Transpl Int (1998) 11 [Suppl 1]: S377–S382

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Changes at the extracellular matrix during acute and chronic rejection in human liver transplantation

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Abstract We have previously observed changes at the extracellular matrix (ECM) which significantly correlated with the extent of preservation and reperfusion injury. In the present study, we attempted to investigate whether the ECM may be also involved in the pathophysiological sequelae of acute and chronic rejection. Of 81 patients monitored for the ECM parameters laminin, hyaluronic acid, fibronectin receptor, and transforming growth factor (TGF)-β, 28 patients developed acute rejection (< 1 month), in 14 patients (17.4%) acute rejection was steroid resistant, 4 patients (4.5%) developed early chronic rejection following acute steroid-resistant rejection. Acute and chronic rejection were confirmed by established clinical and histological criteria. Laminin levels were significantly increased in patients experiencing acute steroid-resistant rejection (4204 ± 133 ng/ml; $P \leq 0.01$) compared with patients with steroid-sensitive rejection (1059 ± 27.3 ng/ml) and with an uneventful postoperative course (1214 ± 17.4 ng/ml). No increase in laminin was observed in those four patients who developed early chronic rejection (1099 ± 58.7 ng/ml). Hyaluronic acid, fibronectin receptor, and TGF-β levels also increased in patients with acute steroid-resistant rejection; hyaluronic acid: 290 ± 10.8 μg/l *vs 154 ± 13.6 μg/l and 131 ± 11.7 μg/l* in patients with steroid-sensitive and no rejection, respectively; fibronectin receptor: 1003 ± 23.5 ng/ml vs 573 ± 24.8 ng/ml and 428 ± 13.6 ng/ml in patients with steroid-sensitive and no rejection, respectively; and TGF-β: 393 ± 14.9 pg/ml versus 315 ± 10.7 pg/ml and 233 ± 8.9 pg/ml in patients with steroid-sensitive and no rejection, respectively. A further increase in hyaluronic acid levels was observed in patients who developed early chronic rejection, while fibronectin receptor and TGF-β levels remained low, similarly to laminin levels. The increase in laminin, hyaluronic acid, fibronectin receptor, and TGF-β during acute steroid-resistant rejection may be stimulated by the rejection-related release of cytokines and adhesion molecules which paralleled the increase in ECM parameters. The lack of increase in laminin and fibronectin receptor levels in those patients who developed early chronic rejection may reflect an inability to recover from acute rejection.

Key words Extracellular matrix · Liver transplantation · Acute rejection · Early chronic rejection

Introduction

We have previously observed changes at the extracellular matrix (ECM) which significantly correlated with the extent of preservation and reperfusion injury [1]. Previous investigations have shown that endothelial cells are significantly involved in the early pathophysiological events of preservation/reperfusion injury and acute rejection [2, 3]. However, the function of the ECM during acute and chronic rejection is not well elucidated. The pivotal role of the ECM in embryogenesis and in determining cell–cell interaction and cell-function [4–8], may render the ECM a central target in regulating repair or ultimate tissue damage during acute and chronic rejection.

Severe acute and chronic rejection are still compromising the outcome after liver transplantation, despite improvements in immunosuppression. Although the main mechanisms of acute rejection are well understood, the involvement of all structures, especially the ECM, has not been well investigated. Furthermore, the main mechanisms leading to chronic rejection are still under discussion. Therefore, in the present study, we attempted to investigate whether the ECM may be involved in the pathophysiological sequelae of acute and chronic rejection.

Materials and methods

Patients

Between August 1993 and July 1994, 81 patients receiving 85 orthotopic liver transplants were prospectively monitored for various ECM parameters, including laminin, fibronectin receptor, hyaluronic acid, and transforming growth factor (TGF)-β. Indications for liver transplantation were alcoholic cirrhosis (n = 16), hepatitis B (n = 13), hepatitis C (n = 13), primary biliary cirrhosis (n = 11), cryptogeneic cirrhosis (n = 8), autoimmune cirrhosis (n = 4), 15 other liver diseases, and 5 retransplantations. The study was approved by the ethics committee of the Humboldt University of Berlin and informed consent was received from each patient prior to participation in the study. Surgical procedure, antibiotic treatment, and various other prophylaxes were performed perioperatively, as previously reported [9, 10].

Immunosuppression

Immunosuppression was commenced as quadruple therapy comprising cyclosporine A, azathioprine, prednisolone, and anti-lymphocyte immunoglobulin (Merrieur, France; n = 24) or interleukin-2 receptor antagonist BT563 (Biotest, Dreieich, Germany; n = 47) for the first 7 or 12 postoperative days, respectively, and subsequently continued as triple therapy [9]. Twelve patients received no induction therapy. Two patients undergoing retransplantation received FK506 (Prograf; Fujisawa, Osaka, Japan) in conjunction with prednisolone.

Management of rejection

Diagnosis of acute rejection was based on clinical (fever, change of color, and amount of bile production) and laboratory (aspartate and alanine transaminases, bilirubin, γ-glutamate transaminase, and alkaline phosphatase) findings and was confirmed by histological evaluation of graft biopsies. Liver biopsies were performed routinely on postoperative day (POD) 7 and whenever rejection was suspected. Histological classification of acute rejection was used as previously reported [10–12]. Patients received methylprednisolone for treatment of acute rejection at a dosage of 500 mg/day for 3 days and FK506 (n = 8) or a combination of FK506 and OKT3 monoclonal antibody (n = 6) (Cilag, Sulzbach, Germany) simultaneously for steroid-resistant or severe recurrent rejection. Acute rejection was defined as steroid resistant by a missing response or second increase in liver enzymes after one course of methylprednisolone in combination with histological signs of ongoing rejection in repeated liver biopsies prior to initiation of rescue therapy. Early chronic rejection was classified according to established criteria and treated with high-dose FK506-rescue therapy [11, 12].

Experimental monitoring

Experimental parameters were determined at predefined time points during and after transplantation and subsequently on a daily basis. Heparinized blood was immediately stored on ice and centrifuged at 4°C for 10 min within 30 min of retrieval. Plasma was stored at – 70°C until measured. Commercially available immunoassays with 96-well microtiter plates were used: laminin, fibronectin receptor, and TGF-β (DPC Biermann, Bad Nauheim, Germany). Hyaluronic acid was determined by radioimmunoassay (Kabi Pharmacia Diagnostics, Uppsala, Sweden). The normal ranges (n = 45) of various plasma levels for healthy individuals between 21 and 55 years were 497 ± 53 ng/ml for fibronectin receptor, 19.4 ± 2.5 µg/l for hyaluronic acid, and 57 ± 10.4 ng/ml for TGF-β.

Statistical analysis

Kaplan-Meier estimates, Wilcoxon, chi-squared, and Kruskal-Wallis tests and analysis of variance (one-way ANOVA and multivariate analysis) were used as indicated. Linear regression analysis was used to determine if changes of various parameters correlated with the incidence and severity of acute and early chronic rejection. Multivariate analysis was additionally used to test for the significant impact of individual parameters for acute and early chronic rejection. Results were expressed as means ± standard error of the mean.

Results

Survival and incidence of acute and chronic rejection

Actuarial 1-month and 1-year patient and graft survival were 97.5% (79/81) and 88.9% (72/81) for patient survival, and 94.1% (80/85) and 84.7% (72/85) for graft survival, respectively. During the first year after transplantation, nine patients died (11.1%); in five patients, death was related to serious infections; two patients died because of fulminant HBV recurrence, one patient

Table 1 Extracellular matrix parameters in patients with acute steroid-resistant, acute steroid-sensitive, and no rejection. Laminin, fibronectin receptor, hyaluronic acid and transforming growth factor *(TGF)-β* levels in patients with an uneventful postoperative course were significantly higher than in non-transplanted individuals

Parameter	Steroid-resistant ($n = 14$)	Steroid-sensitive ($n = 14$)	No ($n = 37$)
Laminin	4204 ± 133 ng/ml*	1059 ± 27.3 ng/ml	1214 ± 17.4 ng/ml
Fibronectin receptor	1003 ± 23.5 ng/ml*	573 ± 24.8 ng/ml	428 ± 13.6 ng/ml
Hyaluronic acid	290 ± 10.8 µg/l*	154 ± 13.6 µg/l	131 ± 11.7 µg/l
TGF-β	393 ± 14.9 pg/ml**	315 ± 10.7 pg/ml	233 ± 8.9 pg/ml

* $P \leq 0.01$ for acute steroid-resistant vs acute steroid-sensitive and no rejection (patients with an uneventful postoperative course)
** $P \leq 0.01$ for acute steroid-resistant and acute steroid-sensitive vs no rejection (patients with an uneventful postoperative course)

because of fulminant HCV, and one because of tumor recurrence.

Twenty-eight patients (34.6%) developed acute rejection during the first month after transplantation; in 14 cases (17.3%) rejection was steroid resistant and required treatment with FK506 (eight patients) or a combination of FK506 and OKT3 simultaneously (six patients). The latter approach was performed in clinically more severe acute rejection. Four patients (4.5%) developed early chronic rejection; two patients recovered completely with FK506-rescue therapy, one patient required retransplantation, and one patient died. Three of the four patients with early chronic rejection suffered from acute steroid-resistant rejection prior to development of chronic rejection, while in one patient on previous episode of acute rejection was observed.

Extracellular matrix parameters

Mean laminin levels (3 days prior to rejection and 14 days after the onset of rejection) were significantly increased in patients experiencing acute steroid-resistant rejection (4204 ± 133 ng/ml; $P \leq 0.01$) compared with patients with acute steroid-sensitive rejection (1059 ± 27.3 ng/ml) and with an uneventful postoperative course (1214 ± 17.4 ng/ml; Table 1). The main increase in laminin levels was observed during the late period of acute steroid-resistant rejection, commencing at POD 4 after onset of rejection. No increase in laminin was observed in those four patients who developed early chronic rejection (1099 ± 58.7 ng/ml; $P \leq 0.01$ versus patients with acute steroid-resistant rejection; Fig. 1).

Similarly to laminin levels, mean hyaluronic acid, fibronectin receptor, and TGF-β levels also increased significantly in patients experiencing acute steroid-resistant rejection; hyaluronic acid, 290 ± 10.8 µg/l vs 154 ± 13.6 µg/l and 131 ± 11.7 µg/l in patients with steroid-sensitive and no rejection, respectively; fibronectin receptor, 1003 ± 23.5 ng/ml vs 573 ± 24.8 ng/ml and 428 ± 13.6 ng/ml in patients with steroid-sensitive and no rejection, respectively; and TGF-β, 393 ± 14.9 pg/ml vs 315 ± 10.7 pg/ml and 233 ± 8.9 pg/ml in patients with steroid-sensitive and no rejection, respectively (Table 1). A further increase in hyaluronic acid levels was observed in the four patients who developed early

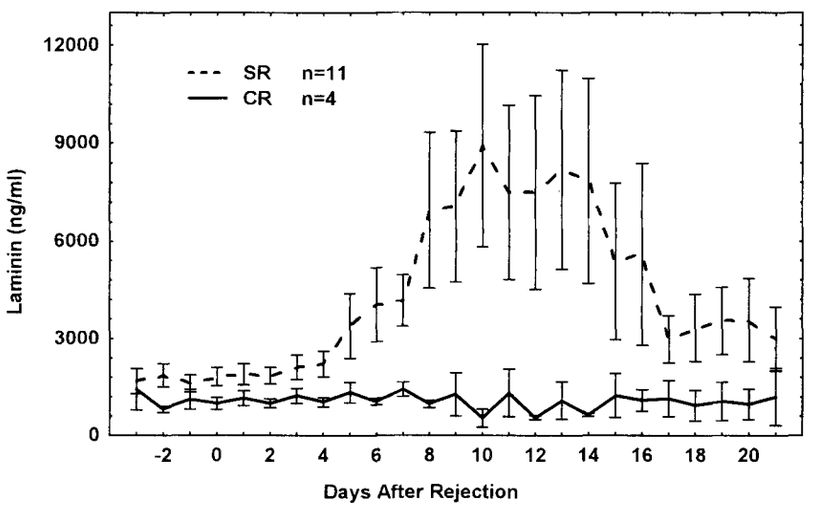

Fig. 1 Laminin levels in patients with acute steroid-resistant *(SR)* and early chronic rejection *(CR)*. $P \leq 0.01$ for acute steroid-resistant versus early chronic rejection

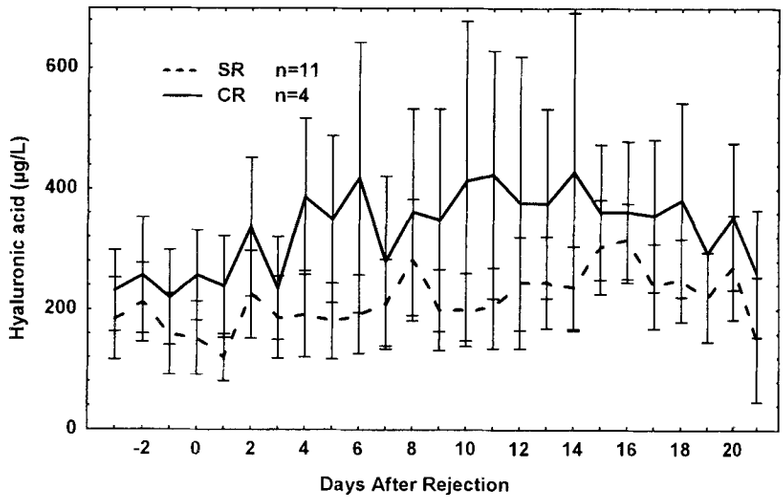

Fig. 2 Hyaluronic acid levels in patients with acute steroid-resistant *(SR)* and early chronic rejection *(CR)*. *P* not significant

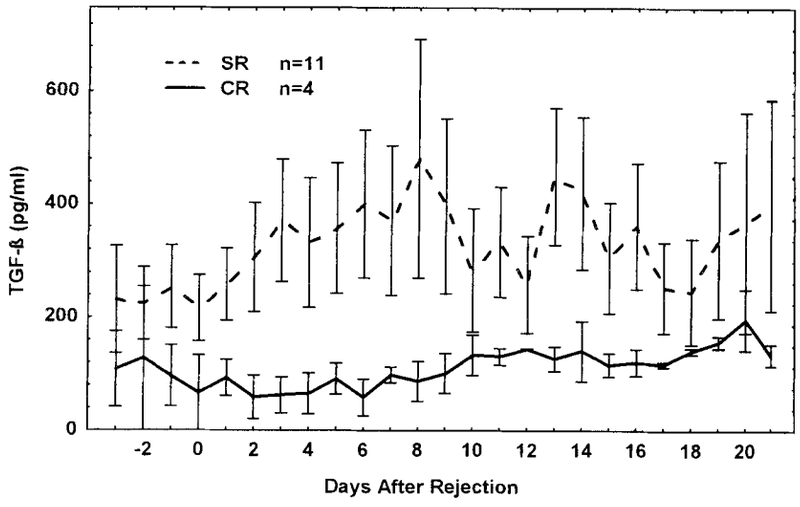

Fig. 3 Transforming growth factor-β *(TGF-β)* levels in patients with acute steroid-resistant *(SR)* and early chronic rejection *(CR)*. $P \leq 0.01$ for acute steroid-resistant versus early chronic rejection

chronic rejection (272 ± 12.1 µg/l; Fig. 2), while fibronectin receptor (662 ± 32.2 ng/ml) and TGF-β levels (112 ± 11.8 pg/ml; Fig. 3) remained low in these patients, similarly to laminin levels.

Discussion

ECM parameters have been shown to be responsible for cell–cell interactions, endothelial cell–basement membrane interactions, and probably for the function of endothelial cell monolayers [4, 5]. The pivotal role of ECM components in the regulation of cell–cell interactions may enable the ECM to participate significantly in the sequelae of acute and chronic rejection. Laminin, the most abundant glycoprotein of the endothelial and epithelial cell basement membrane, promotes adhesion, migration, and differentiation of endothelial cells [7, 8]. Fibronectin, which is possibly more closely associated with the epithelial cell membrane, regulates epithelial cell function and repair [13].

TGF-β is probably the most complex parameter in this scenario and has an extremely wide range of biological actions. TGF-β is an important mediator of the formation of the ECM, generally promoting proliferation of the ECM and inhibiting degradation of ECM components [4]. Stimulation of TGF-β therefore leads to liver fibrosis and cirrhosis [14]. However, TGF-β also has immunomodulatory effects; it is a potent suppressor of T- and B-lymphocyte proliferation and has inhibitory effects on the killing activity of natural killer cells and activated macrophages and monocytes [15, 16]. Converse-

ly, TGF-β shows stimulatory effects on the activation and secretion of several growth factors.

Hyaluronic acid is located in the loose connective tissue closely related to the basement membrane and endothelial cell monolayers [17, 18]. Similarly to the other ECM components, hyaluronic acid can be stimulated by a variety of inflammatory and immunostimulatory cytokines [4, 8] which have been shown to increase in patients with acute steroid-resistant and chronic rejection [19]. However, hyaluronic acid is, furthermore, exclusively degraded by liver endothelial cells which elaborate hyaluronic acid as a parameter of liver endothelial cell dysfunction [20].

Significant changes in laminin and fibronectin receptor levels were observed during the late phase of acute steroid-resistant rejection, possibly indicating a regulatory role in repair of the microvasculature and basement membrane structures (laminin) and epithelial cells (fibronectin). The maximal increase in laminin and fibronectin receptor levels was accompanied by a significant increase in various cytokines and adhesion molecules [19]. The timing of laminin and fibronectin receptor increase indicates that activated cytokines and adhesion molecules are the most prominent stimulators of the increase in these ECM components [4, 19], although there is also evidence in experimental small bowel transplantation that these ECM components, as well as hyaluronic acid, may be liberated into the vasculature through impaired basement membrane structures [21–23].

TGF-β and hyaluronic acid increased with the onset of acute steroid-resistant rejection. A further increase in hyaluronic acid was observed in those patients who developed early chronic rejection. In addition to the stimulation of hyaluronic acid production by activated TGF-β, cytokines, and adhesion molecules, the increase in hyaluronic acid levels may result from impaired endothelial cell function, which contributes to decreased degradation of hyaluronic acid.

To date, the function of the ECM during acute and chronic rejection is rather unclear. However, the lack of increase in laminin, fibronectin receptor, and, possibly, TGF-β in those patients who developed early chronic rejection most likely indicates a protective role of these ECM components in regulating tissue repair [1, 24, 25], although previous investigations on TGF-β have demonstrated an increase in TGF-β in renal tissue of patients with chronic rejection and during development of liver fibrosis [4, 26]. Further studies on liver tissue are required to evaluate the local changes and function of the ECM during acute and chronic rejection.

In conclusion, the increase in laminin, hyaluronic acid, fibronectin receptor, and TGF-β during acute steroid-resistant rejection may be stimulated by the rejection-related release of cytokines and adhesion molecules, which paralleled the increase in ECM parameters. Laminin and fibronectin have been known to regulate cell–cell interaction and are able to mediate tissue repair. The lack of increase in laminin and fibronectin receptor in those patients who developed early chronic rejection may reflect an inability to recover from acute rejection.

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