dodecyl sulfate (SDS) sample buffer consisting of 25 mM Tris–HCl (pH 6.8), 0.8 % SDS, 4 % glycerol, 0.008 % bromophenol blue and 2 % β-mercaptoethanol. Lysates were boiled for 6 min and separated by SDS polyacrylamide gel and electrotransferred onto nitrocellulose membrane (GE Healthcare, Buckinghamshire, UK). The blot was blocked with 5 % bovine serum albumin in Tris-buffered saline containing 0.1 % Tween-20 for 1 h and probed overnight at 4 °C using the following antibodies : rabbit monoclonal anti-total ERK1/2, anti-phosphorylated ERK1/2 and anti-β-actin antibodies (1:1,000, #4695/#4370/#4970, Cell Signaling Technology, Danvers, MA, USA). Appropriate horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, CA, USA) were used for secondary reaction. Finally, protein bands were visualized by the enhanced chemiluminescence detection reagents (GE Healthcare, Buckinghamshire, UK).

Assessment of activated KC numbers in regenerative livers

The status of KCs in remnant regenerative livers was also analyzed immunohistochemically by using CD69 antigen, which is among the earliest antigen to appear after activation of macrophage cells [25]. The immunohistochemical procedure was the same as described above. Anti-rat CD69 antibody (1:100, sc-15365, Santa Cruz, CA, USA) was used for the primary antibody. The cells with brown cytoplasms located in the sinusoids were morphologically considered to be activated KCs. The assessment of CD69 staining was undertaken by a pathologist in our facility. The number of CD69 positive KCs was counted at a magnification of 400× and 5 fields were randomly chosen. Then the average number of CD69 positive KCs in each liver specimen was determined [26].

Biochemical test of liver function

To evaluate the liver injury, the levels of serum aspartate aminotransferase (AST), serum alanine aminotransferase (ALT) lactase dehydrogenase (LDH) and total bilirubin (T-Bil) were measured using the Japan Society of Clinical Chemistry standardization matching method. All measurements were performed by Shikoku Chuken, INC. Kagawa, Japan.

Determination of serum MDA levels

Serum MDA levels were measured with the MDA assay kit from Northwest (Northwest Life Sciences Specialities, Vancouver, Canada) following the company protocol. This assay is based on the reaction of MDA with thiobarbituric acid (TBA); forming a MDA-TBA2 adduct that absorbs strongly at 532 nm. This reaction is the most popular method for estimating MDA in biological samples.

RNA isolation and quantitative real time RT-PCR for iNOS, anti-oxidative enzymes, inflammatory markers

Harvested livers were homogenized with a multi-beads shocker (Yasui-Kikai, Osaka, Japan). The RNA was extracted using RNeasy mini kit (Qiagen, Valencia, CA). The RNA was reverse transcribed with high capacity cDNA reverse transcription kit (Applied Biosystems, Tokyo, Japan). Quantitative real-time RT-PCR was performed using the Applied Biosystems 7500 real-time PCR system, TaqMan gene expression assays-on-demand, and Taq-Man universal master mix (Applied Biosystems). The following assays (assay identification number) were used: iNOS (Rn00561646_m1), SOD (Rn00566938_m1), CAT (Rn00560930_m1), GSH-Px (Rn00574703_m1), COX-2 (Rn01483828_m1), NFκB (Rn01502270_m1) and TNF-α (Rn01492022_m1). GAPDH gene (4352338E) was used as an endogenous expression control (Applied Biosystems). The thermal cycler conditions were as follows: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Amplification data were analyzed with an Applied Biosystems Prism 7500 Sequence Detection System ver. 1.3.1 (Applied Biosystems).

Statistical analysis

All results were presented as mean ± SD. Comparisons between 2 groups were performed using the Student's t test and Mann–Whitney U test using statistical software (JMP 8.0.1., SAS Campus Drive, Cary, 27513 NC, USA). A p value of less than 0.05 was considered statistically significant.

Results

Liver regeneration

The Lw/Bw at day 1 and 3 in the GTEHx group were significantly increased compared to the Hx group (0.93 ± 0.20 vs. 0.62 ± 0.09 % at day 1, $p < 0.01$, 2.18 ± 0.47 vs. 1.59 ± 0.26 % at day 3, $p < 0.05$). There were no significant differences at day 7 and 14 (3.00 ± 0.16 vs. 3.23 ± 0.51 % at day 7, $p = 0.37$, 3.35 ± 0.14 vs. 3.42 ± 0.31 % at day 14, $p = 0.68$) (Fig. 2a). PCNA LI at day 1 in the GTEHx group was also significantly increased compared to the Hx group (89.9 ± 9.4 vs. 67.9 ± 26.0 % at day 1, $p < 0.05$) (Fig. 2b).

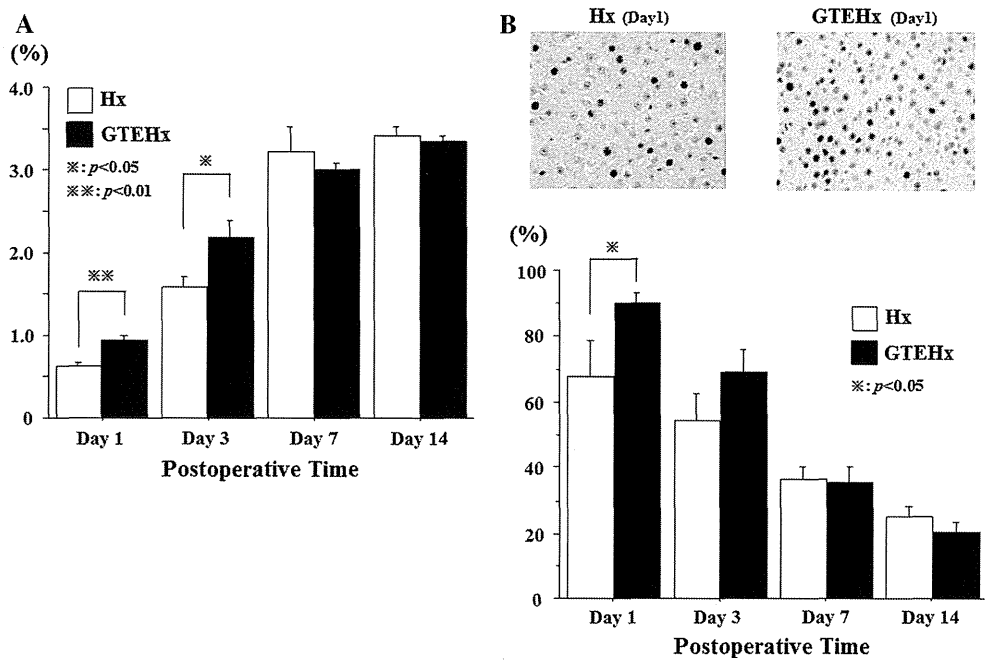

Fig. 2 Liver regeneration after operations. Liver regeneration was defined as Lw/Bw (**a**) and PCNA LI (**b**). The pictures showed immunohistochemistry of PCNA at day 1 in both Hx and GTEHx groups (×400). The Lw/Bw at day 1 and 3 were significantly increased (day 1: $p < 0.01$, day 3: $p < 0.05$) in the GTEHx group. PCNA LI at day 1 was also significantly increased ($p < 0.05$) in the GTEHx group

Hepatic total and phosphorylated ERK 1/2 levels

Phosphorylated ERK/total ERK ratio at day 1 was significantly higher in the GTEHx group (0.65 ± 0.05 vs. 0.42 ± 0.20, $p < 0.05$) (Fig. 3).

The status of KCs in regenerative liver

The average numbers of CD69 positive KCs at day 1 and 3 were significantly larger in the GTEHx group (37.7 ± 9.5 vs. 20.8 ± 4.0 % at day 1, $p < 0.05$, 64.0 ± 21.8 vs. 28.0 ± 5.7 % at day 3, $p < 0.05$) (Fig. 4a, b).

Serum biochemical test of liver function

Serum AST levels at day 1 and 3 were significantly improved in the GTEHx group (2,403 ± 734 vs. 4,737 ± 1,707 U/l at day 1, $p < 0.05$, 298 ± 68 vs. 1,573 ± 1,762 U/l at day 3, $p < 0.05$) (Fig. 5a). ALT and LDH levels at day 1 were also significantly improved in the GTEHx group (ALT 1,125 ± 482 vs. 2,094 ± 1,000 U/l, $p < 0.05$, LDH 1,829 ± 1,090 vs. 4,308 ± 2,313 U/l, $p < 0.05$) (Fig. 5b, c). T-Bil levels at day 3 tended to be lower in the GTEHx group (0.6 ± 0.3 vs. 3.6 ± 3.5 U/l, $p = 0.09$) (Fig. 5d).

Serum oxidative marker levels

Serum MDA levels at day 1 were significantly lower in the GTEHx group (12.3 ± 2.8 vs. 16.5 ± 3.6 μM, $p < 0.05$) (Fig. 6).

Hepatic mRNA levels of iNOS

iNOS levels at day 1 were significantly lower in the GTEHx group (1.03 ± 0.31 vs. 2.99 ± 1.97, $p < 0.01$) (Fig. 7).

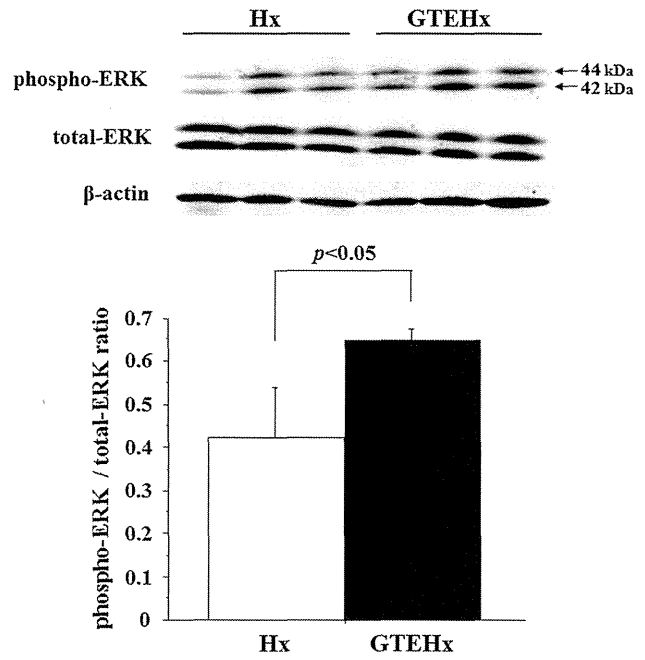

Fig. 3 Hepatic total and phosphorylated ERK 1/2 levels. Phosphorylated ERK/total ERK ratio at day 1 was significantly higher ($p < 0.05$) in the GTEHx group

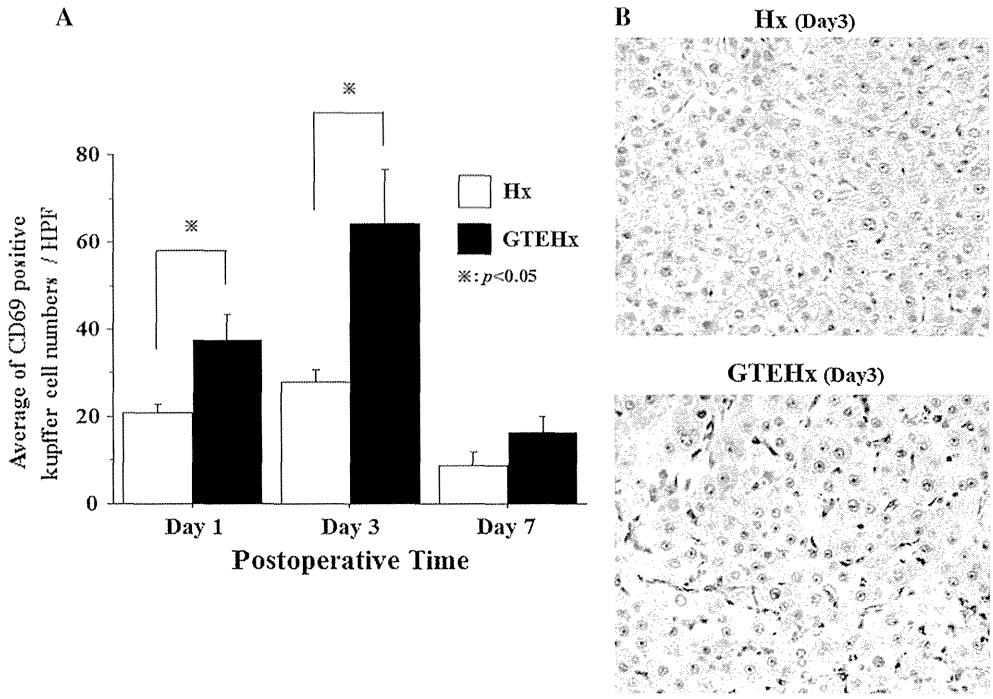

Fig. 4 The status of KCs in regenerative liver. The average numbers of CD69 positive KCs at day 1 and 3 were significantly larger ($p < 0.05$) in the GTEHx group (**a**). The pictures showed immunohistochemistry of CD69 at day 3 in both Hx and GTEHx groups (**b** ×400)

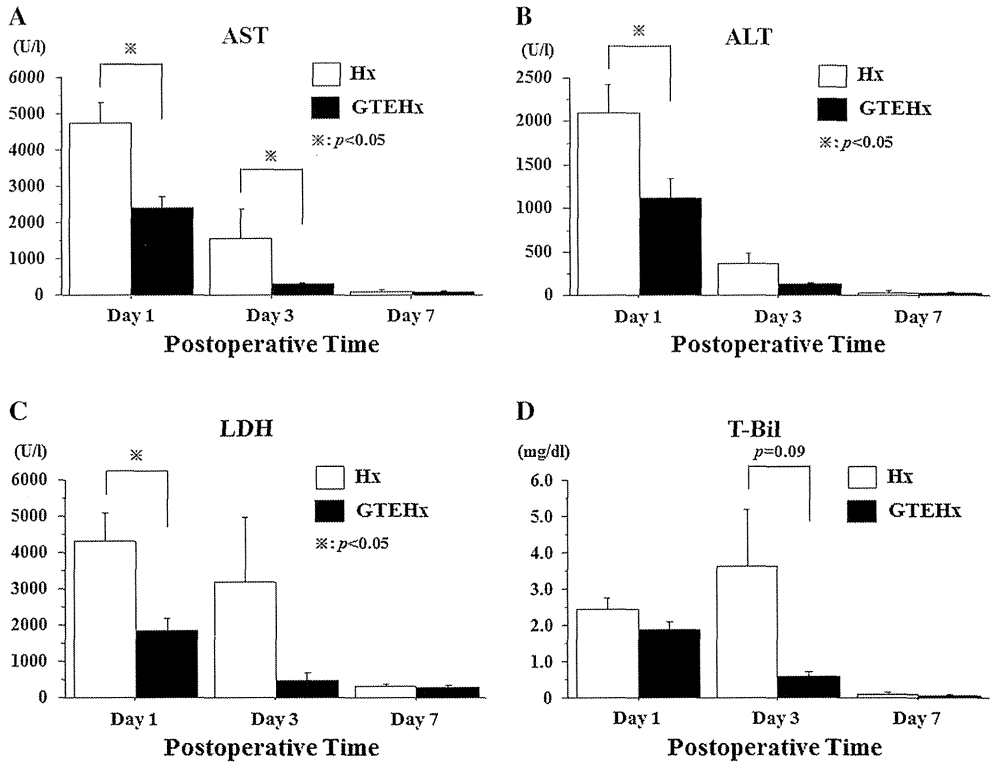

Fig. 5 Serum biochemical test of liver function. AST (**a**), ALT (**b**), LDH (**c**) and T-Bil (**d**) levels. Serum transaminases levels at day 1 were significantly improved ($p < 0.05$) in the GTEHx group

Hepatic mRNA levels of anti-oxidative enzymes and inflammatory markers

Regarding anti-oxidative enzymes, SOD levels at day 1 were significantly higher in the GTEHx group (1.37 ± 0.11 vs. 1.13 ± 0.11, $p < 0.01$) (Fig. 8a). CAT levels at day 1 and 3 were also significantly higher in the GTEHx group and the ones at day 7 tended to be higher as well (1.69 ± 0.95 vs. 0.78 ± 0.51 at day 1, $p < 0.05$, 3.67 ± 1.45 vs. 1.68 ± 0.57 at day 3, $p < 0.05$, 10.86 ± 1.22 vs. 9.05 ± 0.85 at day 7, $p = 0.06$) (Fig. 8b). GSH-Px levels at day 1 and 3 were also significantly higher in the GTEHx group (1.31 ± 0.43 vs. 0.85 ± 0.23 at day 1, $p < 0.05$, 3.66 ± 1.84 vs. 1.51 ± 0.52 at day 3, $p < 0.05$) (Fig. 8c).

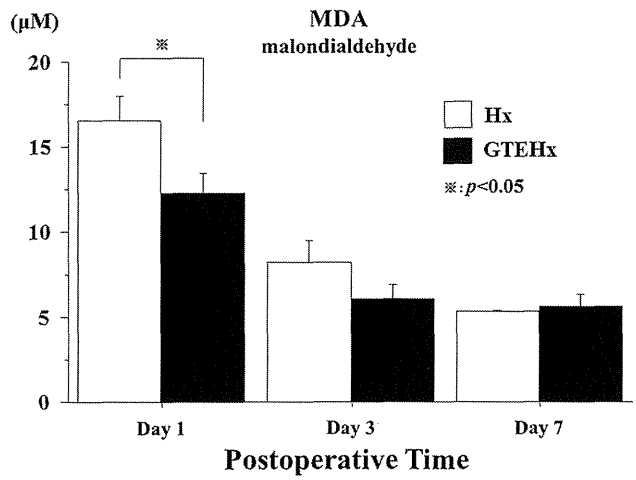

Fig. 6 Serum oxidative marker levels. MDA levels at day 1 significantly decreased ($p < 0.05$) in the GTEHx group

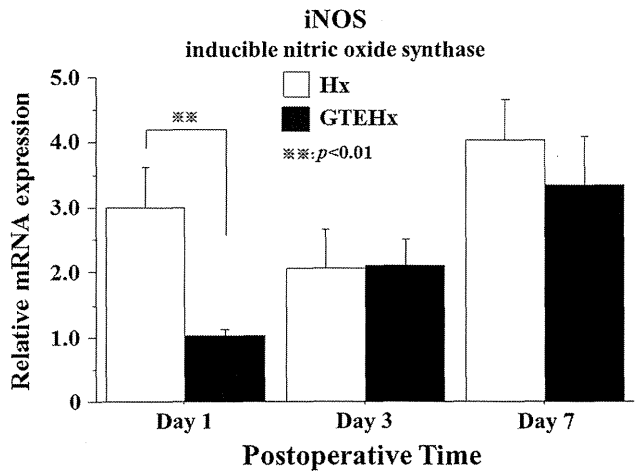

Fig. 7 Hepatic mRNA levels of iNOS. iNOS levels at day 1 significantly decreased ($p < 0.01$) in the GTEHx group

Inflammatory markers including COX-2, NFκB and TNF-α levels at day 1 were all significantly lower in the GTEHx group (COX-2 1.09 ± 0.29 vs. 3.41 ± 1.24, $p < 0.01$, NFκB 1.16 ± 0.49 vs. 1.92 ± 0.66, $p < 0.05$, TNF-α 0.68 ± 0.37 vs. 2.15 ± 1.69, $p < 0.05$) (Fig. 9a–c).

Discussion

In the current study, it has been reported that green tea catechin ameliorates liver injury or liver fibrosis in an experimental rat model such as CCl_4-induced liver injury model [20, 21] and bile duct ligation model [27] by various beneficial effects. To the best of our knowledge, this is the first report regarding effects of green tea catechin on massive hepatectomy model in rats. Herein, we demonstrated that preoperative administration of GTE improved liver regeneration and liver damage after massive hepatectomy in rats through the mechanism of up-regulating hepatic anti-oxidative enzymes and attenuating hepatic iNOS and inflammatory molecules.

Excessive production of pro-inflammatory cytokines and ROS/RNS in the liver has been reported for one of the mechanisms for hepatic failure after major hepatectomy [4, 5]. We found that GTE pretreatment significantly attenuated the up-regulation of representative inflammatory molecules at day 1, which are considered to be responsible for liver failure [28]. In addition, GTE also regulated hepatic mRNA levels of iNOS and serum MDA levels at day 1. MDA is an oxidative product which is the most abundant individual aldehyde resulting from lipid peroxidation and has been implicated in the pathogenesis of numerous diseases, including atherosclerosis, diabetes, and aging [29, 30]. Moreover, GTE pretreatment increased hepatic mRNA levels of antioxidant enzymes such as SOD, CAT and GSH-Px during a few days after operations. It was supposed that, in addition to inhibition of the inflammatory response, this phenomenon was also an important role of GTE pretreatment in preventing liver injury following massive hepatectomy.

Green tea extract pretreatment also had a beneficial effect for liver regeneration. It was actually proved that hepatocytes proliferations at day 1 in the GTEHx group were significantly stimulated by PCNA LI and hepatic phosphorylation of ERK 1/2. We also demonstrated KCs in remnant regenerative livers at day 1 and 3 were also significantly activated in the GTEHx group. The impact of activated KCs after massive hepatectomy to liver regeneration remains controversial. Wang et al. [31] reported that the decrease in function of the reticuloendothelial system including KCs suppressed liver regeneration after 90 % hepatectomy due to systemic circulation of endotoxin. On the other hand, Mochida et al. [32] reported that activated KCs might contribute to massive hepatic necrosis as a result of sinusoidal fibrin deposition provoked by endotoxin in partially hepatectomized rats. However the detailed mechanisms utilized by GTE to stimulate liver regeneration remained to be elucidated, we speculated that prevention of liver injury by inhibiting the oxidative and inflammatory response leaded to up-regulate hepatic phosphorylation of ERK 1/2 or activate KCs and finally to stimulate liver regeneration after massive hepatectomy.

It should be noted that a previous study reported that inflammatory cytokines such as TNF-α, NFκB and interleukin-6 (IL-6), play important roles in the early stage in liver regeneration after hepatectomy [33]. However, Kawai et al. [34] reported that over-production of such cytokines was cytotoxic rather than cytoprotective under the condition of severe hepatic stress. In our study, GTE pretreatment down-regulated hepatic mRNA levels of inflammatory

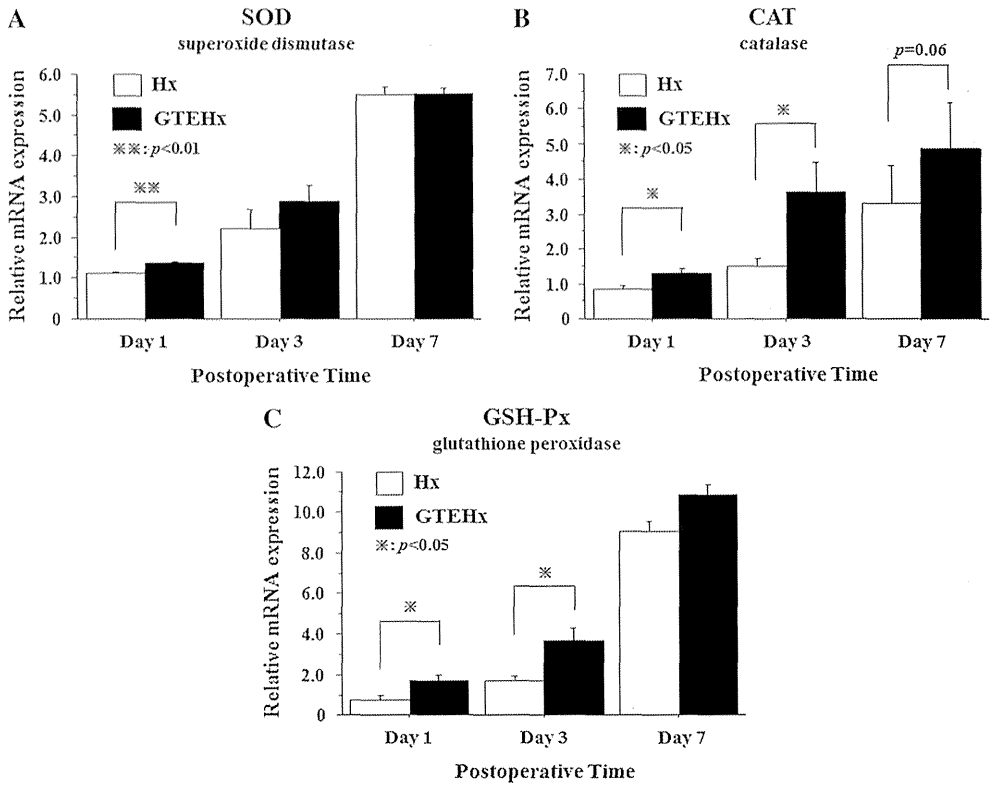

Fig. 8 Hepatic mRNA levels of anti-oxidative enzymes. SOD (**a**), CAT (**b**) and GSH-Px (**c**). SOD levels at day 1 and CAT, GSH-Px levels at day 1 and 3 significantly increased (SOD at day 1: $p < 0.01$, CAT and GSH-Px at day 1 and 3: $p < 0.05$) in the GTEHx group

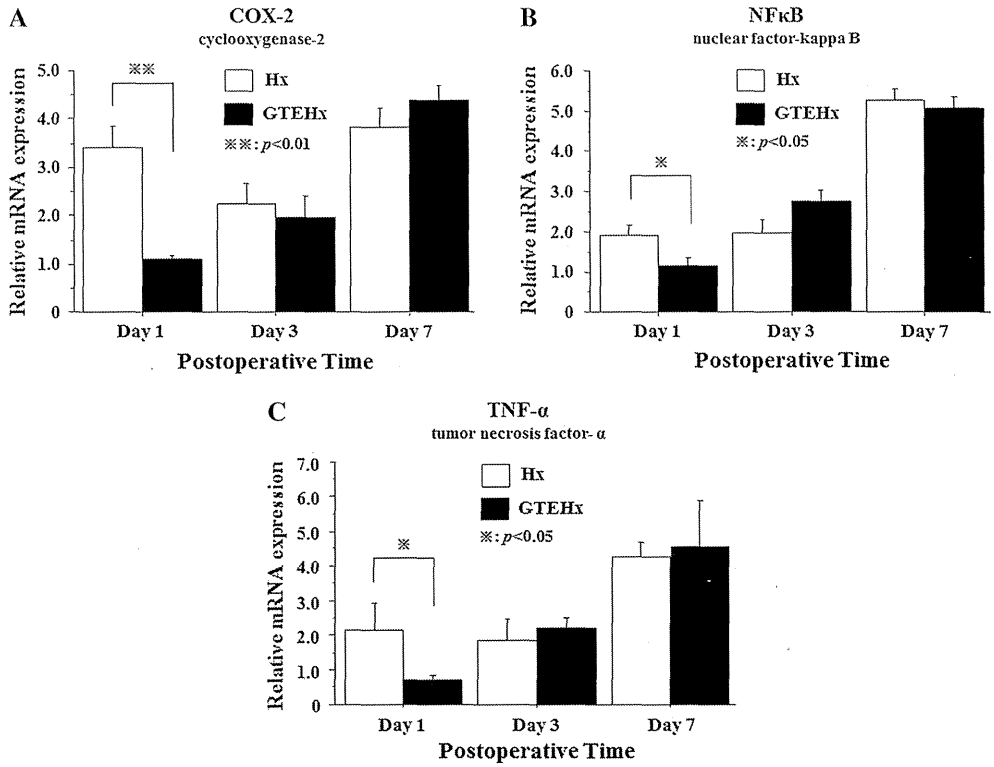

Fig. 9 Hepatic mRNA levels of inflammatory markers. COX-2 (**a**), NFκB (**b**) and TNF-α (**c**). COX-2, NFκB and TNF-α levels at day 1 all significantly decreased (COX-2: $p < 0.01$, NFκB and TNF-α: $p < 0.05$) in the GTEHx group

markers at the early stage of liver regeneration, especially at day 1, so this phenomenon might be beneficial from the point of view of liver regeneration.

Green tea is a beverage widely consumed around the world especially in Japan. The economic and social interest of tea is clear and its consumption is part of many people's

daily routine, as an everyday drink. In a fact, the daily GTE intake of rats in our study was equivalent to approximately 100–200 times as Japanese in average per day. But there were no adverse effects due to a large amount of GTE intake in all rats and there has been few reports regarding severe adverse effects of green tea catechin so far. Therefore, based on our findings, we propose to perform a large-scale, randomized, controlled study to evaluate the beneficial effects of GTE pretreatment for cases of major hepatectomy.

In conclusions, green tea extract pretreatment had beneficial effects in a massive hepatectomy model in rats owing to anti-oxidative and anti-inflammatory effects. We believe that green tea catechin might have the potential to attenuate liver dysfunction in early stage after massive hepatectomy.

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Conflict of interest The author and all co-authors declare that they have no conflict of interest.

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Trophic effect of adipose tissue–derived stem cells on porcine islet cells

*Shinichiro Yamada, MD, Mitsuo Shimada, MD, PhD,**
Tohru Utsunomiya, MD, PhD, Tetsuya Ikemoto, MD, PhD,
Yu Saito, MD, PhD, Yuji Morine, MD, PhD, Satoru Imura, MD, PhD,
Hiroki Mori, MD, PhD, Yusuke Arakawa, MD, PhD,
Mami Kanamoto, MD, PhD, and Shuichi Iwahashi, MD, PhD

Department of Digestive and Transplant Surgery, The University of Tokushima, Tokushima City, Tokushima, Japan

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ABSTRACT

Background: Adipose tissue–derived stem cells (ADSCs), which are widely known as multipotent progenitor cells, release several cytokines that support cell survival and repair. The aim of this study was to investigate whether ADSC-secreted molecules could induce a trophic effect in pancreatic islet culture conditions *in vitro*.

Materials and methods: We cocultured porcine islet cells with ADSCs using a transwell system for 48 h and evaluated the viability of islet cells. We also determined the concentration levels of cytokines and insulin in the supernatant of the culture medium. We used anti-vascular endothelial growth factor (VEGF) and anti-interleukin (IL)-6 receptor antibodies to investigate the effect of VEGF and IL-6 on islet cells.

Results: ADSCs improved the viability of islet cells in the absence of cell-cell contact ($P < 0.05$). VEGF and IL-6 levels in the culture medium increased when islet cells were cocultured with ADSCs ($P < 0.05$). Furthermore, inhibition of VEGF decreased the viability of islet cells ($P < 0.05$); however, inhibition of IL-6 did not affect islet cell viability.

Conclusions: These results suggested that trophic factors, particularly VEGF, secreted by human ADSCs enhanced the survival and function of porcine islet cells.

1. Introduction

Type 1 diabetes is a chronic metabolic disorder that results from the progressive destruction of pancreatic β cells, resulting in insulin insufficiency and hyperglycemia, which is the main cause of chronic vascular complications. Intensive insulin therapies reduce the onset and progression of diabetic complications but are associated with an increased risk of life-threatening hypoglycemic episodes. Islet cell transplantation (ICTx) is a potential treatment for type I diabetes mellitus. Recently, developed protocols have enhanced the short-term success rates of ICTx [1,2], but concerns regarding the deterioration of the metabolic capacity of transplanted islets in the long term remain. Furthermore, this deterioration cannot be compensated by the transplantation of a massive amount of islets [3]. The principal causes include apoptosis and a decreased function of islet cells due to hypoxic stress and poor nutrient factors during the isolation and culture stages [4–8].

Conflict of interest: S. Y. and the coauthors have no financial interest linked to this study.

* *Corresponding author.* Department of Surgery, Institute of Health Biosciences, The University of Tokushima Graduate School, 3-18-15 Kuramoto-cho, Tokushima, 770-8503, Japan. Tel.: +81 88 633 9276; fax +81 88 631 9698.
E-mail address: mshimada@clin.med.tokushima-u.ac.jp (M. Shimada).

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Instant blood-mediated inflammatory reaction, which is characterized by platelet consumption, and activation of the coagulation and complement systems are also involved in islet cell loss [9]. Thus, many strategies used to enhance islet culture conditions to prevent islet cell loss and the functional promotion of isolated islet cells have been reported [9–13].

Recently, stem cells have attracted considerable attention for their ability to support cell survival. Mesenchymal stem cells (MSCs) have been referred to as pluripotent stromal progenitor cells, which can be obtained from different sources of human tissue [14–19]. It has also been reported that MSCs exhibit both multipotentiality and semi-infinite proliferation properties [14–20]. Recently, "adipose tissue-derived stem cells" (ADSCs) have been isolated [21,22] and have the potential to differentiate into cells and organs of mesodermal and nonmesodermal origin. ADSCs have shown beneficial effects in healing tissue damage and have been investigated in phase I clinical trials focused on the cure for recurrent fistula in Crohn disease [23]. Furthermore, abundant ADSCs may be obtained from lipoaspirate with a complication rate of approximately 0.1% [24], and 1% of adipose cells are estimated to be ADSCs, compared with 0.001%–0.002% of ADSCs found in bone marrow [25]. Thus, ADSCs are considered to have a wide range of potential clinical applications in many fields of surgery [26].

Currently, the beneficial effects related to MSC-secreted bioactive molecules have been reported [27]. MSCs release insulin-like growth factor 1 and fibroblast growth factor 2 via the activation of nuclear factor-kappa B, extracellular signal-regulated kinase, and c-Jun-N-terminal kinase [28]. Furthermore, MSCs increase the viability and proliferative capacity of fetal intestinal epithelial cells, following hypoxic injury via paracrine secretion of hepatocyte growth factor, interleukin-6 (IL-6), and vascular endothelial growth factor (VEGF), and downregulation of apoptotic signaling molecules, including caspase-3 and 8 [29]. Furthermore, it has also been reported that trophic factors, particularly VEGF, secreted by human MSCs, enhance islet survival and are functional after transplantation [30]. As previously described, MSCs have a beneficial effect on cell survival via the paracrine release of IL-6 and VEGF.

The aims of this study were to investigate the beneficial effects of ADSCs on islet cells and determine whether VEGF and IL-6 are important factors for the cytoprotective effects on islet cells *in vitro*.

2. Materials and methods

2.1. Isolation and culturing of ADSCs

We purchased STEMPRO human ADSCs from Life Technologies (Tokyo, Japan) [31]. Briefly, ADSCs were isolated from human adipose tissue collected during liposuction procedures and were cryopreserved from primary cultures. Before cryopreservation, the ADSCs were expanded for one passage in MesenPRO RS Medium (Life Technologies). ADSCs contain cells that can differentiate into multiple mature cell phenotypes *in vitro*, including adipocytes, osteoblasts, and chondrocytes. These cells express a flow cytometry cell surface protein that is positive for CD29, CD44, CD73, CD90, CD105, and CD166 (>95%) and negative for CD14, CD31, CD45, and Lin1 (<2%). ADSCs were seeded into tissue culture flasks and cultured with MesenPRO RS basal medium (Life Technologies) containing 2% fetal bovine serum, MesenPRO RS growth supplement (Life Technologies), 2 mM of L-glutamine at 37°C, 5% CO_2, and 90% humidity. When the cells become attached to the growth surface, the medium was replaced with an equal volume of fresh, prewarmed complete MesenPRO RS medium.

2.2. Porcine islet isolation

Pancreata from approximately 2-y-old female pigs were obtained by Kobe Medical Device Development Center (Kobe, Japan), with a warm ischemic time of 0 min using a solution obtained from the University of Wisconsin. We began the procurement of the pancreas approximately 30 min after the cessation of the heartbeat. After removing the duodenum from the pancreas, we immediately inserted a cannula into the main pancreatic duct and infused approximately 200–250 mL of extracellular-type trehalose-containing Kyoto (ET-KYOTO) solution (Otsuka Pharmaceutical Factory, Inc, Naruto, Japan) for ductal preservation [32]. Islet isolation was performed according to the modified Ricordi method, as previously reported [33]. After isolation of the islets, the cells were preserved in culture media. Briefly, this culture media consisted of 500 mL CMRL (Connaught Medical Research Laboratories) 1066 culture media (Sigma Chemical Co, St Louis, MO), 10% fetal bovine serum, 1.5 mL 1 M sodium hydroxide, and 1.1 g sodium bicarbonate. Isolated islets were preserved using a stepwise cooling system to −80°C for 72 h to generate the injured islet model.

2.3. Coculture of porcine islets with ADSCs

We used a transwell system (0.4 μm pore size membrane; Corning, Acton, MA) to inhibit cell-cell contact. Human ADSCs (1.0×10^4) were seeded in the lower chamber of the well in growth medium (Dulbecco minimum essential medium-low glucose with the addition of 10% fetal calf serum with 2 mmol/L L-glutamine and 1% penicillin-streptomycin), and porcine islets (1.0×10^4) were seeded in the upper chamber in culture medium (Roswell Park Memorial Institute 1640 with the addition of 10% fetal calf serum with 2 mmol/L L-glutamine and 1% penicillin-streptomycin). We cocultured islet cells with ADSCs for 48 h ($n = 4$) and then evaluated the viability of islet cells. We also measured the protein levels of cytokines and growth factors, such as IL-6, VEGF, tumor necrosis factor-α (TNF-α), and insulin in the medium using enzyme-linked immunosorbent assay, which was performed at Shikoku Chuken, Inc (Kagawa, Japan).

2.4. Cell viability

Forty-eight hours after coculture, islet cell viability was assessed using trypan blue staining. The cell viability in each group was calculated by measuring the average in five random low power fields.

2.5. Administration of anti-VEGF antibody and anti-IL6R antibody

To confirm the effect of VEGF and IL-6 on islet cells, humanized anti-VEGF antibody (bevacizumab) and humanized

monoclonal antibody against IL-6 receptor (tocilizumab) were used. These antibodies were purchased from Chugai Pharmaceutical (Tokyo, Japan). We administered bevacizumab (0.25 mg/mL) and tocilizumab (0.25 mg/mL) into the culture medium and compared the viability of islet cells in the presence or absence of these drugs.

2.6. *Cell culture groups*

We divided the cell cultures into five groups (Fig. 1); cocultures of ADSCs with islet cells (coculture group, $n = 4$), single ADSC cultures (ADSC group, $n = 4$), single islet cell cultures (islet group, $n = 4$), cocultures of ADSCs with islet cells treated with bevacizumab (Bev group, $n = 4$), and cocultures of ADSCs with islets treated with tocilizumab (Toc group, $n = 4$).

2.7. *Statistical analysis*

All results were presented as the mean ± standard deviation. Multiple group comparisons were performed using one-way analyses of variance followed by the Scheffe procedure to compare the means. Comparisons between the two groups were performed using the Mann-Whitney *U*-test using statistical software (JMP 8.0.1.; SAS Institute Inc, Cary, NC). A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. *Quality assessment of islet cells cocultured in the presence and absence of ADSCs*

First, we evaluated the viability of islet cells and insulin concentration levels in the supernatant of culture medium to determine whether ADSCs exert a trophic effect on islet cells. The cell viability of the coculture group was significantly enhanced compared with the islet group (84.0% ± 5.3% *versus* 65.4% ± 5.1%, $P < 0.05$, Fig. 2A). Furthermore, the insulin concentration levels in the coculture group were significantly higher compared with the islet group (34.5 ± 10.3 *versus* 2.1 ± 0.9 mIU/mL, $P < 0.05$, Fig. 2B). Considering good viability, insulin secretion in the coculture group was not caused by islet destruction. Dulbecco minimum essential medium contains a glucose concentration of 100 mg/dL, and RPMI 1640 of 200 mg/dL. Thus, medium in the coculture group contain a lower glucose concentration compared with the islet group because this transwell system does not inhibit liquid migration. Despite the lower glucose concentration, the coculture group showed a higher insulin secretion. These results suggested that ADSCs have a beneficial effect on the viability of porcine islets, and this effect was determined by a liquid factor and not by cell-cell contact.

3.2. *Measurement of cytokines in the supernatant of the culture medium*

Next, we measured the levels of VEGF, IL-6, and TNF-α in the supernatants of the culture medium to identify cytokines that exert a trophic effect. In the coculture group, the concentration levels of VEGF and IL-6 in the culture medium were significantly higher compared with the ADSC group (888.7 ± 42.4 *versus* 55.5 ± 5.0 pg/μL, 3500 ± 68.7 *versus* 175 ± 27.5 pg/μL, $P < 0.05$, respectively, Fig. 3A and B). VEGF and IL-6 concentration levels in the islet group were significantly lower compared with the other two groups ($P < 0.05$, Fig. 3A and B). However, there were no significant differences in the TNF-α levels between the three groups (Fig. 3C).

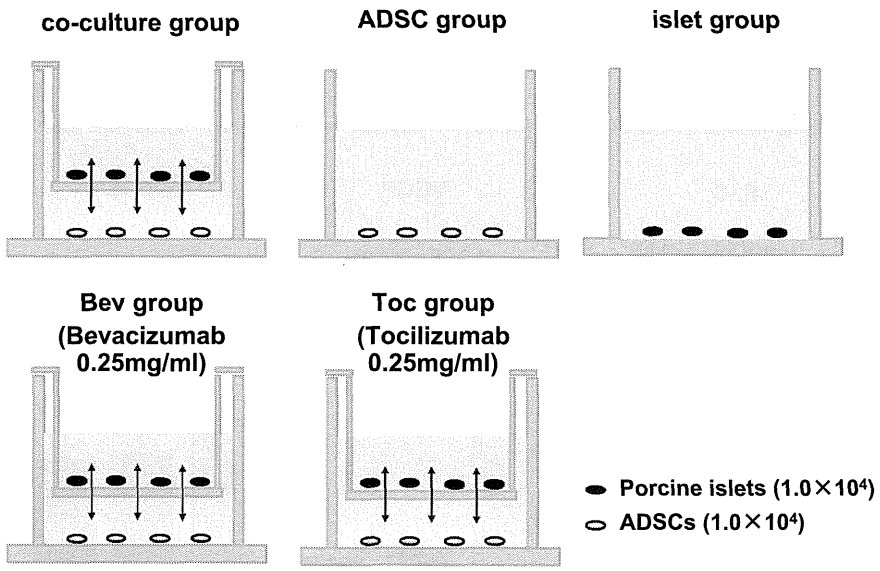

Fig. 1 – We divided the cell cultures into five groups; coculture of ADSCs with islet cells using a transwell system (coculture group, $n = 4$), single ADSC culture (ADSC group, $n = 4$), single islet cell culture (islet only, group $n = 4$), coculture of ADSCs with islet cells treated with bevacizumab (Bev group, $n = 4$), and coculture of ADSCs with islet cells treated with tocilizumab (Toc group, $n = 4$). (Color version of figure is available online.)

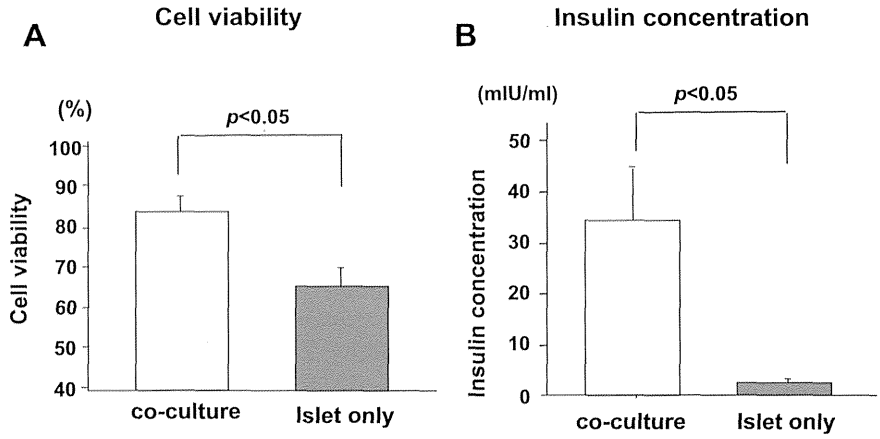

Fig. 2 – Quality assessment of islet cells. Cell viability (A) and insulin concentration levels (B) of the coculture group were higher compared with the islet only group ($P < 0.05$).

3.3. *The effect of VEGF and IL-6 signals on the quality of islets*

To identify which cytokines are more important for preserving islets, we evaluated the quality of islets using anti-VEGF and anti-IL6 receptor antibodies. In the Bev group, the cell viability was significantly decreased compared with the coculture group (66.7 ± 6.1 *versus* 84.0 ± 5.3%, $P < 0.05$, Fig. 4A). Furthermore, the insulin concentration levels in the Bev group were significantly lower compared with the levels found in the coculture group (18.0 ± 2.7 *versus* 34.5 ± 10.3 mIU/mL, $P = 0.05$, Fig. 4B). However, the cell viability and insulin levels in the Toc group showed no significant difference compared with the coculture group (Fig. 4A and B). Thus, the protective effects of ADSCs on islets were provided by VEGF signaling and not by IL-6 signaling.

4. Discussion

ICTx is a promising therapy for type 1 diabetes mellitus; however, the limitation of its widespread use is due to several critical issues [34]. One of these issues is that ICTx requires large quantities of islets to achieve insulin-free status [35]. Multidonor-1–recipient transplantation is still required due to the need for large quantities of islets to yield effective ICTx. Another issue is a severe ICTx donor shortage in some countries, such as Japan [36]. In such countries, it is sometimes difficult to find deceased donors both for clinical ICTx and basic research. As porcine islets are thought to be unlimited, xenotransplantation using porcine islets is one potential method for these countries. Successful ICTx is dependent not only on the number or mass of the islets but also on the stable engraftment and prevention of immune-mediated rejection of transplanted islets. Moreover, high islet quality, as defined by viability, metabolic activity, and function, is necessary [37,38]. Various factors, including a lack of trophic factors and instant blood-mediated inflammatory reaction [6], have been proposed to account for the low survival rate of transplanted islet grafts.

Recent studies have suggested that MSCs have a beneficial effect on cell survival, wound healing, and organ functional improvement. However, to the best of our knowledge, there have been no reports regarding the effect of human ADSCs on porcine islets *in vitro*. Thus, we showed that human ADSCs

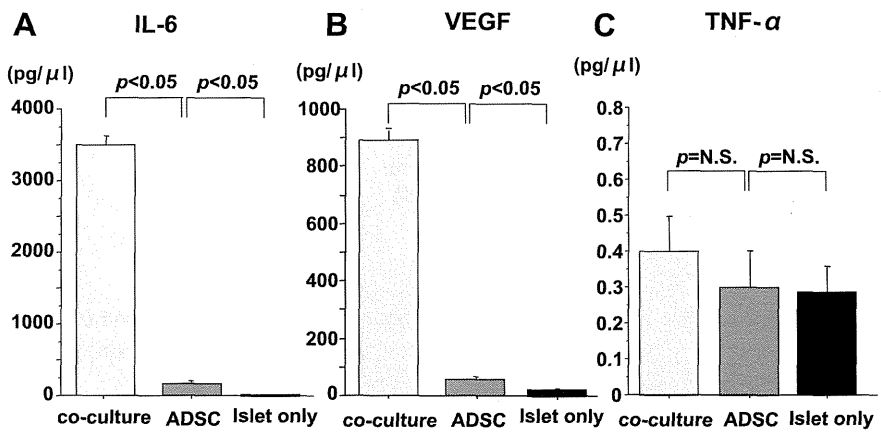

Fig. 3 – Measurement of the levels of cytokines in the supernatant in the culture medium. The concentrations of IL-6 (A) and VEGF (B) in the coculture group were higher compared with the ADSC and islet groups ($P < 0.05$). However, the concentration of TNF-α (C) showed no significant difference between the three groups.

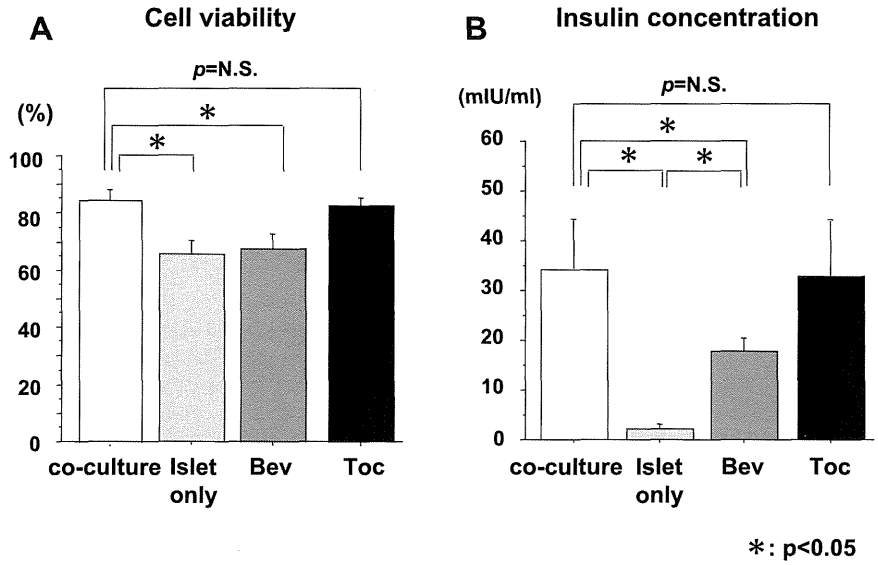

Fig. 4 – Quality assessment of islet cells with inhibition of VEGF or IL-6. In the Bev group, the cell viability (A) was significantly decreased compared with the coculture group, and the insulin concentration levels (B) in the Bev group were significantly lower than in the coculture group. However, the cell viability and insulin levels in the Toc group showed no significant difference compared with the coculture group.

have a trophic effect on porcine islets *in vitro* due to a liquid factor and not due to cell-cell contact. Moreover, VEGF, and not IL-6, might play an important role for cell survival. VEGF is a well-known signaling protein secreted by cells that stimulates vasculogenesis and angiogenesis. The most important member of this family is VEGF-A [39,40]. However, it has also been reported that VEGF exhibits another function, such as its anti-apoptotic effect.

Park *et al.* [30] reported that VEGF signaling plays a key role in the cell protective effects of MSCs. In this study, human MSCs were cocultured with mouse islet cells and VEGF was secreted at high concentrations and played an important role in the improvement of islet quality. This study also referred to xenotransplantation, from mouse to human. Furthermore, cocultured islets showed increased levels of VEGF-receptor 2, which was induced by VEGF-A signaling. In addition, phosphorylation of the Akt signaling molecules, XIAP and HSP-32, showed increased expression [30]. It was also reported that VEGF might inhibit the apoptosis of acute leukemia cells via an increase in the expression of Bcl-2 and Mcl-1 proteins [41]. Furthermore, VEGF suppressed the apoptosis of granulosa cells by inhibiting the release of caspase-activated DNase [42]. We showed the importance of VEGF for cell survival *in vitro* via the inhibition of the function of VEGF using bevacizumab. Thus, VEGF might correlate with vasculogenesis and angiogenesis as well as anti-apoptotic effects.

For IL-6, there have been several reports on the beneficial effect of IL-6 signaling [43,44]. IL-6 has been shown to protect mice and rats from ischemia-induced reperfusion injury by increasing bcl-2 protein expression and decreasing p53 expression [43]. Husain *et al.* [44] showed a role for bcl-2 in the protection of enterocyte against apoptosis. However, in this study, the inhibition of IL-6 signaling by tocilizumab did not affect the viability of islet cells. Thus, the protective effects of ADSCs on islets might be mainly provided by VEGF signaling. However, in addition to VEGF and IL-6, various cytokines are secreted from MSCs, such as IL-10, IL-1Ra, hepatocyte growth factor, nerve growth factor, fibroblast growth factor, among others [29,45]. ADSCs may also secrete these cytokines, as well as trophic effects exerted via the interaction of these cytokines. ADSCs can be recovered in large amounts from human subcutaneous fat using less invasive techniques [21,22]. This study suggests that ADSCs might have a beneficial effect on transplanted islets for type 1 diabetes, although the method and timing of the transplantation require further considerations. ADSCs are currently being applied in a clinical setting, and ICTx using porcine islets is also under clinical application. Thus, ADSCs for ICTx can be used in a clinical setting.

In conclusion, ADSCs have a trophic effect on the survival of porcine islets in the absence of cell-cell contact. VEGF secreted from ADSCs may play an important role in providing for this beneficial effect. Thus, an ADSCs-based strategy might be a breakthrough for achieving the clinical output of ICTx for type 1 diabetes.

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HEPATOLOGY

Gene profile in the spleen under massive partial hepatectomy using complementary DNA microarray and pathway analysis

Yusuke Arakawa, Mitsuo Shimada, Tohru Utsunomiya, Satoru Imura, Yuji Morine, Tetsuya Ikemoto, Hiroki Mori, Mami Kanamoto, Shuichi Iwahashi, Yu Saito and Chie Takasu

Department of Surgery, Institute of Health Biosciences, The University of Tokushima, Tokushima, Japan

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EGR1, GRO1, massive hepatectomy, microarray, splenectomy.

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Correspondence
Yusuke Arakawa, Institute of Health Biosciences, Department of Surgery, The University of Tokushima Graduate School, 3-18-15 Kuramoto, Tokushima 770-8503, Japan.
Email: yarakawa@clin.med.tokushima-u.ac.jp

Abstract

Background and Aim: In general, the spleen is one of the abdominal organs connected by the portal system, and a splenectomy improves hepatic functions in the settings of partial hepatectomy (Hx) for portal hypertensive cases or living donor liver transplantation with excessive portal vein flow. Those precise mechanisms remain still unclear; therefore, we investigated the DNA expression profile in the spleen after 90% Hx in rats using complementary DNA microarray and pathway analysis.

Methods: Messenger RNAs (mRNAs) were prepared from three rat spleens at each time point (0, 3, and 6 h after 90% Hx). Using the gene chip, mRNA was hybridized to Affymetrix GeneChip Rat Genome 230 2.0 Array (Affymetrix®) and pathway analysis was done with Ingenuity Pathway Analysis (IPA®).

Results: We determined the 3-h or 6-h/0-h ratio to assess the influence of Hx, and cut-off values were set at more than 2.0-fold or less than 1/2 (0.5)-fold. Chemokine activity-related genes including Cxcl1 (GRO1) and Cxcl2 (MIP-2) related pathway were upregulated in the spleen. Also, immediate early response genes including early growth response-1 (EGR1), FBJ murine osteosarcoma (FOS) and activating transcription factor 3 (ATF3) related pathway were upregulated in the spleen.

Conclusions: We concluded that in the spleen the expression of numerous inflammatory-related genes would occur after 90% Hx. The spleen could take a harmful role and provide a negative impact during post Hx phase due to the induction of chemokine and transcription factors including GRO1 and EGR1.

Introduction

The results of hepatobiliary surgeries for malignant tumors such as hepatocellular carcinoma or bile duct cancer have steadily improved;[1,2] however, liver failure after a partial hepatectomy (Hx) was still the major cause of morbidity and mortality.[3]

It is well recognized that the spleen is one of the abdominal organs, which connected with the portal system. Kim *et al.* reported the negative relation between liver and spleen after Hx.[4] In the settings of Hx for portal hypertensive cases or living donor liver transplantation, it had been reported that a splenectomy induced significant reductions in excessive portal system pressure, resulting in improving hepatic functions including serum albumin level, prothrombin time and number of platelets.[5–7] Furthermore, recent reports have suggested that a splenectomy might cause some anti-inflammatory effects in the portal systems on massive Hx or hepatic ischemia reperfusion injury due to a reduction of the number of splenic monocytes or macrophages, although the exact mechanism was still unclear.[8] We have previously reported that a splenectomy had beneficial effects owing to the protection of the hepatocytes by the induction of heat shock proteins in a massive Hx rat model.[9] However, the large "black-box" still remained. No research has focused on what happened in the spleen after Hx, although many reports and studies mentioned about the gene profile in the liver under massive Hx.[10,11] This was because that a new study was needed to be performed in this field to reveal the molecular mechanism and role of the spleen under that condition.

Nowadays, large-scale gene expression analysis with complementary DNA (cDNA) microarray has been gaining importance in biology and chemistry. The quantity of information obtained from analysis of the expression of thousands of genes at once not only created unique opportunities for research but also posed substantial challenges.[10] As we described recently, numerous genes and pathways were expressed in the liver of the rat with massive Hx using cDNA microarray and pathway analysis.[12]

In the current study, the molecular changes in the spleen after massive Hx were investigated using cDNA microarray and pathway analysis methods.

Materials and methods

Animals. Six-week-old male Wister rats, weighing 180–220 g were obtained from Charles River Laboratories (Kanagawa, Japan). Animals were provided with water and standard laboratory diet for at least 7 days before use. Throughout the experiment, the animals were maintained behind barriers under controlled conditions and had free access to tap water and food before and after operation. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Experimental protocols. Animals were randomly divided into following three groups ($n = 3$, each); sham operation group, 3 h after 90% partial Hx (3-h group) and 6 h after 90% Hx (6-h group). Surgeries were performed under ether anesthesia. In sham operation group, mouse underwent only simple laparotomy without hepatic resection. Hepatic resection was performed according to the technique of Higgins and Anderson.[9] Then splenic artery and vein were ligated with 4-0 silk and spleen was removed from the abdominal space after the hepatic resection was performed. Consecutively, animals were killed at 3 and 6 h after surgery. Immediately before the killing, the spleens for further study were obtained.

RNA isolation and purification. Total RNA was isolated from frozen spleens manually by Trizol reagent (Invitrogen Corporation, Carlsbad, CA, USA)[13] and then purified using RNeasy Mini Kit (QIAGEN, Inc, Valencia, CA, USA, Cat.No.74106.).[14] Total RNA samples concentration and purity were examined and estimated by optical density measurements at 260/280 nm (NanoDrop, ND-1000 Spectrophotometer, NanoDrop Technologies, Inc, Wilmington, DE, USA). After RNA extraction, equal amounts of samples from three rats were mixed together in each group for further microarray analysis.

Preparation of sample and gene chip analysis. We committed Bio Matrix Research Inc (Nagareyama-shi, Chiba, Japan) to the cDNA microarray. Isolated total RNA were amplified and labeled as described in the GeneChip Expression Analysis Technical Manual (Affymetrix, Santa Clara, CA, USA). First, total RNA (1 μg) was converted into double-stranded cDNA using the One-Cycle cDNA Synthesis Kit (Affymetrix). Double-stranded cDNA was purified by using a GeneChip Sample Cleanup Module (Affymetrix). *In vitro* transcription reactions were performed using a GeneChip IVT Labeling Kit, which includes T7 RNA polymerase and Biotin-labeled ribonucleotides. Biotin-labeled cRNA was purified using a GeneChip Sample Cleanup Module. The concentration of cRNA was calculated from light absorbance at 260 nm using a UV spectrophotometer. Next, cRNA (15 μg) was fragmented at 94°C in the presence of a fragmentation buffer (Affymetrix). Fifteen micrograms of the cRNA was hybridized to Affymetrix GeneChip Rat Genome 230 2.0 Array (Affymetrix). The array was incubated for 16 h at 45°C, then automatically washed and stained with GeneChip Hybridization, Wash and Stain Kit (Affymetrix). The Probe Array was scanned using a GeneChip Scanner 3000 7G. The data was analyzed with Microarray Suite version 5.0 (MAS 5.0).

Normalization and analysis of microarray data. Further analysis was carried out with GeneSpring®7.3.1 (Agilent Technologies, Palo Alto, CA). Additionally, gene function was evaluated using web tools. In per-chip normalization, a raw intensity value was divided by the median value of the chip measurements. In per-gene normalization, each gene was normalized to the respective control, to enable of relative changes in gene expression levels between samples.

Then the normalized signal values and the fold changes of 3 h or 6 h group/sham group could be obtained. Cut-off-values were set at more than 2.0-fold or less than 1/2 (0.5)-fold.

Ingenuity Pathway Analysis (IPA) pathway analysis. The IPA application (Ingenuity Systems, Mountain View, CA, USA) was used to organize differentially expressed genes into networks of interacting genes and to identify modules of functionally related genes that correspond to pathways. A detailed description is given in the online repository (http://www.ingenuity.com).

Quantitative real-time polymerase chain reaction (PCR) for EGR1. After that, the mRNA expression level of early growth response-1 (EGR1) was evaluated by quantitative real-time PCR. Total RNA was extracted from 30 mg of rat liver and spleen tissue using RNeasy Mini Kit (QIAGEN, Inc, Valencia, CA, USA, Cat.No.74106). Quantitative RT-PCR was performed by using an ABI 7500 Real-time PCR system (PE Applied Biosystems, Foster City, CA, USA). TaqMan gene expression systems (PE Applied Biosystems) for EGR1 (assay ID Rn00561138) was used for quantification of mRNA expression of the respective genes. To normalize, amplification of glyceraldehyde-3-phosphate dehydrogenase (Taq man ribosomal RNA control reagents, assay ID Rn9999916) was performed as an endogenous control.

Statistical analysis. Real-time PCR result was presented as mean ± SD and Mann–Whitney *U*-test was used for statistical analysis. The probability of a relationship between each biological function and the identified genes was calculated by Fisher's exact test. A *P*-value of less than 0.05 was considered statistically significant.

Results

Microarray data analysis and biostatistics. Microarray data analysis identified 31 099 probe sets that were differentially expressed. Among these, 760 (upregulated genes: 572, downregulated genes: 188) after 3 h and 1452 (upregulated genes: 1018, downregulated genes: 434) after 6 h uniquely known genes were identified as "post Hx specific genes in the spleen." Analysis of these genes with IPA indicated that the spleen under the massive hepatectomy was likely to have altered the activities of "disease and disorders pathways," "molecular and cellular functions," "physiological system development and function," and "Tox lists" (Tables 1 and 2).

Top canonical pathway from IPA in spleen. The top 10 high-level canonical pathways were identified by IPA as being

Table 1 The list of top bio functions of the splenic genes in 3 h after hepatectomy

	P-value	Number of molecules
Disease and disorders		
Cancer	1.54E-17	167
Inflammatory response	9.58E-15	96
Organismal injury and abnormalities	3.06E-12	62
Dermatological disease and conditions	1.72E-10	71
Reproductive system disease	2.78E-10	86
Molecular and cellular functions		
Cell death	3.99E-12	132
Cellular growth and proliferation	5.71E-12	145
Cellular movement	1.89E-11	94
Cellular development	6.07E-10	129
Cell morphology	1.73E-08	53
Physiological system development and function		
Hematological system development and function	9.72E-09	88
Immune cell trafficking	9.72E-09	59
Tissue morphology	1.63E-08	75
Organismal survival	4.00E-08	56
Nervous system development and function	2.73E-07	48
Tox lists		
LXR/RXR activation	1.49E-07	79
Cardiac hypertrophy	1.28E-06	259
Liver proliferation	1.42E-06	133
Renal necrosis/cell death	1.92E-06	314
Cardiac fibrosis	7.64E-06	95

LXR, liver X receptor; RXR, retinoic X receptor.

Table 2 The list of top bio functions of the splenic genes in 6 h after hepatectomy

	P-value	Number of molecules
Disease and disorders		
Cancer	1.78E-34	343
Inflammatory response	2.14E-34	205
Inflammatory disease	1.58E-32	316
Dermatological diseases and conditions	2.16E-27	163
Respiratory disease	2.37E-26	144
Molecular and cellular functions		
Cellular movement	1.54E-31	206
Cell-to-cell signaling and interaction	2.53E-31	230
Cell death	7.95E-26	275
Cellular growth and proliferation	1.81E-25	293
Cellular development	5.08E-21	263
Physiological system development and function		
Tissue development	2.53E-31	209
Organismal survival	4.14E-28	152
Hematological system development and function	6.11E-27	209
Immune cell trafficking	6.11E-27	153
Tissue morphology	4.89E-22	142
Tox lists		
Cardiac hypertrophy	3.69E-12	259
Liver necrosis/cell death	4.31E-12	166
Liver proliferation	1.04E-11	133
LXR/RXR activation	3.86E-11	79
Hepatic fibrosis	1.73E-10	85

LXR, liver X receptor; RXR, retinoic X receptor.

significantly overrepresented by differentially expressed genes in this dataset. In 3 h, inflammatory response related pathways including "acute phase response signaling," "hepatic fibrosis/hepatic stellate cell activation," "IL-10 signaling" and "IL-6 signaling" were affected under the massive Hx (Fig. 1a). In 6 h, inflammatory response related pathways including "IL-10 signaling," "IL-6 signaling," and "hepatic fibrosis/hepatic stellate cell activation" were also strongly affected (Fig. 1b).

Gene ontology (GO) analysis on chemokines. The detailed GO analysis revealed that genes of the chemokine activity, which has a crucial role in the acute phase response showed significant different patterns between the two groups. In GO "chemokine activity," eight unique genes were affected (upregulated: Cxcl1 [GRO1], Cxcl2 [MIP-2], Ccl7, Ccl2 [MCP-1], Ccl24, Cxcl5 and Ccl20, downregulated: Xcl1) after 3 h and 13 unique genes were affected (upregulated: Cxcl1 [GRO1], Ccl2 [MCP-1], Ccl20 [ST38], Cxcl2 [MIP-2], Ccl9, Ccl7, Cxcl5, Ccl3 [MIP-1a], Ccl4 [MIP1-B], Cxcl9, Ccl6 [Mrp-1], Cxcl10 [IP-10], Ccl22) after 6 h (Tables 3 and 4). We constructed several networks using these genes. IPA revealed that chemokine activity formed an acute phase response network of "chemokine activity." IPA represented upregulated (red) and downregulated (green) genes in the gene network. This pathway included many kinds of chemokine genes. Interestingly, all of these genes were upregulated except XCL1, so this meant these pathways were upregulated (Fig. 2a,b).

GO analysis on transcription factor. In addition, another GO analysis revealed that genes of the transcription factor activity, which has a crucial role in the acute phase response, showed significantly different patterns between the two groups. In GO "transcriptional factor activity," 30 unique genes were affected (upregulated: 24, downregulated: 6) after 3 h, and 56 unique genes were affected (upregulated: 44, downregulated: 12) after 6 h (Tables 5 and 6). Those genes included key transcription factors, such as EGR1, FBJ murine osteosarcoma (FOS), Activating transcription factor 3 (ATF3), JUN and JUN-B belonging to immediately early response genes. We constructed several networks using these genes. These pathways included these stress response genes (Fig. 3a,b). The qRT-PCR showed that in the spleen under the massive Hx constitutively expressed a higher amount of EGR1 mRNA, which was a key molecule of the pathway (Fig. 3c).

Discussion

In general, the major known functions of the spleen were removal of aging erythrocytes and recycling of iron, elicitation of immunity, removal of blood-borne microorganism and cellular debris, and a supply of erythrocytes after hemorrhagic shock.[15] In response to inflammation, splenic monocytes such as dendritic cells or macrophages increased their motility, exited the spleen, accumulated in the injured tissue, and participated in wound healing.[16] The blood flow was significantly lower and vascular

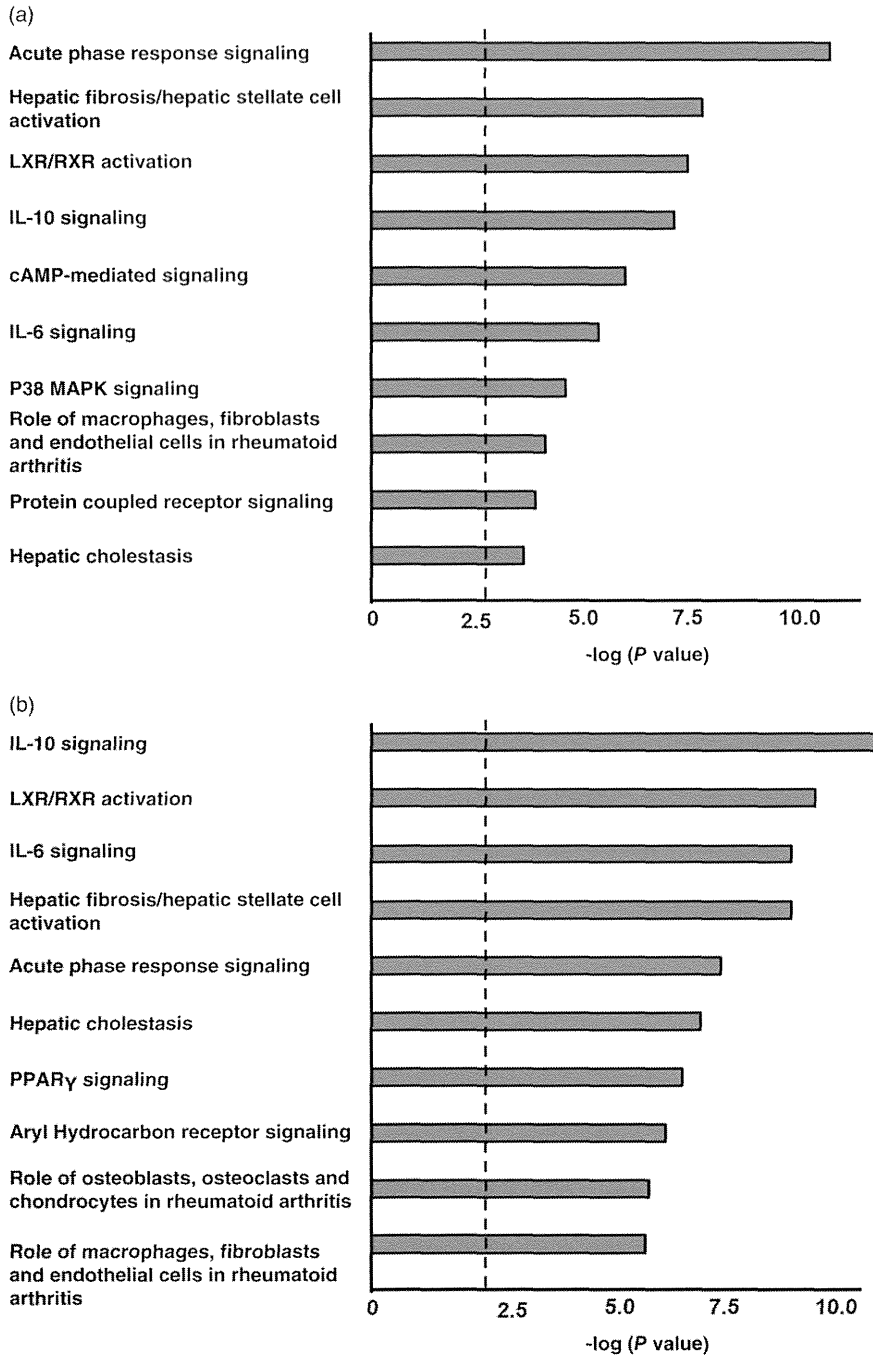

Figure 1 Canonical pathway analysis with the Ingenuity Pathway Analysis (IPA) application showed the pathways of specific genes in the spleen significantly modified by 90% partial hepatectomy (Hx) using Fisher's exact test. The threshold lines represent a *P* value with 0.05. (a) 3 h and (b) 6 h after Hx.

resistances became higher in the spleen after Hx; therefore, the congestion and ischemia injury might occur in the spleen.[17] As a result, it could affect the gene expression in the spleen. Despite these reports, molecular changes in the spleen after Hx have not been investigated and reported yet. Remarkably, this was the first report to investigate the genetic change in the spleen after Hx, so we could know "what happens in the spleen" expectantly.

First of all, the results of top bio functional gene analysis (Tables 1 and 2) suggested that the altered genes included inflammatory response, inflammatory disease, cell death, liver necrosis/cell death and hepatic fibrosis. The results of top canonical pathways (Fig. 1a,b) also suggested that the altered pathways included acute phase response signaling, hepatic fibrosis/hepatic stellate cell activation and IL-6 signaling. These features of genes and pathways expression showed an inflammatory response could occur in the spleen, and they might provide a negative and harmful impact to the liver via the portal vein. Actually, Kuriyama *et al.* reported splenectomy could not only decrease the portal vein flow but also induce cytoprotective effect by decreased ET-1 and increased HO-1.[18] Takuya *et al.* has also reported the limitation of

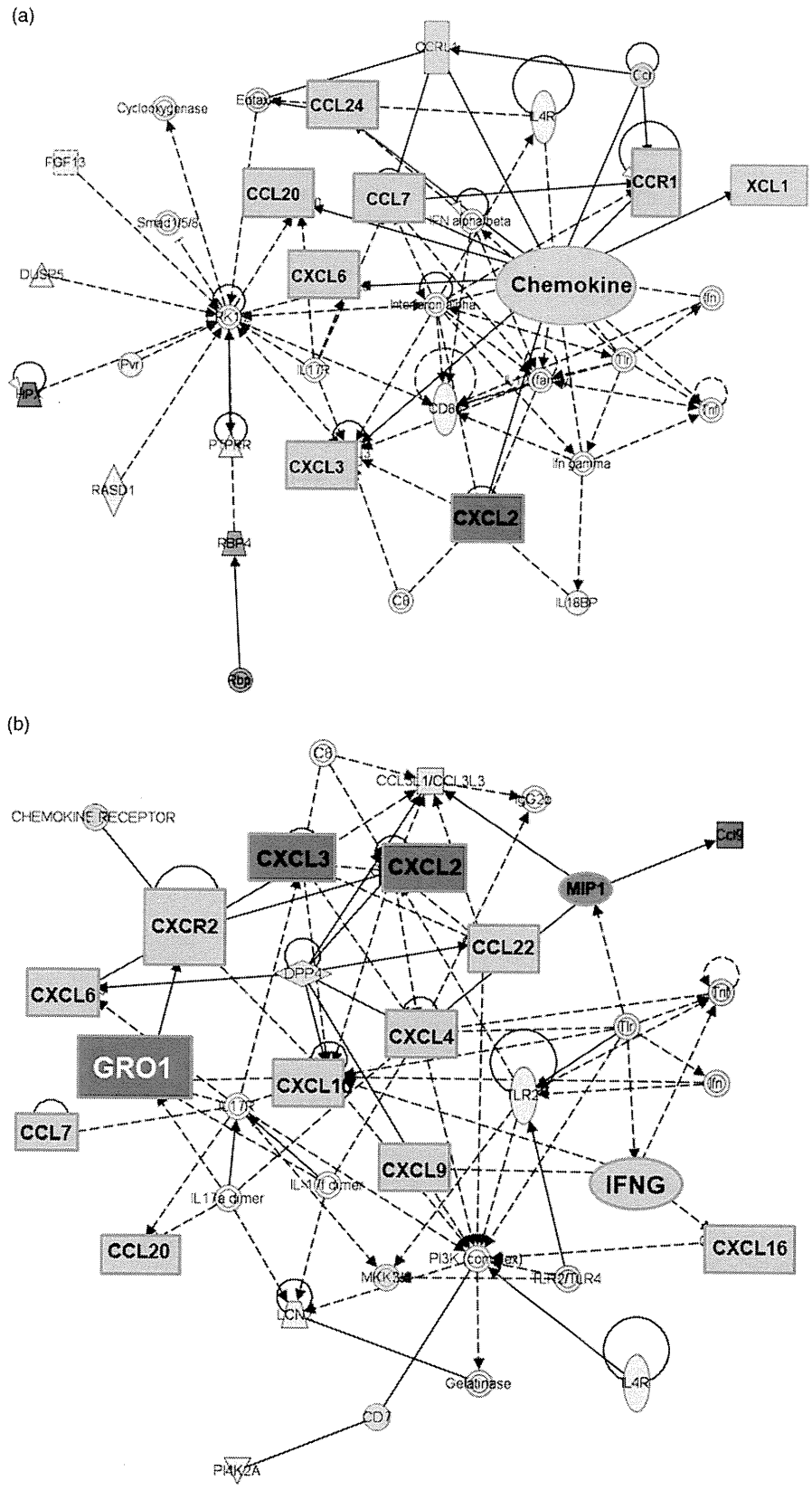

Figure 2 Isolation 90% Hx-induced changes in chemokine networks. Using the chemokine-related genes network. (a) 3 h and (b) 6 h after Hx. Genes up-regulated and down-regulated by 2-fold were expressed in red and in green (online only), respectively.

Table 3 Gene ontology (GO) analysis on "chemokine activity"-related genes in 3 h

Gene name	Fold change	Percentile (median 2.3685356)	GenBank number
Cxcl1 (GRO1)	48.04672	0.998	NM_030845
Cxcl2 (MIP-2)	6.53484	0.906	NM_053647
Ccl7	3.706583	0.806	BF419899
Ccl2 (MCP-1)	3.393003	0.776	NM_031530
Ccl24	2.973374	0.706	BI294084
Cxcl5	2.925218	0.657	NM_022214
Ccl20	2.18812	0.382	AF053312
Xcl1	0.338448	0.018	NM_134361

Table 4 Gene ontology (GO) analysis on "chemokine activity"-related genes in 6 h

Gene name	Fold change	Percentile (median 2.2727032)	GenBank number
Cxcl1 (GRO1)	439.649	1.000	NM_030845
Ccl2 (MCP-1)	70.00393	0.996	NM_031530
Ccl20 (ST38)	62.80219	0.995	AF053312
Cxcl2 (MIP-2)	54.54451	0.995	NM_053647
Ccl9	47.29132	0.992	AI169984
Ccl7	37.39671	0.989	BF419899
Cxcl5	27.58101	0.984	NM_022214
Ccl3 (MIP-1a)	11.08792	0.951	U22414
Ccl4 (MIP-b)	4.445999	0.845	U06434
Cxcl9	3.993681	0.821	AI044222
Ccl6 (Mrp-1)	2.310009	0.516	BE095824
Cxcl10 (IP-10)	2.304766	0.513	U22520
Ccl22	2.287212	0.506	AF432871

splenic inflammatory cell recruitment into the liver was the cytoprotective effect of splenectomy in small-for-size liver transplantation.[19] Furthermore we have already reported gene changes in the liver after 90% Hx and protective effect of splenectomy.[12]

In the current study, we could achieve the investigation or the gene-expression changes in the rat spleens under the situation of 90% Hx on the two time points. Firstly, we could find numerous "chemokines" related genes. Along with the accelerated rate of the discovery of chemokines had come the realization that these molecules not only controlled hemopoietic cell migration, but also they were involved in a number of other physiological and pathological processes including induction of either monocytes, macrophages or fibroblastic cells in inflammation.[20] So it was expected that they were one of the triggers in inflammatory response in the spleen. The message for the chemoattractants Ccl2 (MCP-1), Ccl7, Cxcl2 (MIP-2), Cxcl1 (GRO1), Ccl4 (MIP1-B) and Ccl3 (MIP-1A) were extremely increasing. Especially, GRO1 showed the highest upregulation in chemokine related genes in this study. GRO1 encoding a potent chemoattractant belonging to the CXC chemokine subfamily was most rapidly and markedly upregulated after 90% Hx; therefore, GRO1 played a vital role in acute liver injury after Hx.[21] Cxcl4 and Cxcl3 were MIP-1 (macrophage

Table 5 Gene ontology (GO) analysis on "transcription factor"-related genes in 3 h

Gene name	Fold change	Percentile (median 2.3685356)	GenBank number
Top 20 genes			
RGD1559697_predicted	26.59984	0.992	BE105492
Fos	9.197226	0.943	BF415939
Hit39	8.171527	0.928	AF277902
Crem	6.371599	0.902	NM_017334
Cebpd	6.35065	0.898	BF419200
Phox2a	6.192782	0.895	NM_053869
Ccl7	3.706583	0.806	BF419899
Ccl2	3.393003	0.776	NM_031530
Sox9	3.199223	0.750	AI548994
Ccl24	2.973374	0.706	BI294084
Gadd45g	2.763646	0.657	AI599423
Bhlhb2	2.443606	0.541	AI548256
Nfkbiz_predicted	2.354374	0.492	AI176265
Fkhl18	2.224701	0.401	AI008883
Zfp36l2	2.2081	0.395	AA943730
Fev	2.183003	0.379	U91679
Atf3	2.099832	0.313	NM_012912
Jundp2	2.070742	0.288	NM_053894
EGR1	2.063132	0.279	NM_012551
Mtf1_predicted	2.001628	0.223	BG375691

Table 6 Gene ontology (GO) analysis on "transcription factor"-related genes in 6 h

Gene name	Fold change	Percentile (median 2.2727032)	GenBank number
Top 20 genes			
Cebpd	9.097913	0.933	NM_013154
Neurod1	7.304114	0.917	NM_019218
Nfkbiz_predicted	5.75559	0.886	AI176265
Phox2a	5.332282	0.878	NM_053869
Batf_predicted	4.482911	0.848	AA819819
Ccl4	4.445999	0.845	U06434
Cebpb	4.111673	0.829	NM_024125
Fkhl18	3.672931	0.799	AI008883
EGR1	3.628126	0.795	NM_012551
Tfec	3.415391	0.768	L08812
Trim28	3.299043	0.754	BE098563
Fos	3.20988	0.743	BF415939
Gadd45g	3.055698	0.726	AI599423
Junb	3.027802	0.721	NM_021836
Crem	3.015733	0.718	NM_017334
Lhx1	2.862468	0.693	BI282093
Atf3	2.854969	0.691	NM_012912
Zfhx1b	2.729652	0.661	BG377397
Stat3	2.567718	0.620	BE113920
Jundp2	2.49348	0.596	NM_053894

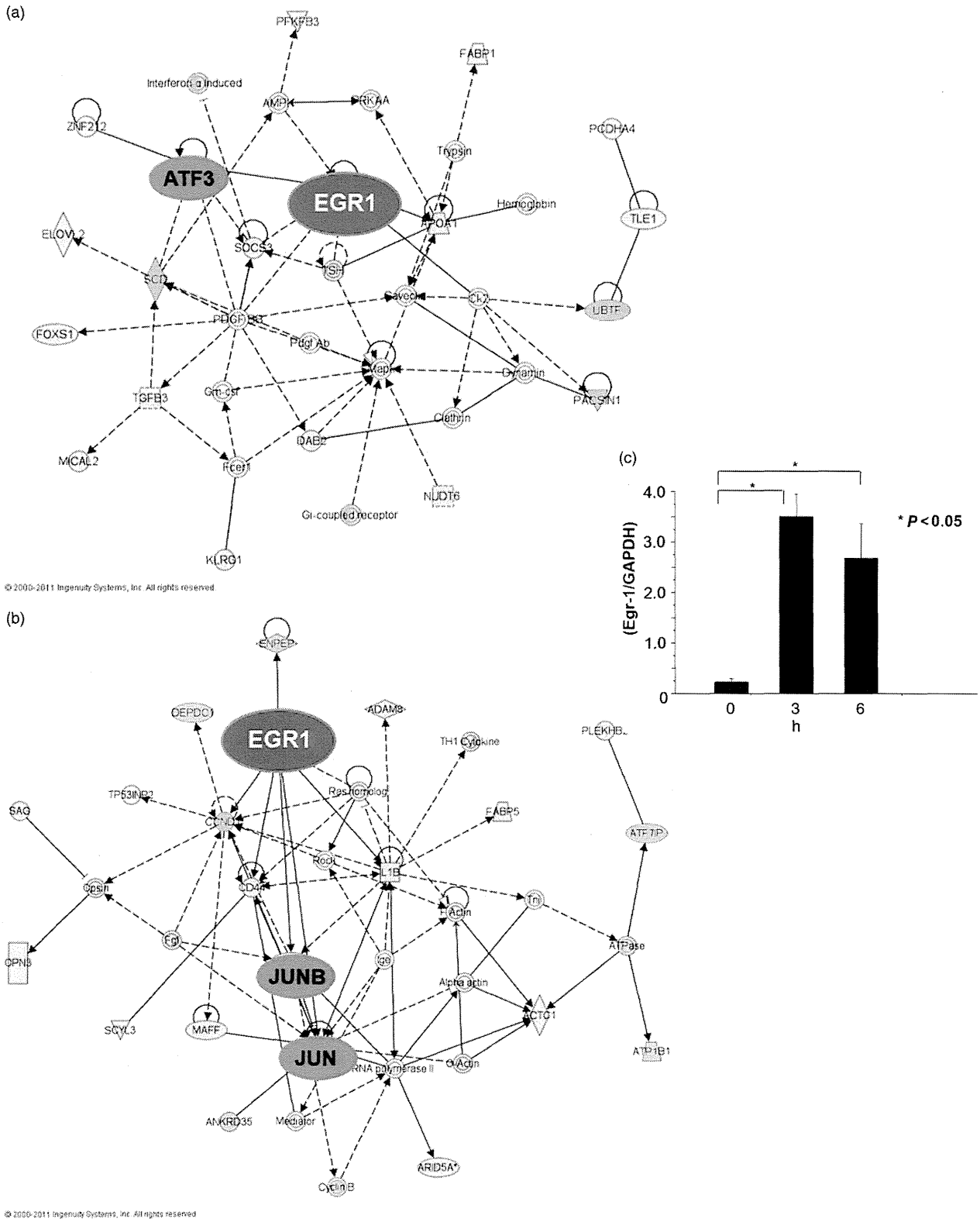

Figure 3 Isolation 90% Hx-induced changes in transcriptional factor networks. Using the transcription factor-related genes including EGR1, activating transcription factor 3 (ATF3), JUNB, and JUN network. (a) 3 h and (b) 6 h after Hx. Genes upregulated and downregulated by twofold were expressed in red and in green (online only), respectively. The gene expressions of EGR1 (c) in the spleen were determined by real-time polymerase chain reaction (PCR). glyceraldehyde-3-phosphate dehydrogenase RNA serves as an endogenous quantity control. Values were means ± SD. (* $P < 0.05$)