Distribution of Pyruvate Dehydrogenase Dihydrolipoamide Acetyltransferase (PDC-E2) and Another Mitochondrial Marker in Salivary Gland and Biliary Epithelium from Patients with Primary Biliary Cirrhosis

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Previous studies in which quantitative immunofluorescence was used have shown that certain biliary epithelial cells in liver with primary biliary cirrhosis show increased levels of pyruvate dehydrogenase dihydrolipoamide acetyltransferase compared with controls. This study was designed to determine whether the increase in intensity of pyruvate dehydrogenase dihydrolipoamide acetyltransferase in biliary epithelial cells is accounted for by an increase in the number of mitochondria in the same cells. A double-antibody staining technique was used with antibodies specific for pyruvate dehydrogenase dihydrolipoamide acetyltransferase and another mitochondrial inner membrane marker, recognized by the mouse monoclonal antibody MCA151A. Distribution of the antigens was studied in sections of liver and salivary gland, an additional site that is frequently involved in primary biliary cirrhosis. Confocal microscopy was used to quantify the intensity of fluorescence resulting from binding of fluorochrome-labeled antibody. In both liver and salivary glands MCA151A binding was similar in normal and sections with primary biliary cirrhosis and corresponded to the predicted distribution of mitochondria in these tissues. In the liver staining was less intense in biliary epithelial cells than in hepatocytes. In salivary gland binding of both antibodies was predominantly localized to duct cells, with those forming striated ducts, known to be rich in mitochondria, being most intensely stained. There was high coincidence of the two antigens in salivary glands (p < 0.01) and in biliary epithelial cells from normal liver (p = 0.01). However, in liver with primary biliary cirrhosis, despite high coincidence between the antigens on hepatocytes, biliary epithelial cells showed high intensity of pyruvate dehydrogenase dihydrolipoamide acetyltransferase but not MCA151A. The results indicate that an increase in mitochondria does not account for high intensity of pyruvate dehydrogenase dihydrolipoamide acetyltransferase in biliary epithelial cells in liver with primary biliary cirrhosis. (HEPATOLOGY 1994;19:1375-1380).

PBC is characterized by circulating antimitochondrial antibodies (AMAs), which recognize the central, E2 components of the 2-oxo-acid dehydrogenase complexes. Of these, the major antigen is the core enzyme of the mitochondrial inner membrane pyruvate dehydrogenase complex, dihydrolipoamide acetyltransferase (PDC-E2) (1, 2). However, the reason for the close association between PBC and production of specific AMAs remains unclear. Previous studies demonstrated that biliary epithelium of certain bile ducts in primary biliary cirrhotic liver contain high levels of immunoreactive material, which is detected by antibodies specific for the E2 component of PDC (3, 4). The reason for this is unknown and unexpected because biliary epithelial cells (BECs) are reported to contain few mitochondria relative to hepatocytes (5).

When transmission electron microscopy was used, an increase in the number of mitochondria in BECs in some ducts of primary biliary cirrhotic liver was noted (6). In another study enlargement of mitochondria in BECs was reported in PBC (7). Thus it is possible that high intensity of PDC-E2 seen in BECs of some bile ducts in liver with PBC (3, 4) may be directly attributable to an increase in the number of mitochondria in the cells lining these ducts.

This study was designed to test the hypothesis that the high level of PDC-E2 in BECs is a result of an increase in mitochondria in these cells. In view of problems associated with adequate representative sampling when electron microscopy is used, particularly in tissue, such as liver, in which biliary epithelium is a minor component, a double-antibody labeling method

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has been used for comparing the distribution and intensity of PDC-E2 with that of another mitochondrial marker recognized by the antibody MCA151A (8). Antibody binding was quantified by laser scanning confocal microscopy of sections of liver and salivary gland obtained from patients with PBC and normal subjects. Patients with PBC frequently show symptoms associated with polyglandular disease including pancreatic insufficiency and sicca syndrome (9). Thus salivary gland was used in this study to determine whether elevation of PDC-E2 was localized to the liver or whether there was evidence for increased levels in another epithelial cell system.

MATERIALS AND METHODS

**Antibodies.** Antibodies specific for the denatured E2 subunit of bovine heart PDC were raised in rabbits (3, 10), and the specificity and cross-reactivity of the affinity-purified antibodies were checked by Western blotting against a variety of tissues and cell lines including human, porcine and bovine cell lines (3, 11, 12). The mouse monoclonal antibody, MCA151A (Serotec, Oxford, UK) was originally generated by immunization with mitochondrial membranes prepared from normal human liver (8). This antibody is specific for mitochondrial inner membrane and shows typical mitochondrial distribution on normal tissue sections (8).

**Source of Tissue.** To obtain sufficient bile ducts for evaluation, we used hepatocytectomy specimens from the orthotopic liver transplantation program. Normal liver specimens (n = 7) were obtained from graft reductions for pediatric liver transplantation recipients, and liver with PBC (n = 8) was obtained from hepatocytectomy specimens removed from patients undergoing liver allografting. In all cases the diagnosis of PBC had been confirmed by histological and serological investigation. Labial salivary gland biopsy was performed under local anesthesia on patients with PBC (n = 8, with symptoms of xerostomia present in six) and normal healthy control subjects (n = 7). One-centimeter cubes of liver were snap-frozen in liquid nitrogen and stored at -70°C. Salivary gland biopsy specimens were stored in liquid nitrogen.

**Staining Sections.** Cryostat sections (5 μm) were fixed in acetone for 10 min at room temperature. Sections were incubated simultaneously with a mixture of the primary antibodies (anti-E2, 1:50 and MCA151A, 1:20; determined as optimal concentrations in preliminary tests) at room temperature for 1 hr. After washing (three times for 5 min in PBS), the sections were incubated with the secondary antibodies (goat antirabbit FITC, 1:100; biotinylated goat antimouse, 1:100), previously absorbed with normal human liver homogenate to remove cross-reactivity (13). After washing (three times for 5 min in PBS) the sections were incubated in streptavidin Texas Red (Vector Laboratories Ltd., Peterborough, Cambridgeshire, UK) (1:100). Sections serial to the double-stained sections were labeled singly with anti-E2 and treated with biotinylated goat antimouse and streptavidin Texas Red, whereas others were labeled singly with MCA151A and treated with goat antirabbit FITC to establish the level of cross-reactivity between primary and secondary antibodies.

**Scanning Laser Confocal Microscopy.** Sections were coded and read without knowledge of their identity. Fluorescence images were collected and analyzed by laser scanning confocal microscopy, with the MCR-500 system (Bio-Rad, Microscience Div., Cambridge, MA) in the dual-channel mode, based on the laser line at 514 nm. Under these conditions single labeling appears red (Texas Red) and green (FITC); areas of coincident labeling appear yellow. Optical sectioning in the z axis was performed at 0.5-μm increments. Relative fluorescence intensity was quantified by color banding, and the section showing the highest level in each Z series was used for the analysis. The threshold for positive staining was based on the level of background staining recorded on control slides treated with inappropriate primary antibodies.

Although the procedure for collection and storage of salivary gland biopsy specimens was identical for normal subjects and patients with PBC, there were differences between the handling of livers from patients with PBC and those from normal subjects. Normal liver was perfused with tissue preservation fluid and maintained at 4°C for up to 12 hr before specimens were taken, whereas samples from liver with PBC were from the unperfused organ and were processed within 1 hr of hepatectomy. Thus because of variation in preparation procedure between samples of liver from different subjects, direct comparison between antibody binding in sections from different subjects was not possible. To circumvent this problem, measurements on sections of liver were standardized by comparison of antibody binding on bile ducts with that on an adjacent area of hepatocytes (Fig. 1, f). The fluorescence intensity within a defined area occupied by bile duct cells or hepatocytes was color-coded (intensity 0 to 255, Fig. 1, g-h), and the area showing high fluorescence (146-255) was expressed as a percentage of the total stained area (37-255). Unstained, nonfluorescent areas were excluded from the calculation so that results relate directly to antibody binding to antigen in cells and are unaffected by the presence of the lumen of the ducts or the sinusoids between hepatocyte cords.

**Statistical Analysis.** PDC-E2-related fluorescence was correlated with MCA151A-related fluorescence by Kendall’s correlation. Differences between fluorescence intensity of the two markers on individual sections and between disease and control samples as appropriate were determined by Wilcoxon signed rank test. P Values of less than 0.05 were considered significant.

**RESULTS**

The typical distribution of antibody binding on sections of salivary gland and liver is shown in Figure 1. In all tissues studied, distribution of the two antigens (PDC-E2 and MCA151A) showed a high degree of coincidence. A notable exception was the biliary epithelium lining bile ducts in liver with PBC. Differences in staining pattern on BECs in liver with PBC relative to the other epithelia studied are subject to quantitative analysis in Figures 2 and 3.

**Salivary Gland Sections.** In salivary glands (Fig. 1, a-e) MCA151A and PDC-E2 positivity was consistent with the predicted distribution of mitochondria in this tissue (14) and was similar in both patient and control tissue. In double-stained sections a high degree of coincident staining was seen within ductal epithelium. Striated ducts, which contain numerous mitochondria (14), exhibited the strongest staining for both antigens (Fig. 1, c-e). Weak, patchy staining of acini appeared to be mainly localized to myoepithelial cells, which expressed PDC-E2 more intensely than MCA151A in both PBC and control specimens (Fig. 1, a-e). A significant positive correlation was found between intensity of fluorochrome label binding to anti-
PDC-E2 and MCA151A in salivary ducts ($p = 0.01$; 21 ducts analyzed in seven sections, three normal and four with PBC). Significant difference between the fluorescence intensity of the two antigens was not found ($p = 0.65$).

**Liver Sections.** In both normal liver and liver with PBC the distribution of MCA151A staining was weak on BECs compared with staining on hepatocytes (Fig. 1, g, Fig. 2). In normal liver MCA151A and PDC-E2 showed a high degree of coincident staining on both hepatocytes and BECs lining bile ducts (Figs. 2 and 3). However, in liver with PBC, although high coincidence between the two markers was seen on hepatocytes (Fig. 1, i), this was not seen on BECs; high intensity of PDC-E2 was observed on BECs, whereas MCA151A intensity was low (Fig. 1, g-i, Fig. 3). Periportal hepatocytes in liver with PBC showed stronger staining intensity than centrilobular hepatocytes with both anti-PDC-E2 and MCA151A antibodies. However, despite the higher staining intensity of PDC-E2 on periportal hepatocytes with PBC, the percentage area of high PDC-E2–related fluorescence on BECs relative to hepatocytes was significantly higher in patients with PBC than in normal control subjects (Fig. 2).

The high intensity staining for PDC-E2 on BECs in liver with PBC was patchy; some ducts showed very high intensity, whereas others were moderately stained (Fig. 3). In one duct analyzed in liver with PBC, the intensity of MCA151A was observed to be higher than that of PDC-E2 (Fig. 3). BECs in liver with PBC showed significantly more PDC-E2–related fluorescence than hepatocytes in the same specimen ($p < 0.01$, Wilcoxon signed-rank test). This was not seen when MCA151A was analyzed in this way. With one exception, BECs showed significantly less MCA151A-related fluorescence than hepatocytes in the same specimen ($p < 0.01$), and the ratio of MCA151A-related fluorescence on bile ducts relative to hepatocytes was not significantly different in patients with PBC from the ratio in control subjects (Fig. 2) ($p = 0.68$, Wilcoxon signed-rank test).
DISCUSSION

In this study we examined the possibility that the previously reported high intensity of PDC-E2 in the biliary epithelium of liver with PBC (3, 4) may result from an increase in mitochondria in these cells. In previous studies the periportal hepatocytes of liver with PBC showed higher intensity of PDC-E2 than centrilobular hepatocytes (3). In this study higher intensity of PDC-E2 in periportal hepatocytes was accompanied by higher intensity of MCA151A in these cells. These observations suggest that localized mitochondrial metaplasia may contribute to the higher intensity of PDC-E2 in periportal hepatocytes. However, no evidence was found for a significant increase of mitochondria in BECs in liver with PBC as judged by the distribution of MCA151A. Because the two antibodies used are specific for antigens normally found on the mitochondrial inner membrane, a similar distribution for both was predicted, with a high degree of coincident labeling. Indeed, this was the case in most of the cell types studied. Although the identity of the antigen recognized by MCA151A is unknown (8), MCA151A was selected as a mitochondrial marker because its reaction is limited solely to mitochondrial inner membrane (8). In preliminary studies MCA151A binding was found to have a distribution similar to that of the mitochondrial outer membrane enzyme, monoamine oxidase, in both liver and salivary gland sections. However, for a colocalization study, it was considered important that a mitochondrial inner membrane marker be used. Thus despite uncertainty about the precise antigen recognized by MCA151A, for the double labeling study, the mitochondrial inner membrane marker was used.

Sections from hepatectomy specimens were used to increase the number of bile ducts available for evaluation. The possibility that use of liver with end-stage cirrhosis was suboptimal in that results may be a consequence of advanced hepatic disease as opposed to PBC-related phenomena is considered unlikely for several reasons. First, in previous work, samples of liver from patients with end-stage cirrhosis of other types, including sclerosing cholangitis and cryptogenic cir-
rhosis, were studied (3). It was shown that abnormal distribution of PDC-E2 on BECs was restricted to PBC; other cirrhotic conditions showed no difference from normal controls (3, 4). Second, it has been shown, with biopsy specimens, that the abnormal distribution of PDC-E2 on BECs is a feature of early disease (4). Finally, even in liver with end-stage cirrhosis there is continued inflammatory activity in portal tracts, suggesting that stimulation by antigen is still occurring.

Although many patients with PBC have symptoms of sicca syndrome (9), it is not clear whether this and other extrahepatic features of PBC represent associated conditions or are part of the primary disease process. In this study we have found elevation of PDC-E2 to be restricted to BECs in liver with PBC. No evidence for elevated PDC-E2 was found in the epithelium of salivary glands. Although the possibility of sampling error because of use of biopsy material from salivary glands cannot be ruled out, the data suggest that elevated PDC-E2 is restricted to the liver.

Previous reports suggested increases in both size and number of mitochondria in BECs of liver with PBC (6, 7). In this study one duct of those analyzed in liver with PBC stained with high intensity for MCA151A, indicating that some ducts may indeed contain more mitochondria. However, the duct with strong positivity for MCA151A did not show correspondingly higher intensity for PDC-E2 than ducts that were only weakly stained with MCA151A. Thus we conclude that high intensity of PDC-E2 on bile ducts in PBC is not explained by an increase in mitochondria in BECs lining these ducts. Although our results demonstrate a clear difference in the distribution of PDC-E2 between BECs in the livers of patients with PBC and control BECs, it is not possible to determine from the available data whether these observations are of primary disease processes or are secondary consequences of PBC.

Intracellular traffic and targeting of mitochondrial proteins is mediated through specific leader sequences, which are subsequently cleaved once their destination is reached (15). It is possible that the diffuse high density of PDC-E2 on BECs in liver with PBC could be a consequence of changes to the sequence responsible for successful targeting of PDC-E2 to mitochondria. Failure in correct localization may lead to accumulation of enzyme at an inappropriate intracellular location. Thus defective targeting of PDC-E2 provides one possible hypothesis for the nonmitochondrial distribution of PDC-E2 in PBC BECs. It is also possible that high density of intracellular enzyme could lead to the inappropriate appearance of PDC-E2 at the plasma membrane as reported for BECs from patients with PBC (16, 17). It is also equally possible that a cross-reactive epitope present in a different (non-PDC) protein may be recognized by anti-PDC-E2 antibody. Further work is required to characterize the BEC proteins that react with PDC-E2 antibody.

In conclusion, this study provides further evidence suggesting abnormal concentration of PDC-E2 in biliary epithelium of liver with PBC. This could be accounted for by a defect in the molecular mechanisms involved in mitochondrial import of the polypeptide, resulting in an
accumulation of antigen unrelated to the distribution of mitochondria. Further studies are required to determine the precise subcellular localization of PDC-E2 in BECs.

REFERENCES


