Changes in cell surface expression of fibronectin and fibronectin receptor
during liver regeneration

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Summary

The surface expression of fibronectin and its major integrin receptor in liver, integrin α5β1, was studied during liver regeneration after partial hepatectomy. Using immunoblotting, plasma membranes isolated from livers at different regeneration stages were found to contain 6- to 8-fold elevated levels of fibronectin, α5 and β1 at 12-24 h after the operation. Normal levels were gradually restored during the following 8-9 days. The membrane-associated fibronectin lacked the ED-A domain, suggesting that it consisted of plasma fibronectin. A prominent fibronectin fragment (180 kDa) was present at 12-24 h after surgery, possibly reflecting turnover of the pericellular matrix during cell division.

Indirect immunohistochemical staining of liver sections revealed β1 and fibronectin mainly in the sinusoidal region of the hepatocyte plasma membrane. The distribution was not markedly altered during liver regeneration. The results suggest that the fibronectin-mediated contacts between the cells and the extracellular matrix increase during the pre-replicative and proliferative phases of liver regeneration. The significance of these results for the growth and for the structure of the liver during regeneration and development is discussed.

Key words: fibronectin, fibronectin receptor, liver regeneration.

Introduction

Liver cells initiate the transition from resting state (Go phase) to the proliferative state after specific stimuli such as partial hepatectomy (PHX), when about 70% of the organ is removed. Loss of parenchyma rapidly induces a wave of cell proliferation so that the normal mass of the liver is restored within 8-10 days (in the rat). Although all cells of the liver participate in the regeneration, most studies have been focused on the main functional cells of the liver, the parenchymal hepatocytes (Michalopoulus, 1990). They represent about 65% by number of liver cells and about 90% of the liver mass. These cells are polarized with different functions and structures associated with their three plasma membrane domains: (i) the sinusoidal domain, which has direct contact with the blood and where most receptors for growth factors and hormones are located; (ii) the lateral domain, which contains several types of cell-cell contacts, including desmosomes and gap junctions; (iii) the canalicular domain, involved in the formation and secretion of bile. In addition to hepatocytes, the liver also contains significant numbers of endothelial cells, Ito cells and Kupffer cells (Arias et al., 1988) that contribute to the function of the organ. Obviously, the mitotic activity of each cell type has to be coordinated in order to yield a normal structure of the regenerated tissue. Furthermore, the hepatocytes have to obtain their polarization after mitosis if the intricate canal systems of the organ are to be maintained. These aspects of liver growth may be similar during regeneration and embryonic development.

The kinetics of the regenerative response in hepatocytes have been well described (Grisham, 1962; Rabes et al., 1976); DNA synthesis starts 12-16 h after PHX and reaches a peak within 22-24 h, followed by a mitosis wave 6-8 hours later. The proliferative activation process is quite synchronized and about 30% of the hepatocytes enter into the first reproduction cycle (Alison, 1986). A second, less pronounced peak of parenchymal DNA synthesis occurs around 48 hours after PHX. At about this time DNA synthesis in the nonparenchymal cells also starts.

Cell division and organ growth require that existing contact points between neighbouring cells and between cells and extracellular matrix are remodeled and that new contacts are formed. Whereas some information is available on how cell-to-cell contacts are affected during liver regeneration (Hughes and Stamatoglou, 1987; Odin and Öbrink, 1988), little is known about
how the matrix contacts may be modulated. Fibronectin is a cell adhesive protein of extracellular matrices (Hynes, 1986) and a major component associated with the hepatocyte cell surface (Enrich and Evans, 1987; Coll et al., 1986). Although several membrane components have been suggested to contribute to the binding of FN (Stamatoglou et al., 1990), integrin α5β1 appears to be a dominating FN receptor on hepatocytes (Johansson, 1985; Forsberg et al., 1990). As with all members of the integrin protein family, this receptor is a heterodimeric, transmembrane protein that mediates cell adhesion (Hynes, 1987; Ruoslahti and Pierschbacher, 1987; Hemler, 1990; Humphries, 1990).

During liver regeneration and fibrosis, increased amounts of fibronectin have been reported to be associated with the cell surface (Enrich et al., 1988; Hahn et al., 1980). Further, striking changes in the adhesive properties of hepatocytes after having entered into the proliferative stage have been demonstrated (Hynes and Yamada, 1982; Carlsson et al., 1981). The basis for these observations has not been resolved, and no detailed studies have been performed on specific cell surface receptors for matrix proteins during the regenerative process. In this investigation, FN and the major FN receptor integrin α5β1 have been studied during the proliferation process in vivo after PHX.

Materials and methods

Isolation of integrins α5β1 and α5β1

Integrins α5β1 and α5β1 were purified as previously described (Forsberg et al., 1990) except that whole rat liver was used as starting material instead of isolated hepatocytes. A 30 g sample of rat liver was homogenized with a Potter-Elvehjem homogenizer in 150 ml of solubilization buffer containing 2% Triton X-100, 10 mM EDTA, 10 mM Tris-HCl, pH 7.4. After stirring for 30 min at 4°C, the homogenate was centrifuged at 25,000 g for 45 min at 4°C, the supernatant was applied to a column of WGA-Sepharose previously equilibrated with 0.2% Triton X-100, 2 mM MnCl2, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4 (integrin buffer). After washing, the column was eluted with 0.3 M N-acetyl-D-glucosamine in the same buffer, and the eluate was applied sequentially to Sepharose conjugated with a 105 kDa FN (fibronectin) fragment and to laminin-Sepharose. The latter columns were washed with integrin buffer and eluted with 0.2% Triton X-100, 10 mM EDTA, 150 mM NaCl, 10 mM Tris-HCl, pH 7.4. Protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM N-ethyl maleimide (NEM)) were included in the buffers.

Antibodies

A mouse monoclonal antibody (mAb) specific for the ED-A domain of FN (Borsi et al., 1987) was kindly provided by Dr. L. Zardi (Genova, Italy). Rabbit antisera against FN, integrin subunit β1, and integrin subunit α5, respectively, were the same as described previously (Johansson and Hook, 1984; Forsberg et al., 1990; Stamatoglou et al., 1991). A rabbit antisera against integrin subunit α5 was raised by intramuscular injections of α5 which had been cut out from an SDS-polyacrylamide gel. This antiserum reacted specifically with α5 in western blots (see Fig. 3).

Partial hepatectomy

Male Sprague-Dawley rats weighing 200-250 g were used for all experiments. Partial hepatectomies (PHX) were performed between 8 a.m. and 10 a.m. The surgery was carried out according to Higgins and Anderson (1931) on ether-anesthetized animals. Sham-operated rats were used as controls.

Plasma membrane fractions

Hepatocyte plasma membranes were isolated following the protocol established by Bachmann et al. (1977). Whole livers from normal, sham-operated and partially hepatectomized rats, respectively, were homogenized in a hypotonic buffer (1 mM NaHCO3, 0.5 mM CaCl2, 0.2 mM PMSF pH 7.4). After differential centrifugation, the pellet was applied onto a discontinuous sucrose gradient and centrifuged at 96,000 g for 2 h. Finally, the plasma membranes were washed with 10 mM Tris-HCl, pH 7.4. Protein content of the fractions was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. Analysis of the fractions by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that the concentration of the major proteins was similar in all membrane preparations (data not shown).

Immunostaining

Rat livers were fixed in vivo by perfusion with 3% paraformaldehyde solution and were subsequently paraffin embedded. Sections of 5 μm thickness were cut, deparaffinized, rehydrated and incubated with primary antibodies (1:200 dilution) overnight at 4°C, followed by washing in phosphate-buffered saline (PBS) (3 x 10 min). After incubation with swine anti-rabbit immunoglobulins (DAKOPATS, Denmark) diluted 1:30 for 60 min at room temperature, the sections were washed as described above and incubated with soluble complex of horseradish peroxidase and rabbit anti-swine immunoglobulins (DAKOPATS, Denmark) diluted 1:70 during 60 min. After thorough washing the staining was developed with diaminobenzidine (DAB) and mounted in DPX. Controls were carried out by incubating the sections with antibodies absorbed with the antigen.

Electrophoresis and immunoblotting

Electrophoresis of proteins was performed in SDS-polyacrylamide slab gels according to Blobel and Dobberstein (1975). For immunoblotting analysis, the proteins were electrophoretically transferred (3 h at 60 V) to nitrocellulose sheets (Burnette, 1981). The nitrocellulose was preincubated in Tris-buffered saline (TBS) containing 1% bovine serum albumin (BSA) for 1 h at room temperature, followed by incubation for 1 h at room temperature with the same buffer containing antisera against FN (1:200 dilution), subunit β1 (1:200 dilution), subunit α5 (1:100 dilution) or subunit α1 (1:100 dilution). After several washings with TBS containing 0.01% Triton X-100, the sheets were incubated with TBS containing 1% BSA and 125I-labelled protein A (0.5 x 106 to 1.0 x 106 cts/min per ml) for 1 h at room temperature, and extensively washed. When the mAb was used as primary antibody, an incubation with rabbit anti-mouse IgG serum was included before the 125I-protein A step. The recognized antigens were visualized by autoradiography using X-ray film (X-OMAT AR, Kodak). Quantification of stained bands was done in a laser scanner (UltraScan XL, LKB-Pharmacia).
Results

Cell surface content of FN and FN receptor α5β1 during liver regeneration

Immunoblotting analysis of parenchymal plasma membrane fractions isolated at different time points after PHX revealed an increased amount of plasma membrane-associated FN at 12 h after surgery, corresponding to the G1 phase of the cell cycle. High levels of FN were detected until the fourth day of the regenerative process. From this time on, cell surface expression of FN decreased progressively until the 9th day post-operation, when the amount appeared equal to that in resting, normal liver (Fig. 1). At 12 h and 24 h after surgery, the antiserum detected a component with an Mr of 180,000 in addition to FN (Fig. 1). This component was consistently found at these time points in several series of partially hepatectomized rats. Most likely, the 180 kDa band represents a proteolytic fragment of FN. A monoclonal antibody specific for FN isoforms containing the ED-A domain did not react with the membrane-associated FN at any time point during the regeneration (data not shown). This result indicates that the bound FN was of the type synthesized by normal adult hepatocytes ("plasma FN"). Plasma membranes from sham-operated rats did not contain elevated levels of FN or the 180 kDa fragment (Fig. 1).

Binding to integrin α5β1 is one possible mechanism by which FN could be associated with the plasma membranes. Therefore the presence of integrin subunits β1 and α5 in the membrane fractions was studied. In the case of subunit β1 a markedly increased amount (≈8-fold) was seen 12 h after PHX. Except for a second smaller peak appearing around day 4, the amount of β1 declined until the original level was reached 10 days after PHX (Fig. 2). Similarly, the α5 subunit was significantly increased (≈6-fold) 12 h after PHX. The level of α5 in the plasma membrane remained elevated up to day 4 after the operation, and returned to normal levels at the end of the regeneration process (Fig. 3). For comparison, the amount of the major receptor for collagen and laminin on hepatocytes, integrin α1β1 (Forsberg et al., 1990; Gullberg et al., 1990; Stamatoglou et al., 1991), was analysed analogously. The content of α1 in plasma membranes was elevated approximately 3-fold at 12 h after PHX, and returned to normal levels after 4 days (Fig. 4). Sham-operated control rats showed no differences from normal non-operated rats in the plasma membrane content of the integrin subunits β1, α5 and α1.

Localization of FN and integrin subunit β1 in normal and regenerating liver tissue

The distribution of FN and the β1 subunit in liver tissue sections was studied using immunohistochemical methods. In normal liver, FN was localized around the cells, with the predominant staining present along the sinusoidal plasma membranes of hepatocytes (Fig. 5a) as previously shown by Martinez-Hernández (1984). The integrin subunit β1 was also located at the sinusoidal face with a pattern resembling that of fibronectin (Fig. 5c). When the distribution during regeneration was analyzed no obvious changes in the localization of the two proteins were seen (Fig. 5b, d, e, f). It should, however, be noted that 24 h after PHX, the cellular volume had doubled, thus causing a shrinking of the sinusoidal space (Fig. 5e). Changes in the staining intensity during the time course of regeneration were observed, which correlated with the results obtained in the immunoblotting experiments. At
Fig. 2. Immunoblot analysis of integrin subunit $\beta_1$ in hepatocyte plasma membranes. Identical samples of hepatocyte plasma membranes to those in Fig. 1 were run in unreduced form in a 8% SDS-PAGE, transferred to nitrocellulose sheets and incubated with anti-$\beta_1$ antiserum. A sample of isolated FN receptor was run as a standard in the first lane and as a control of the antiserum specificity in the second lane. The immunoreactive bands were: (A) visualized and (B) quantified as in Fig. 1.

9 days of regeneration the organization of cells as well as the labelling intensity for $\beta_1$ (Fig. 5f) and for FN (not shown) was similar to that in normal livers.

Discussion

Liver regeneration provides an experimental model with which to study the transition from the quiescent to replicative state of cells in vivo. During this transition the cells undergo a series of changes, some of which are similar to those observed in the hyperplastic nodules in the early phase of liver carcinogenesis, and they also acquire some characteristics of fetal and neonatal hepatocytes (Curtin and Snell, 1983). These changes include alterations in cell surface properties, such as the composition of glycoconjugates (Coll et al., 1986), and the level of receptors for hormones and growth factors (Leffert et al., 1975; Rubin et al., 1982) and of proteins involved in cell-cell contacts (Odin and Öbrink, 1986).

Previous studies on the latter type of proteins are of particular interest in relation to our results on cell-matrix contacts. Whereas the increased amounts of FN and integrins $\alpha_5\beta_1$ and $\alpha_1\beta_1$ present in isolated plasma membranes during regeneration suggest that cell-matrix contacts are transiently reinforced, cell-cell contacts are partly dissolved during this phase of the process. For instance, the membrane concentration of the gap junction protein becomes dramatically reduced for a short period coinciding with the first mitotic wave of hepatocytes at 28-35 h after PHX, and is restored 15-20 h later (Traub et al., 1983). Cell CAM-105, an intercellular adhesion molecule (Ocklind and Öbrink, 1982), is also found in reduced amounts in plasma membranes during liver regeneration (Odin and Öbrink, 1986), but its concentration decreases somewhat later than the gap junction protein and reaches a
Fig. 5. Staining for FN (a, b) and integrin subunit β1 (c-f) was performed on normal liver (a, c) and on regenerating liver 12 h (d), 24 h (b, e) and 9 days (f) after PHX. For further experimental details, see Materials and methods. Bar, 10 μm.
minimum level 1.5-3 days after PHX at the time of cell division (Odin and Öbrink, 1986). Compared to the kinetics of these changes, the increased surface expression of the FN receptor appears to occur early, with maximal amounts found 12-24 h after PHX (at or before the peak of DNA synthesis). This rapid increase was followed by a slow decline to the normal level. Notably, a similar result has been reported in a different model of cell growth where changes in mRNA concentrations were studied during the transition from G0 to G1 of growth-arrested fibroblasts in vitro. In this system the integrin α5β1 mRNA was identified as one of a group of mRNA species that increased markedly (Tominaga, 1988). These results support the general ideas of a coupling between matrix adhesion and DNA synthesis (Hedin et al., 1988).

The integrin β1 and FN were mainly distributed along sinusoids in normal liver, and this pattern did not significantly change during the regenerative process. In contrast, cell CAM-105 was previously shown to become redistributed during regeneration, from the normal restricted location at the apical cell surface (around bile canaliculi) to an even distribution on all surfaces of the hepatocytes 3-5 days after PHX (Odin and Öbrink, 1988). This redistribution was interpreted to reflect a depolarization of the cells, but it is not yet clear if this is a general effect affecting also other membrane components. Since receptors for extracellular matrix remained unevenly distributed on the hepatocyte surface during the proliferation period, they may be of importance for the restoration of a polarized organization of hepatocytes.

Except for some minor differences, the kinetics of the surface expression of the integrin subunits α5 and α1 after PHX followed a similar pattern. The amount of FN associated with the plasma membranes changed closely in parallel with the α5 subunit, in accordance with other studies showing that integrin α5β1 is a major FN receptor of hepatocytes (Forsberg et al., 1990; Johansson et al., 1987), and that it is involved in deposition of FN fibrils on the surface of fibroblasts (Fogarty et al., 1990). The isotype of FN that bound to the cells was not fully determined. However, it lacked the ED-A domain, as the FN derived from normal adult hepatocytes does (Tamkun and Hynes, 1983). The ED-A domain is often present in FN of embryonic origin (Norton and Hynes, 1987), and it has been reported to be expressed by fetal liver and by malignant liver tumors (Oyama et al., 1989). Thus, in this respect the regenerating hepatocytes did not acquire the neoplastic or fetal character.

The proliferative response during liver regeneration is believed to be regulated mainly by a number of soluble growth factors, including TGFα and TGFβ (Michalopoulus, 1990; Mead and Fausto, 1989). In fibroblast cultures, TGFβ has been reported to cause a marked increase in mRNA and protein levels of several connective tissue components and integrin subunits, including FN, β1, α5 and α1 (Roberts et al., 1988; Heino et al., 1989). However, the concentration of TGFβ mRNA in liver increases late during regeneration and reaches more than 10 times the normal level 3 days after PHX (Mead and Fausto, 1989), consistent with data which implicate TGFβ as a paracrine inhibitor of hepatocyte DNA synthesis (Russell et al., 1988). At this time the amounts of β1, α5 and α1 in liver plasma membranes are decreasing. These results suggest that other factors than TGFβ are responsible for the increased surface expression of integrins α5β1 and α1β1 in hepatocytes.

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References


Blobel, G. and Dobberstein, B. (1975). Transfer of proteins across membranes. I. Presence of proteolytically processed and


