ABSTRACT

Background. Liver ischemia-reperfusion (I/R) injury is of great importance in primary graft dysfunction after transplantation, and could be more severe in transplantation using aged donor livers. In order to alleviate the I/R injury in aged donor livers, we transferred exogenous human telomerase reverse transcriptase (hTERT) gene into aged rat's livers before liver transplantation. After transplantation, the effect of the gene for aged rats on cell apoptosis caused by I/R injury was evaluated.

Methods. The experiment was divided into 2 parts: comparative experiment between aged rats and adult rats, and exogenous induction experiment of aged rats. In the first part, Wistar rats were divided into 2 groups; group I was composed of adult rats (5 months) and group II was composed of aged rats (16–18 months). After successful transplantation, chronic oxidative stress and lipid peroxidation-related indicators (contents of vitamin C and vitamin E; activities of superoxide dismutase, catalase, and methane decarboxylic aldehyde) and alanine aminotransferase activity were examined. In the second part, additional aged rats were divided into 3 groups: group A included the donors pretreated with exogenous hTERT gene; group B included the donors pretreated with adenovirus vector; and group C was composed of the donors pretreated with physiological saline. Various indicators were detected to analyze the effect of the gene on I/R injury of the aged rats.

Results. The lower vitamin C, vitamin E, SOD, and CAT contents in the aged group than those in the adult group ($P < .05$), and the higher MDA and ALT contents in the aged group than those in the adult group ($P < .05$) were observed. The apoptotic index and ALT levels in the hTERT gene-pretreated group were significantly lower than those in the adenovirus vector group and the physiological saline group ($P < .05$). Meanwhile, mild histological injury and increased telomerase activity were also observed in the hTERT gene-pretreated group.

Conclusion. Compared with the adult rats, I/R injury in the aged liver donor is more severe. The induction of exogenous hTERT gene offers protection against I/R injury in the aged liver.
intraoperative anesthetic management, better understanding of the pathophysiology in liver diseases, and better preoperative and postoperative care [1], however, the shortage of liver donors has hindered its implementation. To expand the donor pool, many liver donors that were traditionally regarded as marginal donors have also been gradually used in clinical treatment. The application of elderly donors is among them [2–4], but this can lead to several problems. Elderly liver graft is now an identified cause of nonfunction for primary graft and an independent cause of mortality among them [2, 4]. Relevant studies showed that ischemia-reperfusion (I/R) injury is one of the major contributors to the dissatisfactory results in elderly liver transplantation [7,8]. If the I/R injury in elderly donor liver transplantation could be released or eliminated, it would greatly promote its implementation. Hepatocyte apoptosis is a critical pathological feature in liver I/R injury [9–11]. Many investigators have applied gene recombination technology to construct Bcl-2 (B-cell lymphoma-2), SOD-1 (superoxide dismutase-1), HO-1 (heme oxygenase-1) and other apoptosis-inhibitory genes into proper vectors, and transferred them into cells to enhance their antiapoptotic capacity [12–14]. Our former studies have clarified that exogenous human telomerase transcriptase (hTERT) can effectively inhibit apoptosis in adult donor liver [15]. However, the effect of hTERT genes on I/R injury of aged donor livers has rarely been reported. In this study, the I/R injury degrees in both aged and adult rats were compared and discussed. At the same time, the defective adenovirus AdhTERT (including exogenous hTERT gene) and AdEasy (without exogenous hTERT gene) were constructed, and then transfected into the livers of aged donor rats to observe the effect of the hTERT gene on I/R injury in aged rat’s livers.

MATERIALS AND METHODS

Experimental Design

Replication defective adenovirus AdhTERT (expressing exogenous hTERT and green fluorescent protein) and AdEasy (without exogenous hTERT gene) were constructed. After amplification and purification, virus titer was detected using caesium chloride density gradient centrifugation and preserved in –80°C. Adult (5 months) and aged (16–18 months of age) Wistar rats were used as donors and recipients. Animal housing and procedures were carried out according to “Principles of Laboratory Animal Care” (National Institutes of Health publication, vol. 25, no. 28, revised 1996). Group I (adult rats, n = 10) and group II (aged rats, n = 10) were subjected to no hTERT gene induction. Additional aged rats were divided into 3 groups (n = 25) for the injection via vena of dorsalis penis at 48 hours before liver transplantation: group A, AdhTERT viral suspension (3 × 10^9 pfu/mL; 1 mL); and group C, physiological saline (1 mL).

Liver Transplantation

Orthotopic liver transplantation was performed using the 2-cuff technique [9] after injection. Inhalation anesthesia was used during the operation with ether. A median and transverse abdominal incision was performed. The donor liver was freed from its ligaments and resected from the donor. University of Wisconsin (UW) solution was used to perfuse the liver through the portal vein. The liver was preserved at 4°C for maximally 2 hours before being transplanted into the recipient’s abdomen. The suprahepatic vena cava anastomosis was established between donor and recipient in conjunction with the recipient’s cuff diaphragm. The portal vein and infrarehepatic vena cava anastomosis between donor and recipient was performed by pulling the recipient’s vein over a cuff that was secured with a circumferential silk suture. Biliary continuity was reconstructed by inserting a stent into the common bile duct.

Determination of Lipid Peroxidation-Related Indicators

Liver tissues of group I and group II were prepared into cell homogenates 48 hours after successful transplantation, then, the contents of vitamin E and vitamin C were determined using the high-performance liquid chromatography (HPLC) method; superoxide dismutase (SOD) and catalase (CAT) were measured using the xanthine oxidase chemiluminescence method, ultraviolet spectrospectroscopy, or chemical colorimetry; the content of methane decarboxyl aldehyde (MDA) (representing lipid peroxide content) was measured using the thiobarbituric acid method. The operation proceeded according to the manufacturer’s instructions.

The Determination of Alanine Aminotransferase

Venous blood was harvested from the inferior vena cava of all rats and the content of alanine aminotransferase (ALT) was measured.

Table 1. Contrasts Between Adult and Aged Rats After Liver Transplantation in Oxidative Stress and Lipid Peroxidation

<table>
<thead>
<tr>
<th>Indicators</th>
<th>Group</th>
<th>n</th>
<th>Measured Value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C (µg/mg)</td>
<td>Adult 10</td>
<td>2.81 ± 0.59</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aged 10</td>
<td>1.89 ± 0.22</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin E (µg/mg)</td>
<td>Adult 10</td>
<td>0.32 ± 0.08</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aged 10</td>
<td>0.20 ± 0.06</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SOD (U/mg)</td>
<td>Adult 10</td>
<td>628.30 ± 45.81</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aged 10</td>
<td>551.96 ± 85.82</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAT (U/mg)</td>
<td>Adult 10</td>
<td>24.94 ± 7.95</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aged 10</td>
<td>18.56 ± 3.13</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MDA (µg/mg)</td>
<td>Adult 10</td>
<td>0.98 ± 0.19</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aged 10</td>
<td>1.31 ± 0.31</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note: Protective factors (Vitamin C, Vitamin E, SOD, and CAT) against oxidative stress and lipid peroxidation were higher in adult rats than that of aged rats at 48 h after transplantation. At the same time, risk factor MDA was lower in adult rats.

Table 2. Serum ALT of Aged and Adult Rats After Liver Transplantation for 3, 6, 12, 24, and 48 Hours (U/L)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
<th>24 h</th>
<th>48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aged rats</td>
<td>10</td>
<td>1062.3 ± 146.5</td>
<td>1694.5 ± 194.3</td>
<td>899.2 ± 221.5</td>
<td>481.3 ± 112.9</td>
<td>341.5 ± 63.8</td>
</tr>
<tr>
<td>Adult rats</td>
<td>10</td>
<td>824.5 ± 106.5</td>
<td>1329.4 ± 254.9</td>
<td>698.2 ± 183.6</td>
<td>355.0 ± 67.9</td>
<td>273.2 ± 76.8</td>
</tr>
</tbody>
</table>

Note: Serum ALT of aged rats were significantly higher than that of adult rats (P < 0.05) at 3, 6, 12, 24, and 48 h after transplantation.
using automatic biochemical analyzer at 3, 6, 12, 24, and 48 hours after liver transplantation.

*The evaluation of telomerase activation.* Twenty milligrams of donor liver tissues were harvested at 48 hours after liver transplantation from 3 aged donor groups, respectively. The relative magnitude of telomerase activation was measured using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s instructions (Telomerase PCR ELISA Kit, Roche).

*Western blot analysis.* Protein extracts from donor liver at 48 hours after liver transplantation was subjected to SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis (9% gel) with the Laemmli discontinuous buffer system (Bio-Rad, America), and the resolved proteins were electrotransferred for 2 hours to nitrocellulose membrane using the HorizBlot semi-dry blotter. The membranes were blocked for 1 hour at 22°C with TBS-T [20 mmol/L Tris/HCl (pH 8.0), 500 mmol/L NaCl, and 0.1% (v/v) Tween-20] containing 5% (w/v) skimmed milk powder. The membranes were then incubated with a polyclonal or monoclonal anti-(hTERT) antibody (1 μg/mL in TBS-T) with 5% skimmed milk powder and 0.02% NaN₃ overnight at 4°C with shaking, followed by 5 washes (5 minutes each) in TBS-T. For peptide blocking, a polyclonal anti-(hTERT) antibody (Abcam Corporation, Cambridge, England) was incubated for 2 hours at 22°C with the peptide (1 μg/mL in TBS-T) against which the antibody was raised. The antibody-peptide mixture was then centrifuged at 10,000 g for 10 minutes at 4°C, and the resultant supernatant was incubated with the membranes as described above. Membranes were further incubated with secondary antibody for 2 hours at 22°C, followed by 5 washes in TBS-T buffer. Bound peroxidase was detected using Western blotting detection reagents according to the manufacturer’s instructions, followed by the exposure of the membrane to hyperfilm.

*Histological evaluation.* The tissues from liver samples fixed in 4% formalin were embedded in paraffin after liver transplantation for 48 hours. Hematoxylin and eosin staining was performed for morphological analysis under a light microscope.

*Terminal-deoxynucleotidyl Transferase Mediated Nick End Labeling (TUNEL) analysis.* Liver tissues (approximately 0.5 cm × 0.5 cm) fixed in formalin and embedded in paraffin after liver transplantation for 48 hours were sectioned into 5-μm slices. TUNEL analysis was performed according to the manufacturer’s instructions (Boehinger Mannheim Corporation, Indianapolis, Ind, United States). The slices with buff color in the cell nucleus were defined as positive. In total 5 × 100 cells were observed in each slice at the high power of 400 Pa, and positive cell number in every 100 cells was recorded.

*Statistical analysis.* For all animals survival was the endpoint. All measurements and calculations were analyzed using SPSS 20.0 (IBM, New York, United States). The data between the groups were analyzed using Student t test and the significant difference was considered at P < .05.

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**Fig 1.** Comparison of ALT contents between aged and adult rats after liver transplantation. The contents of ALT in Group II after liver transplantation for 3, 6, 12, 24, and 48 hours were significantly higher than those in Group I (P < .05).

**Fig 2.** Comparison of ALT in aged rats after liver transplantation. The contents of ALT in Group A after liver transplantation for 3, 6, 12, 24, and 48 hours were significantly higher than those in Group B and Group C (P < .05). On the contrary, the variation could be used to explain the difference of ALT between Group B and Group C (P > .05). The peak level of ALT content was observed at the time point of 6 hours after liver transplantation.
RESULTS
Comparison Between Aged and Adult Rats After Liver Transplantation

Vitamin C, Vitamin E, SOD, CAT, and telomerase activity revealed a significantly lower level in aged rats than that in adult rats \((P < .05; \text{Table 1})\). In contrast, the MDA content in aged rats was significantly higher than that in adult rats \((P < .05; \text{Table 1})\). ALT content among the aged rats after liver transplantation for 3, 6, 12, 24, and 48 hours was significantly lower than that in the adult rats at corresponding transplantation time point \((P < .05; \text{Table 2}; \text{Fig 1})\).

ALT and Telomerase Activity in Groups A, B, and C

Serum ALT levels in Group A after liver transplantation for 3, 6, 12, 24, and 48 hours were 587.4 ± 83.5, 997.2 ± 123.4, 425.4 ± 161.3, 254.8 ± 92.8, and 124.4 ± 25.3 U/L, respectively, which were significantly lower than those in Group B and Group C \((P < .05)\). In contrast, the variation could be used to explain the difference in ALT between Group B and Group C \((P > .05; \text{Fig 2})\). At the same time, the relative magnitude of telomerase activation after perfusion at 48 hours in Group A was 179.07 ± 6.54\% of Group B and 181.04 ± 6.43\% of Group C, which showed a significant difference \((P < .01)\).

Western Blotting

A band with an apparent size of 3.4 kd, identified as the exogenous hTERT protein, was observed in rAd-hTERT-intervened liver tissue (lane 2), and none was observed in Group B (lane 3) and Group C (lane 4; \text{Fig 3}).

DISCUSSION

Liver I/R injury is commonly observed in clinical pathology and in complex liver operations, and it is of great importance because of primary graft nonfunction and inactivation [16]. During the transplantation, the donor liver is in ischemia condition due to long surgery time and liver transportation. After reperfusion, the dysfunction of the primary graft leads to vascular and biliary complications [17] such as intrahepatic cholestasis, a pathological state of reduced bile formation or flow, and posthepatic jaundice [18].

Investigations showed that I/R injury is related to cell apoptosis [19]. Reactive oxygen species (ROS) is one of the major contributors for cell apoptosis [20,21]. As a result, antioxidants are effective for the inhibition of this process. Except for antioxidant drugs, gene engineering has been used to prevent apoptosis by intervening oxidation pathways. In the liver I/R model, transfection of Bcl-2 genes into liver cells using genetic engineering technology has revealed the inhibition of cell injury and apoptosis [22].

Telomerase is a hotspot for researchers, and it is believed to play an important role in maintaining telomerase stability, gene integrity, cellular immortality, and proliferation [23]. Telomerase is a special reverse transcriptase, and it can repeatedly extend the 3' terminal of chromosome. Telomerase is composed of human telomerase RNA component (hTERC), dyskeratosis protein (Dyskerin), and hTERT. Among them, hTERT has the decisive effect on telomerase [24]. According to the report by Cook et al, the reason for telomere DNA shortening is...
the inactivation of telomerase in normal cells [25]. A number of factors, including hormones, cytokines, vitamins, and herbal extracts can accelerate the activation of telomerase and extension of telomere length [26], thereby exhibiting the effect of slowing down the aging process [27]. With thousands of telomere DNA losses, cells will result in fission termination or senescence. Previous studies have demonstrated hTERT genes into embryonic stem cells can maintain its proliferative potential, cell cycle regulation, and differentiation capability in vitro [28]. These studies indicate that the transfection of exogenous Bcl-2 and HO-1 into rat liver will cause the inhibition of I/R injury. Our former studies have confirmed that the exogenous hTERT gene can improve adult rat I/R injury, but the effect of the hTERT gene on aged rats has less been reported. Based on previous studies, the transfection of the exogenous hTERT gene 48 hours before liver transplantation into aged Wistar rats was conducted in this study. In the AdhTERT group, the activation of telomerase and the expression of the hTERT protein were significantly increased. At the same time, lower apoptotic index and necrotic rate were observed. The transfection of the exogenous hTERT gene can apparently improve aged rats’ I/R injury, and its mechanism may be related to the activation of telomerase.

REFERENCES


