Application of Keratin Immunocytochemistry and Sirius Red Staining in Evaluating Intrahepatic Changes with Acute Extrahepatic Cholestasis Due to Hepatic Duct Carcinoma

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Summary

A series of 10 cases of biliary obstruction due to primary cholangiocarcinoma has been studied with histological and immunocytochemical means. The total duration of cholestasis (as manifested by jaundice) was between 2 and 11 weeks with variable periods of preoperative drainage. Liver biopsy specimens taken during surgery for cholangiocarcinoma were investigated for the presence of ductular proliferation and the development of fibrosis, as demonstrated by Sirius Red F3BA collagen staining. The differentiation of epithelial components was evaluated by AEC-immunostaining with chain-specific monoclonal antibodies specifically directed against human keratins type 7, 18 and 19. Keratin 7, normally occurring only in the ductular system, was expressed in hepatocytes at the periphery of the hepatic lobule (zone 1) following about 4 weeks' cholestasis, when an increase of ductular profiles in the enlarged portal areas had become manifest. Such keratin 7 positive cells, however, still retained all morphological aspects of hepatocytes. Keratin 19, normally also restricted to the ductular system in liver, is not expressed by zone 1 hepatocytes even after longer duration (up to 11 weeks) of cholestasis. It is concluded that the increase in ductular profiles during the first weeks is mainly due to proliferation of pre-existing ductules, while ductular metaplasia occurs in more chronic cholestasis. Development of fibrosis, not always strictly paralleling the multiplication of ductular profiles in sections through a portal tract, represents an early change, and is clearly apparent after 2 weeks of obstruction.

Key words

Cholestasis – Hepatic duct carcinoma – Immunocytochemistry – Staining

Introduction

In man various causes of obstruction of the extrahepatic bile ducts are known (reviewed by Desmet, 1). Among the most frequent diseases causing such cholestatic conditions are gallstones, strictures of the biliary tree, and cancers of the extrahepatic bile ducts. Benign obstructions due to gallstones, strictures or pressure on the duct by enlargement of the pancreatic head entail complete and lasting obstruction of bile flow in a minority of cases (2). Bile duct carcinoma, however, is frequently found as an adenocarcinoma of the hepatic duct at its bifurcation in the porta hepatitis, the so-called Klatskin tumor (3), and as a rule leads to complete obstruction of bile flow in an early phase. Owing to the death of the patients from cachexia, metastases or other causes, long-term follow-up studies of intrahepatic changes in acute obstructions due to malignancy have seldom been possible (4).

The introduction of modern surgical techniques enabling resection of early cases of bile duct carcinoma (5) made it possible to study early intrahepatic changes caused by complete extrahepatic obstruction in surgical biopsy specimens.

For the reasons mentioned above, changes in the intrahepatic bile ducts caused by extrahepatic obstruction have been studied mainly in cases with long-standing intermittent or partial obstruction due to strictures, stones or extrinsic compression of the bile ducts (6, 7). In these cases, ductular proliferation and periductular fibrosis have been described as typical morphologic features (reviewed in 1, 2, 8, 9, 10). The same morphological changes as observed in human pathology have been found in experimentally induced extrahepatic obstructions in rabbits, rats and dogs (11, 12, 13, 14, 15, 16).

Multiplication of ductules in portal areas as an early feature of cholestasis may be due – at least in part – to mitogenic effects of epidermal growth factor, which is secreted by hepatocytes into the bile and accumulates in the obstructed ductular system (17). On the other hand, in long-standing incomplete or intermittent obstruction of extrahepatic bile ducts, phenotypic changes in the hepatocytes around terminal portal tracts seem to indicate a gradual transformation of hepatocytes into ductular cells. This has been supported by the immunohistochemical demonstration of keratins specific for ductules (18, 19, 20) and of the S-100 protein (21). However,
Table 1

<table>
<thead>
<tr>
<th>Age</th>
<th>Sex</th>
<th>Duration of jaundice (weeks)</th>
<th>Liver histology</th>
<th>Collagen configuration</th>
<th>Block No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td>m</td>
<td>2</td>
<td>Preserved lobular architecture, discrete cholangioliisis, slight increase of ductules in portal fields</td>
<td>Slight increase in portal fields, discrete radiation into parenchyma</td>
<td>1368</td>
</tr>
<tr>
<td>53</td>
<td>f</td>
<td>4</td>
<td>Variable increase of ductuli in portal fields surrounded by polymorphonuclears. Centrilobular cholestasis with intercellular bilirubin plugs</td>
<td>Slight increase in collagen density in portal fields</td>
<td>1367</td>
</tr>
<tr>
<td>69</td>
<td>m</td>
<td>4</td>
<td>Widespread increase of ductuli and cholangioliisis; centrilobular accumulation of bilirubin with intra- and interlobular localization</td>
<td>Considerable thickening of collagen strands in portal fields, radiating into the lobules</td>
<td>1213</td>
</tr>
<tr>
<td>69</td>
<td>m</td>
<td>1 + 4*</td>
<td>Increase of ductular profiles in portal tracts with infiltration of mononuclear cells; centrilobular cholestasis. Macrovesicular steatosis</td>
<td>Coarse collagen accumulation in portal areas penetrating into lobules</td>
<td>1234</td>
</tr>
<tr>
<td>54</td>
<td>f</td>
<td>4 + 2*</td>
<td>Portal fields edematous with ductular proliferation and cholangioliisis. Centrilobular cholestasis</td>
<td>Radiating strands of collagen from the portal fields</td>
<td>1191</td>
</tr>
<tr>
<td>62</td>
<td>m</td>
<td>5 + 2*</td>
<td>Centrilobular cholestasis, slight increase of ductular structures, bilirubin in macrophages; mononuclear infiltration of portal fields</td>
<td>Coarse bands of collagen radiation from the portal fields</td>
<td>1229</td>
</tr>
<tr>
<td>63</td>
<td>f</td>
<td>7</td>
<td>Portal fields edematous with noticeable increase of ductules and cholangioliisis</td>
<td>Strong fibrotic strands radiating into parenchyma</td>
<td>1230</td>
</tr>
<tr>
<td>55</td>
<td>m</td>
<td>7 + 1</td>
<td>Severe periportal fibrosis. Variable degrees of cholangioliisis. Centrilobular cholestasis; macrovesicular steatosis</td>
<td>Periportal connected trabeculae of collagen</td>
<td>1242</td>
</tr>
<tr>
<td>52</td>
<td>m</td>
<td>5 + 3*</td>
<td>Edematous portal tracts with noticeable increase of ductular profiles; cholangioliisis, bile infarcts</td>
<td>Thick periportal collagen bands, discrete radiation from portal fields</td>
<td>1196</td>
</tr>
<tr>
<td>47</td>
<td>f</td>
<td>1 + 10*</td>
<td>Lobular architecture with ductular proliferation and cholangioliisis centrilobular accumulation of bilirubin</td>
<td>Concentration of dense collagen in portal fields, strands into lobules less obvious</td>
<td>1211</td>
</tr>
</tbody>
</table>

* Second figures: numbers of weeks of pre-operative drainage

the situation has not been systematically investigated in acute extrahepatic cholestasis in man, undoubtedly because of the scarcity of material.

This paper describes findings obtained with histological and immunohistological methods using monoclonal keratin antibodies in livers with acute cholestasis due to hepatic duct carcinoma.

Material and methods

Ten patients who underwent surgery for a histologically confirmed tumor of the common bile duct were selected for this study. All patients had a history of permanent jaundice of sudden onset. Details of the surgical procedure applied are described elsewhere (22, 23). The exact duration of the jaundice was known for all patients. However, a majority of the patients had an endoprosthesis inserted into the common bile duct for one to several weeks prior to operation, in order to restore bile flow. As this drainage (never a complete re-establishment of the bile flow) had variable success, it was difficult to ascertain its influence on the classification of patients with regard to the duration of complete cholestasis.

Histological techniques

Surgical biopsies of approximately 0.5 cm³ were taken from the lower border of the liver. One half of the biopsies were immediately frozen in liquid nitrogen and stored at −80°C until further use. The other half were fixed in Heidenhain’s USA mixture (24) for 4 hours, dehydrated in an alcohol series and embedded in Paraplast (Gurr, High Wycombe, England). Sections of 5 µm were cut and stained with the polychrome method of Shroeder (25) for histological examination. Sirius Red F3BA (Chroma AG, Stuttgart, FRG) in saturated picric acid was used for collagen staining in accordance with James et al. (26).

For immunocytochemistry, 8 µm, thick cryostat sections were cut, incubated with the different antisera and treated with an indirect immunoperoxidase method as follows:
Frozen sections were air-dried and fixed in pure acetone for 1/2 min at room temperature. Thereafter, the slides were rinsed in phosphate-buffered saline (PBS) and incubated for 45 min with the primary antisera in a moist incubation chamber at room temperature. After three rinses of 2 min in PBS the slides were incubated with horseradish-peroxidase labeled rabbit-anti-mouse IgG (Dakopatts, Glostrup, Denmark), diluted 1:200 with 3% normal human serum for 30 min at room temperature and rinsed again.

Peroxidase activity was detected by incubation with 3-amino-9-ethylcarbazol (AEC). For this purpose, 10 ml dimethylformamide in which 40 mg AEC (Sigma, St. Louis, USA) had been dissolved, were added to 190 ml Na-acetate buffer (0.05 M pH 4.90). The solution was mixed with a glass pipette and finally 70 ml 30% H2O2 were added to the mixture, resulting in an end concentration of 0.01%.

The sections were incubated in this freshly made AEC solution for 10 min. at room temperature and the reaction was stopped with distilled water. Sections were counterstained with diluted hematoxylin and mounted in glycerin-gelatin.

The following monoclonal keratin antibodies have been used:

1. RCK 105 (IgG2a; 27) reacts only with keratin 7 in immunoblotting and stains a subgroup of glandular epithelia and their tumors, as well as transitional bladder epithelium and bladder carcinomas.

2. RCK 106 and CK 18-2 (IgG1; 27) are monospecific for keratin 18 in immunoblotting. In general these two antibodies recognize columnar epithelial cells from digestive, respiratory, and urogenital tracts, endocrine and exocrine tissues, transitional epithelium and mesothelial cells, as well as their tumors. Generally, no reaction is found in squamous epithelia or squamous cell carcinomas.

3. LP2/4 (28) binds to most simple epithelia and basal cells in non-keratinizing stratified squamous epithelia. The antibody recognizes only keratin 19 in immunoblotting assays.

Control reactions (complete immunostaining procedure without primary antiserum) showed a completely negative picture, with the exception of macrophages in portal tracts, which showed AEC staining as a result of endogenous peroxidase activity.

Results
A summary of the patient population with qualitative estimation of the findings with Sirius Red staining is shown in Table 1.

Restriction to patients showing a clear-cut onset of jaundice lasting until their arrival at the department of surgery permits the classification of cases in accordance with the duration of jaundice. This gives only an approximate value for the actual period of cholestasis, but nevertheless yields a workable indication. Since the drainage was not always successful, and even when it was, could not be considered to provide complete relief of the cholestasis, the drainage period has been added to the period of jaundice before admission to the surgical department. A correlation between the cholestatic period and the degree of invasion of the parenchyma by connective tissue strands was apparent (Figs. 1a and 2b).

With regard to the results of the frozen sections incubated with the monoclonal keratin antibodies, the following observations have been made.

The use of antibodies CK 18-2 and RCK 106, both specifically recognizing keratin No. 18, yielded a uniformly positive reaction in both ductuli and hepatocytes. Among the individual cases investigated, no significant differences were observed with increasing duration of cholestasis.

The situation is different for antibody RCK 105 recognizing keratin type 7, which showed a reaction exclusively in bile duct epithelium, but not in hepatocytes or hepatocellular carcinoma (29, 30). In cases 1 and 2 (Table 1) the localization of keratin 7 appeared to be restricted to the ductal system (Fig. 1b), but in cases 3 and 4 a faint immunoreactivity was found in a few cells from the limiting plates bordering the expanded portal tracts. With increasing duration of cholestasis, this phenomenon became more prominent. As a result, in cases 5 and 6 discrete groups of hepatocytes showing a clear and sometimes intense staining reaction for keratin 7 were observed. No essential difference were found, however, when cases 3 and 4 were compared with cases of 4 weeks of continu-
ous jaundice without drainage and one week of jaundice followed by 4 weeks of drainage.

The immunohistochemical staining pattern for keratin 7 in cases 7–10 with jaundice of longer duration was essentially similar to that in cases 3–6, although more obvious (Fig. 2b). Sometimes a more patchy staining of groups of hepatocytes in the limiting plate was observed, with no tendency of these cells to show a ductular arrangement. In later stages it was occasionally noted that the staining of hepatocytes extended more into the lobule, which was not observed in cases 1–6.

Keratin 19, as detected by monoclonal antibody LP2-K, labeled only bile ducts and ductules in all cases. This remained true through the entire series from 1 to 10, and thus contrasted with the findings for keratin 7.

In this respect, it must be mentioned that in surgical liver biopsies from patients not suffering from cholestasis and which are considered as controls, staining for keratin 19 (LP2), as for keratin 7 (RCK 105) was limited to bile ducts. This has been described in the literature as representing the normal situation (20).

Discussion

An increased number of ductular profiles in portal areas, pericentral fibrosis as expressed in Shoobridge and Sirius Red preparations, and the appearance of keratin 7 in hepatocytes in the periphery of the hepatic lobule appear to be interrelated changes which increase with the duration of cholestasis. The two former points have been described qualitatively and quantitatively in experimental animals (16) and in humans with cholestasis due to various causes (reviewed by Desmet, 1:31). In our series of cases of extrahepatic cholestasis of recent onset caused by a tumor of the extrahepatic duct system, an increase in ductules and peritubular fibrosis became manifest as early as 2 weeks after the onset of jaundice.

Our findings seem to suggest that during the early phase of obstructive changes, the expansion of the ductular complex would be primarily due to pre-existing ductules. The appearance of keratin 7-positive hepatocytes from 4 weeks of jaundice onwards, which is progressive with increasing duration of cholestasis, indicates a tendency towards ductular metaplasia. The hepatocytes expressing keratin 7 retained their normal aspect and arrangement, whereas the ductular keratin No. 19 did not appear in these cells. The situation in our cases is clearly different from that in patients suffering from chronic cholestasis (20, 32) or alcoholic liver disease (33), where an irregular outline of the lobular periphery with an unsharp border towards the ductular portal complexes in the portal areas is seen, and ductular metaplasia apparently contributes to the “ductular proliferation” (see also Desmet, 4).

The application of these combined histological and immunocytochemical techniques to surgical biopsies apparently contributes to an understanding of the intriguing series of events in the cholestatic liver.

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References


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