPAC-1 supernatants often contained only the high molecular weight form. The eluate from GCTM-5 affinity columns was subjected to mass spectrometry. The only protein represented by multiple peptide bands in the sample was the mucin-like protein FCGBP (Supporting Information Table S3). Commercially available antibodies against protein epitopes from either the C-terminal or N-terminal of FCGBP reacted with a high molecular weight band in the acid eluate of GCTM-5 affinity columns (Fig. 7D, lanes 1 and 3). Immunoblotting of concentrated condition medium from CFPAC-1 cells showed that the antibody against the C-terminal epitope reacted with a protein band of approximately 100 kDa (Fig. 7D, lane 2) and the antibody directed against the N-terminal epitope reacted with a band of approximately 55 kDa (Fig. 7D, lane 4). These 100 and 55 kDa bands approximate roughly to the sizes of fragments of FCGBP protein isolated from intestinal mucous that are thought to be generated by a process of autocatalytic cleavage of the intact FCGBP molecule [41].

Neither commercially available anti-FCGBP antisera recognized the native form of the GCTM-5 antigen in concentrated conditioned medium from CFPAC-1 cells and neither reagent immunoprecipitated the high molecular weight form of the GCTM-5 antigen from CFPAC-1 cells (not shown). Neither reagent reacted in bile duct cells in liver tissue sections, although transcripts for FCGBP were readily detected in adult liver nonparenchymal fractions (Supporting Information Fig. S8). However, anti-FCGBP C-terminal antisera reacted with the 100 kDa band seen in conditioned medium in GCTM-5 immunoprecipitates of CFPAC1 concentrated conditioned medium (Fig. 7e).

FCGBP was originally isolated from the small intestine as a protein that bound the Fc portion of immunoglobulins [42, 43]. In our study, secondary antisera against mouse or rabbit Ig showed a degree of reactivity with the high molecular weight form of purified GCTM-5 antigen (Fig. 7F, compare lanes 1 and 3). Secondary antisera did not react with the 100 or 50 kDa fragments recognized by the C- and N-terminal anti-FCGBP antibody in concentrated conditioned medium (C-terminal antiserum, Fig. 7F, compare tracks 2 and 4) or the native high molecular weight form of the antigen. This reactivity of secondary antibodies was greatly decreased using $F(ab')_2$ conjugates instead of intact immunoglobulins to probe the immunoblots (Fig. 7F, lanes 5 and 6).

DISCUSSION

The GCTM-5 Epitope Is Associated with the Mucin-Like Glycoprotein FCGBP

Mass spectrometry sequencing data along with immunoprecipitation and immunoblotting indicate that the GCTM-5 epitope is associated with the mucin-like glycoprotein FCGBP. GCTM-5 bound peptides of ~55 and 100 kDa in conditioned medium from CFPAC-1 pancreatic adenocarcinoma cells that were cross-reactive with antisera to the N and C termini of FCGBP, respectively; these fragment sizes are consistent with previously described autocatalytic degradation fragments of FCGBP [41, 44]. FCGBP was originally isolated from the small intestine as a protein that bound the Fc portion of immunoglobulins [42, 43]. In our study, secondary antisera against mouse or rabbit Ig showed a degree of reactivity with purified GCTM-5 antigen, and predictably this reactivity was greatly decreased using $F(ab')_2$ conjugates as probes instead of intact Ig. The anti-mouse or anti-rabbit Ig secondary antibodies reacted with the high molecular weight form of FCGBP isolated by acid elution from affinity columns, but not the native antigen in concentrated conditioned medium, or the 100 or 55 kDa bands.

Neither commercial antisera to FCGBP could immunoprecipitate the native GCTM-5 antigen and neither reagent decorated bile ducts in liver sections. These antisera did not recognize native high molecular weight GCTM-5 antigen in conditioned medium. However, both antisera recognized fragments of FCGBP in conditioned medium and in immunoaffinity purified material, and both reacted with high molecular weight bands in immunopurified GCTM-5 eluted by acid from a GCTM-5 affinity column. Therefore, it appears likely that the peptide epitopes recognized by the commercial antisera are not exposed on the native intact form of FCGBP expressed in CFPAC-1 cells or in liver. Instead, the relevant epitopes become exposed in degraded fragments of the intact native protein. As noted above, secondary antisera containing intact immunoglobulins bind the GCTM-5 antigen eluted by acid from immunoaffinity columns but not the native antigen or the putative degradation products. It has been proposed that FCGBP undergoes autocleavage at specific sites at acidic pH to generate these fragments [41, 44], and it is thus likely that the acidic buffer (glycine, pH 2.5) used to elute the antigen from the affinity column exposes these sites.

FCGBP is encoded by a 16 kbp transcript that is transcribed to yield a 300 kDa protein with multiple Von Wille-brand factor and mucin-like repeats [43]. FCGBP, like GCTM-5, is found on the apical surface of intestine [45], and expression of both FCGBP and GCTM-5 is downregulated in colorectal carcinoma [46]. FCGBP protein has also been identified in bile [47] and salivary glands [48]. In the intestine, FCGBP exists in a large complex with Muc2 and Trefoil peptide [41, 44]. Like FCGBP, the expression pattern of the GCTM-5 epitope resembles that of mucins. However, the tissue specificity of the GCTM-5 epitope distinguishes it from that of known mucins. The only mucin expressed by both bile ducts and pancreas is Muc-1 [49, 50], and Muc-1 is far more broadly expressed in epithelia and in carcinomas than the GCTM-5 epitope. It is however possible that Muc-1, the GCTM-5 epitope, and FCGBP are present in some form of complex in bile duct and liver.

The GCTM-5 epitope clearly contains sialic acid, and since FCGBP likely exists in a supermolecular complex in its secreted form, it is only possible to conclude that the epitope is associated with FCGBP. Further studies are required to define the exact location of the epitope on the FCGBP poly-peptide, which contains multiple repeats and multiple *N*- and *O*-glycosylation sites. Our results are consistent with a model in which FCGBP is secreted from CFPAC-1 cells in a form that cannot bind immunoglobulin and in which the C- and N-terminal epitopes recognized by commercial antisera against the peptide sequence are concealed. Mucins are known to exert cytoprotective effects, and in the context of tissue

regeneration in the face of microenvironmental damage (gastrointestinal reflux, pancreatitis, and biliary obstruction), progenitor cells may upregulate their expression of surface mucins to enhance their capacity for survival and repair.

Progenitor Cells in Liver Express the GCTM-5 Antigen

Histological studies here have confirmed previous work showing consistent GCTM-5 antigen expression in a subpopulation of cells in the normal biliary ducts and in an expanded population of biliary ductular reaction cells of cirrhotic liver. In this ductular reaction, we demonstrated coexpression of GCTM-5 with EpCAM, and with albumin at the periphery of regenerative nodules. These studies suggest that the GCTM-5 antigen marks a subset of cells in the heterogeneous population of facultative progenitors of the biliary ductular reaction. Although the finding of coexpression of albumin and GCTM-5 at the margin of regenerating foci of hepatocytes suggests that GCTM-5 cells can give rise to hepatocytes, the exact lineages that the GCTM-5-expressing ductular cells contribute to are unknown at this time. The lack of complete overlap of expression between GCTM-5, EpCAM, and NCAM in normal or regenerating liver suggests that this progenitor cell compartment may in fact be quite heterogeneous.

The GCTM-5 monoclonal antibody was used to isolate the biliary ductular cells that express the antigen on the membrane surface. Gene expression showed that these cells express transcription factors characteristic of endodermal progenitors, including GATA-6, HNF1beta, HNF6, HNF4, HNF3a, and PDX1. Some of these transcription factors are found in progenitor populations in the developing liver, in the ductal plate, the cell type thought to correspond to adult liver stem cells [9, 15]. When these GCTM-5-positive cells were cultured in vitro under conditions that promote growth of hepatocyte progenitors, they maintained a pattern of protein markers consistent with hepatic stem cells.

Further studies using multiple markers including GCTM-5 to isolate subpopulations of cells within this compartment will aid in defining those cells with the greatest capacity for repopulation. These studies will also assist in the isolation of progenitor cells from embryonic stem cells or induced pluripotent stem cells undergoing differentiation into the hepatic lineage.

Intrahepatic cholangiocarcinoma is a highly aggressive malignancy of the biliary epithelia with a poor prognosis. The GCTM-5 antigen is expressed variably and heterogeneously in these tumors. If in fact GCTM-5 marks a ductal progenitor population, this may indicate that the cell of origin of a subset of these tumors [51] is GCTM-5 positive.

The GCTM-5 Antigen Is Expressed by the Putative Precursors of Barrett's Esophagus

We have detected expression of GCTM-5 antigen in the metaplasia to adenocarcinoma sequence of the columnar lined esophagus and characterized the immunoreactive populations. In general, GCTM-5 is expressed on sialomucin containing epithelia that are proliferating.

The multilayered epithelium is the candidate precursor of intestinal metaplasia of the esophagus. The GCTM-5 antigen is variably expressed in the multilayered epithelium (ME), but its patterns of expression suggest it marks tissue that is progressing toward intestinal metaplasia. Those foci that express abundant GCTM-5 antigen are typically adjacent to or contiguous with metaplastic columnar epithelia that are also strongly immunoreactive. Further studies confirmed that when this occurs, these epithelia share a common profile of mucins and differentiation markers (not shown). This indicates a direct transition from multilayered epithelium into metaplastic columnar epithelia that is marked by GCTM-5 expression.

The GCTM-5 Antigen Is a Novel Marker for a Subpopulation of Epithelial Cells in Normal Pancreatic Ducts, PanIN, and Pancreatic Ductal Adenocarcinoma

The GCTM-5 antigen is expressed on the surface membrane of a subpopulation of epithelia in the developing and adult pancreas. The normal pancreas demonstrates GCTM-5 expression restricted to a minor proportion of the large ducts and terminal ductules. As in the liver, this population in the pancreas overlaps with those cells expressing Sox-9, thought to be a marker for pancreatic progenitor cells (S. Bonner-Weir, personal communication). In chronic pancreatitis, the proportion of positive cells increases, with severe disease demonstrating GCTM-5 expression in a subpopulation of the massive collection of ductules, often referred to as the tubular complex.

The tubular complex is thought to represent a progenitor population with experimental and circumstantial evidence suggesting that these structures may originate from a variety of sources including centroacinar and the terminal ductule cells [19]. Our data not only demonstrate GCTM-5 expression in the terminal ductules but also demonstrate GCTM-5 expression in the tubular complex. This suggests that GCTM-5 may mark the progenitors in the normal terminal ductules that give rise to the tubular complex found in chronic pancreatitis. Additionally, the PanIN lesions and pancreatic adenocarcinoma express the GCTM-5 antigen strongly and consistently. As in the liver, a progenitor cell population in the termini of the pancreatic ducts marked by GCTM-5 may be the cell of origin for pancreatic adenocarcinoma. Identification and isolation of GCTM-5-positive cells will help dissect out relationships between normal, preneoplastic, and cancer progenitors in the pancreas [52].

GCTM-5 was strongly reactive in a high percentage of tumor cells and mucinous material from all cases of pancreatic adenocarcinoma that we examined. CFPAC-1 pancreatic adenocarcinoma cells secrete or shed GCTM-5 antigen into the culture medium. Therefore, GCTM-5 antigen is a potential serum-based biomarker for diagnostic or screening tests for pancreatic adenocarcinoma. FCGBP maps to chromosome 19q13, a region of the genome that is often overrepresented in a subset of patients with pancreatic adenocarcinoma [53]. The FCGBP locus lies within a 650 kbp minimal amplicon on 19q13 defined on a panel of pancreatic cancer cell lines and patient tumors, and two-third cell lines that harbored the amplicon expressed FCGBP at levels >20-fold above levels in controls that did not contain the amplicon [49]. Although these limited data do not of course implicate FCGBP in tumor progression, it is possible that as in other types of cancer, mucin overexpression is characteristic of the disease state.

SUMMARY

The GCTM-5 monoclonal antibody is specific for tissue derivatives of the definitive endoderm in the fetus and adult. In particular, the GCTM-5 antigen is expressed in regenerating and pathological states of gastrointestinal and hepatopancreatic ductal epithelia in fetal and normal adult. The distribution of the GCTM-5 antigen localizes to many known or putative progenitor compartments such as the biliary ductular reaction, colonic crypts, pancreatic tubular complex, and the esophageal multilayered epithelium. Prospective isolation of these progenitors in liver, pancreas, and esophagus will enable elucidation of their role in tissue regeneration and neoplasia. Expression of FCGBP on the cell surface may protect progenitor cells from adverse environmental conditions during tissue repair. Antibodies to the GCTM-5 epitope may find application in the diagnosis or treatment of pancreatic cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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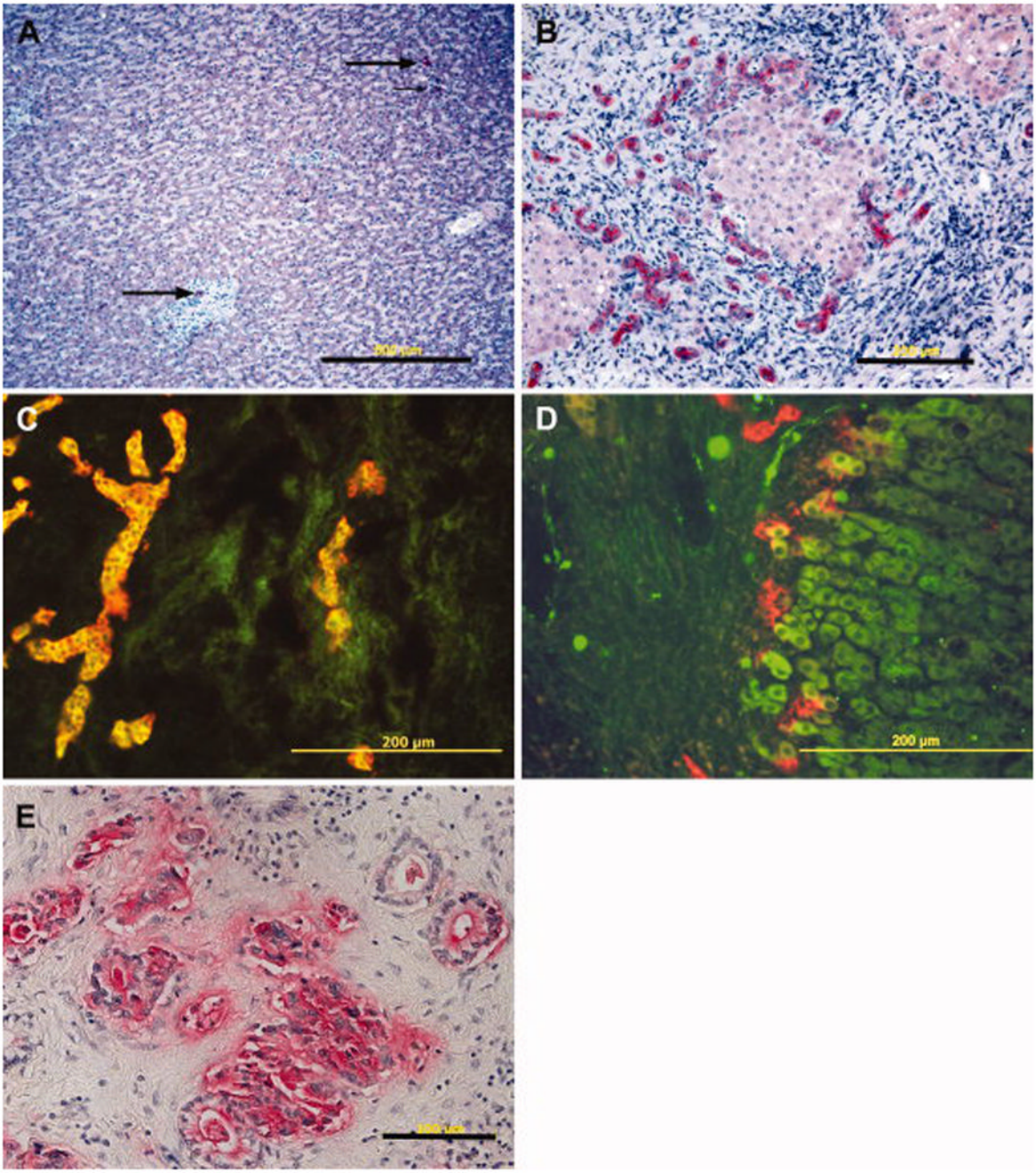

Figure 1.

GCTM-5 marks putative progenitors of liver regeneration and neoplasia of biliary origin. **(A):** Normal liver shows GCTM-5-reactive biliary ducts (red; arrows) confined to the small areas of the portal tracts (scale bar = 500 μm). **(B):** Cirrhotic liver demonstrates a regenerative hepatocyte nodule (center) surrounded by an expanded population of GCTM-5-expressing biliary ducts (scale bar = 200 μm). **(C):** Cirrhotic liver demonstrates overlapping coexpression (yellow) of EpCAM (red) and GCTM-5 (green) on a subpopulation of biliary ducts by immunofluorescence; note that not all the GCTM-5-expressing cells are also expressing EpCAM (scale bar = 200 μm). **(D):** Immunofluorescence showing GCTM-5 (red) and albumin (green) coexpression (yellow) at the periphery of a regenerating nodule

(scale bar = 200 μm). **(E):** Intrahepatic cholangiocarcinoma variably express the GCTM-5 antigen (red) (scale bar = 100 μm).

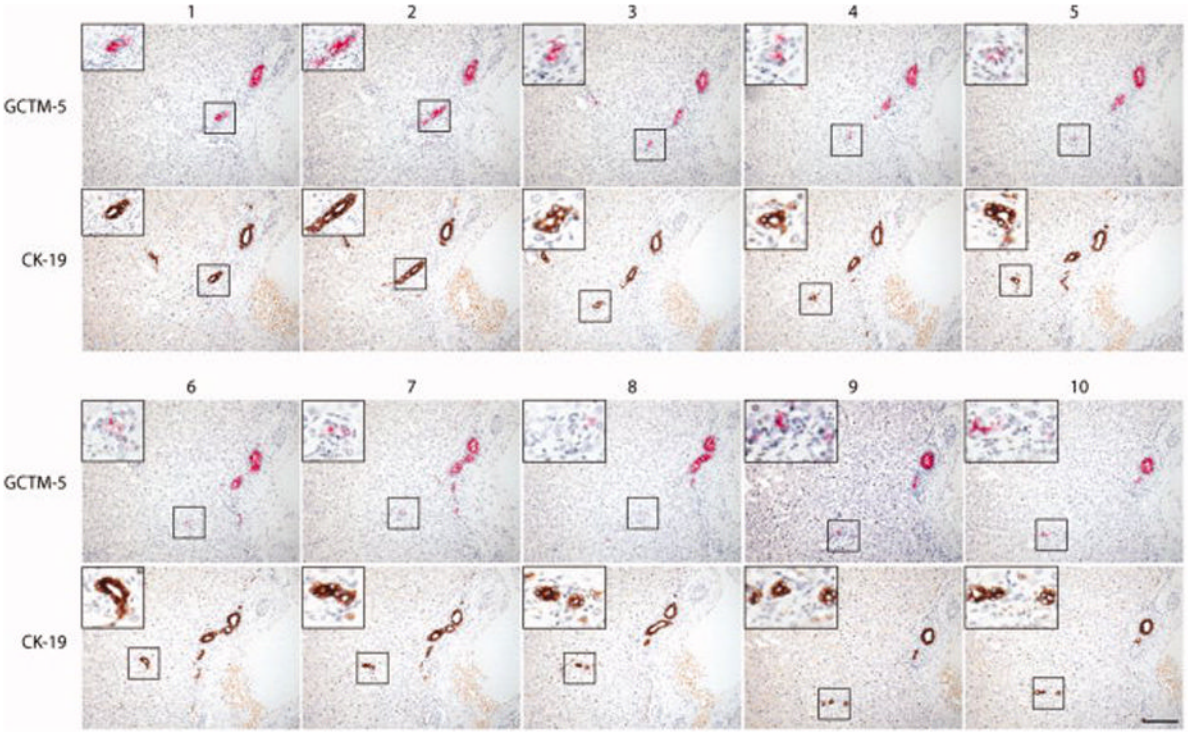

Figure 2.
Distribution of GCTM-5 staining in the biliary tract. Serial sections of normal pediatric liver stained with GCTM-5 (top, pink) and CK19 (bottom, brown) antibodies. Each section is 5-μm thick. Scale bar = 100 μm.

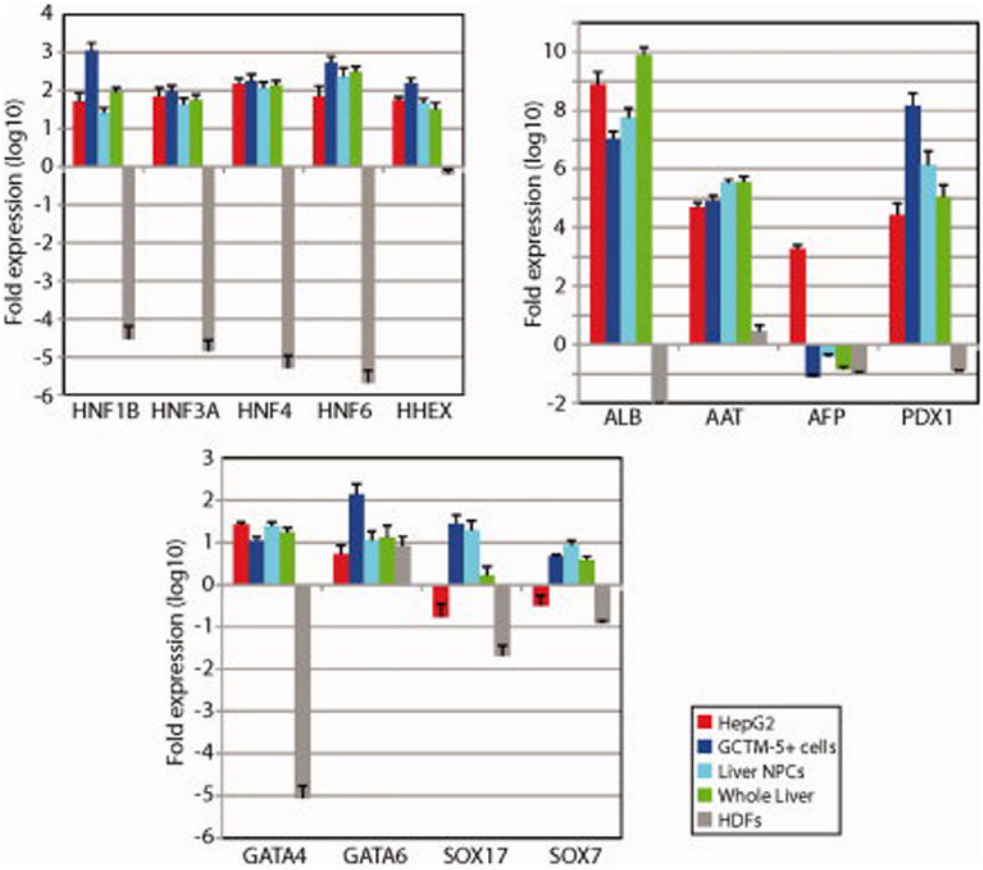

Figure 3.

QRT-PCR analysis of gene expression in GCTM-5-positive cells from nonparenchymal fraction of adult liver. Nonparenchymal cells were isolated by immunomagnetic affinity, and QRT-PCR was carried out on positive cells, the total nonparenchymal cell fraction, and whole liver. HepG2 hepatocellular carcinoma cells and human dermal fibroblasts were included as controls. Graphs display log of relative expression. Abbreviations: HDF, human dermal fibroblasts; NPC, non-parenchymal cells.

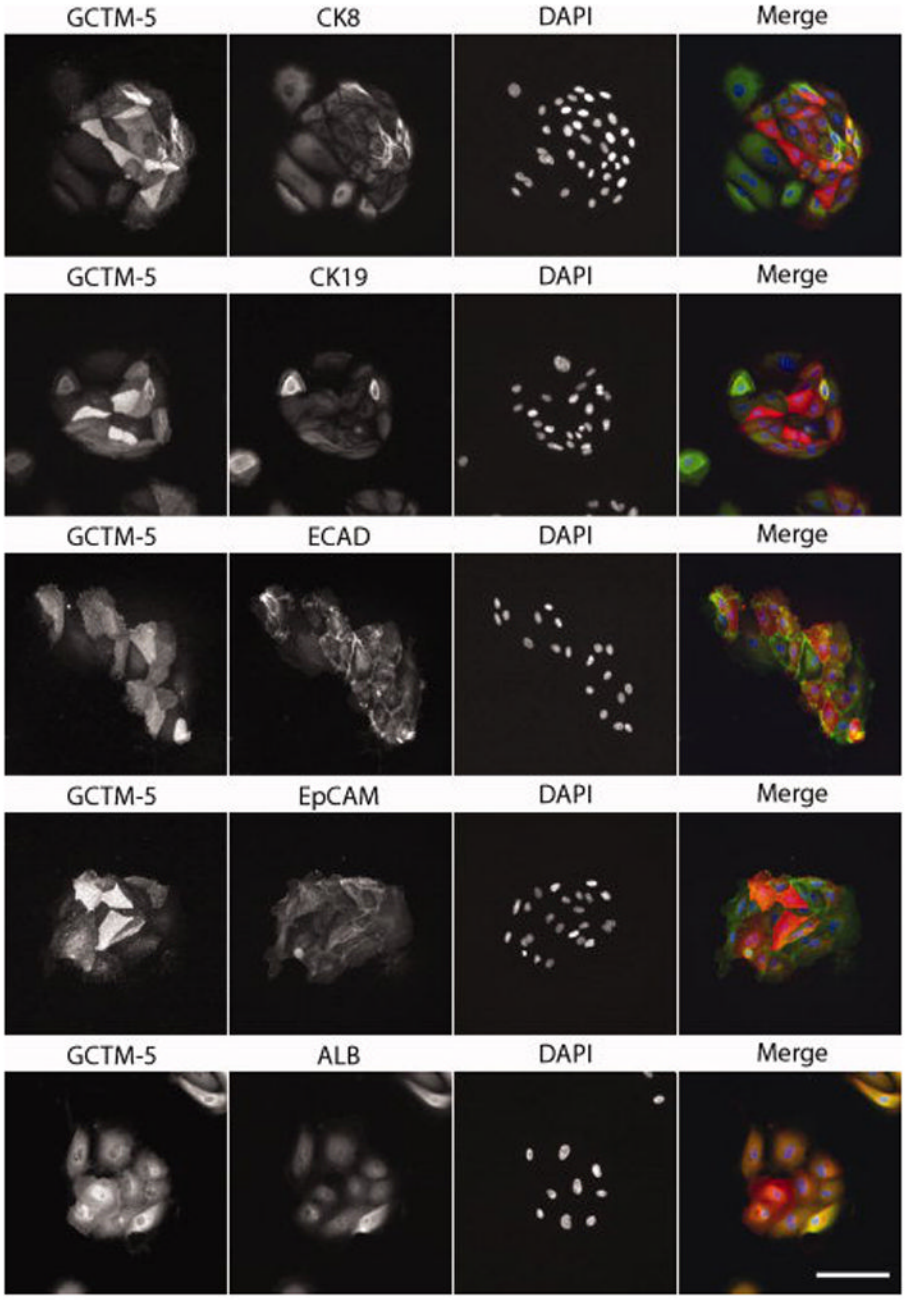

Figure 4.

Indirect immunofluorescence microscopy of hepatic marker expression in cultured cell colonies derived from GCTM-5-positive cells in the nonparenchymal fraction of adult liver. Fixed colonies were stained with GCTM-5 and various cell surface and intracellular markers, then counterstained with DAPI (scale bar = 50 μm). Abbreviation: DAPI, 4′,6-diamidino-2-phenylindole.

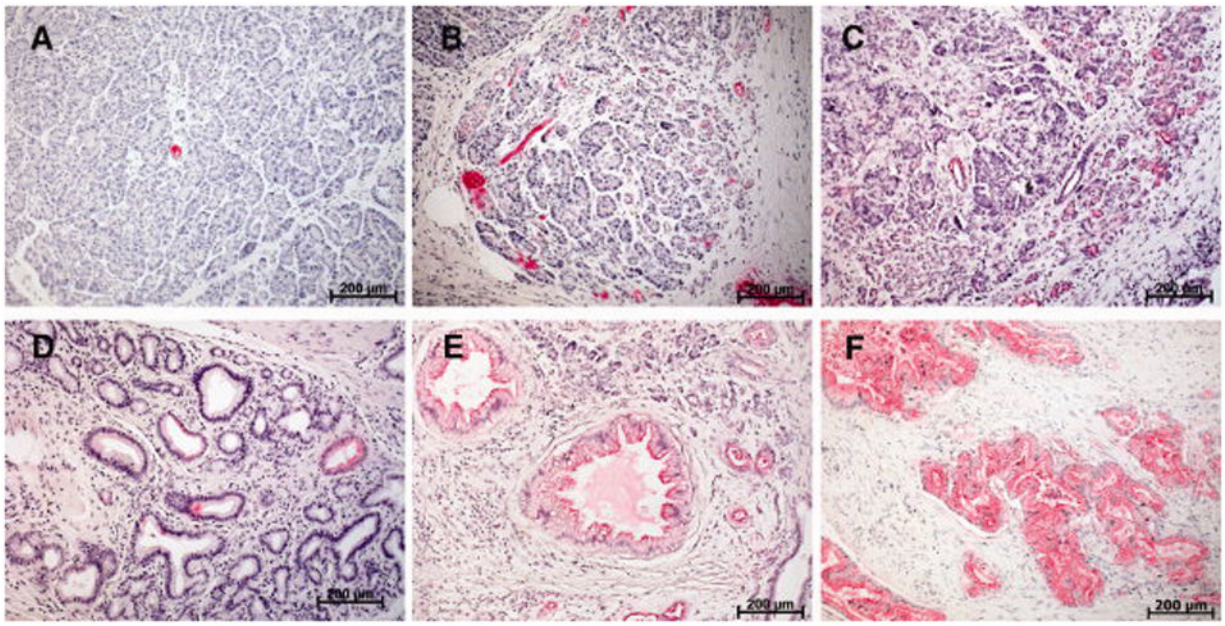

Figure 5.

GCTM-5 antigen expression in normal and pathologic pancreatic tissues. **(A):** An exocrine lobe shows a single focus of GCTM-5 staining (red) in a small ductule in cross-section among a larger field of acini. **(B):** In an area of mild fibrosis due to chronic pancreatitis, the expression of GCTM-5 antigen in ductules is expanded. **(C):** In severe chronic pancreatitis, GCTM-5 expression detected a subpopulation of cells in an expansion of ductules also known as a tubular complex formation or acinarductal metaplasia. **(D):** An area of acinar lobe replaced by a range of irregular duct-like structures that express the GCTM-5 antigen. **(E):** Pancreatic intraepithelial neoplasia lesions consistently express the GCTM-5 antigen. **(F):** Adenocarcinoma strongly expresses GCTM-5 antigen in the cytoplasm, membrane, and mucinous material.

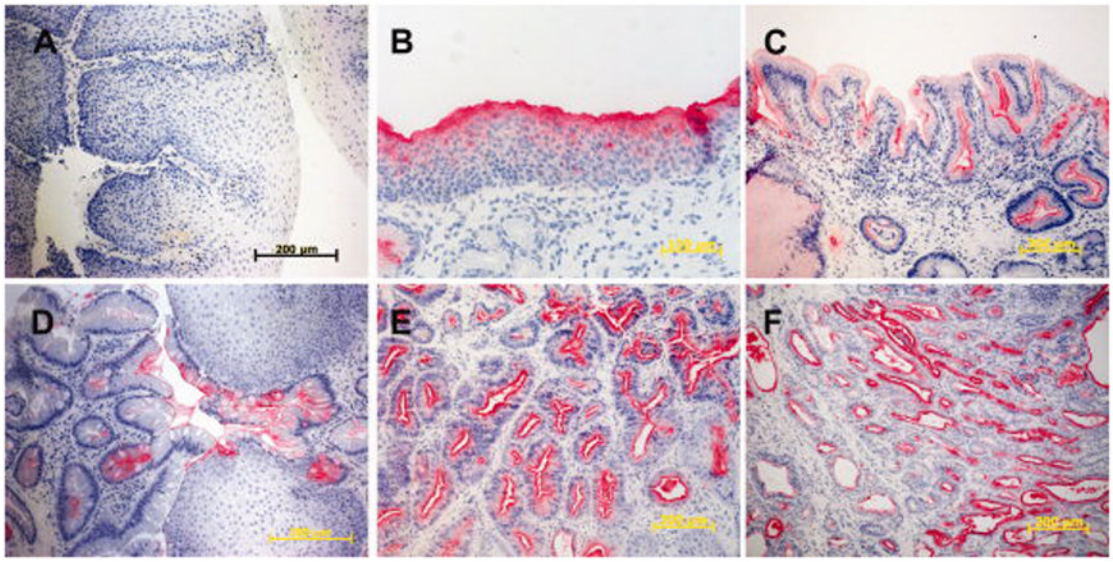

Figure 6.

Distribution of the GCTM-5 antigen in metaplastic columnar epithelia of the esophagus. **(A):** Squamous epithelium does not express the antigen. **(B):** The multilayered epithelium shows surface mucous cells with abundant GCTM-5 antigen (red). **(C):** Cardia-type metaplasia expresses the antigen in a subpopulation of the epithelia. **(D):** Intestinal metaplasia expresses the GCTM-5 antigen in the apical membrane mainly in the deep pits and glands. Dysplasia **(E)** and adenocarcinoma **(F)** react more extensively with GCTM-5.

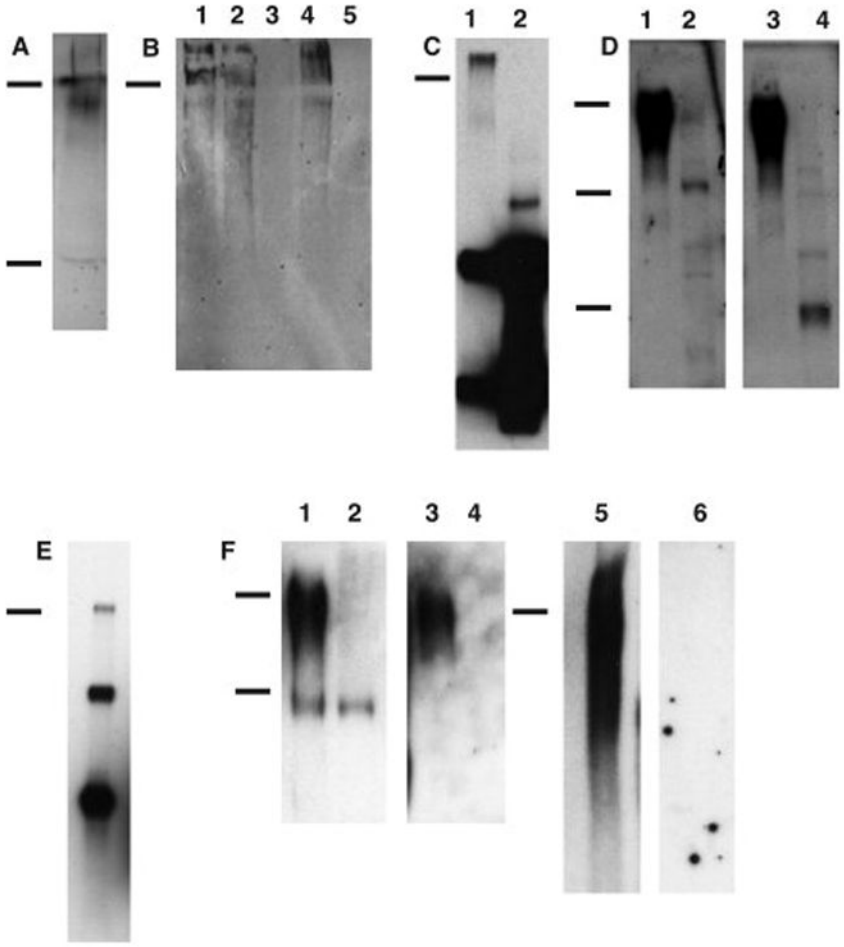

Figure 7.

Biochemical characterization of the GCTM-5 antigen. **(A):** Immunoblot of CFPAC-1 cell lysate with GCTM-5. Positions of 200 and 55 kDa markers are shown. **(B):** GCTM-5 immunoblot of CFPAC-1 conditioned media treated with no enzyme control (lane 1), *N*-Glycanase (lane 2), sialidase A (lane 3), *O*-Glycanase (lane 4), and all three enzymes (lane 5). Position of 200 kDa marker is shown. **(C):** Immunoprecipitate of concentrated CFPA-1 conditioned medium blotted with GCTM-5. Lane 1, GCTM-5 immunoprecipitate; lane 2, control IgG1. Position of 250 kDa marker is shown. Lower bands are immunoglobulins reacting with the secondary antibody. **(D):** Immunoblot of affinity purified GCTM-5 antigen (lanes 1 and 3) or CFPAC-1 concentrated conditioned medium (lanes 2 and 4) with antisera to C-terminal (lanes 1 and 2) or N-terminal (lanes 3 and 4) peptide sequences of FCGBP. Positions of 250, 105, and 50 kDa markers are shown. **(E):** Immunoblot of GCTM-5 immunoprecipitate of CFPAC-1 conditioned medium probed with antiserum against C-terminal peptide sequence of FCGBP. Position of 105 kDa marker is shown. Lower bands are immunoglobulins reacting with secondary antibody. **(F):** Immunoblots of affinity purified GCTM-5 antigen (lanes 1, 3, 5, and 6) or concentrated CFPAC-1 antigen (lanes 2 and 4) probed with C-terminal peptide sequence of FCGBP (lanes 1 and 2 detected with anti-rabbit Ig, lane 5 with anti-rabbit Ig $F(ab')_2$ precleared with Protein A) or anti-rabbit Ig alone (lanes 3 and 4) or anti-rabbit Ig $F(ab')_2$ pre-cleared with Protein A alone (lane 6). Positions of 250 and 105 kDa markers are shown.