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Polymeric Ig Receptor Expression in Hepatocellular Carcinoma

Mireille Rossel, Estelle Seilles, Jean-Jacques Voigt, Dominique Vuitton, Norbert Legait and Jean-Pierre Revillard

The cellular localisation of the polymeric Ig receptor (pIg-R) and carcinoembryonic antigen (CEA), hepatic and biliary cell markers, were investigated in patients with hepatocellular carcinoma (HCC) and high serum levels of secretory component. Serum SC were increased 6–20-fold in 8 HCC patients compared with normal subjects. Serum free SC was positively correlated bilirubin ($r = 0.95$, $P < 0.04$). In normal liver tissue, cytokeratin (CK) 8 and 18 were localised in hepatocytes and biliary cells while pIg-R and CK 19 expression was restricted to biliary cells. In tumoral liver tissue, malignant cells expressed CK 8 and 18 weakly; pIg-R and CK 19 were not detected in tumoral cells. CEA was expressed by biliary cells in normal and proliferating ducts. In peritumoral fibrosis, proliferating biliary cells were strongly stained by anti-cytokeratins and anti-pIg-R antibodies. In one case, pIg-R was localised in isolated cells close to fibrosis without co-staining of anti-CK 19. Thus increased serum SC is not associated with pIg-R expression by tumoral cells, and pIg-R may be considered an additional marker of biliary cells. High SC might be explained either by reflux from bile to serum and/or release of unbound SC from the vascular pole of non-functional, proliferating biliary structures.
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INTRODUCTION

THE POLYMERIC immunoglobulin receptor (pIg-R) is a molecule of the Ig superfamily which is synthesised by mucosal epithelial cells. When it transports p-Ig across the cell, it is then released, bound to p-Ig as secretory Ig (S-IgA and S-IgM) [1], or in its free form, as secretory component (SC). Increased S-IgA levels have been found in the serum from patients with liver metastases [2] and from patients with hepatic diseases such as cirrhosis and chronic cholestasis [3, 4]; quite elevated serum levels of S-IgA, S-IgM and free SC have been reported in American and South African patients with hepatocellular carcinoma (HCC) [5, 6]. The cellular origin, however, of these elevated levels in the above diseases remains to be determined. In epithelial neoplasia such as lung tumour, colorectal carcinoma [7] or bladder carcinoma [8], it has been shown that tumoral cells are able to synthesise pIg-R.

Delacroix *et al.* [9] and Nagura *et al.* [10] have localised pIg-R in the epithelial cells of the biliary ducts of the normal human liver tissue. However, in animal species such as mice, rats and rabbits, pIg-R is synthesised by parenchymal hepatic cells. Liver regeneration after massive liver necrosis has also been shown to be associated with increased SC serum levels [11]. In HCC, the tumoral process modifies the rate of synthesis of albumin and other proteins while inducing the neo-synthesis of fetal proteins like alphafoetoprotein (AFP) [12]. In cholestatic diseases [13], alcoholic liver diseases [14] and HCC [15], some hepatocytes express cytokeratin polypeptides which are restricted to bile duct cells in normal livers. Malignant hepatic cells of HCC might be able to synthesise pIg-R as a result of the tumoral differentiation. Since no study on pIg-R expression by HCC cells has yet been reported, we investigated the expression of pIg-R in HCC cells from patients with elevated SC serum levels. Other cell markers such as cytokeratins (CK) 8, 18, 19 and carcinoembryonic antigen (CEA) were also investigated and their localisation was compared to that of pIg-R.

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MATERIALS AND METHODS

Patients and controls

Sera were obtained from 8 patients with HCC. Routine liver function tests for serum gamma glutamyl transferase (GGT) and phosphatase alkaline activities, and albumin, conjugated bilirubin and AFP concentrations were performed. The control group comprised 36 age-matched healthy volunteers. Results obtained from the two groups were analysed using the non-parametric Mann–Whitney test and Spearman's correlation test.

Surgical liver biopsy specimens were obtained from 5 patients with adenocarcinomas of the hepatocellular carcinoma type for routine histopathological examination and immunohistochemistry. Five paraffin-embedded sections from normal livers were studied as normal control tissues.

Immunoassay for free SC, S-IgA and S-IgM measurement

Differential quantification of free SC, S-IgM and S-IgA was performed by an enzyme-linked immunosorbent assay (ELISA), using monoclonal antibodies specific to SC in free or bound form [16].

Immunohistochemical procedures

Serial sections (5 μm) from paraffin-embedded and frozen liver samples were analysed by indirect immunofluorescence and immunoperoxidase methods. Indirect immunofluorescence staining was performed using the following antibodies: the antihuman SC, a goat antiserum specific for free and bound secreted forms of pIg-R (Prof. J.P., Vaerman, ICP, Brussels; its specificity has already been tested [11]); the anti-CK19: a monoclonal antibody specific for cytokeratin 19 (Boerhinger). The indirect immunoperoxidase method, a three-step method, has been described [17]. The antibodies used in this method were KL1, a monoclonal antibody directed against a polypeptide determinant present in cytokeratins 8 and 18 (Immunotech) and anti-CEA, a rabbit antiserum specific for human CEA (Dakopatts).

To allow the use of monoclonal antibodies on deparaffinised sections, samples were incubated 30 min at 37°C in a moist chamber with proteinase K (5 μgm/ml). Sections were then washed three times in phosphate-buffered saline (PBS) and the primary antibody was applied. For co-staining of pIg-R and CK 19, the sections were incubated first with the anti-CS antibody, and second with the anti-CK 19 antibody. The anti-goat FITC (Dakopatts) and the anti-mouse TRITC (Boerhinger) antibodies were applied, respectively in the third and fourth steps. Each of the above incubation periods was 45 min in length at room temperature. Control sections included the use of PBS with 5% fetal calf serum, and normal mouse, rabbit or goat serum instead of the primary antibody.

RESULTS

Serum levels of free SC, S-IgA and S-IgM

Free SC and S-IgA levels were 6–20-fold higher in HCC patients than the serum values of control subjects (Table 1). Free and bound SC levels were not correlated with alkaline phosphatase activity, GGT activity, albumin or AFP concentrations. However, a significant positive correlation was found between free SC and conjugated bilirubin levels ($r = 0.95$, $P<0.04$).

Immunohistochemical results

In the normal liver biopsy samples, hepatocytes and biliary duct cells were stained by the monoclonal antibodies directed against CK 8 and 18, whereas only biliary epithelial cells of ductules in the portal area were stained with the anti-CK 19 antibody and anti-SC antiserum (Fig. 1a). Expression of CEA was observed in normal medium-sized biliary ducts.

In the tumoral tissues, the anti-CK 8 and 18 antibodies stained normal hepatocytes when islets of normal cells were present together with tumoral hepatocytes on a given section. The signal obtained on tumoral cells was very faint. However, expression of CK 8 and CK 18 was demonstrated using the KL1 antibody with the immunoperoxidase technique (Fig. 1b). The epithelial cells of normal bile ducts as well as the proliferating duct-like structures were also stained with the anti-CK 8 and 18 antibody. Expression of pIg-R and CK 19 was never observed in tumoral parenchymal cells. In most cases, numerous proliferating ducts were observed in the connective tissue around the tumoral nodules. These neo-ductules were strongly stained by the anti-SC and the anti-CK 19 antibodies. Some cholangiocyte-like cells in the peritumoral areas, isolated or sometimes arranged in rosettes, were strongly stained with these two cell markers (Fig. 1c,d). These cells with a bile duct-like phenotype had not yet formed ductules and were in close contact with the collagen fibres of the connective tissue. In 1 patient, some isolated cells of normal hepatocyte size were stained by anti-SC antibodies in the area of bile duct proliferation (Fig. 1e). None of these isolated cells expressed CK 19. There was no difference in the pattern of expression of pIg-R between the HCC that had developed in the cirrhotic livers (3 out of the 5 patients) and those which had developed in the non-cirrhotic livers.

No CEA expression was observed by tumoral cells in the patients studied. In 1 case, an intense staining was observed in polymorphonuclear cells infiltrating the tumoral area. Some proliferating biliary cells were stained with anti-CEA antibodies.

Table 1. Free and bound secretory component levels in the serum of control subjects, and patients with hepatocellular carcinoma

	Free SC (mg/l)	S-IgA (mg/l)	S-IgM (mg/l)	IS	Cirrhosis*
Control subjects					
Median (range)	0.13 (0.06–0.25)	3.2 (1.0–11.5)	4.6 (0.7–18.5)		
Patients (No.)					
1	0.81	19.9	7.9	–	–
2	0.76	30.6	3.0	+	+
3	0.62	4.5	24.9	+	+
4	1.74	26.6	74.8	+	–
5	0.87	15.1	9.0	+	–
6	3.13	132.0	13.4	–	+
7	1.09	55.0	14.6	–	+
8	2.22	100.0	42.8	+	+
Median	0.98	28.1	13.6		

IS:Immunostaining was realised (+) in 5 patients using the indirect immunofluorescence and indirect immunoperoxidase methods.
*5 patients have developed hepatocellular carcinoma on cirrhotic liver tissue.

DISCUSSION

We confirmed that serum levels of free SC, S-IgA and S-IgM are raised in patients with HCC. This study of a small series of cases is in agreement with previous reports on HCC in American [6] and South African patients [5]. This increase appears irrespective of either the presumed aetiological factors involved in HCC or the presence or absence of cirrhosis. However, SC serum levels in HCC patients were much higher than those reported in patients with other epithelial tumours [2, 8, 18].

In normal liver tissue, our results on the localisation of cytokeratins are in agreement with previous studies [13, 19, 20]. The localisation of pIg-R was restricted to bile duct cells,

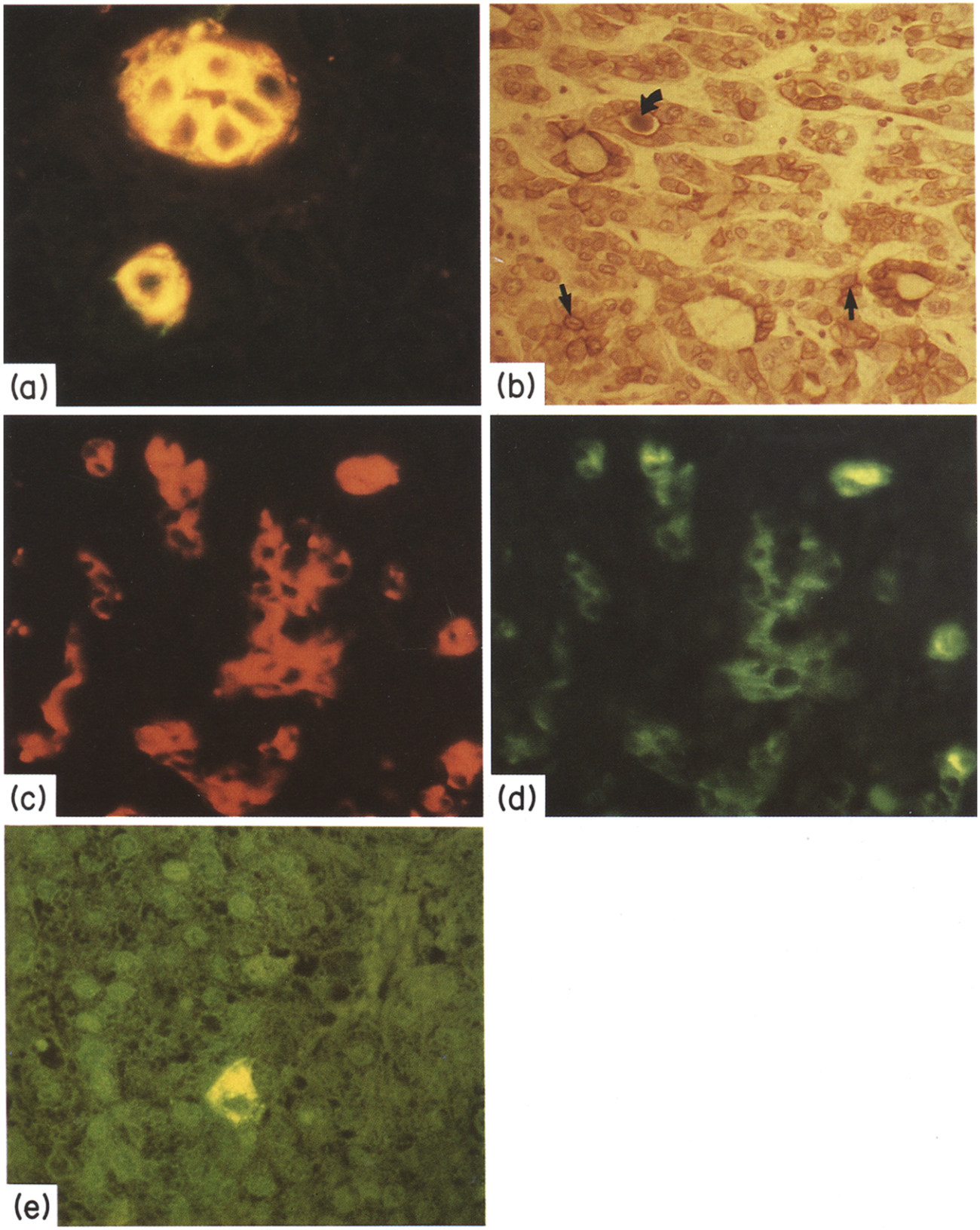

Fig. 1. (a) Double immunofluorescence staining of a normal biliary duct in normal liver using the antihuman SC antibody (yellow) and the antihuman CK 19 antibody (red). × 500. (b) Immunoperoxidase staining of tumoral hepatocytes using the anti CK 8-18 monoclonal antibody. Note the staining of the tumoral cell membranes (→) and the presence of bile material (↶). × 500. (c) Immunofluorescence staining of biliary neoductules in the peritumoral connective tissue using the antihuman CK 19 antibody and the antimouse Ig TRITC-conjugated antiserum. Some proliferating cells were not yet arranged in duct-like form. × 250. (d) On the same section as (c), these cells were stained by the antihuman SC antibody and revealed by an antigoat Ig FITC-conjugated antiserum. The localisation was exactly the same as that of CK 19. × 250. (e) Immunofluorescence staining of SC in an isolated cell, in the tumoral parenchyma, close to the bile duct proliferating area. This cell gave no staining with an anti-CK 19 antibody. × 250.

as was that of CK 19. This particular localisation of pIg-R in cholangiocytes has been previously reported by several authors using immunohistochemical and ultrastructural methods with polyclonal antibodies against SC [1, 9, 10]. In addition, northern blot analysis with a cDNA specific of pIg-R has failed to detect any mRNA in normal human liver tissue [21]. However, several studies have reported the expression of pIg-R by human hepatocytes [22–24]. These study results might be due to a very limited expression of the receptor or to a crossreactivity between pIg-R and other glycoproteins of the hepatocyte membrane, such as the asialoglycoprotein receptor, since pIg-R expression by hepatocytes has been demonstrated with only 1 monoclonal antibody antihuman SC and with polyclonal antibodies against S-IgA. Whether or not cross-reactive pIg-R-like structures are expressed on the human hepatocyte membrane, their role in pIgA transport has not been demonstrated; up to now, it has been accepted that cholangiocytes are solely responsible for the SC-mediated transport of polymeric Ig in the human liver [25].

In HCC, tumoral cells did not express pIg-R. These results suggest that high SC levels in HCC are not due to its secretion by tumoral cells. The same localisation of pIg-R and CK 19 in bile duct cells and proliferating biliary cells was demonstrated by double immunostaining. There was no expression of either CK 19 or pIg-R in normal or tumoral hepatocytes. We did not observe, as van Eyken *et al.* [15] did, hepatocyte islets stained with CK 19 antibodies in the tumoral liver tissue. Van Eyken *et al.* studied 34 patients with HCC and observed CK 19 expression by hepatocytes in 15 subjects [15]; the small size of the patient group in our study might be responsible for the negative results concerning CK 19. Our findings are very similar to those of Delacroix *et al.* [3] who observed that, during biliary obstruction, SC was localised in biliary duct epithelial cells and lumen as well as in cholangiocyte-like cells of the proliferating structures. In one case, there was evidence that some cells with a morphological aspect intermediate between hepatocytes and biliary epithelial cells were able to express pIg-R. However, further investigations are needed to assess the capacity of these cells to synthesise pIg-R independently of CK 19. Nevertheless, our results obtained with anti-SC antibodies suggest that "ductular metaplasia" of hepatocytes may contribute to the ductular proliferation as demonstrated by van Eyken *et al.* in other liver diseases [13, 14]. PIg-R expression could thus be an additional marker for phenotyping cell populations in HCC compared with CK 19 and the epithelial membrane antigen. We showed CEA expression in normal biliary duct cells and in proliferating ducts as did Nakanuma and Ohta [26], who found that CEA-like molecules can be detected in the same biliary structures.

The correlation observed between SC levels and conjugated bilirubin suggests a relationship between increased SC concentrations and cholestasis. Cholestasis induced by biliary obstruction has been reported to result in increased levels of serum levels of S-IgA and free SC [2–4, 27]. Increased levels of free SC have recently been shown in patients with elevated levels of alkaline phosphatase [28] and we have found a significant correlation between free SC levels and GGT and total bilirubin levels in liver transplant rejection [29]. The strong correlation between serum levels of bilirubin free SC levels on one hand, and the intense cytoplasmic staining of cells belonging to neoductules on the other hand, suggest a biliary origin of free SC. High SC levels could result from a reflux of free and bound SC from bile to serum and/or from the direct release of free SC by normal or newly developed biliary structures at their vascular poles. Such free SC might bind to polymeric IgA and IgM in plasma, because of the molecular equilibrium between free and bound forms [30]. We are investigating such mechanisms in other models of hepatobiliary dysfunction, such as primary biliary cirrhosis, liver graft rejection and chronic biliary obstruction.

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β-Carotene-mediated Inhibition of a DNA Adduct Induced by 7,12-Dimethylbenz(a)anthracene and 7-Hydroxymethyl-12-methylbenz(a)anthracene in Mouse Mammary Gland *in vitro*

Sanjoy K. Das, Ting Z. Jia, Abhik M. Bandyopadhyay and Mihir R. Banerjee

The influence of β-carotene on the formation of DNA-adducts induced by 7,12-dimethylbenz(a)anthracene (DMBA) and 7-hydroxymethyl-12-methylbenz(a)anthracene (7-OHM-12-MBA) during transformation of mouse mammary cells in organ culture was analysed. Treatment with β-carotene (10^{-8}–10^{-5} mol/l) caused inhibition (48.8–94.4%) of an adduct (VI), which was detectable in DNA samples from DMBA-treated mammary glands. Out of six adducts, derived from further analysis of DNA samples from 7-OHM-12-MBA-treated glands, adduct f eluted in the same fraction as adduct (VI), indicating these adducts were analogous. Likewise, adduct f was also inhibited by β-carotene. Boronate chromatographic analysis revealed this particular adduct was a syn-dihydrodiol epoxide product. Adduct inhibition was detectable both at the start and after DMBA treatment. α-Tocopherol and canthaxanthin were ineffective in inhibiting adducts. It is reasonable to conclude that β-carotene-mediated modification of adducts is associated with the inhibition of a syn-adduct, which is derived from further metabolism of a 7-OHM-12-MBA intermediate.
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INTRODUCTION

EPIDEMIOLOGICAL STUDIES have indicated that higher levels of dietary β-carotene and other carotenoids may reduce the tumour incidence in human populations [1]. Although the association between high levels of β-carotene in the diet and reduced tumour incidence may not be causally related, β-carotene may provide a protective influence against tumour incidence [2]. In contrast to the extensive studies [3] on the chemoprevention properties of the compounds having vitamin A-like activity, little is known about the mechanism of the preventive action of β-carotene against the neoplastic disease. There has been interest in elucidating the mechanism by which dietary β-carotene may exert such a protective action [4]. A recent study on biological activities of α-carotene has indicated that when human neuroblastoma cells GOTO were exposed to α-carotene, they were arrested in G_0–G_1 phase of their cell cycle [5]. Earlier, we reported that β-carotene can inhibit 7, 12-dimethylbenz (a) anthracene (DMBA)-induced mammary cell transformation both at the initiation and the promotion stages of carcinogenesis in organ culture of the whole mammary gland in a hormonally-defined serum-free medium [6]. This inhibitory effect is likely to be due to the action of β-carotene itself, since no retinol was measurable in the glands in culture after β-carotene treatment [6]. β-carotene also modifies carcinogen-induced DNA damage and

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