Improvement of Rat Liver Graft Function After Storage in University of Wisconsin Solution Containing Testicular Hyaluronidase

Vassilios Papanikolaou,[*,†] *Dionisios Vrochides,*[*,†] *Dimitrios Gakis,*[*] *Kalliopi Patsiaoura,*[*] *Paraskevi Margari,*[*] *Fani Dogramatzi,*[*] *Antonios Antoniades,*[*] *and Paraskevi Heldin*[†]

Hyaluronan accumulates at sites of inflammation, which affects the organization of matrix and thereby the proliferation, migration, and adherence of cells. In this study we investigated possible beneficial effects of the hyaluronan-degrading enzyme hyaluronidase on rat liver graft viability. Orthotopic rat liver transplantation was performed using a cuff technique in Wistar AL Bacharach Glaxo (WAG) rats grafted with WAG livers, which had been stored in the University of Wisconsin (UW) solution or in UW solution enriched with testicular hyaluronidase. Liver tissue architecture, as well as tissue and serum hyaluronan levels, were determined using immunohistochemistry and biochemical assays. Addition of testicular hyaluronidase (0.4 mg/mL) to livers preserved for 24 hours in cold UW solution followed by brief exposure to Ringer's lactate both prolonged the function of the grafted livers and improved their viability (4 of 10 grafts survived, compared with 0 of 10 in the control group). Hyaluronidase treatment did not damage the liver tissue architecture, and a reduced edema was observed in the survivors. Furthermore, 10 minutes after restoration of circulation, higher serum hyaluronan levels were observed in non-successful compared with successful transplantations, whereas no differences in the levels of other serum viability markers were detected. We conclude that addition of testicular hyaluronidase to storage UW solution limits liver cell damage and considerably improves graft function. Furthermore, our data suggest that serum hyaluronan level is a better marker than other serum markers for early evaluation of postoperative graft function. *{Liver Transpl 2002;8:1028-1035.)*

*From the *Organ Transplantation Unit, Department of Surgery, Hippokration Hospital, Aristotle University, Thessaloniki, Greece; the †Department of Medical Biochemistry and Microbiology, Unit of Biochemistry, Biomedical Center, Uppsala University, Uppsala, Sweden.*

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The first two authors (V.P. and D.V.) contributed equally to this work.

Address reprint requests to Paraskevi Heldin, Department of Medical Biochemistiy and Microbiology', Unit of Biochemistry, Biomedical Center, Uppsala University, Box 582, SE-751 23 Uppsala, Sweden. Telephone: 0046-18-471-4261; FAX: 0046-18-471-4209; E-mail: Paraskevi. Heldin @imbim. uu.se

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Hyaluronan is a high-molecular-weight glycosaminoglycan composed of repeated disaccharide units of *N*-acetyl glucosamine and glucuronic acid. It is ubiquitously distributed in the extracellular space and has been assigned various physiological and biological functions, e.g., in water and plasma protein homeostasis, cell proliferation, migration, and differentiation.[1] This wide range of biological functions is the result of hyaluronan interactions with hyaluronan-binding proteins such as versican, CD44, and liver endothelial cell clearance receptor. The most common hyaluronan receptor and the most studied to date is CD44, which has diverse functions, including cell adhesion and recruitment of lymphocytes during inflammation.[2] Hyaluronan is synthesized by hyaluronan synthases and is degraded by hyaluronidases.[3-5] In inflammatory conditions, an accumulation of hyaluronan is seen that may be caused by both increased synthesis of hyaluronan by cells of mesenchymal origin in response to inflammatory mediators and growth factors, and an impaired elimination of hyaluronan by lymph nodes, sinusoidal endothelial cells, and macrophages.[6-8] Hyaluronan has been used as a diagnostic marker, for example in liver injuries; decreased liver function leads to higher serum hyaluronan levels.[9]

Liver transplantation surgery is used in patients with irreversible liver diseases, but the success is dependent on graft viability. Various solutions for hypothermic preservation of liver grafts have been developed, among them the University of Wisconsin (UW) solution, introduced by Wahlberg et al in 1987,[10] which allows better preservation and significant extension of the liver cold storage time.[11] However, there is a need for better quality of liver grafts by further improving the composition of storage solutions to prevent the primary nonfunction that is a prominent problem in liver transplantations. Previous studies revealed that the sinusoidal endothelial cells are particularly vulnerable when exposed to cold ischemia and reperfusion, and the impairment of their endocytotic function is well correlated to increased serum and tissue levels of various components such as hyaluronan and bilirubin.[12-15] In

addition to early impairment of endothelial cell function, most likely other extracellular matrix (ECM) components and ECM-leukocyte interactions are important for graft quality and viability. Heterotopic heart transplantations showed better morphology after daily hyaluronidase therapy compared with untreated controls,[16] and more recent studies by us revealed that hyaluronidase promotes endothelial cell morphogenesis in a CD44-dependent manner.[17] These observations prompted us to investigate the effects of testicular hyaluronidase, as a supplement to UW preservation solution, on the function and morphology of liver grafts in a rat liver transplantation model. Furthermore, the serum hyaluronan level was evaluated as an indicator of early graft function.

Materials and Methods

Animals

Male WAG/OlaHsd rats weighing approximately 400 g (HARLAN, Blackthorn, Bicester, UK) were used for all experiments. All animals were housed in chip-bedded Plexiglas cages with controlled light/dark cycles, temperature, and humidity. They were allowed free access to water and normal rat chow (Amylum, Thessaloniki, Greece) until 12 hours before surgery, when they had access to water only. The project was approved by the Ethical Committee for Laboratory Animals in Uppsala, Sweden, and by the Veterinary Committee in Thessaloniki, Greece.

Experimental design. First a large number of orthotopic rat liver transplantations (n = 70) were performed to obtain maximal surgical proficiency; none of these experiments were included in the study group. Then, 18 livers were divided into six groups (n = 3) and preserved in UW solution alone or UW solurion supplemented with testicular hyaluronidase (bovine testes, type I-S, H-3506, Sigma-Aldrich) at concentrations of 0.1,0.2,0.4, 0.8, and 1.6 mg/mL (300 to 500 units per mg). After 24 hours of preservation at 4°C, the hyaluronan content was measured and visualized immunohistochemically. In a second series, treatment with the optimal concentration hyaluronidase (0.4 mg/mL; 10 animals) was compared with controls not treated with hyaluronidase (10 animals).

Orthotopic Liver Transplantation

Donor operation. Liver Transplantations were performed with the isogenic combination of WAG donor and recipient rats using the cuff technique originally described by Kamada and Calne.[18] Briefly, donors were anesthetized with diethyl ether and the abdomen was opened with a transverse, subcostal incision. Falciform, left triangular, and other minor ligaments as well as right renal and suprarenal veins, pyloric vein, left diaphragmatic vein, and common hepatic artery were exposed and divided. Bile duct was cannulated. At this time point, blood (0.5 mL) was taken from each donor and serum was prepared by centrifugation at 2000g for 19 min, at 4°C. Livers were perfused in situ through the portal vein (by gravity from a height of 20 cm) with 10 mL lactated Ringer's solurion, excised, and transferred into an ice-cold box and infused again with 20 mL of ice-cold UW solution (n = 10) or UW solution supplemented with 0.4 mg/mL hyaluronidase (n = 10). Portal vein and intrahepatic inferior vena cava cuffs were inserted, and the livers were preserved in UW solution with or without hyaluronidase for 24 hours at 4°C. In all livers, the preservation solution was rinsed with 10 mL of cold lactated Ringer's solution just before implantation. Prior to implantation, a small part of left lateral lobe was ligated, dissected free, fixed in 4% formaldehyde solution, and examined immunohistochemically for hyaluronan content.

Recipient operation. Recipients were treated with atropine (400 µg intramuscularly) and anesthetized with diethyl ether. They received 0_2 (by tent) and 5% dextrose solution (up to 3 mL, via femoral vein) throughout the operation. Liver excision of the recipient rats was performed in a similar way as for the donors. The suprahepatic vein anastomosis was performed using running nylon suture (7-0), and the other anastomoses were performed by means of cuffs. The mean unhepatic phase for the recipient was 22 minutes, and the time between reperfusion of liver graft with lactated Ringer's solution and the completion of all the vascular anastomosis was about 10 minutes. Then, 0.5 mL blood was taken from each recipient and serum was prepared. Right after blood sampling, the caudate lobe was ligated and dissected free. The frontal caudate lobe was fixed with formaldehyde and stained for hyaluronan. The dorsal caudate lobe was immediately weighed at room temperature (i.e., liver wet weight) with a precision balance and stored at — 20°C until lyophilization followed by pronase digestion and analysis of hyaluronan content (see below). The animals were then treated with cefuroxime (75 mg/mL solution, 0.2 mL, intraperitoneally), buprenorphine (400 µg, intramuscularly) and kept under an infrared lamp for 2 hours with free access to tap water and food. Postoperatively, all animals were examined clinically and weighed daily. Animals that died within the first 48 hours postoperatively were considered to suffer from liver primary nonfunction (nonsurvival group). Animals that survived the first 48 hours after operation and had a normal clinical evaluation were considered as survivors and were killed by exsanguination 14 days after operation, and the right lateral lobe was dissected free and fixed with formaldehyde. The paraffin sections were processed for hematoxylin staining and hyaluronan localization in the tissue.

Quantitative Analysis **of Tissue** Hyaluronan and **Water Content**

The frozen dorsal caudate lobes were put at — 70°C for 1 hour, lyophilized for 3 days, and weighed to obtain liver dry weight. The samples were then digested under vigorous stirring in 1 mL of 0.05 M Tris buffer, pH 7.5, containing 10

mM $CaCl_2 \cdot H_20$ and 5 U/mL pronase (type XIV, P-5147, Sigma Chemical Co, St Louis, MO) pet gram dry weight liver, for 24 hours at 55°C. The digested tissues were then heated for 10 minutes at 95°C. After centrifugation for 20 minutes at 15,000g, at 22°C, the hyaluronan-containing supernatants were collected and their contents (liver tissue hyaluronan) were determined using a commercially available radiometric assay (HA Kit, Pharmacia Diagnostics, Uppsala, Sweden). The same kit was also used to determine hyaluronan serum levels. The relative water content of liver tissue, expressed as percent water of the total weight of the graft tissue, was calculated from the formula 100 X (wet wt — dry wt) / wet wt.

Immunohistochemical Detection of Hyaluronan

The formaldehyde fixed and paraffinized left and right lateral lobes and frontal caudate lobe were sectioned and deparaffinized. The slides were then incubated with 1 % bovine serum albumin solution for 30 minutes at 22°C to block unspecific binding, and then washed with phosphate-buffered saline. Detection of hyaluronan in situ was performed using a specific probe, the biotin-labeled hyaluronan binding protein (b-HABP), which was prepared essentially as described by Tengblad.[19] Some slides were incubated with 100 units/mL of *Streptomyces* hyaluronidase (Seikagaku kogyo Co, Tokyo, Japan) for 2 hours at 37°C to test the specificity of b-HABP. Then, the sections were incubated with 3% H_20_2 for 10 minutes at 22°C to destroy exogenous peroxidase activity, washed and incubated overnight with b-HABP (17 µg/mL) at 22°C. After phosphate-buffered saline washings, the sections were incubated with the Vectastain-Elite avidin-biotin complex (ABC) reagent (Vectastain ABC Elite kit, PK-6100; Vector Laboratories, Inc, Burlingame, CA) for 60 minutes at 22°C. Then the slides were incubated with a 0.1% diaminobenzidine tettahydrochloride solution (Sigma) for 10 minutes at 22°C, stained with Mayer's hematoxylin and eosin, and mounted with glycerol gelatin solution. Processed cells were examined and photographed with a light microscope (Nikon; Tokyo, Japan).

Liver Graft Viability Marker Determinations

Commercially available kits were used to determine graft function 10 minutes after vascular anastomosis by measuring serum levels of aspartate aminotransferase (AST), serum alanine aminotransferase (ALT), alkaline phosphatase (AP), albumin and bilirubin (System reagent 500, Olympus Diagnostica, GmbH; Hamburg, Germany). Serum levels of total protein were determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hamel Hamstead, Herts, England).

Statistics

Comparisons among more than two data groups were performed using an ANOVA test corrected with Bonferroni's post hoc. Comparisons between two data groups in which tissues were examined for hyaluronan content (see below) were performed with an unpaired Student's test, after checking the variances with Fisher's exact test. Statistical significance error was set to less than 5%.

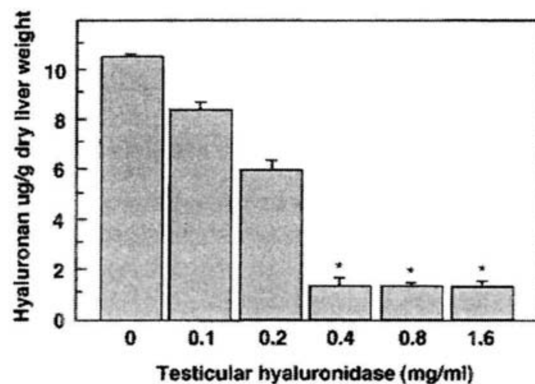
Results

Testicular Hyaluronidase Improved Graft Viability

Previous studies have shown that increased levels of tissue hyaluronan in various diseases leads to impairment of organ function. Therefore, we have investigated the effect of storing grafts in the presence of testicular hyaluronidase on the outcome of liver graft transplantations in rats. We first investigated the optimum concentration of hyaluronidase able to degrade hyaluronan in liver grafts without affecting tissue architecture. Testicular hyaluronidase added to the storage UW solution at a concentration of 0.4 mg/mL decreased the amounts of hyaluronan to about 10% compared with that of liver grafts preserved in UW solution only. This reduction was statistically significant ($P < .0001$). Further increase did not lead to more elimination of tissue liver hyaluronan (Fig. 1).

The viability of grafts treated with 0.4 mg/mL hyaluronidase was then compared with controls not treated with hyaluronidase. None of the recipient rats (n = 10) survived the orthotopic liver transplantation after 24 hours of graft preservation in cold UW solution only. Interestingly, a 40% survival rate (4 of 10 recipients survived) was achieved when grafts were preserved in

Figure 1. Optimization of hyaluronidase concentration.



Liver grafts were stored for 24 hours at 4°C in UW solution alone or in UW solution containing various concentrations of testicular hyaluronidase. The hyaluronan content in the graft liver tissues was measured as described in the Materials and Methods sections. *Significantly different from non—hyaluronidase-treated grafts.

cold UW solution containing *0.4* mg hyaluronidase/mL (data not shown). Of the recipient rats in which this failed, the time period to primary nonfunction was significantly shorter *(P* < .0001) when grafts were preserved in UW solution only (about 16 hours) than when preserved in UW solution supplemented with hyaluronidase (about 22 hours). Thus, preservation in UW solution containing hyaluronidase both increases the survival rate and prolongs the function of the transplanted liver.

Using immunohistochemistry, a positive staining **for** hyaluronan perivascularly and periductaly was observed (Fig. 2A), which was removed efficiently after liver graft treatment with 0.4 mg/mL hyaluronidase. Under the experimental conditions used, testicular hyaluronidase had degraded hyaluronan completely already after 6 hours (data not shown). Light microscopy of the hematoxylin—eosin stained liver tissues preserved in UW solution and supplemented with 0.4 mg/mL hyaluronidase showed normal liver tissue architecture (Fig. 2B), except a slight dilatation of the central veins (data not shown). However, livers stored in UW solution containing 0.8 to 1.6 mg/mL hyaluronidase showed deterioration of their normal hepatic architecture (data not shown).

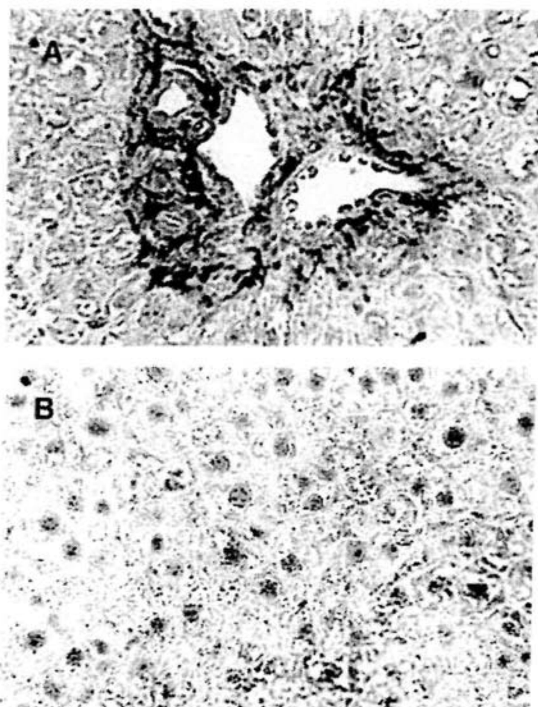


Figure 2. Staining of graft liver tissues for hyaluronan and with hematoxylin—eosin. Staining of liver graft sections for hyaluronan after 24 hours of storage in cold UW solution (A) and hematoxylin—eosin staining (B) of a liver graft section in UW solution containing 0.4 mg hyaluronidase/mL. Original magnification x400.

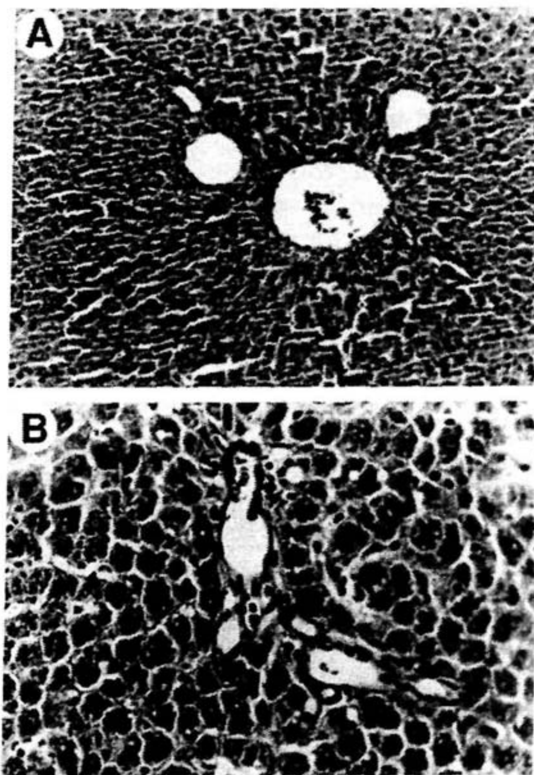


Figure 3. Morphology of liver graft tissues. The sections show the morphology of the liver after restoration of circulation (A, original magnification X200) and the morphology 14 days after transplantation (B, original magnification X400).

Liver specimens were taken for histopathologic studies immediately after the vascular anastomosis. All biopsies showed sinusoid dilatation, red blood cell stasis, deposition of eosinophilic substance, and minor hepatocyte atrophy (Fig. 3A). Figure 3B shows that the liver architecture of the hyaluronidase-treated grafts was normal 14 days after transplantation. At that time point, no pronounced hyaluronan staining was detected (data not shown).

Correlation Between Hyaluronan Levels and Graft Outcome

Because serum hyaluronan levels have been shown to be a sensitive test for liver function, we determined serum hyaluronan levels in the donors as well as grafted animals (Fig. 4). The mean hyaluronan levels in the donors

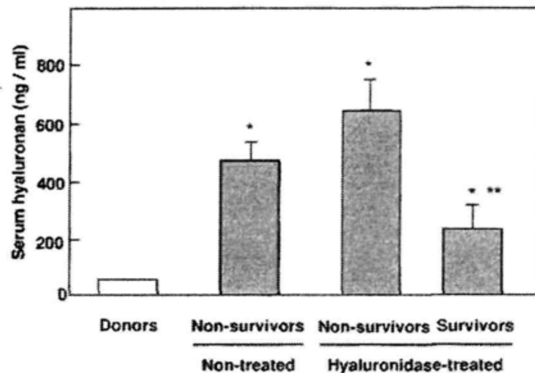


Figure 4. Serum hyaluronan levels in donors and recipients. Blood was taken from donors (□) and recipients (■) 10 minutes after restoration of circulation. After centrifugation at 2000g for 10 minutes at 4°C, the hyaluronan content in serum was measured using a HA kit. Data represents the mean ± SD. *Significantly different from donors *(P* < .0001). **Significantly different from non-treated *(P* =.0074) and hyaluronidase-treated *(P* = .0001) nonsurvival groups.

was estimated to be approximately 40 ng/mL. Higher serum hyaluronan levels were detected 10 minutes after restoration of circulation in the recipient animals of the non-hyaluronidase-treated grafts (477 ng/mL, *P* < .0001 versus donors); none of the recipients survived. High mean hyaluronan levels (648 ng/mL) were also observed in the nonsurvival group of hyaluronidase-treated grafts *(P* < .0001 versus donors; *P* = .0235 versus nontreated grafts). The serum hyaluronan concentration in the survival group was considerably lower (243 ng/mL) compared with nonsurvival groups. The differences in the hyaluronan levels in recipient sera of this group are statistically significant from donor rats *(P* = .0116) and the two nonsurvival groups of non-treated *(P* = .0074) and hyaluronidase-treated *(P* < .0001) grafts. Thus, high serum hyaluronan levels could possibly indicate a risk of early graft failure.

As shown in Figure 5A, the relative water content of the grafts after implantation in the rejection groups, i.e., nontreated and hyaluronidase-treated nonsurvivals, was 76.8% ± 0.9% and 75.5% ± 0.8%, respectively. The graft water content in the survival group was 71.5% — 2.7%, which was significantly lower compared with non-surviving animals grafted either with non-hyaluronidase-treated livers *(P* = .0001) or with hyaluronidase-treated livers *(P* =.0075).

Generally, there was a higher hyaluronan content in liver tissues of non-hyaluronidase-treated grafts compared with those that had been treated with hyaluronidase. Ten minutes after restoration of circulation, the hyaluronan content in recipient liver tissues of non-hyaluronidase-treared grafts was estimated to be about 8.9 μg/g dry liver weight, whereas the hyaluronan content in hyaluronidase-treated grafts, both in the nonsurvival and survival groups, was about fourfold lower (Fig. 5B). Statistically significant differences were observed between non-treated and hyaluronidase-treated grafts both in the nonsurvival group *(P* = .0264) and survival group *(P* = .0425). No statistically significant differences in hyaluronan content were observed between the survivors and non-survivors of the hyaluronidase-treated group.

Other Liver Graft Viability Markers

The hepatocyte function was also evaluated by measuring the serum levels of AST and ALT 10 minutes after restoration of circulation. The levels of both enzymes increased considerably in recipients from non-treated and hyaluronidase-treated grafts compared with donors;

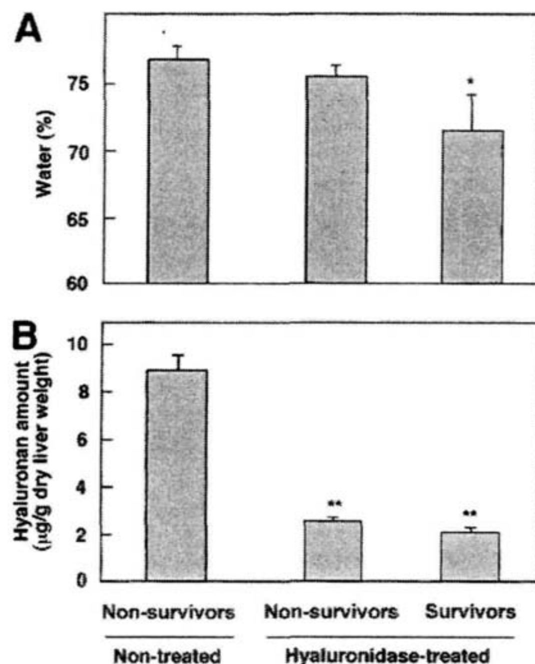


Figure 5. Quantification of water and hyaluronan content. After implantation and vascular anastomosis, the caudate lobes were removed and the water content (A) and amounts of hyaluronan (B) in graft livers were calculated as described in the Materials and Methods section. The **data show the mean ± SD. *Significant differences versus non-surviving animals grafted either with non—hyaluronidase-treated livers *(P* = .0001) or with hyaluronidase-treated livers *(P* = .0075). **Significant differences versus nontreated grafts *(P* < .05).**

however, the values for the different recipient groups were not significantly different. The AP levels in donors and recipients were also not statistically different (Fig. 6A). Furthermore, about a 25% decrease in the total concentrations of protein and albumin was observed in recipients compared with donors (Fig. 6B). No difference in the levels of bilirubin between donors and recipients was observed. Thus, the common viability markers **did** not differentiate between successful and non-successful rat liver transplantations.

Discussion

In the present study, we show that addition of testicular hyaluronidase to UW storage solution considerably improves the function of the liver grafts. Testicular hyaluronidase has been shown to have cardioprotective effects, probably by reducing tissue water accumulation

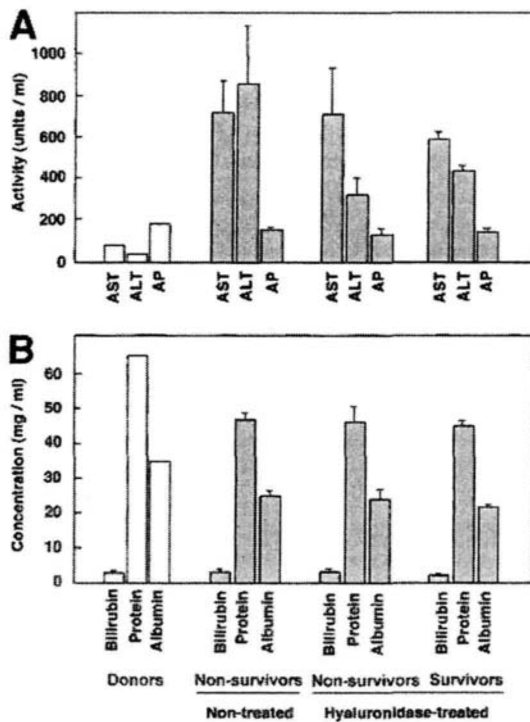


Figure 6. Graft viability markers. The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (AP) (A), as well as bilirubin, total protein, and albumin (B), were measured in the sera of donors and recipients 10 minutes after reperfusion using commercial kits. The data show the mean ± SD of the donors (□) as well as of the recipients (■) of the non-hyaluronidase-treated grafts and those of hyaluronidase-treated grafts.

and thereby tissue pressure, allowing better perfusion.[16, 20-22] In all these studies, it has been suggested that hyaluronidase exerts its effect primarily by degrading the excessive amounts of hyaluronan accumulated in inflammatory states, such as ischemic injury and surgical trauma. Within the liver tissue, hyaluronan is mainly located perivascularly, and its tissue content in nontreated grafts was estimated to be about 0.01% of the liver dry weight. Treatment of a graft with hyaluronidase resulted in a 75% reduction of the tissue hyaluronan independently of its viability (Fig. 5B). Thus, the elimination of perivascular hyaluronan is important but not essential for graft viability. However, testicular hyaluronidase acts also on other glycosaminoglycans, such as chondroitin sulfates, that show an ability to bind water. Thus, in the survival group of hyaluronidase-treated livers (Fig. 5A), the reduction of water seen may also be caused by the degradation of chondroitin sulfates. The role of the extracellular matrix components in modulating the movement of water and solutes between tissues and blood is not fully understood. However, it is becoming clear that signaling molecules such as cytokines and growth factors and the organization of the extracellular matrix influence tissue homeostasis. Taken in consideration that the water content in the grafts of the hyaluronidase-treated survival group was significantly lower than both the non-survival and nontreated grafts (Fig. 5A) and that the tissue hyaluronan content was equal in both hyaluronidase-treated groups, it is likely that other factors than the relatively low hyaluronan content of liver grafts influence the water volume in the liver compartment.

In the liver, Ito cells are the principal cell type that synthesize hyaluronan.[23,24] Under physiological conditions, the capacity of hyaluronan uptake by liver endothelial cells is higher than the rate of hyaluronan production by Ito cells.[23] However, during injury in either the preoperative and/or postoperative period of liver transplantation, Ito cells, activated by cytokines released by inflammatory cells invading the graft, may increase their synthesis of hyaluronan. This may coincide with impairment of the endocytotic function of endothelial cells, leading to a decreased clearance and thereby to accumulation of hyaluronan followed by an increased influx into the circulation. Our results suggest that a serum hyaluronan level equal to or higher than about 480 ng/mL soon after liver transplantation (10 minutes after reperfusion) may be an early risk factor for graft failure; in successful transplantations, the serum hyaluronan levels were about twofold less. In comparison with the other liver viability markers, which did not show such early significantly differences between suc-

cessful and nonsuccessful transplantations, hyaluronan clearance seems to be a more sensitive and rapid marker, which could predict the onset of graft primary dysfunction.

The preservation solution used contains substances that suppress cold-induced cell swelling and interstitial space expansion, reduce superoxide radical-induced injury, and regenerate phosphate compounds.[11] Still, previous studies have shown that postoperative survival after rat liver transplantation, after 24 hours cold storage in UW solution and portal vein clamping time exceeding 20 minutes, is almost zero.[25] In addition, a brief exposure of rat livers to Ringer's lactate, before cold storage, reduced considerably the protective effects of UW solution.[26] Consistent with these observations, we did not observe any survivals in the non—hyaluronidase-treated grafts, as opposed to 40% survival of grafts preserved in UW solution containing hyaluronidase. The beneficial effects of commercial preparations of testicular hyaluronidase on heart transplantation have been reported to be caused by suppression of intragraft tissue pressure.[22] We have recently observed that certain populations of testicular hyaluronidase induce angiogenesis in a CD44-dependent manner, and that this effect is accompanied by a decreased apoptotic rate.[17] Such an effect of hyaluronidase may also contribute to the improvement detected in liver graft viability. The expression of CD44 on endothelial cells has been reported by other laboratories[27] and us,[28] and has been shown to be involved in endothelial cell morphogenesis. Interactions between CD44 and hyaluronan in inflammatory disease and their implications in leukocyte recruitment and cell-matrix remodeling have been well documented, although their biological significance is not fully understood (for review see reference 2). The exact mechanism whereby some populations of testicular hyaluronidase induce endothelial cell morphogenesis and survival, and how hyaluronidase modulates transplant rejection, remain to be elucidated and is the subject of ongoing studies.

Our observations suggest that the addition of testicular hyaluronidase to UW solution can considerably improve the viability of nonfunctioning liver grafts in rats, and that the serum hyaluronan levels early after transplantation can be useful for the assessment of graft function. It will be important to investigate whether these results can be applied to a clinical situation to improve the viability of liver grafts and to guide in the decision early in the immediate post-transplant period regarding the necessity for retransplantation in cases of failing grafts.

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