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Basic Studies

Hepatocyte survival depends on β1-integrin-mediated attachment of hepatocytes to hepatic extracellular matrix

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Abstract: *Background:* A major drawback of allogeneic hepatocyte transplantation is the lack of sustained survival of the transplanted cells in the recipient liver parenchyma. The purpose of this study was to determine the effect of the presence or absence of hepatic extracellular matrix (ECM) molecules on hepatocyte survival and function following hepatocyte isolation for transplantation purposes, and the role of β1-integrin molecules therein. *Methods:* Hepatocytes, either untreated or treated with anti-β1 integrin antibodies or RGD peptides, were seeded on wells precoated with collagen type I, type IV, laminin, fibronectin or polyhydroxyethylmehacrylate. The extent of attachment and apoptosis was evaluated. *Results:* When hepatocytes were added into wells precoated with either fibronectin, or collagen type IV, rapid spreading and prolonged survival occurred, in contrast to hepatocytes that were seeded in wells precoated with collagen type I or polyhydroxyethylmehacrylate. Pretreatment of the cells with anti-β1-integrin antibodies resulted in reduction of cell attachment to laminin, fibronectin, collagen I, and collagen IV. Synthetic RGD (arginine–glycine–aspartate)-peptides and anti-β1 antibodies inhibited apoptosis of cultured hepatocytes. *Conclusions:* Our findings indicate that embedding of hepatocytes within their normal liver ECM surroundings maintains their survival. When detached from their natural surrounding hepatocytes enter into apoptosis, unless treated with anti-β1-integrin antibodies or RGD peptides. This knowledge will allow improvement of hepatocyte transplantation efficiency.

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Hepatocyte transplantation offers an attractive alternative to total liver replacement in the treatment of congenital enzyme deficiencies in the liver. A major drawback of allogeneic hepatocyte transplantation is the lack of sustained survival of the transplanted cells in the recipient liver parenchyma. When hepatocytes are allogeneically transplanted by intraportal injection in a rat model, adherence of the transplanted cells to the endothelium of the portal vein occurs, followed by intravascular aggregation and massive cell death (1, 2). We showed earlier that these processes could be inhibited by adding blocking antibodies against the α1 and β1 integrins present on the transplanted hepatocytes. This leads to a significant increase of intra-hepatic arrival and survival of allo-transplanted hepatocytes from 2% up to 45% at day 2, and from 1.8% up to 21% at day 10 after transplantation (1, 2). To further improve the efficiency of hepatocyte transplantation, a detailed understanding of the molecular mechanisms involved is necessary.

Prior to transplantation, hepatocytes are removed from their natural environment. Physiologically, cells in multicellular organisms are influenced by their microenvironment through interactions with neighbouring cells and with the extracellular matrix (ECM) that surrounds them. Most of the cell receptors involved in cell–matrix interactions belong to the integrin family of adhesion molecules, and provide signals for cell proliferation, differentiation, and migration (3). Recent reports have shown that certain integrin chains provide a cell survival signal upon binding to RGD (Arg–Gly–Asp) sequences on ECM molecules (4). For example, adhesion to

collagen IV and laminin has been reported to protect rat mesangial cells from apoptosis by a β1-mediated mechanism (5). The α1, α5, and αv integrin chains are also able to suppress apoptosis, as has been shown in experiments using α-subunit-blocking monoclonal antibodies. Other integrin chains have been reported to mediate attachment, but do not seem to protect against apoptosis (6).

With respect to the mechanisms underlying these processes, it has been shown that clustering of integrins on the cell surface after exposure to ECM proteins creates focal adhesions between the cells and the ECM (7). Upon detachment from the ECM, focal adhesion kinase (FAK) is dephosphorylated resulting in activation of downstream cysteine proteases, named caspases, inducing irreversible cell damage and apoptosis (8). This process is termed 'anoikis' (9), and has been shown to be of relevance for epithelial cells and mesangial cells (10, 11). Integrin-linked kinase (ILK) can phosphorylate the cytoplasmatic domain of β1-integrins subunit *in vitro* (12). One of the consequences of constitutive ILK activation is suppression of apoptosis and anoikis (13–15). In the present study we wondered how the attachment and survival of hepatocytes is influenced by the presence of various ECM components and the blocking of integrin molecules.

Materials and methods

Hepatocyte isolation

Female rats (weighing 150–200 g) of the Brown Norway (BN) strain were obtained from Harlan (Horst, The Netherlands). All animals received humane care in compliance with the National Research Council's criteria for humane care as outlined in the 'Guide for the Care and Use of Laboratory Animals' prepared by the National Academy of Sciences and published by the National Institutes of Health. They were anaesthetized by a subcutaneous injection containing a mixture of ketamin, thalomonal and hypnorm in phosphate-buffered saline (PBS). The hepatocytes were isolated using a modification of the Seglen collagenase digestion technique (16). In brief, after cannulation of the portal vein, the liver was perfused with a calcium-free Hepes-based buffer (pH 7.4) at 37 °C at a flow rate of 30 ml/min, until the perfusate was free of erythrocytes. Recirculation was established for 9 min with Hepes buffered at pH 7.4 containing 0.5 mg/ml collagenase type IV (Sigma Chemicals Co., St. Louis, MO). The cells were dispersed in Ca^{2+}-free Hepes buffer and filtered through a 40 μm pore diameter sieve. The cells were then washed three times by gentle centrifugation at 50g for 1 min. After resuspension with RPMI-1640, medium the hepatocytes were counted, and viability was determined by 0.02% trypan blue dye exclusion and was at least 90%.

Antibodies

Immunohistochemical stainings were performed using antibodies against collagen type I (Sera-Labs, Loughborough Leicestershire, UK), collagen type IV (Sera-Labs), laminin (E-Y laboratories, San Mateo, CA), and fibronectin (Sigma Chemicals). Functional blocking monoclonal antibodies against CD29 (β1-integrin subunit), CD49a (α1 integrin), CD49b (α2 integrin), CD49d (α4 integrin), and CD49e (α5 integrin) were purchased from Pharmingen (Palo Alto, CA). Hybridomas against rat β2 (CD18, clone WT-3), rat LFA-1α (CD11a, clone WT-1), and rat ICAM-1 (CD54, clone 1A29) were generous gifts from Dr. M. Miyasaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan (17, 18).

Immunohistochemistry

Four μm cryostat sections were cut from snap frozen liver tissue. Sections were air-dried overnight, fixed in acetone for ten minutes, and washed three times in PBS. All dilutions of antibodies and conjugates were performed in PBS containing 1% (w/v) BSA. After 1 h incubation with the primary antibody, the sections were washed three times in PBS for 5 min. Sections were incubated for 30 min with HRP-conjugated secondary antibody. After washing with PBS, visualization of the immune complexes was performed by a 20 min incubation with 3,3′-diaminobenzidine (DAB). The sections were counterstained with hematoxylin.

Culture of hepatocytes

A hepatocyte suspension of 1×10^6 viable BN cells/ml was incubated with functional blocking monoclonal antibodies in concentrations of 40 μg/ml for 10 min at 37 °C or with 100 μM RGD (H–Arg–Gly–Asp–OH) peptides (Brunswig Chemicals, Amsterdam, The Netherlands) for 30 min. The scrambled peptide Arg–Gly–Glu–Ser (Sigma-Aldrich Chemicals, Zwijndrecht, The Netherlands) was used as control for the RGD peptides and normal hamster IgM isolated from hamster serum (Jackson Immunoresearch Laboratories, West Grove, PA, USA) was used as non-relevant IgM. Hepatocytes were suspended

in RPMI-1640 medium containing 100 μg/ml penicillin, 100 μg/ml streptomycin, 10 ng/ml insulin and 10% faetal calf serum, and plated at a density of 0.8×10^5 cells/cm^2 (19). Adhesion experiments were performed in 24-well culture plates. For these experiments culture plates precoated with collagen I, collagen IV, laminin, or fibronectin (Biocoat, Micronic systems, Lelystad, The Netherlands) were used, or culture plates were coated with (12 mg/ml) polyhydroxyethylmethacrylate (polyHEMA) (Sigma-Aldrich chemicals) in order to prevent attachment of the cells (20). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% oxygen. Photographs were taken after 24 h of culture with a Leitz inverted phase contrast microscope (Leica, Rijswijk, the Netherlands).

Cell adhesion assay

After a 5 h culture, hepatocytes were scraped from the coated wells with a cellscraper (Costar, LA), centrifuged for 10 min at 1000g, and washed twice with ice-cold PBS. DNA was extracted with 0.5% Triton X-100, 10 mM EDTA, and 10 mM Tris, pH 7.4. A phenol–chloroform extraction was performed three times, followed by sodiumacetate and ethanol precipitation. The DNA concentration was measured with picogreen (Molecular Probes, Leiden, The Netherlands) in a Wallac Victor^2 fluorescence spectrometer (Perkin-Elmer, Groningen, The Netherlands).

Measurement of caspase-3 activity

The enzymatic activity of caspase-3 (CPP32)-proteases was measured as described (21). Briefly, after 5 or 24 h of culture, hepatocytes were scraped from the coated wells, centrifuged, and washed twice with ice-cold PBS. The cells were resuspended in a lyses buffer consisting of 10 mM HEPES at pH 7.0, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM MgCl2, and 5 mM EGTA. After 10 min on ice, the cells were disrupted by four cycles of freezing and thawing and stored at −80 °C until use. Protein concentration was determined using Bradford reagent (Pierce, Rockfor, IL). For the caspase-3 assay, samples containing 50 μg protein were incubated with 40 nmol of the enzyme substrate DEVD-AMC (Bachem, Heidelberg, Germany) in 100 mM HEPES buffer at pH 7.25, containing 10% (w/v) sucrose, 0.1% (v/v) Nonidet-P40, and 10 mM dithiothreitol. Released fluorescent AMC was monitored at an excitation of 360 nm and emission of 460 nm using a Wallac Victor^2 plate reader. Calibration curves were constructed using free AMC (Bachem). Caspase-3 activity was calculated as pmol AMC/min/mg protein. As positive control for the caspase-3 assay, hepatocytes were treated with anti-Fas antibodies (JO-2, Pharmingen, San Diego, CA), and cultured on collagen IV. Differences in caspase-3 activities were compared by using Student's t-test. $P<0.05$ was considered to be statistically significant. All data are expressed as means ± SD.

DNA fragmentation analysis by gel electrophoresis

Hepatocytes were cultured for 5 or 24 h, either with or without anti-β1 MoAbs, RGD peptides, the non-relevant IgM, and control peptides. The cells were scraped from the coated wells, centrifuged, and washed twice with ice-cold PBS. The cells were resuspended in a lyses buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 0.5% Triton X-100). The samples were treated with the lyses buffer containing 10 μg/ml of RNAse at 37 °C. After 1 h incubation, 0.6% SDS and 0.4 mg/ml proteinase K were added. This was followed by an incubation for 1 h at 37 °C. DNA was precipitated with 5 M NaCl and ethanol. A 100 bp DNA ladder (GibcoBRL, Breda, The Netherlands) was used as a marker. Electrophoresis was performed at 50 V for 60 min on a 1% agarose gel. DNA was stained with SYBRgreen (Molecular Probes, Leiden, The Netherlands) and visualized by ultraviolet light.

Results

Immunohistochemistry

To determine to which ECM components liver parenchyma cells are attached *in vivo*, we analysed the ECM distribution in the normal rat liver. To this purpose, rat liver cryosections were immunostained with a panel of antibodies against various ECM components and their corresponding β1 integrins. The ECM proteins were predominantly present in two distinct areas in the liver: the vascular network and the peri-sinusoidal space. Collagen I (Fig. 1a), and collagen IV (Fig. 1b) were localized both in areas around the vascular network and peri-sinusoidal regions. Laminin (Fig. 1c) was only present around the vascular network. Fibronectin was present in the intracellular area and lateral regions of the hepatocyte (Fig. 1d). The results of the β1 staining (Fig. 1e) were comparable with those of the collagen IV staining. The stainings for α1, α2, and α5 showed an almost complete colocalization with the β1-staining (Fig. 1f–h). The ICAM-1 staining was positive on sinusoidal endothelial cells (data not shown), whereas hepatocytes are

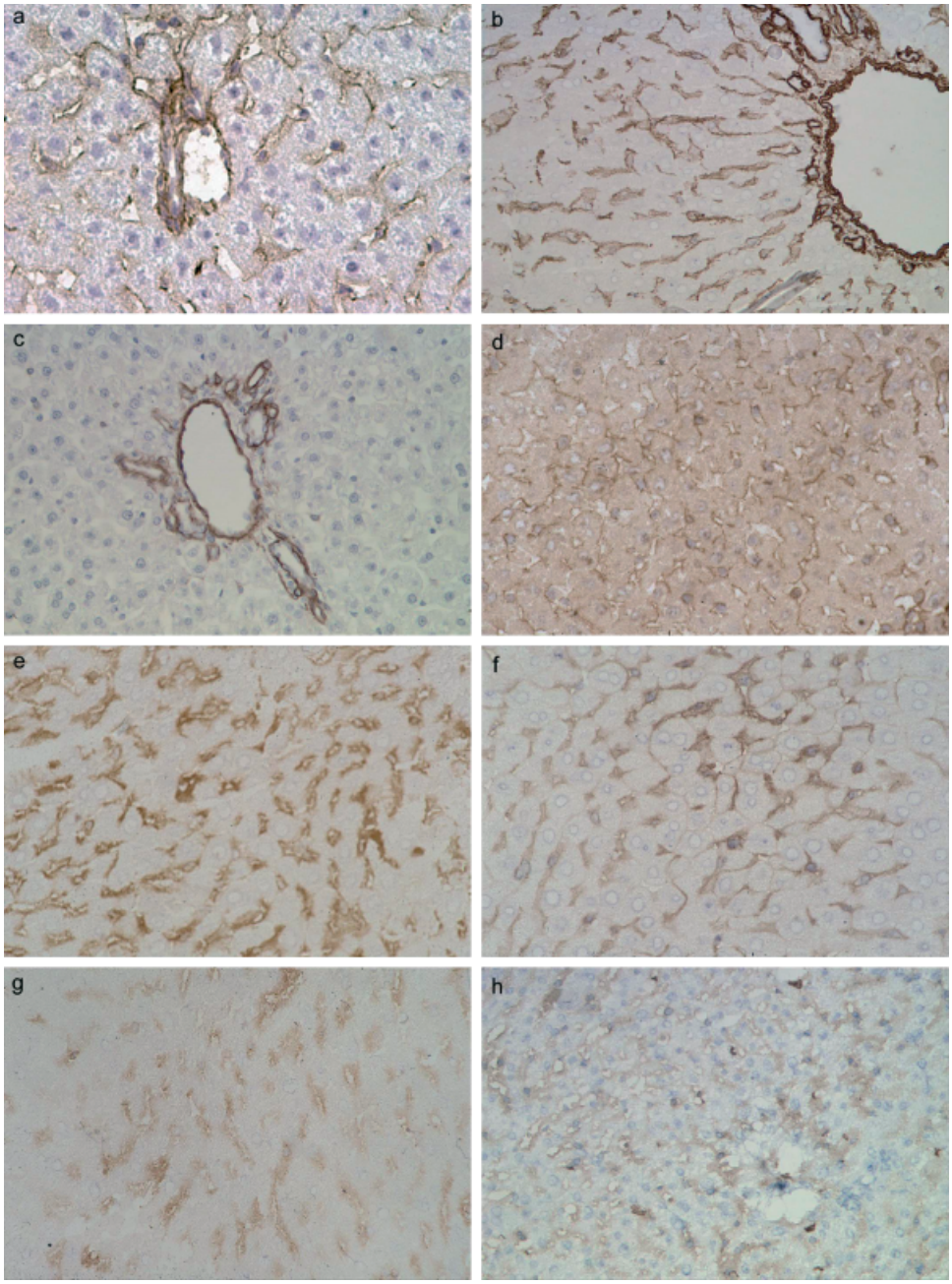

Fig. 1. Immunohistochemical stainings of extracellular matrix molecules and integrins in the rat liver. Collagen I (a), and collagen IV (b) are strongly positive in the basement membrane of the portal area and the central vein, and is seen as an almost continuous lining along the walls of the sinusoids. Laminin (c) is present at the basement membrane in the portal areas and the central vein. Fibronectin (d) is present in the stroma in the portal areas and the central vein. Hepatocytes are positive for fibronectin. Staining for the integrin chains β1 (e), α1 (f), α2 (g), and α5 (h) is seen in the membranes of the hepatocytes. Magnification × 400.

not stained at all. The β2 and the LFA-1α staining were only positive in some areas between the hepatocytes (data not shown).

In vitro attachment and spreading of hepatocytes on ECM proteins

Attachment and subsequent spreading of hepatocytes to various components of the ECM was investigated *in vitro*. Hepatocytes cultured on wells coated with collagen type I, collagen type IV (Fig. 2a), laminin, or fibronectin demonstrated cell attachment within several hours, and the formation of a monolayer within 24 h. Percentages of attachment are shown in Table 1. When cultured on wells precoated with collagen I, collagen type IV, or fibronectin hepatocytes showed rapid attachment of 60–70% of the seeded cells, while only 43.4% of the cells attached to laminin-coated wells. Hepatocytes cultured on collagen I

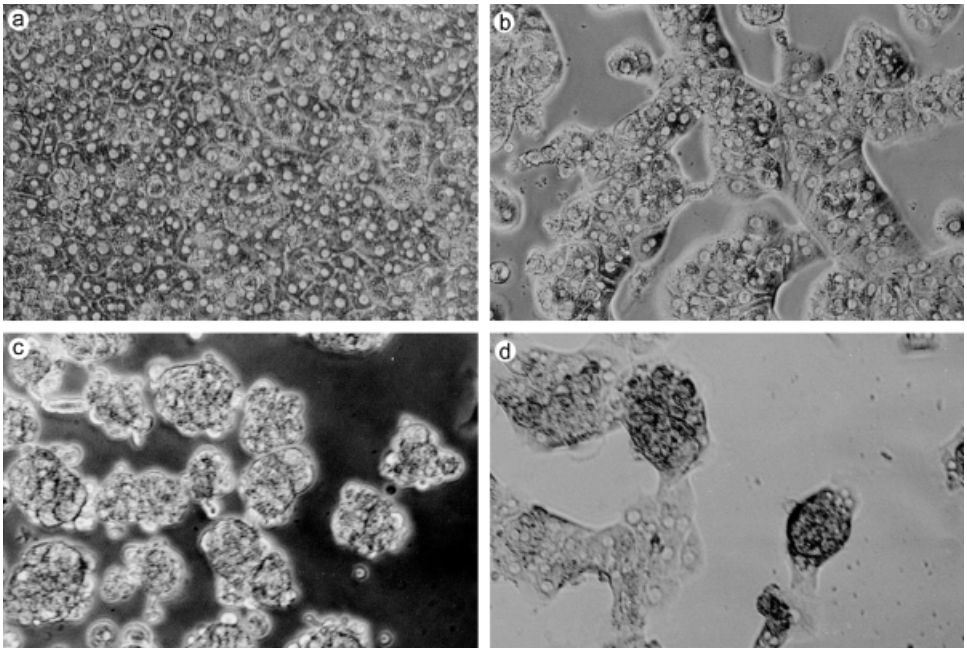

Fig. 2. Rat hepatocytes after 24 h of culture on (a) collagen IV, (b) collagen I, (c) polyHEMA, and (d) collagen IV after pretreatment with anti-β1 antibodies.

Table 1. Attachment and spreading of hepatocytes after 24 h of culture

ECM	Collagen I		Collagen IV		Laminin		Fibronectin	
Antibody/peptide	% Attachment	Spreading	% Attachment	Spreading	% Attachment	Spreading	% Attachment	Spreading
Untreated	63.2 ± 4.5	+	58.3 ± 10.3	++	43.4 ± 2.1	+	72.1 ± 3.5	++
Anti-β1	0.7 ± 0.1[a]	−	4.5 ± 0.8[a]	−	1.9 ± 0.2[a]	−	3.2 ± 0.1[a]	−
Anti-α1	39.5 ± 2.6[a]	+	27.5 ± 1.8[a]	+	38.9 ± 9.1	−	70.0 ± 3.5	++
RGD	45.7 ± 5.3	++	57.0 ± 6.7	++	32.2 ± 1.5[a]	±	45.7 ± 3.4[a]	++

Attachment and spreading of hepatocytes after 24 h of culture on different ECM components preceded by pretreatment with anti-beta 1, anti-alpha 1 antibodies, RGD-peptides, or by none treatment. After 24 h of culture, DNA was extracted, and the DNA concentration was measured with picogreen. The amount of attachment is expressed as a percentage of the amount of DNA of cultured hepatocytes, which remained attached after washing. [a]$P < 0.05$ compared with untreated hepatocytes. Spreading of the hepatocytes was indicated as: −, none; ±, mild; +, moderate; ++, extensive.

(Fig. 2b) showed less spreading than hepatocytes cultured on collagen IV. Hepatocytes grown on polyHEMA were inhibited in their ECM attachment, and they became rounded and formed aggregates (Fig. 2c). To define the role of different integrin chains in hepatocyte cell attachment and spreading, inhibition assays were performed using function-blocking mAbs against β1, α1, α2, α4, α5, β2, LFA-1α, and ICAM-1. Preincubation with the anti-β1 antibody caused a significant reduction in cell attachment of the hepatocytes plated onto collagen I (0.7% residual attachment), collagen IV (4.5%) (Fig. 2d), laminin (1.9%), and fibronectin-treated wells (3.2%) (Table 1). Cell attachment was also significantly reduced by anti-α1-integrin antibodies on collagen type I (39.5% residual attachment) and collagen type IV (27.5% residual attachment). Incubation with antibodies against the α2, α4, α5, LFA-1α, β2 chains, and incubation with normal IgM and control peptides had no effect on attachment or spreading (data not shown). Treatment of hepatocytes with RGD peptides led to a significant decrease in attachment to fibronectin (45.7% residual attachment), and laminin (32.2%). The RGD-treated hepatocytes adhered equally normally to collagen I and collagen IV precoated wells.

Analysis of caspase-3 activity

Caspase-3 activity was measured at different time points after culturing the hepatocytes on different ECM components. After 5 h, the caspase-3 activity of the cultured hepatocytes on collagen I was approximately 1.3-fold and on polyHEMA 1.7-fold increased compared with hepatocytes cultured on collagen IV. After 24 h, the caspase-3 activity of the cultured hepatocytes on collagen I was 1.6-fold and on polyHEMA 2.5-fold increased compared with hepatocytes cultured on collagen IV. Hepatocytes cultured on fibronectin

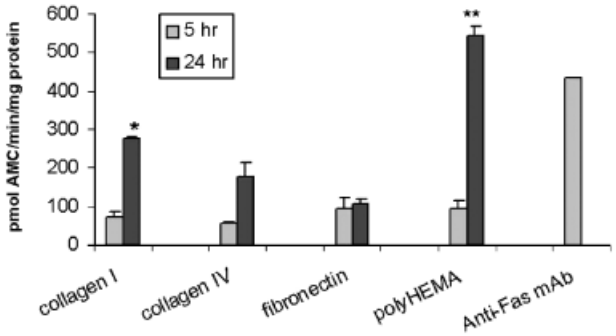

Fig. 3. Effect of the presence of different ECM components on hepatocyte survival. Freshly isolated hepatocytes were cultured on collagen I, collagen IV, fibronectin or polyHEMA. At 5 and 24 h culture, caspase-3 activity was assessed in lysates prepared from rat hepatocytes. The enzyme activity is expressed as pmol AMC per minute per mg protein. Data shown are the means of three independent experiments ± SD. $*P<0.05$ compared with collagen IV, and fibronectin, $**P<0.05$ compared with collagen I, collagen IV, and fibronectin.

showed a decrease in caspase-3 activity of 2.6-fold compared with hepatocytes cultured on collagen I and a decrease of 5.0-fold compared with hepatocytes cultured on polyHEMA (Fig. 3). These levels were significantly different. Hepatocytes pretreated with the anti-Fas antibody, which were cultured on collagen IV, served as the positive control for apoptosis, and showed identical values as the caspase-3 activity of the hepatocytes cultured on polyHEMA.

DNA fragmentation analysis by gel electrophoresis

Hepatocytes cultured on polyHEMA showed more DNA fragmentation than hepatocytes cultured on ECM proteins. Big differences are seen after 5 h of culturing, where untreated hepatocytes showed a distinct DNA laddering as compared with anti-β1-treated hepatocytes cultured on different ECM components (Fig. 4). After 24 h, still no DNA fragmentation was detectable in the anti-β1-treated cells. Hepatocytes cultured on polyHEMA showed less DNA fragmentation when treated with anti-β1 antibodies before culture. When hepatocytes were treated with RGD-peptides and cultured on polyHEMA, DNA fragmentation was completely prevented after 5 h culture (Fig. 4). There was no effect using normal IgM, or the scrambled peptide on hepatocyte apoptosis (data not shown).

Discussion

Hepatocyte transplantation has been proposed as a treatment for patients with inherited metabolic diseases of the liver. In a rat model, allogeneic hepatocyte transplantation is complicated by the formation of intravascular hepatocyte aggregates,

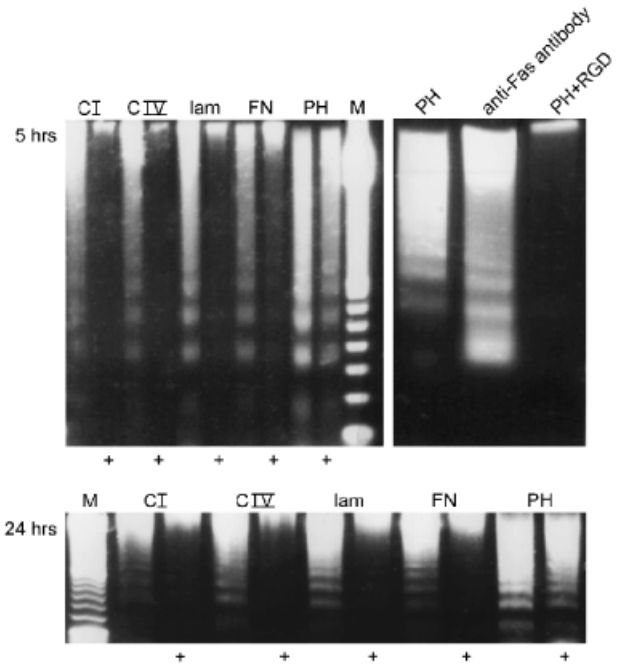

Fig. 4. Effect of the pretreatment with anti-β1 antibodies or RGD peptides on hepatocyte survival. Freshly isolated hepatocytes were treated with anti-β1 antibodies (+) or RGD peptides and cultured on collagen I (CI), collagen IV (CIV), laminin (lam), fibronectin (FN), or polyHEMA (PH) for 5 or 24 h. DNA fragmentation was analysed by agarose gel electrophoresis and SYBR green staining. M = marker. The anti-Fas antibody-treated hepatocytes, which were cultured on collagen IV, served as a positive control.

and a low intrahepatic homing and survival of the transplanted cells (22). However, heparin treatment does not prevent the aggregation (1), suggesting the involvement of adhesion mechanisms other than, or in addition to coagulation. Blocking of α1- and β1-integrin molecules on the hepatocyte surface significantly inhibited this intravascular aggregation, and improved survival of allo-transplanted hepatocytes (1).

Our present findings provide several novel insights into the mechanisms that regulate hepatocyte survival and apoptosis during the transplantation process. We demonstrate *in vitro* that hepatocytes attach readily to ECM components normally occurring in the liver, that is, collagen IV, fibronectin, and collagen I. After attachment to these molecules, the cultured hepatocytes start to spread. After attachment to laminin, in contrast, the cells show considerably less attachment and subsequent spreading. We showed that hepatocytes cultured on poyHEMA or collagen I are more susceptible to apoptosis than hepatocytes cultured on collagen IV or fibronectin. It is known that in normal liver lobules type I, and type IV collagens are present in discrete, small bundles in Disse's space, whereas fibronectin is abundantly present there

as a discontinuous layer. Laminin is absent within the liver lobules, but present in all hepatic basement membranes and around the central vein of the hepatic lobule (23–25). Our ECM distribution analysis seems to confirm these previous reports (Fig. 1). Apparently, under physiological conditions, hepatocytes are directly in contact with collagen type I, collagen type IV, and fibronectin. However, in disease states, the balance between the amounts of different molecules composing the hepatic ECM is dramatically altered. In particular, collagen type I accumulates massively in liver fibrosis and cirrhosis (26–28), which may be associated with hepatocyte loss through apoptosis. Suggesting that the induction of hepatocyte apoptosis during liver fibrosis is a multifactorial process, which include generation of reactive oxygen species, inflammatory cytokines and inappropriate ECM–integrin interactions.

It is known that the effects of ECM components on cells are for a major part mediated by cell surface receptors, especially those of the integrin subfamily (29). These molecules deliver a cell-survival signal during cell–ECM contact, while absence of this signal after cell detachment induces apoptosis (4, 7, 9, 30, 31). In the study reported here, we used function-blocking mAbs to study the role of integrins in attachment, spreading, and survival of hepatocytes on different ECM substrates. Treatment with anti-β1-integrin antibodies led to a significant inhibition of cell attachment and cell spreading on all substrates. Cell spreading on laminin and collagen IV was also inhibited by the use of function blocking mAb directed against the α1-integrin subunit. No inhibition was obtained after incubation with antibodies against the α2, α4, α5, LFA-1α, and β2 chains. These results suggest a heterogeneous role for integrin adhesion molecules in hepatocyte homeostasis. As stated above, in our model of hepatocyte transplantation, treatment with anti-β1 antibodies is applied to inhibit vascular aggregation after intraportal infusion of the donor cells (1). Our results show that this treatment may however inhibit subsequent attachment of the transplanted cells to the recipient liver, depending on whether the antibodies remain present on the cells when they enter the recipient liver.

Of all known integrins, the receptors for fibronectin (i.e. $\alpha_5\beta_1$ and $\alpha_v\beta1$) share the unique property of recognizing the arg-gly-asp (RGD) sequence in their ligands (32, 33). Their function can be inhibited with RGD-bearing peptides. Treatment with these RGD peptides led to a significant decrease in attachment and spreading of the cells when cultured on fibronectin, whereas treatment with anti-α5 antibodies did not. This indicates that attachment of hepatocytes to fibronectin is mediated in particular by the $\alpha_v\beta1$-integrin receptor. On the other hand, attachment and spreading of hepatocytes on collagen I and collagen IV, which could both be inhibited by using either the anti-β1 MAb or the anti-α1 MAb, were not affected by preincubation with the RGD peptides. This indicates that attachment and spreading of hepatocytes on collagen is mediated by α1β1, and slightly less by α5β1 or αvβ1 integrins. Thus, while integrins α1β1 (34, 35), α2β1 (34), α3β1 (34), α5β1, LFA-1 (36), and the β2-integrin ICAM (36) are all expressed on rat hepatocytes, their involvement in attachment and spreading on ECM molecules is heterogeneous. Our results indicate that only certain integrins on hepatocytes mediate cell attachment and cell spreading, such as the α1β1 and $\alpha_v\beta1$ (RGD receptor) integrins. Thus, preventing intravascular aggregation of transplanted hepatocytes by blocking integrin receptors should ideally be accomplished by blocking those receptors, which are not involved in attachment and spreading.

Analysis of DNA-fragmentation showed that treatment with antibodies against the β1-integrin or RGD peptides inhibits apoptosis of cultured hepatocytes. It is reasonable to assume that cross-linking of the integrin adhesion receptors by multivalent antibodies will activate FAK and/or integrin linkage kinase (ILK) (3, 13, 14, 37–39). Likewise, studies of apoptosis in mammary epithelial cells demonstrated that caspases are activated during apoptosis as a result of the absence of a β1-integrin-mediated signal (40). Numerous studies have suggested that RGD interacts with integrins to activate several tyrosine kinases and protein kinase signal transduction pathways (41). Cell attachment mediated by α5β1- and αvβ3 integrins promotes cell survival by upregulation of BCL-2, which is not elevated in cells attaching through the αvβ1 integrin (42, 43). This survival signal can be mimicked by RGD peptides. In earlier reports (1) blocking of the β2-integrin chains had no significant effect on hepatocyte survival after transplantation, although it did inhibit intravascular aggregation. This may be explained by a lack of inhibition of anoikis by the anti-β2 MAb. Indeed, in human umbilical vein endothelial cells, the adapter Shc is tyrosine phosphorylated upon cross-linking the α1β1, α5β1, and αvβ3 integrins, but not upon cross-linking the α2β1, α3β1, α6β1, and β2 integrins (44).

Taken together, our studies show that ultimate survival of transplanted hepatocytes is a function of (1) intravascular aggregation, (2) signals affecting survival and anoikis, and (3) attachment

and spreading within the recipient liver. In particular, the present study demonstrates that the ECM proteins collagen IV and fibronectin promote hepatocyte survival *in vitro* via a β1-integrin-dependent mechanism. Our data also indicate that the surrounding ECM regulates the survival and death of the hepatocytes. Treatment with antibodies against the β1-integrin chain improves survival of transplanted hepatocytes by decreasing hepatocyte aggregation (1). Potentially, cross-linking of integrins might confer a protective effect that prevents apoptosis in transplanted hepatocytes. It remains to be investigated in future studies, however, whether ligation of all β1-integrins will be beneficial for hepatocyte transplantation *in vivo*. The use of RGD peptides might well be instrumental in preventing apoptosis of isolated hepatocytes. These factors must be taken into account when further improving hepatocyte transplantation techniques by modulating hepatocyte surface receptors *in vivo*.

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