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An animal model of benign bile-duct stricture, sclerosing cholangitis and cholangiocarcinoma and the role of epidermal growth factor receptor in ductal proliferation

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Objective: To adapt an animal model of benign bile-duct stricture, sclerosing cholangitis and cholangiocarcinoma in order to determine if the expression of epidermal growth factor receptor (EGFr) could be used to differentiate these lesions.

Design: A prospective control study with blinded interpretation of liver biopsy histology and immunohistochemical staining as the criterion standards.

Setting: A university-affiliated research centre.

Subjects: Male Syrian Golden hamsters (40 for benign duct stricture, 29 for sclerosing cholangitis and 27 for cholangiocarcinoma).

Interventions: Ligation of the common bile duct with 6-0 catgut for benign duct stricture; injection of the biliary tree with 0.15 mL of formalin for sclerosing cholangitis; and weekly subcutaneous injections of 500 mg/kg of di-isopropanolnitrosamine for 10 weeks followed by ligation of the common bile duct with 6-0 catgut for cholangiocarcinoma. Routine histologic preparation of liver biopsies obtained at autopsy 10 weeks postoperatively then immunohistochemical staining of specimens for EGFr.

Main Outcome Measures: The development of benign or atypical biliary ductal proliferation, including adenoma and carcinoma formation. The presence or absence of immunohistochemical staining for EGFr.

Results: Benign ductal proliferation without atypia was seen in 15 of 21 animals in the bile-duct-stricture group that were sacrificed, in 15 of 24 animals in the sclerosing cholangitis group and in 17 of 18 animals in the cholangiocarcinoma group. Atypical proliferation was seen in 13 of 18 animals with cholangiocarcinoma but not in the other two groups. The differential occurrence of atypical ductal proliferation was statistically significant ($p < 0.00001$) for both groups. No evidence of EGFr expression was found in any group.

Conclusion: Although the animal model was valid histologically for comparing benign and malignant biliary disease, EGFr does not play a role in biliary ductal proliferation and so cannot be used to differentiate between benign and malignant lesions.

Objectif : Adapter un modèle animal de rétrécissement bénin du canal cholédoque, de cholangite sclérosante et de carcinome cholangiocellulaire afin de déterminer si l'on peut utiliser l'expression du récepteur du facteur de croissance épidermique (rFCE) pour différencier ces lésions.

Conception : Étude contrôlée prospective et interprétation à l'insu de l'histologie d'une biopsie du foie et d'une coloration immunohistochimique comme normes critérielles.

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CONTEXTE : Centre de recherches affilié à une université.

SUJETS : Hamsters dorés syriens mâles (40 pour le rétrécissement bénin du canal cholédoque, 29 pour la cholangite sclérosante et 27 pour le carcinome cholangiocellulaire).

INTERVENTIONS : Ligature du canal cholédoque avec du catgut 6-0 pour rétrécissement bénin du cholédoque, injection dans l'arbre biliaire de 0,15 mL de formaline pour la cholangite sclérosante, et injections sous-cutanées hebdomadaires de 500 mg/kg de di-isopropanolnitrosamine pendant 10 semaines, suivies d'une ligature du canal cholédoque avec du catgut 6-0 pour le carcinome cholangiocellulaire. Préparation histologique routinière de biopsies du foie prélevées à l'autopsie 10 semaines après l'intervention, suivie d'une coloration immunohistochimique de spécimens pour le rFCE.

PRINCIPALES MESURES DES RÉSULTATS : L'apparition, dans le canal biliaire, d'une prolifération bénigne ou atypique, y compris l'apparition d'adénomes et de carcinomes. La présence ou l'absence de coloration immunohistochimique pour le rFCE.

RÉSULTATS : On a constaté l'apparition dans le canal d'une prolifération bénigne sans atypie chez 15 des 21 animaux soumis à un rétrécissement du canal biliaire qui ont été sacrifiés, chez 15 des 24 animaux atteints de cholangite sclérosante et chez 17 des 18 sujets atteints de carcinome cholangiocellulaire. On a constaté une prolifération atypique chez 13 des 18 animaux atteints de carcinome cholangiocellulaire, mais non chez les sujets des deux autres groupes. L'occurrence différentielle de prolifération atypique dans le canal était significative sur le plan statistique ($p < 0{,}00001$) dans les deux groupes. On n'a trouvé dans aucun groupe d'indication d'expression du rFCE.

CONCLUSION : Même si le modèle animal était valide sur le plan histologique pour la comparaison d'affections biliaires bénignes et malignes, le rFCE ne joue pas un rôle dans la prolifération dans les canaux biliaires et ne peut donc servir à différencier des lésions bénignes de lésions malignes.

Distinguishing cholangiocarcinoma from nonmalignant biliary stricture is difficult. Accurate preoperative diagnosis can permit appropriate therapeutic planning with potential early operation for malignant disease and avoid unnecessary major surgery for benign disease. Nevertheless, the false-positive rate for radiologic diagnosis (including ultrasonography, computed tomography, endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography) is reported to range from 13.4% to 31%.[1,2] The well-differentiated nature of cholangiocarcinoma and the effect of inflammation on biliary epithelium results in a reported overall sensitivity of only 42% for nonoperative cytology, ranging from 38% (range from 30% to 73%) for exfoliative biliary cytology (negative predictive value ranging from 25% to 50%) to 59% (range from 50% to 66%) for brush cytology (negative predictive value ranging from 39% to 74%). Endobiliary needle aspiration biopsy only has a sensitivity ranging from 60% to 84%. Use of an endobiliary forceps biopsy increases this to a range of from 30% to 100% in small published series.[3] Radiologically guided fine-needle aspiration biopsy of a mass lesion has a sensitivity in the range of only 42% to 67%.[3] Even intraoperatively, the focal and fibrotic nature of the tumour can make fine-needle aspiration biopsy difficult and gives a sensitivity of only 80%.[3]

Immunohistochemical techniques have been used in many cases of malignant masses to detect tumour antigen expression, and some antigens are reported to be useful in differentiating benign from malignant disease.[4–6] Despite the potential clinical value, there have been few reports in the literature examining the application of differential antigen expression in the diagnosis of cholangiocarcinoma,[7–10] and only one of these[10] compared antigen expression by cholangiocarcinoma to that of benign strictures.

Epidermal growth factor receptor (EGFr) is a cell-membrane-based glycoprotein thought to play a role in growth regulation and proliferation. Increased expression of EGFr has been reported in a wide variety of neoplasms.[11,12] It has been expressed at a higher rate in cholangiocarcinoma than in normal controls.[7] Since benign cholestasis and cholangiocarcinoma are both characterized pathologically by ductal proliferation,[13] the potential role of EGFr in the pathogenesis of these conditions is of interest and may be useful in differentiating these lesions. There are no reports comparing EGFr expression by cholangiocarcinoma and by benign strictures.

We have adapted an animal model of biliary-tract disease to determine if the expression of EGFr could be used to differentiate benign from malignant disease, as characterized by ductal proliferation.

MATERIALS AND METHODS

Three animal models of biliary-tract disease were established as follows.

Benign bile-duct stricture

Forty outbred male Syrian Golden hamsters (Charles River, Boston, Mass.) were acclimatized for 1 week, fed a stock diet and tap water *ad libi-*

tum, then fasted for 12 hours. After induction of anesthesia by intraperitoneal injection of 0.1 mL of 65 mg/mL of sodium pentobarbital, the animals underwent laparotomy and ligation of the common bile duct proximal to the duodenum, with a 6-0 plain catgut suture (Ethicon, Peterborough, Ont.). The abdomen was then closed with a single layer of 5-0 Dexon (Davis & Geck, Montreal). At 10 or 15 weeks postoperatively the surviving animals were sacrificed by intracardiac injection of 0.5 mL of 65 mg/mL sodium pentobarbital. The liver was removed and fixed in 10% buffered formalin.

Sclerosing cholangitis

Twenty-nine outbred male Syrian Golden hamsters were acclimatized for 1 week, fed a stock diet and water *ad libitum*, then fasted for 12 hours preoperatively. Anesthesia was induced as already described. The distal common bile duct was clamped with a rubber shod mosquito forceps and the gallbladder was injected with 0.15 mL of either 5% (19 animals) or 10% (10 animals) formalin through a 30-gauge needle. The puncture wound was closed by ligation with a 5-0 Dexon suture. The clamp was retained for 10 minutes while the gallbladder was massaged to reflux the formalin into the intrahepatic biliary tree. The abdomen was then closed with a single layer of 5-0 Dexon. The surviving animals were sacrificed at either 10 or 15 weeks postoperatively, and the liver was removed and fixed in 10% buffered formalin.

Cholangiocarcinoma

Twenty-seven outbred male Syrian Golden hamsters were acclimatized for 1 week, fed a stock diet and water *ad libitum*. They were then given weekly injections of di-isopropanolnitrosamine (Sigma Diagnostics Canada, Mississauga, Ont.), 500 mg/kg subcutaneously for 10 weeks. After that they were fasted and underwent laparotomy as described for the benign biliary stricture group. The survivors were sacrificed at either 10 or 15 weeks postoperatively, and the liver was removed and fixed in 10% buffered formalin.

Preparation of histologic sections

Portions of each fixed liver specimen and two fixed liver specimens from normal hamsters were embedded in paraffin, sectioned at 5 μm and stained with hematoxylin and eosin. They were examined by a gastrointestinal pathologist (D.A.O.) who was blinded to the study group. The specimens were examined for the following features: histologic changes of benign cholestasis (as characterized by simple bile-duct proliferation), atypical ductal proliferation (including papillary hyperplasia, goblet cell metaplasia, cystic proliferation or dysplastic changes), adenomatous proliferation or cholangiocarcinoma.

Immunohistochemistry

Representative specimens from each group were cut to 5 μm, dried, deparaffinized and dehydrated with alcohol. Monoclonal antibody to EGFr (Triton Diagnostics, Alameda, Calif.) at a dilution of 1:10 was applied for 1 hour, then the slides were washed in Tris buffer. Endogenous peroxidase activity was blocked by immersion in 0.6% hydrogen peroxide in methanol for 45 minutes. Following a wash in Tris buffer, a biotinylated link antibody (goat anti-mouse; Biocan Scientific, Mississauga, Ont.) was applied for 30 minutes followed by peroxidase-labelled streptavidin for 80 minutes. Specimens were then washed in Tris buffer followed by acetate buffer. They were developed with 6 mg of 3-amino-9-ethyl carbazole in 1.5 mL *N,N*-dimethyl formamide, 28.5 mL of 0.1 mmol/L acetate buffer and 0.3 mL of 3% hydrogen peroxide and counterstained with Carazzi's hematoxylin. Negative controls consisted of a second section of each specimen processed simultaneously without the primary antibody. Positive controls were known positive specimens of lung carcinoma. Staining was interpreted as present or absent and the degree of staining noted.

Statistical analysis

The occurrence of benign and atypical ductal proliferation was analysed according to Fisher's exact test on 2×2 contingency tables, comparing the malignant model with each of the benign models. A significance level of p less than 0.05 was chosen.

Results

Benign bile-duct stricture

At autopsy, obstruction of the common bile duct was noted, with gross dilatation proximal to the level of the previously placed suture and distension of the gallbladder.

Sclerosing cholangitis

At autopsy, multiple adhesions to the porta hepatis were seen, with a shortened common bile duct and loss of the normal lucency of the tissues. The gallbladder was either markedly shrunken or could not be visualized.

Cholangiocarcinoma

Obstruction of the common bile duct was noted, with distension of the proximal common bile duct and gallbladder. One animal had a grossly

visible tumour mass, which corresponded to a cholangiocarcinoma.

Histology

The histologic findings for the three models are reported in Table I. In each group, data for subgroups were combined because there was no significant difference between them.

Expression of EGFr

There was no difference in staining for EGFr in any of the experimental groups. Specimens showed diffuse mild staining of hepatocytes with little or no staining of bile ducts.

Discussion

Cholangiocarcinoma is an uncommon malignant lesion, constituting only 2% of cancers in autopsy series.[14] This limits the feasibility of adequately sized prospective studies on human tissue even in a multicentre format.

Several studies employing animal models of cholangiocarcinoma have been published.[15–17] These have examined the role of liver fluke infestation and ductal obstruction on nitrosamine-induced cholangiocarcinoma. These factors are all felt to be of etiologic importance in the high incidence of cholangiocarcinoma in Asia.[18]

The occurrence of sclerosing cholangitis in patients treated with scolicidal agents for hydatid hepatic cysts led to the publication of reports on the induction of sclerosing cholangitis in rabbits[19] and rats[20] by exposure of the biliary tree to these agents.

We have successfully adapted these models to a comparative study of benign and malignant ductal proliferation. Benign ductal proliferation was seen in 15 of 21 animals in the benign-stricture group, 15 of 24 animals in the sclerosing-cholangitis group and 17 of 18 animals in the cholangiocarcinoma group. This difference was significant only for the comparison between the sclerosing cholangitis and the cholangiocarcinoma groups ($p = 0.02$), suggesting that the malignant model may enhance ductal proliferation in general. There was no atypical proliferation in either of the benign groups, but 13 of 18 animals in the cholangiocarcinoma group showed evidence of atypical ductal proliferation associated with a spectrum of lesions ranging from dysplastic epithelial proliferation to adenomas and carcinoma. The differential occurrence of atypical ductal proliferation was statistically significant ($p < 0.00001$) for each group.

We were not able to reproduce the high rates of cancer induction reported by others.[15–17] The length of time between initial exposure to the carcinogen and examination of the liver likely plays a significant role in the final tumour induction rate. Although our incidence of cholangiocarcinoma induction (1 of 18) was low we believe that the induction of premalignant lesions (12 of 18) was significant and validates this model. Preneoplastic lesions, including ductal hyperplasia and adenomas, have been described previously in the induction of cholangiocarcinoma in animals[21] and in the pathogenesis of cholangiocarcinoma in humans.[22]

We could not demonstrate any role for EGFr in either benign or malignant ductal proliferation. Nonomura and colleagues[7] reported EGFr expression in 12 of 37 cases of human cholangiocarcinoma, with, however, a wide spectrum of intensity of staining. We previously demonstrated EGFr expression in oral squamous cell carcinoma in Syrian Golden hamsters, which confirms that EGFr is preserved in this species.[23] We believe, therefore, that EGFr does not play a role in either benign or malignant biliary ductal proliferation and cannot be used to differentiate these conditions.

Conclusions

Cholangiocarcinoma and its precursor lesions can be produced by nitrosamine induction in hamsters with bile-duct strictures. Benign ductal proliferation due to stricture alone or exposure to agents that cause secondary sclerosing cholangitis can be used as a comparison group for diagnostic testing. Although EGFr expression does not appear to play a role in

Table I

Histologic Findings in the Three Experimental Models of Biliary-Tract Disease

	Group		
Histologic finding	Benign stricture ($n = 21$)	Sclerosing cholangitis ($n = 24$)	Cholangiocarcinoma ($n = 18$)
Benign ductal proliferation	15	15	17
Atypical ductal proliferation	0	0	13*

*Includes 4 adenomas and 1 carcinoma

biliary ductal proliferation, this combined model can be used in future analyses of the role of tumour antigens in differentiating benign from malignant biliary strictures.

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