Distribution of fibronectin and fibronectin-binding proteins, AGp110 and integrin $\alpha_5\beta_1$, during chemically induced hepatocarcinogenesis in adult rats

STAMATIS C. STAMATOGLOU1*, MARGARET M. MANSON2, JONATHAN A. GREEN2, XAVIER MAYOL3 and R. COLIN HUGHES1

1National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK
2MRC Toxicology Unit, Woodmansterne Road, Carshalton, Surrey SM5 4EF, UK
3Department of Cellular Biology, Faculty of Medicine, University of Barcelona, Av. Diagonal, Barcelona, Spain

*Author for correspondence

Summary

We have used single- and double-label immunocytochemistry to examine the distribution of AGp110, integrin $\alpha_5\beta_1$ and fibronectin in adult rat liver during carcinogenesis induced by aflatoxin B1 or diethylnitrosamine. In normal liver fibronectin and the fibronectin integrin receptor $\alpha_5\beta_1$ are localized on all three domains of the parenchymal cell surface: sinusoidal, lateral and canalicular. In contrast, AGp110, a non-integrin monomeric glycoprotein with fibronectin receptor properties, is confined to the bile canalicular (apical) plasma membrane of hepatocytes. Hepatocarcinogenesis induced by aflatoxin B1 causes altered cell foci to form in the parenchyma, followed by enlargement of these foci to form pre-neoplastic nodules and finally hepatocellular carcinomas of either poorly differentiated, trabecular or adenocarcinoma morphology. Expression of AGp110 decreased to a minimal level, at first selectively in altered cell foci, from the 9th week of treatment, and then indiscriminately in poorly differentiated carcinomas. The same lesions that were deficient in AGp110 also displayed a reduced level of fibronectin and $\alpha_5\beta_1$, although the observed change in AGp110 demarcated altered foci and poorly differentiated tumour lesions more sharply, since expression of $\alpha_5\beta_1$ and fibronectin, though substantially reduced, was still faintly apparent on the cell surface. Small acinar structures, observed in late hyperplastic nodules and in trabecular carcinomas, exhibited even, pericellular staining of fibronectin and $\alpha_5\beta_1$, including prominent staining of the lumen area, whereas staining of AGp110 appeared to be confined to the lumen. In larger ducts of overt adenocarcinomas, fibronectin and $\alpha_5\beta_1$ were distributed along the basal surface of the epithelium and AGp110 on the apical domain. Tumours induced by diethylnitrosamine and promoted with ethinyl estradiol displayed similar histology and staining patterns for all three proteins as that described for aflatoxin B1. Finally, comparisons between AGp110 and cytokeratin 19, a selective tumour marker, indicated that whereas loss of AGp110 occurs in poorly differentiated lesions and tumours, expression of cytokeratin 19 is associated with acinar and glandular structures found in late hyperplasia and with trabecular and pseudoglandular tumours. The results indicate that loss of differentiation in either hyperplastic or neoplastic lesions correlates with reduced expression of fibronectin and of its receptors $\alpha_5\beta_1$ and AGp110. On the basis of morphological similarities and staining patterns in the pre-neoplastic and neoplastic state, we deduce that hepatocellular carcinomas derive from differentially hepatocytes.

Key words: hepatocarcinogenesis, fibronectin, AGp110, integrin $\alpha_5\beta_1$

Introduction

Experimental carcinogenesis in the liver is a multistage process involving a complex structural rearrangement of the parenchyma that results in either morphologically poorly differentiated tumours or in clearly distinct, pseudo-differentiated structures referred to as adenomas and trabecular carcinomas (Stewart and Williams, 1980; Williams, 1980). This transition in the cellular architecture of the liver can be triggered experimentally by chemical carcinogens such as aflatoxin B1 and diethylnitrosamine and is most probably mediated by alterations in cell-matrix and cell-cell adhesion mechanisms. Changes in the expression of extracellular matrix proteins such as fibronectin and laminin (Sell and Ruoslahti, 1982; Jagirdar et al. 1985; Szendroii and Lapis, 1985) and of intracellular adhesion molecules such as cell CAM105 (Hixson et al. 1985) during hepatic carcinogenesis strongly implicate adhesive interactions in phenotypic neoplastic alterations. Modifications in the composition of the extracellular matrix are particularly significant in this respect, since they directly affect the metabolic activity of parenchymal cells: in primary cultures of rat hepatocytes synthesis of liver-specific proteins can be manipulated by inoculation on different extracellular matrix substrata (Sudhakaran et al. 1986; Reid et al. 1988; Ben-Ze'ev et al. 1986).
1988). These cellular interactions with matrix components are mediated by specific receptors, most of which belong to the integrin family of macromolecules (Hynes, 1987), and transformation-associated changes in integrins have already been described in various cells (Buck et al. 1990; Dedhar, 1990; Plantefaber and Hynes, 1989; Virtanen et al. 1990).

In this study our aim has been to investigate changes in fibronectin and fibronectin receptors during hepatocarcinogenesis. So far, two cell surface glycoproteins have been described that mediate adhesion of hepatocytes on fibro-nectin: integrin α5β1 (Johansson et al. 1987) and AGP110, a non-integrin glycoprotein (Stamatoglou et al. 1990a). Under certain experimental conditions these two receptors may act in synergy (Stamatoglou et al. 1990b). In this study we demonstrate that poorly differentiated hepatic tumours are deficient in both receptors and fibronectin whereas adenomas maintain a polarized expression of these proteins. Absence of AGP110 was found to be the most distinct marker for poorly differentiated hepatomas and presence of cytokeratin 19 in parenchyma cells demarcated pseudoglandular carcinomas (adenocarcinomas).

### Materials and methods

#### Chemicals

Aflatoxin B1 was obtained from Makor Chemicals Inc. (Jerusalem, Israel). Diethylthioarsamine was from Merck (Dagenham, Essex, UK). Ethinyl estradiol, Naphthol AS-BI phosphate, Fast Red TR, powdered MRC 41B, contaminated artificially with 2 p.p.m. (parts per million) aflatoxin B1, for times varying from 2 weeks to 10 months.

Integrin α5β1 immunostaining was achieved by incubation first with normal rabbit serum, then with anti-α5β1 IgG (15 μg ml⁻¹) followed by rabbit anti-chicken/alkaline phosphate. Developing and counterstaining were as described above.

#### Fibronectin single label

Polyclonal anti-fibronectin (1:200) was used, followed by goat anti-rabbit/phosphatase. Identical results were obtained with monoclonal anti-fibronectin (100 μg IgG ml⁻¹) using the 'elite' ABC reagents from Vector.

#### Immunocytochemistry

Specimens were fixed in ice-cold acetone and embedded in paraffin wax. Sections were dewaxed in xylene, equilibrated in an ethanol series of decreasing concentrations and hydrated in distilled water. Quenching of endogenous peroxidase or alkaline phosphatase was achieved by incubating for 30 min or by 15 min in 15% acetic acid, respectively. Washes and antibody dilutions were in phosphate-buffered saline, pH 7.5, containing 0.05% Tween 80. After endogenous enzyme quenching, sections were incubated in this buffer for 30 min and then in 1% non-immune goat (or rabbit) serum for 30 min.

**AGP110 staining.** This was performed by incubating sections for 1 h at room temperature with anti-AGP110 (1:200), washing and then incubating with goat anti-rabbit IgG/alkaline phosphatase. Sections were developed with Fast Red (0.5 mg ml⁻¹) and Naphthol AS-BI phosphate (0.5 mg ml⁻¹) in 50 mM veronal acetate buffer, pH 9.2. Sections were counterstained by immersion in haematoxylin for 20 s and mounted in Apathy's mounting medium.

**Integrin α5β1 staining.** This was accomplished by incubation with anti-α5β1 IgG (15 μg ml⁻¹) followed by rabbit anti-chicken/alkaline phosphatase. Developing and counterstaining were as described above.

**Fibronectin single label.** Polyclonal anti-fibronectin (1:200) was used, followed by goat anti-rabbit/phosphatase. Identical results were obtained with monoclonal anti-fibronectin (100 μg IgG ml⁻¹) using the 'elite' ABC reagents from Vector.

**AGP110/fibronectin double label.** Vector ABC reagents were used according to the manufacturer's specifications. AGP110 was detected using our anti-AGP110 serum and the ABC/alkaline phosphatase kit. For fibronectin we used the mouse monoclonal antiserum and the Vector 'elite' peroxidase kit. In the sections the sections were first processed for AGP110 (1:200), then with anti-rabbit/biotin and finally with ABC reagent (freshly prepared complex of biotin and streptavidin/phosphatase). Development was in a substrate solution prepared by adding 33 μl of BCIP (50 mg ml⁻¹ in dimethyl formamide) and 6 μl of NBT (75 mg ml⁻¹ in 70% dimethyl formamide) in 10 ml of 100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM MgCl₂. Sections were then processed for fibronectin staining, using mouse monoclonal IgG (100 μg ml⁻¹), following the same order as in the procedure above. The ABC reagent contained avidin linked to peroxidase and the developing solution was 10 ml of 20 mM acetate buffer, pH 4.0, to which 50 μl of carbazole (20 mg ml⁻¹ stock in DMSO) and 30 μl of 30% H₂O₂ were added. No counterstain was used.

**AGP110/α5β1 double label.** AGP110 was localized using the Vector ABC alkaline phosphatase kit as described in the previous section. Integrin α5β1 immunostaining was achieved by incubating first with normal rabbit serum, then with anti-α5β1 IgG (15 μg ml⁻¹), followed by rabbit anti-chicken IgG/biotin (1:500) and, finally, with extravidin/peroxidase (1:500). Development was as described for AGP110/fibronectin labelling.

**AGP110/cytokeratin 19 double label.** This is described in detail elsewhere (Green and Manson, 1991). Briefly, sections were incubated with anti-AGP110 and anti-cK-19 and then with anti-rabbit/peroxidase and anti-mouse/alkaline phosphatase. Development was as in AGP110/Fn double label.

### Results

Animals fed aflatoxin B1 were killed at different times after initiation of dietary administration (2 weeks to 10 months). Animals subjected to the diethylthioarsamine regimen were killed 9-12 months after the first i.p. injection.

**AGP110**

In adult rat liver AGP110 was found in canalicular plasma (80 mg kg⁻¹ body weight) for 4 weeks or a weekly injection (40 mg kg⁻¹ body injection) for 8 weeks. Animals were killed 9-12 months after the beginning of treatment.
membranes (Fig. 1a) as previously described (Stamatoglou et al. 1990a,b). The expression and distribution of this glycoprotein was drastically altered during carcinogenesis (Fig. 1b–f). Approximately 8–9 weeks after initiation of the aflatoxin B₁ regimen, altered cell foci with markedly diminished expression of AGp110 were noted (Fig. 1b). Such foci were characterized by parenchymal disorganization, closer packing of cells and augmentation of cell size (Fig. 1b and c). Continuation of aflatoxin treatment over longer periods of time resulted in an increase in the number and size of these hyperplastic lesions that lacked significant amounts of AGp110 (Fig. 1c; 24 weeks of aflatoxin in the diet). At the same time, however, from the 5th week onwards, we occasionally observed increased expression in periporal areas, often manifested as pericellular staining (Fig. 1d). The cells that exhibited this apparently non-polarized staining were closely packed together with no discernible plate structure but appeared indistinguishable from hepatocytes in the surrounding parenchyma and did not have the appearance of oval cells. In poorly differentiated hepatic tumours AGp110 was virtually absent (Fig. 1e). The boundaries of such tumours were distinctly demarcated by the lack of AGp110 stain from adjacent morphologically normal parenchyma, as was evident in areas of normal tissue being invaded by carcinoma (Fig. 1e, arrows). Pseudoglandular, adenoma-like tumours exhibited apical membrane staining only (Fig. 1f, arrows). The intensity of that apical stain varied, but mostly appeared weaker than canalicular stain in normal liver. In tumours induced by diethylntrosoamine AGp110 was similarly lacking in poorly differentiated carcinomas but persisted on the apical domain of pseudoglandular hepatocarcinomas, as described for aflatoxin-induced tumours (Fig. 1e and f). Ductular cholangiomas in either aflatoxin B₁ or diethylntrosamine-induced tumours showed variable expression: most were positive on the apical domain, but occasionally minimal staining was observed. Proliferating bile duct cells appeared negative (results not shown).

**Fibronectin**

Normal liver was intensely stained for fibronectin (Fig. 2a1), this matrix protein being particularly prominent on sinusoidal cell surfaces. As previously documented, fibronectin can also be detected on canalicular and lateral surfaces of hepatocytes (Hughes and Stamatoglou, 1987; Enrich et al. 1988), but this is more evident using immunofluorescence on frozen sections (Hughes and Stamatoglou, 1987) rather than enzyme immunocytochemistry on paraffin wax-embedded tissue sections (Fig. 2a1). Strong staining was also observed in the cytoplasm of control parenchymal cells (Fig. 2a1; Hughes and Stamatoglou, 1987) in accordance with results indicating that hepatocytes are the main source of plasma fibronectin (Tamkun and Hynes, 1983). During aflatoxin administration, an overall decline in cytoplasmic staining was observed 3–4 weeks after the initiation of the carcinogenesis regimen and, additionally, marked reductions in both cell surface and cytoplasmic fibronectin occurred selectively in altered foci after the 8th–9th week of treatment (Fig. 2a2; 22 weeks of aflatoxin in the diet). In poorly differentiated hepatocellular carcinomas (Fig. 2a3) the expression of fibronectin was similarly reduced but cell surface staining was frequently conspicuous, particularly in areas that maintained the normal liver cord appearance (Fig. 2a3, arrows). Pseudoglandular hepatocellular carcinomas were invariably weakly positive for fibronectin along the basal surface of the cells lining the lumen (Fig. 2a4, arrowheads) but pericellular staining could, occasionally, be detected (Fig. 2a4, arrows).

**Integrin αβ₁**

This fibronectin receptor was distributed in a manner similar to fibronectin, in this case the pericellular, non-polarized localization of the protein being more distinct (Fig. 2b; b1, control liver; b2, liver from animal fed aflatoxin for 5 weeks, with marked membrane domains, sinusoidal (s), canalicular (c) and lateral (l)). As with fibronectin, overall expression of αβ₁ noticeably declined approximately 4 weeks after initiation of aflatoxin dietary administration (Fig. 2b2). Further marked reductions in αβ₁ expression were detected after 9 weeks of treatment in altered foci and then in poorly differentiated tumours (not shown: see section on double staining) although cell surface staining was usually retained, albeit with reduced intensity.

Small acinar, duct-like structures, frequently seen within trabecular carcinomas (Fig. 2b3), were positive for integrin, the protein being quite prominent in lumina (Fig. 2b3, arrow) that resembled enlarged canaluli, as well as along basolateral cell surfaces. Adenomas with larger pseudoglandular structures (Fig. 2b4), perhaps emanating from the small acinar formations in trabecular carcinomas (Fig. 2b3), were negative for αβ₁ on apical (Fig. 2b4, arrow) and lateral epithelial surfaces; expression on the basal surface was also barely discernible.

**Comparative distribution of AGp110 and fibronectin**

The spacial and temporal expression of AGp110 during aflatoxin-induced hepatocarcinogenesis was compared with that of fibronectin in double-label experiments. As described in control liver AGp110 was detected in both canaluli whereas fibronectin was most conspicuous in sinusoids (Fig. 3a). AGp110 was also visible on the apical surface of bile duct epithelium (Fig. 3a, arrow). By the 6th–8th week after initiation of the aflatoxin treatment, expression of both proteins became slightly reduced in small foci that initially did not appear overly hyperplastic, apart from a slight increase in individual cell size and concurrently increased expression of both proteins was noticed in periporal areas (Fig. 3b). On longer exposure to aflatoxin (from the 9th week onwards), distinct altered or hyperplastic foci appeared (Fig. 3c; 14-week treatment) that, presumably, gave rise to larger pre-neoplastic lesions observed later on (Fig. 3d; 32-week treatment). Such hyperplastic and pre-neoplastic lesions were clearly deficient in both AGp110 and fibronectin (Fig. 3c and d). In altered foci, which were beginning to take on the appearance of adenomas, AGp110 was localized on the apical surface of the luminal epithelium whereas fibronectin was sparsely distributed along the basal cell surface (Fig. 3e). In poorly differentiated hepatocellular carcinomas little staining for either protein was observed (Fig. 3f), although fibronectin persisted, albeit weakly, on the cell surface. Furthermore, fibrillar accumulations of fibronectin were sparsely dispersed in both adenomas and poorly differentiated carcinomas (Fig. 3e and f). Some of these areas were encapsulated by extracellular matrix intensely positive for fibronectin, or by rows of hepatocytes over-producing fibronectin (Fig. 3e and g). It is worth noting that groups of cells in tumours that appear to preserve a differentiated morphology, maintain a normal
pattern of staining for both AGp110 and fibronectin (Fig. 3h).

**Comparative distribution of AGp110 and integrin α5β1**

In double-label experiments of AGp110 and integrin α5β1, we were able to confirm the results we obtained in single-label experiments and to establish that the alterations in the expression of AGp110 during hepatocarcinogenesis coincide with those observed for α5β1 (Fig. 4). Altered cell foci (Fig 4c and d) and poorly differentiated carcinomas (Fig 4e, area marked 1) were deficient in both proteins, although α5β1 could still be detected on the cell surface. The normal distribution pattern and expression of AGp110 (Fig. 4a and b) was lost in such lesions. In adenomas (Fig. 4e, area marked 3, and f) the pseudoglandular lumen (Fig. 4e and f, arrows) was AGp110-positive whereas α5β1 could only be detected on the basal surface of the cells (Fig. 4e and f, arrowheads). Areas in tumours sustaining a morphology reminiscent of normal parenchyma were positively stained for α5β1 antigens (Fig. 4b, area marked 2). Our overall impression from these double-label experiments was that the difference in expression between normal and neoplastic liver was significantly more pronounced for AGp110 than for α5β1. Furthermore, the observed reduction of α5β1 on tumour cell surfaces was less than that seen for fibronectin (Figs 3 and 4).

**Comparative distribution of AGp110 and cytokeratin 19**

The expression of AGp110 during chemically induced carcinogenesis was most conspicuously altered in poorly differentiated foci and tumours. To assess the potential usefulness of AGp110 as an histological marker of such lesions in transformation we compared the distribution of the proteins with that of cytokeratin 19 (ck19), a selective marker for hepatic transformation. In normal liver ck19 is confined to bile duct cells but in altered foci, groups of hepatocytes synthesize this cytokeratin (Green et al. 1990). Loss of AGp110 in hepatocellular foci occurred earlier (from 9 weeks of treatment) than appearance of ck19 (from 14 weeks of treatment). This change in ck19 expression was restricted to a few foci initially but, as the treatment progressed, more and larger lesions were observed. The various patterns of double-staining of AGp110 and ck19 are shown in Fig. 5: (i) in parenchyma with normal morphology AGp110 was located on canalicular membranes and ck19 in bile duct cells (Fig. 5a); (ii) some altered cell foci were AGp110-negative but ck19 was still restricted to bile duct cells (Fig. 5b); (iii) in other foci with minimal AGp110 expression, however, ck19 was detected in hepatocytes being organized around lumina (Fig. 5c) that remained AGp110-positive (see previous sections), although in this case this could not be conclusively confirmed or refuted, since the strong ck19 reaction in the luminal area masked AGp110 stain; (iv) finally, there were foci positive for both AGp110 and ck19 (Fig. 5d).

From the 9th to the 14th week of treatment virtually none of the AGp110-negative altered foci showed any ck19 in hepatocytes (as in Fig. 5b). From then on, the patterns portrayed in Fig. 5c and d appeared, the pattern in Fig. 5c becoming progressively more frequent although we could still observe lesions being negative for both proteins (Fig. 5b). In tumours, ck19 was expressed only in pseudoglandular hepatomas (not shown) as described (Green et al. 1990). In poorly differentiated carcinomas ck19 was occasionally present, although these tumours were usually histologically heterogeneous and ck19 was found in pseudoacinous areas.

**Discussion**

Distinct histological changes were observed during hepatocarcinogenesis, first at the pre-neoplastic stage, early on during aflatoxin B1 dietary administration, and then in tumours induced by either aflatoxin B1 or diethylnitrosamine. The histopathology of induced hepatocyte lesions was as described previously by other investigators (Ogawa et al. 1979; Williams, 1980). In brief, hyperplastic foci that appeared early during AFB1 treatment increased in number and size to form so-called pre-neoplastic nodules with a morphology similar to that of foci. At a later stage, approximately 9 months after initiation of either carcinogenesis regimen, tumours appeared that could be classified as poorly differentiated, trabecular or pseudoglandular (adenoma). In general, the one-cell-thick plate structure of the normal parenchyma would adopt either of the following three morphologies in carcinogenesis: (i) poorly differentiated, apparently non-polarized groups of cells appearing first and persisting throughout; (ii) groups of hepatocytes organizing around a dilated, stellate canaliculus were present in late (14th week onwards) altered foci and nodules and in trabecular carcinomas; (iii) large ducts in pseudoglandular carcinomas (adenomas). Furthermore, a certain degree of structural heterogeneity was observed in most tumours and areas in between tumours often maintained the plate structure of normal liver. Adenomas may arise from small acinar configurations, since the presence of cytokeratin 19 in both structures (see Results, and Green et al. 1990) suggests a common origin. Moreover, the number of acini and the cross-sectional area of the lumen increased progressively at the pre-neoplastic stage. Use of the antiserum against the apical antigen AGp110 indicated a frequent occurrence of acini in trabecular carcinomas where these glandular structures cannot usually be easily recognized with conventional histochemical stains. It is worth noting that similar glandular formations also occur during non-pathological liver growth, in late embryogenesis and during regeneration following partial hepatectomy (Ogawa et al. 1979). In this study, cytokeratin 19, normally absent in parenchymal cells, was always expressed in hepatocytes organizing around a lumen and could be the causal factor in initiating and/or maintaining this rearrangement, perhaps mediated by an association of this intermediate filament protein with desmosomal components.

Significant alterations in the pattern of expression of AGp110, integrin α5β1 and fibronectin were observed during chemical induction of hepatocarcinogenesis. Poorly differentiated hyperplastic, pre-neoplastic and tumour lesions were deficient in all three antigens, particularly AGp110. In small acinar structures, found in hyperplastic lesions and trabecular carcinomas, AGp110 was maintained on the apical (luminal) surface whereas fibronectin and α5β1 were present on all surfaces. In pseudoglandular carcinomas, however, all three proteins appeared polarized: AGp110 persisted on the luminal cell surface but fibronectin and α5β1, (the latter noticeably reduced in expression) were redistributed along the basal surface. Overall therefore, the results indicate that the pattern of distribution and the expression of fibronectin and its receptors relate to, or perhaps induce, distinct structural arrangements of the parenchyma: poor differentiation correlates with deficiency in AGp110, α5β1 and fibronectin, whereas pseudo-differentiated structures such as acini or adenomas with polarized cells preserved the proteins. The
Fig. 1. Distribution of AGp110 in rat liver during AFB\textsubscript{1}-induced hepatocarcinogenesis. Paraffin-embedded acetone-fixed liver sections from rats fed AFB\textsubscript{1} for various times were stained with anti-AGp110 serum followed by secondary antiserum conjugated to alkaline phosphatase (see Materials and methods). Red deposit of alkaline phosphatase substrate shows the localization of AGp110.
(a) Control male Fischer rat. AGp110 is confined to bile canaliculi. (b) 9 weeks of AFB\textsubscript{1} at 2 p.p.m. in the diet. Decreased expression of AGp110 in some altered cell foci (central, delineated area) but normal distribution in most areas. (c) 24 weeks of treatment. Pre-neoplastic lesion with markedly reduced expression of AGp110. (d) Treatment as in c. Periportal region showing increased expression of AGp110 and apparent pericellular, non-polarized localization of the protein. (e) 10 months of AFB\textsubscript{1} at 1 p.p.m. in the diet, followed by 2 months on control diet. Non-differentiated hepatocellular carcinoma negative for AGp110. Carcinoma invades AGp110-positive tissue with normal morphology; the invading cellular front is marked by arrows. (f) Treatment as in e. AGp110 is localized on the lumen of pseudoglandular tumour (arrows). Bar, 100 μm.
Fig. 2. Distribution of fibronectin (a) and fibronectin receptor, integrin $\alpha_5\beta_1$ (b) during AFB$_1$-induced hepatocarcinogenesis. (a1) Fibronectin (Fn) in control male Fischer rat. Strong intracellular and cell surface staining. (a2) 22 weeks of AFB$_1$ in the diet. Overall reduction in fibronectin synthesis (area marked 2) with further decrease shown in altered cell focus (area marked 1). Expression on cell surface may persist. (a3) 9 months of treatment. Hepatocellular carcinomas exhibit reduced expression of fibronectin but cell surface staining still present, especially in regions that display normal morphology (in between arrows). (a4) 9 months of treatment. Pseudoglandular hepatic carcinoma. Variable expression of fibronectin on cells lining the lumen: mostly negative (open arrow), but occasionally pericellular staining, apparent on basolateral surfaces can be distinguished (arrows). Subepithelial fibrillar-like staining is also shown (arrowheads). (b1) Integrin $\alpha_5\beta_1$ distribution in control male Fischer rat. Protein is expressed on all surface domains of hepatocytes and intracellularly, as fibronectin (a1). (b2) 5 weeks of AFB$_1$ in the diet. Overall reduction in $\alpha_5\beta_1$ intracellular expression in liver but surface staining appears equally strong as in control (b1). Pericellular nonpolarized staining is clearly shown: $\alpha_5\beta_1$ is found on sinusoidal (s), lateral (l) and canalicular (c) surface domains. (b3) 8 months of AFB$_1$ in the diet. Hepatocellular carcinoma with reduced expression of $\alpha_5\beta_1$. Arrow points to acinar formation where $\alpha_5\beta_1$ persists in the lumen. (b4) Pseudoglandular hepatic carcinoma. Note absence of $\alpha_5\beta_1$ in lumen (arrow). Bar, 100 $\mu$m.
Fig. 3. Double-label staining of AGp110 and fibronectin in livers of AFB₁-fed rats. AGp110 localization is shown by blue colour and fibronectin by red colour (see Materials and methods). (a) Control liver. AGp110 staining along the biliary tree, whereas fibronectin is mostly obvious on sinusoidal surface. Note AGp110 stain on apical domain of bile duct epithelium (arrow). (b) 8 weeks of AFB₁ in the diet. Normal cell surface distribution for AGp110 and fibronectin is maintained in most areas but focal reductions in expression can occasionally be observed as well as enhanced expression mainly in periportal areas (see text). (c) 14 weeks of treatment. Concurrent reduction of AGp110 and fibronectin in altered foci. (d) 32 weeks of AFB₁ in the diet. Pre-neoplastic nodule with markedly decreased staining for both AGp110 and fibronectin. (e) 10 months of AFB₁ in the diet. Large altered focus with adenoma-like (pseudoglandular) lesions. Overall reduction of AGp110 and fibronectin in this area. Fibronectin staining encapsulating the area and occasionally scattered within it. AGp110 present only in lumen of ducts. (f) 10 months of AFB₁ in the diet. Non-differentiated hepatocellular carcinoma with minimal expression of AGp110 and fibronectin. (g) 10 months of AFB₁ in the diet. Stratum of cells at border of tumour (right) overproducing fibronectin and expressing normal levels of polarized AGp110. (h) 10 months of AFB₁ in the diet. Cord of hepatocytes, traversing morphologically poorly differentiated tumour, that expresses normal levels of AGp110 and fibronectin. Bar, 100 μm.
Fig. 4. Double-label staining of AGp110 and integrin αsβ1 in livers of AFB1-fed rats. αsβ1 is marked by red colour and AGp110 by blue colour (see Materials and methods). Dark mauve colour of canaliculi (as in b) results from co-localization of the antigens. (a) Liver from rat fed AFB1 for 8 weeks. Area showing normal distribution of both proteins as in control livers but a reduction of αsβ1 intracellular stain has already occurred (cf. Fig. 2b1 and b2). (b) Detail from a. αsβ1 is evenly distributed on all hepatocyte membrane domains but AGp110 is found only on canalicular surfaces. (c) 22 weeks of AFB1 in the diet. Altered cell foci. Decrease in the stain intensity for both αsβ1 and AGp110 is observed. (d) Detail from c. In comparison to morphologically normal parenchyma (top left), hepatocytes of the altered focus (bottom right) exhibit reduced αsβ1 and minimal AGp110. Note that αsβ1, though reduced, persists on cell surfaces in the focus. (e) 10 months of treatment. Hepatocellular carcinoma. Adenoma-like region (3) shows apical AGp110 stain (arrows) and basal αsβ1 stain (arrowheads). In areas where some plate structure is preserved both proteins appear to be present (2) but poorly differentiated carcinomas lack AGp110 and show deficiency in αsβ1 (1). (f) 9 months of treatment. Pseudocanalicular carcinoma where cells are virtually negative for αsβ1 (arrowheads) but remain weakly positive for AGp110 on their apical domain (arrow). Bar, 100 μm.

unpolarized state of cells in undifferentiated hepatomas has been documented (Scoazec et al. 1988).

In addition to AGp110, expression of other canalicular glycoproteins has also been shown to be altered by cell transformation (Becker et al. 1985; Hixson et al. 1985; Scoazec et al. 1988). Furthermore, depolarization of a canalicular antigen may precede its eventual disappearance in undifferentiated tumours (Scoazec et al. 1988) and evidence for such depolarization is also presented here (Fig. 1d). The absence of AGp110 in poorly differentiated carcinomas may enhance their tumorigenicity and invasive potential by reducing stabilizing cell–fibronectin interactions.

Our findings concerning the reduction of fibronectin synthesis in hepatocytes, especially in poorly differentiated tumours, corroborate earlier observations on liver (Sell and Ruoslahti, 1982; Jagirdar et al. 1985; Szendroii and Lapis, 1985) and other cells (Hynes and Yamada, 1982). The expression of integrin αsβ1 has not, to our knowledge, been studied previously in liver tumours, but investigations on virally transformed rodent cells (Plantefaber and Hynes, 1989) and on highly tumorigenic human cells (Dedhar and Saulnier, 1990) have produced results in line with our own, i.e. down-regulation of αsβ1. Transformation may not only affect expression but function too. Transformation of chicken embryo fibroblasts by oncogenes encoding tyrosine kinases results in phosphorylation of the chicken fibronectin receptor and consequent impairment of its binding affinity for fibronectin and talin (Hirst et al. 1986; Topley et al. 1989). Reduced expression of αsβ1 has been correlated to high tumorigenicity (Giancotti and Ruoslahti, 1990; Schreiner et al. 1991). Synthetic peptides containing the RGD sequence of fibronectin recognized by some integrins, including αsβ1 (Ruoslahti and Pierschbacher, 1987), can inhibit malignant invasion and metastasis (Gehlsen et al. 1988; Humphries et al. 1986; Saiki et al. 1989). Transformation affects other integrins too (Buck et al. 1990; Virtsen et al. 1990; Dedhar, 1990): for instance, chemical transformation of human cells into highly tumorigenic cells results in increased expression of αsβ1, αsβ3 and αsβ5 receptors for laminin, collagen and type IV collagen and laminin, respectively (Dedhar and Saulnier, 1990).

Our study focused on pre-neoplastic and neoplastic lesions that appeared to be of hepatocellular origin. Only a fraction of altered cell foci appear to develop into tumours (Farber, 1980; Williams, 1980; Pitot and Sirica, 1980; Emmelot and Seherer, 1980) and no definitive single marker for these pre-malignant cells has been identified. An alternative possibility is a de novo genesis of carcinomas from hepatocyte stem cells (Sell and Dunsford, 1989). In our study early hyperplastic foci appeared to originate from parenchymal cells and loss of differentiation correlated with deficiency in AGp110, αsβ1 and fibronectin in early and later hyperplasia and, finally, in tumours. Adenomas, positive for ck19, seemed to emanate from small pseudoacinar structures, also positive for ck19. These acini were formed in foci from hepatocytes organizing around dilated canaliculi. Furthermore, loss of AGp110 and appearance of ck19 in hepatocytes was an event of comparatively infrequent incidence among early hyperplastic foci but occurred in all undifferentiated tumours and adenomas, respectively. AGp110 and ck19 appear therefore to be good cell markers for progenitors of two different types of carcinomas and our study supports the hypothesis that at least some tumours arise from differentiated hepatocytes.

We are grateful to Dr R. O. Hynes (MIT, USA) for the anti-fibronectin monoclonal antibody, to Dr Staffan Johansson (Biomedical Centre, Uppsala, Sweden) for the anti-αsβ1 antibody and to Dr E. B. Lane (University of Dundee, UK) for the anti-cytokeratin 19 antibody. We also thank Dr P. Cartew for much helpful discussion, Mr Neil Papworth for advice on photography and Mr J. Morgan for printing the photographs. The excellent secretarial assistance of Ms Marilyn Brennan is gratefully acknowledged.

References


Fibronectin and its receptors in liver cancer

603


