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Hideki Ohdan
Yasuhiko Fukuda
Takashi Urushihara
Ryo Sumimoto
Toshimasa Asahara
Hisao Ito
Kiyohiko Dohi

Synergistic effects of nafamostat mesilate rinse and Kupffer cell blockade for rat liver preservation

H. Ohdan (✉) · Y. Fukuda · T. Urushihara · R. Sumimoto · T. Asahara · K. Dohi
Second Department of Surgery, Hiroshima University, School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan
Tel. +81 82 257 5222; Fax +81 82 257 5224

H. Ito
First Department of Pathology, Tottori University, School of Medicine, 86 Nishi-machi, Yonago 683, Japan

Abstract We investigated the efficacy of a new rinse solution containing nafamostat mesilate (NM) (a serine protease inhibitor) for liver preservation with modulation of Kupffer cell function. Orthotopic liver transplantation (OLT) was performed in male Lewis rats after 24 h of cold storage in University of Wisconsin organ preservation solution. After OLT, survival was determined, together with assays of blood chemistry, tissue NM metabolites, and histology 3 h after OLT. NM rinse was found to have a cytoprotective effect on liver parenchymal cells, based on enzyme data showing that NM rinse reduced the release of serum alanine aminotransferase significantly in comparison with saline rinse ($P < 0.05$). However, the effect was not sufficient to improve the survival rate. In contrast, when the donor was treated with gadolinium chloride 24–30 h before graft harvest, NM rinse improved the survival rate to around 80 % compared with 25 % for saline. The assay of NM metabolites in grafted liver tissue showed that pretreatment of the donor rats with $GdCl_3$ delayed the degeneration of NM in the liver tissue. These data demonstrate that NM rinse and Kupffer cell blockade exert synergistic effects, leading to increased survival after cold-preserved liver transplantation.

Key words Liver transplantation · Nafamostat mesilate · Preservation · Kupffer cell · Gadolinium chloride

Introduction

Nafamostat mesilate (NM; 6-amidino-2-naphthyl p-guanidinobenzoate dimethanesulfonate), is a synthetic serine protease inhibitor used for the treatment of pancreatitis and disseminated intravascular coagulation. Its main actions are: 1) the suppression of proteases such as thrombin, activated coagulation factors (XIIa, Xa), plasmin, complements ($C1\bar{r}$, $C1\bar{s}$), trypsin, and phospholipase A_2, 2) the inhibition of platelet aggregation, and 3) the stabilization of lysosomal and cellular membranes [1, 3, 4, 17]. We have recently demonstrated that a cocktail of NM added to a prereperfusion rinse solution (NM rinse) can improve graft survival dramatically after transplantation of a long-term-preserved pancreas in the rat [16]. This new strategy is also expected to be potentially useful for attenuating reperfusion injury in cold-preserved liver transplantation, because several reports have shown that proteases are involved in the development of liver ischemia-reperfusion injury [2, 8, 9, 12, 15]. Considering NM can be easily hydrolyzed, however, it is anticipated that hydrolases from activated Kupffer cells would facilitate the degradation of NM in the graft tissue if an NM rinse were used in cold-preserved liver transplantation. Therefore, in the present study, we investigated the efficacy of an NM rinse solution for extended liver preservation together with modulation of Kupffer cell function.

Table 1 Composition of UW solution and NM rinse. Values in mmol/l, unless otherwise stated

	UW	NM rinse
Potassium lactobionate	100	
Sodium lactobionate		110
$NaKH_2PO_4$	25	
NaH_2PO_4		25
Raffinose	30	30
$MgSO_4$	5	
Glutathione	3	3
Adenosine	5	
Allopurinol	1	1
Insulin (IU/l)	100	
Hydroxyethyl starch (g%)	5	
Nafamostat mesilate		0.8
β-Cyclodextrin		3
γ-Cyclodextrin		10
Osmolarity	320–330	273
pH	7.4	7.4

Materials and methods

Composition of the NM rinse

The composition of the University of Wisconsin organ preservation (UW) solution used for preservation and that of the NM rinse solution are listed in Table 1. The NM rinse is potassium-free to avoid potassium loading after reperfusion, and contains cyclodextrin as a solubilizer of NM. Immediately before use, NM (Torii Pharmaceutical Company, Tokyo, Japan) was added.

Liver transplantation

Syngeneic orthotopic liver transplantation (OLT) without rearterialization was performed in male Lewis rats weighing 180–280 g (Japan Charles River Laboratory, Osaka, Japan) as described previously [7]. Donor livers were flushed in situ through the portal vein with 10 ml of UW solution at 4 °C. Grafts were preserved for 24 h in UW solution at 4 °C. Immediately before implantation, grafts were rinsed with 12–15 ml of test solution at 4 °C via the portal vein under a pressure of 10–15 cmH_2O. After revascularization of the graft, each recipient was given 1 ml of lactate Ringer's solution through the penile vein and 100 mg/kg cefazolin sodium was administered intramuscularly. Implantation surgery required less than 50 min, during which time the portal vein was clamped for 11–15 min.

Experimental groups

Experimental animals were divided into four groups, which differed according to the presence or absence of donor pretreatment with Kupffer cell blockade and the type of rinse solution used. In groups 1 ($n = 9$) and 2 ($n = 8$), donor animals received no pretransplant treatment. In donor animals of groups 3 ($n = 8$) and 4 ($n = 9$), gadolinium chloride ($GdCl_3$) ($GdCl_3.6H_2O$, Nacalai Tesque, Kyoto, Japan) was administered intravenously, at a dose rate of 10 mg/kg body weight 24–30 h before harvest, to destroy the Kupffer cells [13]. Immediately before implantation, the preservation solution was flushed out with the test rinse solution. Saline was used for this purpose in groups 1 and 3, whereas NM rinse was used in groups 2 and 4. One-week survival rates were compared and an autopsy was performed on all recipient animals that died before 7 days to determine the cause of death. Five additional recipients in each group were sacrificed 3 h after liver graft reperfusion for the determination of blood chemistry, measurement of tissue NM metabolite concentration in the liver, and histology.

Blood chemistry

Blood samples were collected via the inferior vena cava 3 h after reperfusion. Serum levels of alanine aminotransferase (ALT) were measured using the standard enzymatic procedure to evaluate the degree of liver parenchymal cell damage. Serum levels of hyaluronic acid (HA) were measured with the aid of a radiometric kit (FML Laboratories, Hiroshima, Japan) to evaluate the degree of liver endothelial cell damage [14].

Determination of tissue NM metabolite concentrations

The tissue concentrations of intact NM and its hydrolysis product, 6-amidino-2-naphthol (AN), were measured in grafted livers by fluorometric detection as described previously [11].

Histology

Histological specimens of rat livers were stored in 10 % formalin and embedded subsequently in paraffin, followed by sectioning and staining with hematoxylin and eosin.

Statistics

Statistical evaluation was made by the unpaired t-test. Differences were considered significant at a P value of less than 0.05.

Results

Survival

Survival curves following transplantation surgery are shown in Fig. 1. One-week survival rates in groups 1, 2, 3, and 4 were 22.2 % (2/9), 25.0 % (2/8), 25.0 % (2/8) and 77.7 % (7/9), respectively. Survival rate was not improved when the NM rinse and the pretreatment of donor rats with $GdCl_3$ were employed separately. However, a combination of the two strategies improved the 1-week survival rate.

Blood chemistry

The results of the blood chemical assays are shown in Table 2. The elevation of serum ALT was significantly suppressed in group 2 compared with group 1 ($P < 0.05$), and was more suppressed in group 4. The elevation of serum HA was significantly suppressed in groups 3 and 4 compared with group 1 ($P < 0.05$) (Table 2).

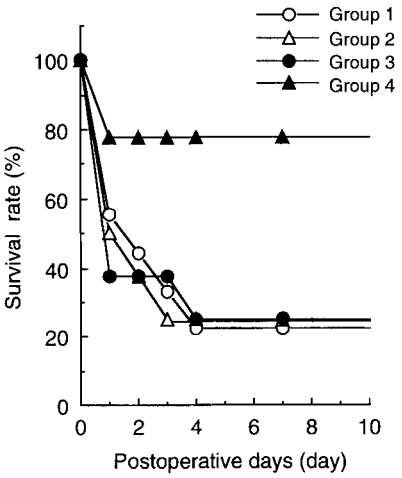

Fig. 1 Postoperative survival rate. Data expressed as percentages (n = 8–9/group)

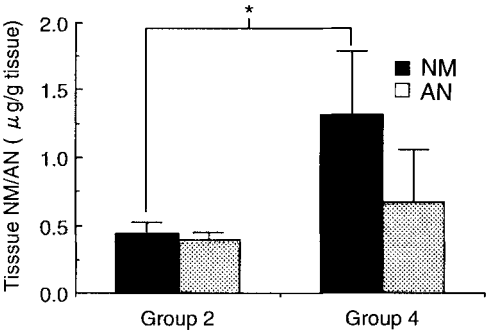

Fig. 2 NM metabolite concentrations in graft liver tissues 3 h after OLT. The tissue concentrations of intact nafamostat mesilate *(NM)* and its hydrolyzed product, amidino-naphthol *(AN)*, were measured in liver tissues. Data expressed as mean ± SEM (n = 3–5/group, * $P < 0.05$)

Table 2 Serum levels of alanine aminotransferase (ALT) and hyaluronic acid (HA) 3 h after OLT in each group. Data expressed as mean ± SE

Group	n	ALT (IU/l)	HA (ng/ml)
1. (Saline rinse)	5	3134 ± 1037	936 ± 272
2. (NM rinse)	5	1069 ± 225*	634 ± 383
3. ($GdCl_3$ + saline rinse)	5	1645 ± 304	303 ± 36*
4. ($GdCl_3$ + NM rinse)	5	814 ± 251*,**	196 ± 52*

* $P < 0.05$ vs group 1; ** $P < 0.05$ vs group 3

Liver tissue NM metabolite concentrations

After 3 h of reperfusion, the concentration of intact NM in the liver tissue was significantly higher in group 4 than in group 2 ($P < 0.05$). Tissue AN concentration in the liver did not differ significantly between groups 2 and 4 (Fig. 2).

Histology

After 3 h of reperfusion in groups 1, 2, and 3, severe congestion and extensive vacuolization of hepatocytes were observed. Hyaline inclusion bodies were frequently recognized in the hepatocytes of the congested areas. In contrast, in group 4, mild injury consisting only of some centrilobular vacuolation was observed, but hyaline inclusion bodies were not evident (Table 3).

Discussion

It has been suggested that ischemia-reperfusion injury to the liver is mediated, at least in part, by graft proteases released immediately after graft revascularization [2, 8, 9, 12, 15]. Proteases appear to be involved in the development of liver ischemia-reperfusion injury and some protease inhibitors have been shown to be cytoprotective in various models of liver transplantation. Aprotinin has been reported to have significant impact on survival when administered continuously to the recipient animal for 6 h following revascularization in a pig OLT model [12], and when given to the donor and added to the preservation solution in a rat OLT model [9, 15]. It has been recently indicated that rat liver ischemia-reperfusion injury is attenuated by pretreatment with urinary trypsin inhibitor, which suppresses neutrophil elastase and cathepsin G, and stabilizes lysosomal and cellular membranes [8]. Thus, protease inhibitors have a beneficial effect on liver preservation by protecting against ischemia-reperfusion injury.

NM is a synthetic serine protease inhibitor, whose main actions include the suppression of various proteases, the inhibition of platelet aggregation, and the stabilization of lysosomal and cellular membranes, these actions being more potent than those of other protease inhibitors such as aprotinin and gabexate mesilate [1, 3, 4, 17]. Recently, we have devised a new strategy that involves the use of a cocktail of NM added to a prereperfusion rinse solution, for extended organ preservation. This strategy seems reasonable because it can deliver a high concentration of the cytoprotective agent to graft tissues during reperfusion; otherwise the agent may be degraded during prolonged storage and may be removed from grafts by prereperfusion rinsing when the agent is added to the preservation solution. In our recent study, NM rinse has been shown to dramatically improve the graft survival rate of rat pancreas, cold-preserved for 48 or 72 h in UW solution [16].

In the present study, NM rinse exerted some beneficial effects on 24 h rat liver preservation, although it did not prolong the survival of liver-transplanted recipients. It obviously reduced the release of ALT from hep-

Table 3 Number of intracytoplasmic hyaline bodies per field in histological specimens of 24 h cold-preserved livers. The number of intracytoplasmic hyaline bodies was determined in a randomly selected field of liver from rats killed 3 h after OLT. The number per field (× 200) was classified as follows: – no hyaline bodies, + 1 ≦ hyaline bodies < 3, ++ 3 ≦ hyaline bodies < 6, +++ 6 ≦ hyaline bodies

Group	Number of hyaline bodies
1. (Saline rinse)	+, +, +, ++, ++
2. (NM rinse)	+, +, ++, +++, +++
3. ($GdCl_3$ + saline rinse)	–, +, ++, ++, +++
4. ($GdCl_3$ + NM rinse)	–, –, –, –, –

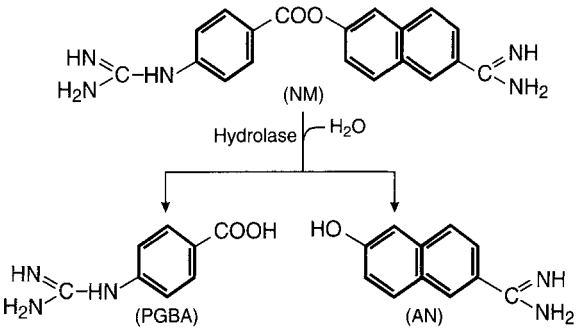

Fig. 3 Structural formula of nafamostat mesilate *(NM)* and its hydrolysis products, 6-amidino-2-naphthol *(AN)* and *p*-guanidino benzoic acid *(PGBA)*

atocytes into the serum after 3 h of reperfusion. In contrast, the elevation of serum HA after 3 h of reperfusion was not substantially suppressed by the use of NM rinse. These findings indicate that NM rinse acts on liver parenchymal cells rather than on endothelial cells.

The liver has resident macrophages, Kupffer cells, that are known to be the predominant source of reactive oxygen formed during the initial reperfusion period. Kupffer cell activity, including reactive oxygen formation, contributes to reperfusion injury in the liver. Previous reports have documented that inactivation of Kupffer cells by blockade with agents such as $GdCl_3$ and methyl palmitate protects the liver against ischemia-reperfusion injury [5, 10]. In the present study, pretreatment of donor rats with $GdCl_3$ prevented endothelial cell damage during reperfusion, as shown by assay of serum HA, although it did not prolong the survival of recipients that received transplants of 24 h cold-preserved liver.

It has been suggested that Kupffer cells, activated by transplantation, release mediators that stimulate the mitochondria of parenchymal cells and enhance drug metabolism by increasing the supply of cofactors [13]. Considering that NM can be easily hydrolyzed by certain cofactors [11], this hypothesis is of particular interest, since the increase of hydrolase supplied by activated Kupffer cells might facilitate the degradation of NM in grafted liver tissue (Fig. 3). In the assay of liver tissue NM metabolites, we observed that pretreatment of donor rats with $GdCl_3$ delayed the degradation of NM in the grafted liver tissue and maintained an effective concentration of intact NM after 3 h of reperfusion. Furthermore, combined treatment with NM rinse and pretreatment with $GdCl_3$ produced a marked improvement in the survival rate of rats that received transplants of 24 h cold-preserved livers. This finding is in good agreement with the histological data that showed obvious prevention of hyaline inclusion body formation due to an increase in membrane permeability caused by hypoxia and excessive inhibition of serum protein [6]. These results are the first documented evidence of a synergistic effect of protease inhibitor and Kupffer cell blockade in preventing ischemia-reperfusion injury in cold-preserved liver transplantation.

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References

1. Aoyama T, Ino Y, Ozeki M, Oda M, Sato T, Koshiyama Y, Suzuki S, Fujita M (1984) Pharmacological studies of FUT-175, nafamostat mesilate. I. Inhibition of protease activity in in vivo and in vitro experiments. Jpn J Pharmacol 35: 203–227
2. Himmelreich G, Muser M, Neuhaus P, Bechstein WO, Slama KJ, Jochum M, Riess H (1992) Different aprotinin applications influencing hemostatic changes in orthotopic liver transplantation. Transplantation 53: 132–136
3. Hitomi Y, Ikari N, Fujii S (1985) Inhibitory effect of a new synthetic protease inhibitor (FUT-175) on the coagulation system. Haemostasis 15: 164–168
4. Iwaki M, Ino Y, Motoyoshi A, Ozeki M, Sato T, Kurumi M, Aoyama T (1986) Pharmacological studies of FUT-175, nafamostat mesilate. V. Effects on the pancreatic enzymes and experimental acute pancreatitis in rats. Jpn J Pharmacol 41: 155–162
5. Jaeschke H, Farhood A (1991) Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. Am J Physiol 260: G 355–362
6. Kajihara H, Yamamoto M, Yamada H, Mochizuki T, Taguchi (1981) Intracytoplasmic inclusion bodies in hepatocytes during prolonged extracorporeal circulation. Path Res Pract 172: 349–362
7. Kamada N, Calne RY (1983) A surgical experience with five hundred and thirty liver transplants in the rat. Surgery 93: 64–69

8. Li XK, Matin AFM, Suzuki H, Uno T, Yamaguchi T, Harada Y (1993) Effect of protease inhibitor on ischemia/reperfusion injury of the rat liver. Transplantation 56: 1331–1336
9. Lie TS, Seger R, Hong GS, Preissinger H, Ogawa K (1989) Protective effect of aprotinin on ischemic hepatocellular damage. Transplantation 48: 396–399
10. Lindert KA, Caldwell-Kenkel JC, Nukina S, Lemasters JJ, Thurman RG (1992) Activation of Kupffer cells on reperfusion following hypoxia: particle phagocytosis in a low-flow, reflow model. Am J Physiol 262: G345–350
11. Nanpo T, Ohtuki T, Jin Y, Matunaga K, Takahashi M (1984) Pharmacokinetic studies of FUT-175 (nafamostat mesilate) (1) – blood level profiles, tissue distribution, metabolism and excretion in rats after intravenous administration (in Japanese). Kiso to Rinsho 18: 467–488
12. Oldhafer KJ, Schuttler W, Wiehe B, Hauss J, Pichlmayr R (1991) Treatment of preservation/reperfusion liver injury by the protease inhibitor aprotinin after cold ischemia storage. Transplant Proc 23: 2380–2381
13. Qu W, Savier E, Thurman RG (1992) Stimulation of monooxygenation and conjugation after liver transplantation in the rat: involvement of Kupffer cells. Mol Pharmacol 41: 1149–1154
14. Shimazu H, He W, Guo P, Dziadkoviec I, Miyazaki M, Falk RE (1994) Serum hyaluronate in the assessment of liver endothelial cell function after orthotopic liver transplantation in the rat. Hepatology 20: 1323–1329
15. Takei Y, Marzi I, Kauffman FC, Currin RT, Lemasters JJ, Thurman RG (1990) Increase in survival time of liver transplants by protease inhibitors and a calcium channel blocker, nisoldipine. Transplantation 50: 14–20
16. Urushihara T, Sumimoto K, Sumimoto R, Ikeda M, Fukuda Y, Dohi K (1994) Rinse solution containing a protease inhibitor and Na lactobionate increases graft survival after acute rat pancreas preservation. Transplant Proc 26: 559–560
17. Yoshikawa T, Murakami M, Furukawa Y, Kato H, Takemura S, Kondo M (1983) Effects of FUT-175, a new synthetic protease inhibitor on endotoxin-induced disseminated intravascular coagulation in rats. Haemostasis 13: 374–378