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Abstract

Controversy exists over whether the predominant cell death of hepatocytes is due to apoptosis or necrosis after ischemia/reperfusion injury. In this study we investigated the predominant cell death of hepatocytes after cold ischemia/reperfusion injury using the Annexin V-based assay, and evaluated the anti-apoptotic effect of ascorbic acid 2-glucoside (AA-2G) added to the University of Wisconsin solution (UW solution) in rat liver transplantation. The retrieved liver was preserved in 4 UW solution for 24 h, and then transplanted orthotopically to the syngeneic Wistar recipient. The animals were divided into 2 groups, a control group (n=10), in which liver grafts were preserved in UW solution (4), and an AA-2G group (n=10), in which liver grafts were preserved in UW solution (4) with AA-2G (100 ug/ml). The serum AST level 4 h after reperfusion in the control group was significantly suppressed in the AA-2G group, and the bile production of the liver graft in the AA-2G group was well recovered. The mean survival time in the AA-2G group was significantly improved compared with that in the control group. Annexin-V and Propidium iodide staining 4 h after reperfusion showed a significantly higher percentage of viable hepatocytes in the AA-2G group compared with the control group (93.4 +/- 2.0 vs. 80.3 +- 2.1%, $P<0.05$). In the control group, the main cell death of hepatocytes was apoptosis (early apoptosis: 10.0 +- 4.7%, late apoptosis: 6.4 +/- 1.7%). The addition of AA-2G to the UW solution significantly inhibited both early and late apoptotic cell death 4 h after reperfusion (early apoptosis: 0.98 +/- 0.88%, late apoptosis: 2.2 +/- 1.1%). The expression of caspase 9 in the immunostaining of the liver graft was suppressed in the AA-2G group compared with in the control group. Our study using the Annexin V-based assay provided evidence that the predominant cell death of hepatocytes was apoptosis after 24 h cold ischemia/reperfusion injury in rat liver transplantation. The addition of AA-2G to the UW solution attenuated 24 h cold ischemia/reperfusion injury by inhibiting the apoptosis of hepatocytes.

KEYWORDS: apoptosis, ischemia/ reperfusion injury, liver transplantation, ascorbic acid 2-glucoside(AA-2G)

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Original Article

Annexin V Assay-proven Anti-apoptotic Effect of Ascorbic Acid 2-glucoside after Cold Ischemia/Reperfusion Injury in Rat Liver Transplantation

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Controversy exists over whether the predominant cell death of hepatocytes is due to apoptosis or necrosis after ischemia/reperfusion injury. In this study we investigated the predominant cell death of hepatocytes after cold ischemia/reperfusion injury using the Annexin V-based assay, and evaluated the anti-apoptotic effect of ascorbic acid 2-glucoside (AA-2G) added to the University of Wisconsin solution (UW solution) in rat liver transplantation. The retrieved liver was preserved in 4 °C UW solution for 24 h, and then transplanted orthotopically to the syngeneic Wistar recipient. The animals were divided into 2 groups, a control group (n = 10), in which liver grafts were preserved in UW solution (4 °C), and an AA-2G group (n = 10), in which liver grafts were preserved in UW solution (4 °C) with AA-2G (100 ug/ml). The serum AST level 4 h after reperfusion in the control group was significantly suppressed in the AA-2G group, and the bile production of the liver graft in the AA-2G group was well recovered. The mean survival time in the AA-2G group was significantly improved compared with that in the control group. Annexin-V and Propidium iodide staining 4 h after reperfusion showed a significantly higher percentage of viable hepatocytes in the AA-2G group compared with the control group (93.4 ± 2.0 *vs.* $80.3 \pm 2.1\%$, $P < 0.05$). In the control group, the main cell death of hepatocytes was apoptosis (early apoptosis: $10.0 \pm 4.7\%$, late apoptosis: $6.4 \pm 1.7\%$). The addition of AA-2G to the UW solution significantly inhibited both early and late apoptotic cell death 4 h after reperfusion (early apoptosis: $0.98 \pm 0.88\%$, late apoptosis: $2.2 \pm 1.1\%$). The expression of caspase 9 in the immunostaining of the liver graft was suppressed in the AA-2G group compared with in the control group. Our study using the Annexin V-based assay provided evidence that the predominant cell death of hepatocytes was apoptosis after 24 h cold ischemia/reperfusion injury in rat liver transplantation. The addition of AA-2G to the UW solution attenuated 24 h cold ischemia/reperfusion injury by inhibiting the apoptosis of hepatocytes.

Key words: apoptosis, ischemia/reperfusion injury, liver transplantation, ascorbic acid 2-glucoside (AA-2G)

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It has been reported in a clinical setting that an ischemia/reperfusion (I/R) injury reduces liver graft viability due to direct damage to the graft and the susceptibility of the patient to acute rejection [1–3]. Together with other factors, such as intracellular calcium and the calpain and tumor necrosis factor, the reactive oxygen species (ROS) has been reported to play an important role in the pathogenesis of I/R injury [4–6]. ROS can damage the membranes via lipid peroxidation, leading to mitochondrial swelling and cell necrosis [7], and can also damage DNA and the fragmentation of the double-strand chromatin, resulting in apoptosis [8, 9]. With regards to hepatocellular damage after I/R injury, it was reported that ROS can initiate the cascades of both necrosis and apoptosis [10]. Therefore, it is still important to protect against ROS-mediated damage induced by I/R injury using free radical scavengers.

There is controversy over whether the predominant cell death of hepatocytes after I/R injury is apoptosis or necrosis. Several laboratories reported, using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay [11, 12], that 50% to 70% of endothelial cells (SEC) and 40% to 60% of hepatocytes undergo apoptosis during reperfusion. In human liver transplantation, a high percentage of apoptotic hepatocytes have also been detected in biopsy specimens [13]. In contrast, Gujral *et al.* reported that only a small minority of SEC and hepatocytes undergo apoptosis after warm I/R injury, using the Annexin V-based assay [14]. This controversy might be influenced by the sensitivity of the methods used to detect apoptosis. Because the Annexin V-assay is the most sensitive technique to detect ongoing apoptosis [15], we investigated the predominant cell death of hepatocytes after 24 h cold ischemia (UW solution)/reperfusion injury in rat liver transplantation, using the Annexin V-based assay.

It has been demonstrated that Ascorbic acid (AA) has ROS scavenging activity not only by protecting the membrane phospholipids against the peroxidative damage of ROS, but also by suppressing ROS production of the membranes [16, 17]. However, the fact that AA is unstable for easy oxidation in the body is a disadvantage, and AA also cannot be synthesized in the body. Ascorbic acid 2-glucoside (AA-2G) was found to be a promising AA derivative. AA-2G has two beneficial properties; it has high stability against thermal and oxidative degradation, and it can be rapidly converted to AA by α-glucosidase in the blood and liver cells [18–20]. Therefore, in this study, we added AA-2G into the UW solution in order to reduce cold I/R injury caused by liver transplantation.

In this study, we investigated the predominant cell death of hepatocytes after cold ischemia/reperfusion injury using the Annexin V-based assay, and also evaluated the anti-apoptotic effect of AA-2G added to UW solution in rat liver transplantation.

Materials and Methods

Experimental animals. All experiments were performed in accordance with the national guidelines for the care and use of laboratory animal and in accordance with the local ethics committee. Male Wistar rat weighing 230–260 g were purchased from Shimizu Laboratory (Shimizu Supplies Co., Ltd., Hamamatsu, Japan). The rats were allowed free access to food and water before, during, and after the transplantation. The animal room was windowless with temperatures maintained at (22 ± 2 °C), and was kept under constant environmental conditions with a 12-h light-dark cycle (light, 7AM-7PM). All of the steps of the experiment, including the surgeries, were performed under sterile conditions.

Experimental groups. The experimental animals were divided into the following two groups according to the preservation procedure used for the retrieved liver graft: control group ($n = 10$); the liver grafts were preserved in UW solution (4 °C) for 24 h; AA-2G group ($n = 10$); the liver grafts were preserved for 24 h in UW solution (4 °C) with AA-2G (100 ug/ml) (from Hayashibara Biochemical Research Institute Co., Ltd., Okayama, Japan).

Liver procurement, preservation, and transplantation. The donor liver procurements were performed according to the standard microsurgical techniques for rat liver transplantation. Briefly, the animals were anesthetized with ether, and the abdomen was opened through a transverse incision. The liver was freed from its ligaments with minimal manipulation and the portal vein and vena cava were divided. A catheter was inserted into the portal vein, and the livers were then flushed *in situ* with 10 ml of 4 °C UW solution or UW solution containing AA-2G, while the supra-and intrahepatic vena cava were transected for venting the flushing solution. The liver grafts in each group were preserved for 24 h in a computer-programmed incubater (MIR-151, Sanyo Co., Ltd., Tokyo, Japan) at 3.9 ± 0.1 °C. After

cold preservation for 24 h, the liver grafts were transplanted orthotopically using the Kamada technique [21].

Bile production, liver enzyme and histological assessment. The bile production in the liver graft was measured as an index of the liver graft function at 4 h after reperfusion. Blood samples were taken at 4 h after reperfusion, and the serum levels of aspartate amino transferase (AST) was measured. Liver biopsies were obtained after cold preservation for 24 h for hematoxylin and eosin (HE) staining. The recipients were sacrificed at 4 h after reperfusion for HE staining and Annexin-V assay of the liver grafts.

Survival time. Ten animals in each group were used for the assessment of survival time.

Annexin-V immunostaining and flow cytometric analysis. Annexin V is a 35- to 36-kD calcium-dependent, phospholipid binding protein that has a high affinity for phosphotidyl serine and binds to cells with exposed phosphotidyl serine [15]. Because the membrane phospholipid phosphotidyl serine is translocated from the inner to the outer leaflet of the plasma membrane at an early stage of apoptosis, an Annexin-V assay can identify apoptosis at an earlier stage than the TUNEL assays based on nuclear changes such as DNA fragmentation [14, 15, 22]. In addition, Annexin-V is conjugated to fluorochrome FITC that served as a sensitive probe for flow cytometric analysis in this Annexin-V assay. The Annexin-V assay was carried out in conjunction with vital dye Propidium iodide (PI) staining in order to distinguish between apoptosis and necrosis, because PI staining can detect DNA that has leaked from the necrotic cell. To detect apoptotic and viable cells, the recipients were sacrificed, and the liver tissues were taken at 4 h after reperfusion from each group (n = 5). The hepatocytes were isolated by gently pressing the livers collected, and by then filtering the cells collected through a 70-μm mesh filter. The cells were washed in cold PBS solution of 4 ˚C, and were sedimented at 1500 Xg for 5 min 3 times. The viable hepatocytes exceeded 95%, as judged by trypan blue exclusion in the normal liver. About 10^6 hepatocytes were incubated in 10 ul of FITC-conjugated Annexin-V (10 ug/ml, Bender Medsystems Diagnostics GmbH, Vienna, Austria) for 15 min, and in PI (50 ug/ml in PBS) on ice for 1 min in the dark. The fluorescence was measured immediately by flow cytometry with FACScan (Beckton Dickinson, San Jose, CA, USA). At least 10,000 hepatocytes were examined. Analysis gates were set to exclude endothelial cells that were acquired concurrently with the hepatocytes collection.

Immunohistochemical staining of caspase 9. At 4 h after reperfusion, the livers were excised, and the tissues were immediately cryopreserved. Five μm sections were prepared and were fixed with 99.5% acetone for 10 min. After being washed 3 times for 5 min with TBST (DW 900 ml, TBS 100 ml, 1% TritonX-100 1 ml), the activity of the endogenous peroxidase was blocked by 0.3% H_2O_2 in methanol for 30 min at room temperature. After 3 rinses in TBST, the sections were incubated with 150 μl normal blocking serum for 20 min at room temperature. Hundred fifty μl of cleaved caspase-9 antibody (Cell Signaling Technology, Beverly, MA, USA) was added to the samples, which were incubated at 4 ˚C overnight. The sections were incubated on the next day for 30 min with diluted biotinylation secondary antibody solution (Vector Laboratories Inc., Burlingame, CA, USA). After being washed 3 times for 5 min each with TBST solution, the sections were incubated for 30 min with ABC reagent at room temperature. Each section was then incubated in peroxidase substrate solution until the desired stain intensity developed at room temperature. Each section was then examined by light microscopy at 200 x magnification.

Statistical analysis. All values are expressed as mean ± SD. The results were analyzed using the Student-*t* test. *P* values less than 0.05 were considered to indicate statistical significance.

Results

Bile production and liver enzyme. The graft liver of the control group had decreased bile production (6.1 ± 1.6 ml/h/100 g liver), whereas the AA-2G group had significantly improved bile production (10.8 ± 2.8 ml/h/100 g liver, $P < 0.05$, Fig. 1). The increase of AST levels 4 h after I/R injury in the AA-2G group were significantly suppressed compared with those in the control group (4292 ± 1401 *vs.* 6944 ± 2143 IU/L, $P < 0.05$, Fig. 2).

Histological assessment. Microvesicular vacuolation in the cytoplasm of the hepatocytes were observed in the control group (Fig. 3A). In contrast, these vacuolations were not prominent in the AA-2G group (Fig. 3B).

Survival time. Table 1 shows the recipient survival time of the 2 groups. The mean survival time in

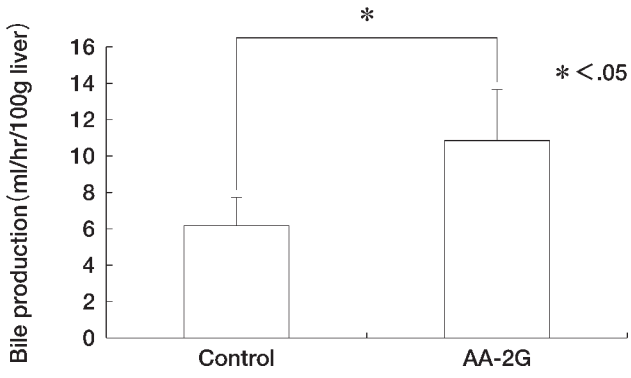

Fig. 1 At 4 h after reperfusion, bile production in the AA-2G group was significantly improved compared with that in the control group (6.1 ± 1.6 ml/h/100 g liver *vs*. 10.8 ± 2.8 ml/h/100 g liver, $P <$ 0.05).

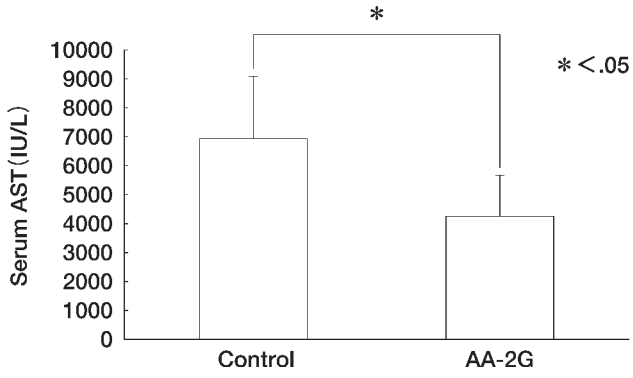

Fig. 2 The increase of AST levels 4 h after reperfusion in the AA-2G group were significantly suppressed compared with those in the control group (4292 ± 1401 *vs*. 6944 ± 2143 IU/L, $P < 0.05$).

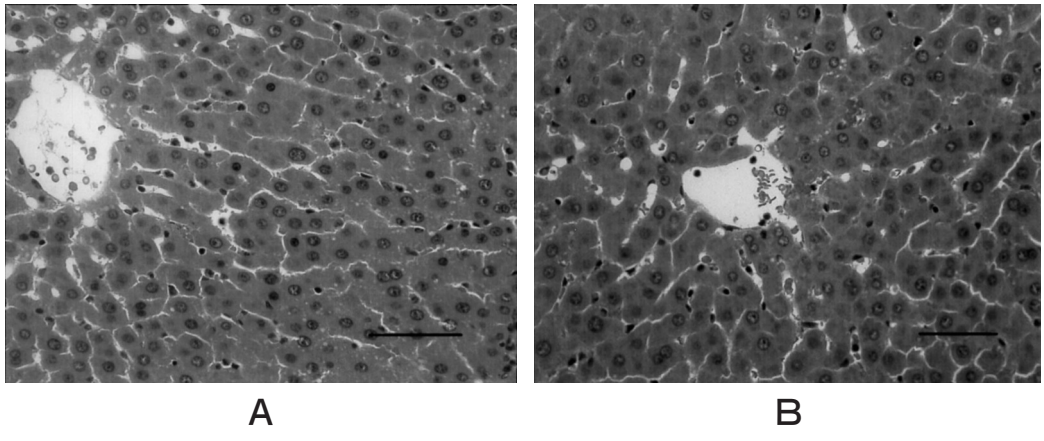

Fig. 3 Light micrographs of the liver graft, obtained 4 h after reperfusion, stained with H&E. (**A**) Control group: microvesicular vacuolation in the cytoplasm of the hepatocytes were observed. (**B**) AA-2G group: these vacuolations were not prominent. Bars indicate 50 μm.

Table 1 The effect of addition of AA-2G to UW solution on survival time

	Survival (days)	Mean Survival Time (days)	
Control	5, 5, 5, 6, 6, 7, 12, 18, 20, 25	10.9 ± 7.5	*
AA-2G	12, 13, 13, 14, 18, 20, 30, 48, 52, 60	28.0 ± 18.5	

*$P < 0.001$

the AA-2G group was 28.0 ± 18.5 days, which was significantly longer than in the control group (10.9 ± 7.5 days) ($P < 0.001$).

Annexin V staining and Flow cytometric analysis. We used Annexin V and PI staining to verify the percentage of viable, early, and late apoptotic and necrotic hepatocytes after 24 h cold preservation and 4 h reperfusion in rat liver transplantation. Fig. 4 displays a representative flow cytometric result. The viable hepatocytes were negative for both Annexin V and PI (Quadrant 1). The cells that were Annexin-V positive,

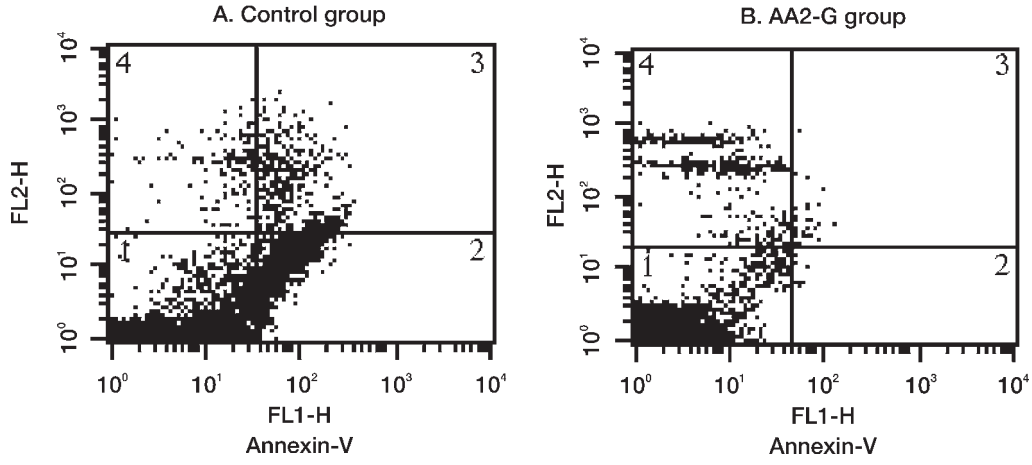

Fig. 4 Dual-parameter flow cytograms of FITC-labeled Annexin V (abscissa) *vs*. PI staining (ordinate) of the control group (**A**) and the AA-2G group (**B**). Viable hepatocytes were negative for both Annexin V and PI (quadrant 1); Early apoptotic hepatocytes were labeled by Annexin V, while being negative for PI (quadrant 2); Late apoptotic hepatocytes were positive for both Annexin V and PI (quadrant 3); Necrotic hepatocytes were labeled by PI, while being negative for Annexin V (quadrant 4). The percentages of hepatocytes in quadrant 1 were $80.3 \pm 2.1\%$ and $93.4 \pm 2.0\%$ in the control group and AA-2G group, respectively, whereas those for early apoptotic hepatocytes in quadrant 2 were $10.0 \pm 4.7\%$ and $0.98 \pm 0.88\%$.

Table 2 The percentage of viable, early and late apoptosis hepatocytes 4 h after reperfusion analysed by flow cytometry of Annexin V and PI staining.

	Viable cell (%)		Early apoptosis (%)		Late apoptosis (%)	
Control	80.3 ± 2.0	*	10.0 ± 4.7	**	6.4 ± 1.7	***
AA-2G	93.4 ± 2.0		0.98 ± 0.88		2.2 ± 1.1	

$^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$

but PI negative, represent the fraction with early apoptosis (Quadrant 2). The cells that were both Annexin-V and PI positive showed the fraction with late apoptosis (Quadrant 3). The cells that were Annexin-V negative, but PI positive, demonstrated the fraction with necrosis (Quadrant 4). The ratio of viable hepatocytes in the AA-2G group was significantly higher than that in the control group (93.4 ± 2.0 *vs.* $80.3 \pm 2.1\%$, $P < 0.005$, Table 2). The rates of early apoptotic and late apoptotic hepatocytes in the control group were $10.0 \pm 4.7\%$, and $6.4 \pm 1.7\%$, respectively, indicating that the main cell death of hepatocytes in the control group was apoptosis. In contrast, both early and late apoptosis of the hepatocytes was significantly inhibited in the AA-2G group (early apoptosis: $0.98 \pm 0.88\%$, late apoptosis: $2.2 \pm 1.1\%$). There was no significant difference in the ratio of necrotic hepatocytes between the AA-2G group and control group (3.52 ± 2.25 *vs.* $2.76 \pm 0.99\%$).

Immunohistochemical staining of caspase 9. The expression of caspase 9 in the hepatocytes was observed in the control group (Fig. 5A). In contrast, the expression of caspase 9 in the hepatocytes was suppressed in the AA-2G group (Fig. 5B).

Discussion

Controversy exists over whether the main cell death of hepatocytes after I/R injury is due to apoptosis or necrosis. As before, cold ischemia appears to cause injury mainly to sinusoidal endothelial cells (SEC), and also activates Kupffer cells, resulting in hepatocellular necrosis after reperfusion [23, 24]. In recent years, several laboratories have reported evidence for apoptotic cell death during I/R injury [11, 12, 25]. According to these studies using the TUNEL assay, 50% to 70% of SEC and 40% to 60% of hepatocytes undergo apoptosis

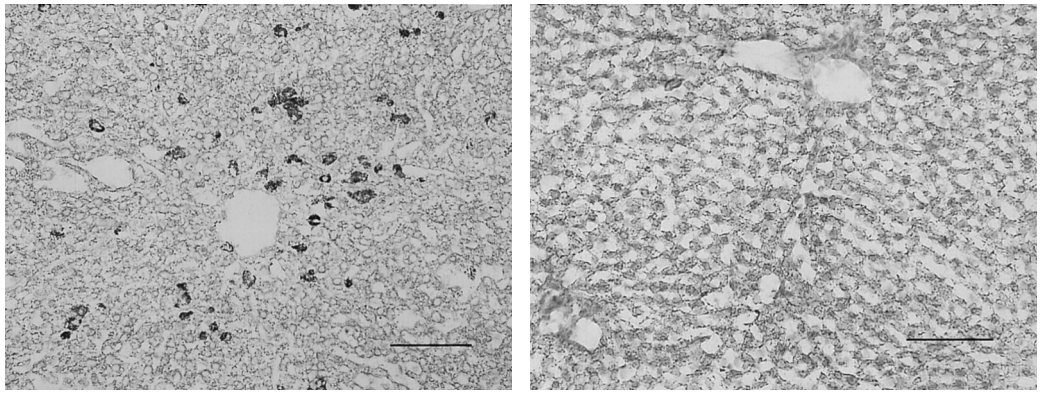

Fig. 5 Immunohistochemical staining of caspase 9. (**A**) Control group. (**B**) AA-2G group. Bars indicate 100 μm.

during reperfusion [11, 12]. In contrast, Gujral *et al.* reported that only a small minority of SEC and hepatocytes undergo apoptosis after warm I/R injury, as the result of using the Annexin V-based assay [14]. Although the main cell death of hepatocytes after I/R injury might be influenced by both the phase after reperfusion and the preservation time [4, 6, 14], we investigated the predominant cell death of hepatocytes after I/R injury (24 h cold ischemia in UW solution and 4 h reperfusion) in rat liver transplantation, using the Annexin V-based assay.

The controversy about the causes of the main cell death of hepatocytes after I/R injury (apoptotic or necrotic) might be influenced by the sensitivity of the methods used to detect apoptosis. Because the TUNEL assay detected the cleaved DNA fragments, which occur not only during apoptosis but also during necrosis, the TUNEL assay cannot be used to quantify apoptotic cell without supporting morphological evidence if the mode of cell death is in question [26, 27]. In contrast, the Annexin V assay can identify apoptosis, based on the detection of cell surface-exposed phosphotidyl serine, because the externalization of phosphotidyl serine occurs in the earlier stages of apoptosis [14, 15, 22]. The exposure of phosphotidyl serine does not depend on the involvement of the nucleus, but requires the activation of caspase 3 and a Ca^{2+} flux over the plasma membrane [28]. Therefore, the Annexin V assay can detect apoptotic cells in various phases ranging from the early stage, in which no morphological changes of the nucleus are detected on the electron microscopy level, until the late stage, with a pyknotic nucleus and condensed cytoplasm [29].

Ascorbic acid (AA), while it possesses strong antioxidative activity and reacts rapidly with many kinds of active oxygen and free radicals, is chemically unstable and easily oxidized by oxygen in aqueous solution or air [16, 17]. In contrast, AA-2G is chemically stable and is hydrolyzed to AA by tissue α-glucosidases [18–20]. Thus, AA-2G can be applied to the additive agent for the preservation solution as a free radical scavenger. We set up the dose of additional AA-2G to the UW solution according to the effective serum level of AA, to reverse the preexisting condition of scurvy animals [18]. In this study, the addition of AA-2G to the UW solution reduced the apoptosis of hepatocytes after 24 h cold ischemia/reperfusion injury, leading to the improvement of the survival time in rat liver transplantation.

In ischemia/reperfusion injury, hepatocytes are subjected to oxidative stress induced by both ROS generated intracellularly in response to cytokines, and ROS produced extracellularly by inflammatory cells [30]. Higher amounts of extracellular ROS can cause necrosis caused by the loss of plasma membrane integrity, whereas low concentrations of extracellular ROS can only induce apoptosis [31, 32]. ROS produced intracellularly are known to act as intermediates in the apoptotic signaling pathway [31, 33, 34]. Both intracellular and extracellular ROS elicites a rapid and massive cytochrome c release from mitochondria, resulting in the activation of caspase-9, followed by the activation of other caspases [35]. In our study, the expression of caspase-9 in the liver graft was suppressed by the addition of AA-2G to the UW solution. Therefore, it was suggested that

adding AA-2G to the UW solution could reduce the apoptosis of hepatocytes after cold ischemia/reperfusion injury, not only by suppressing ROS production, but also by inhibiting cytochrome c release from mitochondria and subsequent activation of caspase-9 due to ROS.

In conclusion, our study using the Annexin V-based assay provides evidence that the predominant cell death of hepatocytes is due to apoptosis after 24 h cold ischemia/reperfusion injury in rat liver transplantation. The addition of AA-2G to the UW solution suppressed the apoptosis of the hepatocytes after 24 h cold ischemia/reperfusion injury by inhibiting the activation of the caspase pathway due to ROS, and contributed to the improvement of survival. AA-2G, which has chemical stability and a unique bioreactive nature, may thus be of great use in clinical liver transplantations.

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