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Liver cell-mediated alleviation of acute ischemic myocardial injury

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1. ABSTRACT

Cardiomyocyte injury occurs in myocardial ischemia, resulting in impairment of cardiac function. As the endogenous protective function of adult cardiomyocytes is limited, nonmyocytic cells may be activated to protect myocardium from ischemic injury. In this investigation, we demonstrated in a mouse model of myocardial ischemia that the liver was able to respond to myocardial ischemia to upregulate a number of genes encoding secreted proteins, mobilize its cells, and release cell contents into the circulatory system. These naturally occurring mechanisms suggested a possible cardioprotective role for myocardial ischemia-conditioned liver cells and inspired us to develop cardioprotective therapies based on these mechanisms. We demonstrated that administration of liver cell extract derived from myocardial ischemic mice, but not sham control mice, resulted in a significant reduction in acute myocardial infarction as well as the density of TUNEL+ cells in ischemic myocardium compared to administration of PBS at 2, 6, 12, and 24 hrs. These observations suggest that liver cells may respond to myocardial ischemia to express cardioprotective factors, which may be identified and used for alleviating myocardial infarction.

2. INTRODUCTION

Myocardial ischemia induces cardiomyocyte injury and death, resulting in impairment of cardiac function. As the adult heart possesses a limited capacity of myocardial protection and regeneration in myocardial ischemia, nonmyocytic cells may be activated to assist the heart in cardioprotection. An example is the involvement of cardiac resident stem and progenitor cells in myocardial ischemia (1-5). These cells can be induced to differentiate to cardiomyocyte-like cells in vitro and transform to cardiomyocytes when transplanted into ischemic myocardium in vivo, contributing to the regeneration of ischemic myocardium (1,3). Furthermore, bone marrow cells may be activated in response to myocardial ischemia or cytokine upregulation (6-11). These cells have been shown to promote myocardial survival and performance (12-14), although the underlying mechanisms remain to be determined (15-19). Another nonmyocytic mechanism is the activation of macrophages in the healing process of injured myocardium. These cells can migrate to ischemic myocardium and release cytokines and angiogenesis-stimulating factors, contributing to myocardial protection and regeneration (20,21). In this investigation, we

demonstrated that liver cells were able to upregulate a number of genes encoding secreted proteins and mobilize to the circulatory system to release cell contents in response to experimental myocardial ischemia. These processes possibly contributed to alleviation of acute ischemic myocardial injury.

3. EXPERIMENTAL PROCEDURES

3.1. Transgenic Mouse Model

The Cre/loxP transgenic mouse model has been widely used for identifying and tracking desired cell types (23). In this investigation, we used an Alb-Cre-EYFP mouse model for identifying liver cells in the circulatory system. The Alb-Cre-EYFP model was established by crossing an Alb-$Cre^{+/+}$ mouse strain expressing albumin promoter-driven Cre recombinase [B6.Cg-Tg(Alb-cre)21Mgn/J, C57BL/6J background, Jackson Laboratory] (24) with a conditional $EYFP^{+/+}$ mouse strain [B6.1.29X1-Gt(ROSA)26Sor$^{tm1(EYFP)Cos}$/J, C57BL/6J background, Jackson Laboratory] (25) expressing the EYFP gene controlled by a loxP-flanked stop sequence that blocks EYFP expression. When the albumin gene promoter-driven Cre recombinase gene is expressed in the liver, the stop sequence of the EYFP gene between the loxP sites is deleted by the Cre recombinase, resulting in liver-specific EYFP expression. In this investigation, EYFP was used as a marker for identifying mobilized liver cells.

3.2. Coronary Arterial Ligation

Myocardial ischemia was induced in the mouse by ligating the left anterior descending (LAD) coronary artery. Mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and ventilated via the trachea by using a rodent respirator. Intercostal thoracotomy was carried out and the LAD coronary artery was ligated permanently at a location about 3 mm below the left atrial appendage. Each ischemic mouse was paired with a gender-matched sham control littermate with identical procedures with the exception that the coronary artery was not ligated. Observation was conducted at 2, 6, 12, and 24 hrs following coronary ligation. All experimental procedures were approved by the Institutional Animal Care and Use Committee.

3.3. Preparation of liver cell extract

Liver cell extract was prepared from mice with 5-day myocardial ischemia as ischemic preconditioning possibly stimulated upregulation of cardioprotective factors. Myocardial ischemia was induced as described above. At day 5, the portal vein of the liver was cannulated and perfused with phosphate buffered saline (PBS) for 5 min to remove circulating blood cells. Large blood vessels and bile ducts were removed mechanically, and the liver parenchyma was homogenized and frozen for 3 cycles. The liver cell homogenates were centrifuged at 20,000 rpm for 20 min to remove cell organelles and debris. The supernatant was filtered through 0.2 micron filters and used for intravenous administration at the dose of 5 micrograms/gm x3/day starting 24 hrs before the induction of myocardial ischemia. Liver cell extract was also prepared from 5-day sham control mice and used for administration at the same dosage. An equal volume of PBS was used for placebo administration. It should be emphasized that the strategy of using liver cell extract for administration was based on the naturally occurring mechanisms by which liver cells were disintegrated to release cell contents to the circulatory system, a process possibly required for cardioprotection in myocardial ischemia (see Results section).

3.4. cDNA Microarray

cDNA microarray analyses were conducted by using the Illumina whole-genome MouseWG-6 v2 Expression BeadChip with 45,200 transcripts. Total RNA was prepared from sham control mice and myocardial ischemic mice at day 0.5, 1, 3, 5, 10, and 30 by using the TRIzol reagent kit of Invitrogen. cDNA and cRNA synthesis, cRNA chip hybridization, and data scanning were carried out according to the standard procedures. An average number of 41+/-8 beads or spots were used for each gene. The target signal intensity values of all spots for each gene were averaged and used for data analyses. The acquisition and initial quantification of array images were achieved using the Illumina Genome Studio Gene Expression Module v1.0 software. The target signal intensity was normalized with reference to the median intensity. Criteria for selecting differentially expressed genes in the liver cells of myocardial ischemic mice were set at a 3-fold change compared to sham controls at any time during the observation period from 0.5 to 30 days (26) and a detection P-value at $p<0.05$ based on the comparison of the target signal with the negative control. Our analyses were focused on the genes encoding liver cell-secreted proteins, as these factors can interact with other cell types via membrane receptors. Although cytoplasmic proteins can be released from mobilized liver cells into the circulatory system, we did not analyze these proteins, since they may not be able to enter the target cells to mediate cell activities. Microarray data compliant to MIAME are currently processed for deposition at http://www.ncbi.nlm.nih.gov/geo.

3.5. Fluorescence Microscopy

EYFP+ hepatic cells in the liver and mobilized to the circulatory system were observed by fluorescence microscopy. For testing circulating EYFP+ cells, a blood sample was collected slowly from the right ventricular chamber of an anesthetized mouse with an 18G needle, applied to a glass slide, fixed in 4% formaldehyde in PBS, and used for examination by fluorescence microscopy. For liver examination, the liver was perfused with 4% formaldehyde in PBS via the portal vein. Liver specimens were collected, cut into 5 micron cryo-sections, and examined by fluorescence microscopy. Fluorescence images were deconvolved to reduce optical distortion and background noise.

A quantitative method was developed and used for identifying EYFP+ cells based on the analysis of fluorescence intensity. A standard level of relative fluorescence intensity was established from EYFP+ liver specimens as $X - 1.962 \times$ standard deviation, where X is

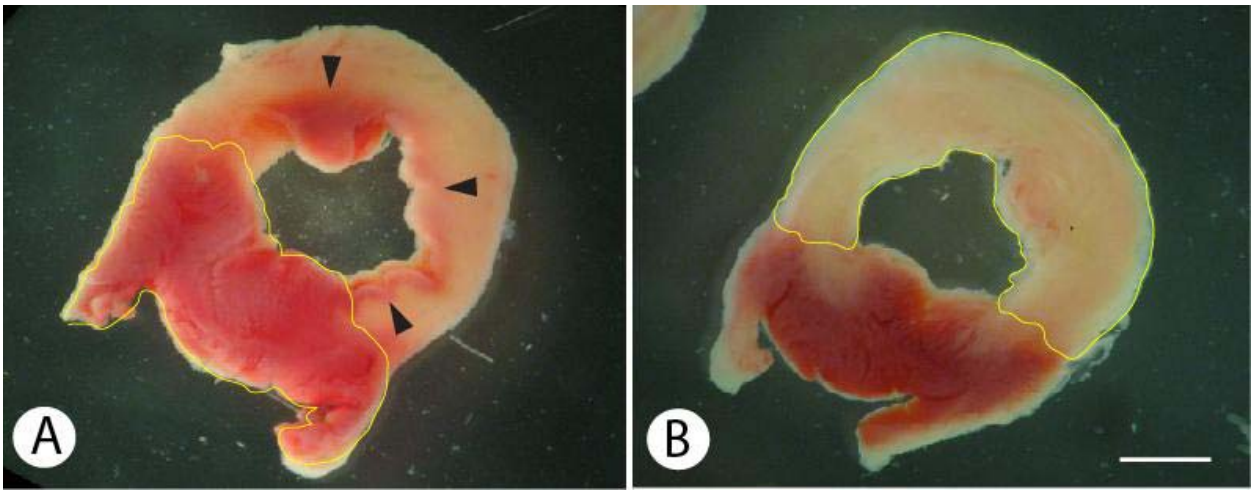

Figure 1. Micrographs showing the measurement of the endocardial layer of TTC-positive or protected myocardium in infarcted regions (arrows, Figure 1A). The thickness of the TTC-positive endocardial layer was estimated based on the measured area and length of the TTC-positive endocardial layer in infarcted regions. Regions with TTC-positive myocardium across the left ventricular wall (circled region, Figure 1A) were not included for measurement. For regions of TTC-negative myocardial infarcts that covered the entire left ventricular wall (circled region, Figure 1B), the thickness of the endocardial layer of protected myocardium was considered zero. Scale: 1 mm.

the mean relative fluorescence intensity measured from the EYFP+ cells of the liver and normalized with reference to EYFP-negative cells. The relative fluorescence intensity of EYFP+ liver cells was measured by using a fluorescence microscope and an image analysis system. Circulating cells with relative fluorescence intensity higher than the standard level were identified as EYFP+ cells.

3.6. TTC assay

The degree of myocardial infarction was assessed using the TTC assay. At a scheduled time, the heart was removed from a deeply anesthetized mouse, quickly frozen at –80^{o} C, and cut into serial transverse sections of ~1 mm in thickness. The specimen sections were immediately incubated in 1% TTC in PBS at 37^{o} C for 30 min, flattened between two glass slides, fixed in 4% formaldehyde in PBS for 20 min, and observed by microscopy. TTC-negative regions (white in color) were defined as myocardial infarcts, and TTC-positive regions (red in color) were defined as intact myocardium. The areas of myocardial infarcts and intact myocardium were measured from each section, the volume of myocardial infarcts was calculated from measured infarct areas and section thickness, the volume fraction of myocardial infarcts was calculated based on the measured infarct volume and the total volume of myocardium below the ligation site of the LAD coronary artery.

We also measured the relative thickness of the endocardial layer of TTC-positive myocardium in regions with myocardial infarction below the ligation site of the LAD coronary artery (see Figure 1 for definition). From each TTC-stained myocardial slice, the thickness of the endocardial layer of TTC-positive myocardium was measured at 6 equally spaced locations along the circumference of an identified myocardial infarct.

3.7. TUNEL assay

The TUNEL assay was used to assess cell death in the myocardial tissue. The heart of each anesthetized mouse was fixed by arterial perfusion of 4% formaldehyde in PBS. Specimens were collected from the ischemic region of myocardium and cut into cryo-sections of 5 microns in thickness. TUNEL assay was carried out to detect cell death by using the Roche cell death labeling kit as described (27).

3.8. Flow cytometry

The population of selected cell types was measured and analyzed by flow cytometry. For circulating cells, a blood sample was collected from the right ventricular chamber of an anesthetized mouse and mixed with equal volume of PBS supplemented with 100U/ml heparin. The red blood cells were remove, and nucleated cells were collected, fixed in 2% formaldehyde, and tested by flow cytometry for detecting EYFP+ cells (excitation 488 nm, emission 525 nm). A standard level of fluorescence intensity was established from the liver cells of Cre-EYFP mice by using the method described in the section "Fluorescence microscopy" and used for assessing the population of circulating EYFP+ cells. Cytometry gates for fluorescent markers were set based on the fluorescence intensity of simultaneously prepared control cells (28). For example, C57BL/6 liver cells were used as negative control for detecting EYFP+ liver cells, nucleated blood cells derived from mice without surgery were used as negative control for circulatory EYFP+ cells, and cells stained with an isotype-matched nonspecific antibody were used as negative control for an antibody marker.

3.9. Statistical Analysis

Means and standard deviations were calculated for each measured parameter. The two-tailed Student t-test

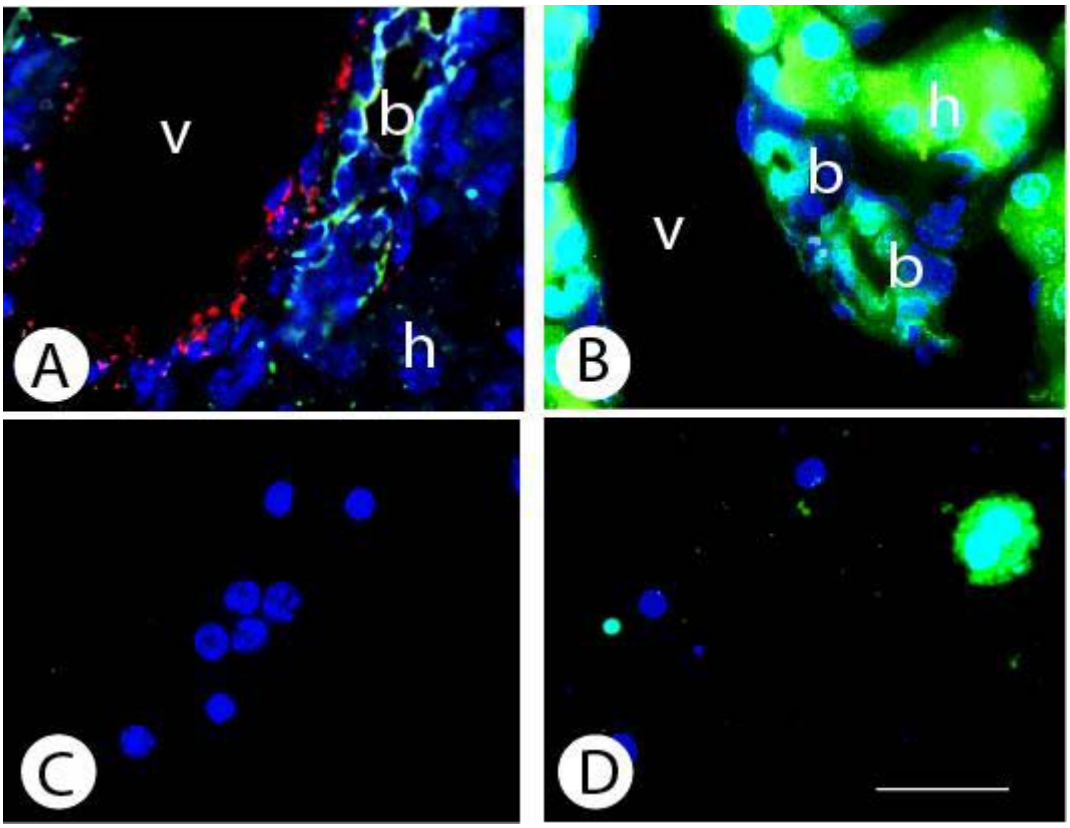

Figure 2. Characterization of the Alb-Cre-EYFP mouse model and mobilization of liver cells to the circulatory system. (A) Fluorescence micrograph of a liver specimen from a wild-type mouse showing the anatomical relationship between a central vein (v) identified by factor VIII labeling of vascular endothelial cells (red), a bile duct (b) identified by cytokeratin 19 labeling of biliary epithelial cells (green), and hepatocytes (h). (B) EYFP expression (green) in hepatocytes (h) and biliary epithelial cells (b) of a liver specimen from an Alb-Cre-EYFP mouse. (C) Leukocytes in a blood sample collected from the right ventricular chamber of an Alb-Cre-EYFP mouse with sham operation, showing the lack of EYFP+ cells. (D) Presence of EYFP+ cells (green) in a blood sample collected from the right ventricular chamber of an Alb-Cre-EYFP mouse with 24 hr myocardial ischemia. The blue color represents cell nuclei and the scale bar is 10 microns for all panels.

was used for analyzing differences between two groups. A difference was considered statistically significant at $p < 0.05$.

4. RESULTS

4.1. Characterization of the Alb-Cre-EYFP mouse model

We established a transgenic mouse model with liver cell-specific expression of EYFP, a marker used for identifying liver cells mobilized to the circulatory system. This model was created by crossing a mouse strain expressing the albumin-Cre recombinase gene (24) with a mouse strain expressing the EYFP gene controlled by a *lox*P-flanked stop sequence (25), which blocks EYFP expression in the absence of Cre recombinase. The crossing of the two strains introduces to the EYFP mouse liver-specific expression of Cre recombinase, which deletes the stop sequence of the EYFP gene between the loxP sites, resulting in liver expression of EYFP. In this model (referred to as the Alb-Cre-EYFP model), EYFP was expressed primarily in hepatocytes and biliary epithelial cells, but not in other liver cell types, including vascular cells, periductular cells, and bone marrow-derived cells (Figure 2). Thus, EYFP can be used for identifying mobilized hepatocytes and biliary epithelial cells (referred to as liver cells in this report) in the Alb-Cre-EYFP model.

4.2. Mobilization of liver cells in response to myocardial ischemia

Following the induction of myocardial ischemia, EYFP+ liver cells were found in the circulatory system (Figure 2). The body and nucleus of these cells were similar to that of hepatocytes or biliary epithelial cells, and can be readily distinguished from that of circulating leukocytes. The population of circulating liver cells was about 0.08 +/- 0.03, 0.19+/-0.09, 0.47+/-0.26, and 0.64+/-0.23% with reference to the population of total nucleated blood cells at 2, 6, 12, and 24 hrs, respectively, following the ligation of the LAD coronary artery (Figure 3), and was significantly larger than that in the sham control group at each time ($p < 0.05$, < 0.01, < 0.01, and < 0.001 at 2, 6, 12, and 24 hrs, respectively). These tests demonstrated liver cell mobilization to the circulatory system in response to myocardial ischemia.

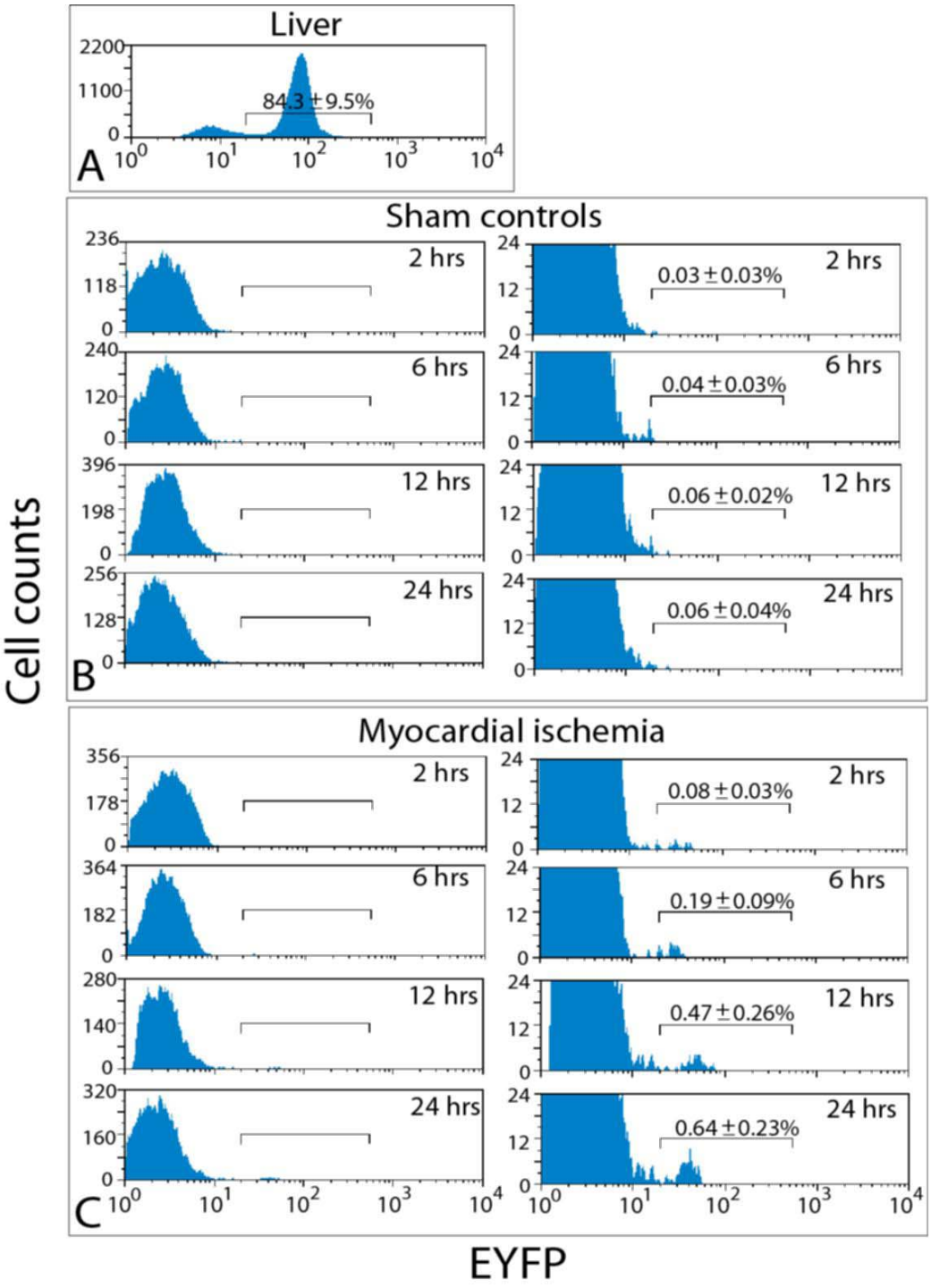

Figure 3. Mobilization of liver cells to the circulatory system of Alb-Cre-EYFP mice with myocardial ischemia. (A) One-dimensional cytometry analysis of liver cells from an Alb-Cre-EYFP mouse, showing the population of EYFP+ cells in the liver. (B, C) Cytometry analyses of EYFP+ cells in blood samples from sham control mice (panel B) and myocardial ischemic mice (panel C) at 2, 6, 12, and 24 hrs. Note that each myocardial ischemic mouse was paired with a littermate sham control mouse with the same gender. A standard level of fluorescence intensity (the left side of the bars) was established from the liver cells of Alb-Cre-EYFP mice as described in the method section and used for assessing the population of circulating EYFP+ cells. The values shown in each panel represents the mean and standard deviation of the relative EYFP+ cell population with reference to the total nucleated cells from 5 tests. For panel B and C, the right column shows magnified portions from the left column.

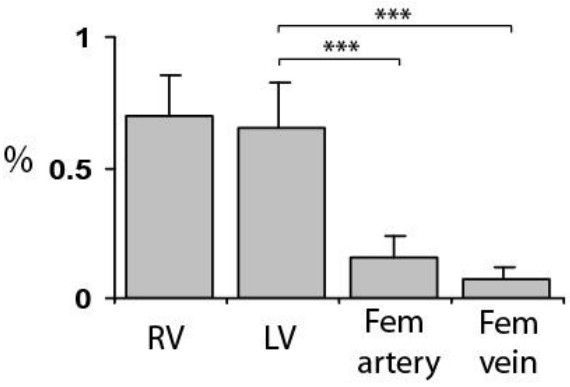

Figure 4. Distribution of mobilized liver cells in the right and left ventricles and the right femoral artery and vein. Blood samples were collected from the right and left ventricles and the right femoral artery and vein of the same Alb-Cre-EYFP mice with 24-hr myocardial ischemia, and the relative density of EYFP+ liver cells was measured with reference to the total nucleated blood cells by fluorescence microscopy. Note that the relative density of EYFP+ cells in the right femoral artery and vein was significantly lower than that in the right and left ventricular chambers. Means and SDs are presented with a sample size 6 at each time point. *** $p < 0.0.01$. Fem: femoral.

4.3. Disintegration of mobilized liver cells in the circulatory system

There might be several possible fates for mobilized liver cells, including disintegration within the circulatory system and engraftment to the peripheral tissues. In this investigation, we tested the first possible fate by measuring and analyzing alterations in the population size of circulating EYFP+ liver cells in blood samples collected from the right and left ventricular chambers as well as the femoral artery and vein. As shown in Figure 4, while the population of EYFP+ liver cells in the right ventricular chamber was comparable to that from the left ventricular chamber, a significantly smaller population of EYFP+ liver cells was detected in the femoral artery and vein. These observations demonstrated that a major loss of mobilized liver cells occurred from the left ventricle to the peripheral arteries, suggesting that mobilized liver cells were disintegrated primarily in the arterial system.

4.4. Alleviation of myocardial infarction in response to administration of liver cell extract

Liver cell mobilization and disintegration in the circulatory system were naturally occurring processes in response to acute myocardial ischemia. Liver cell disintegration resulted in the release of cell contents, which were possibly beneficial to myocardial protection. To test this possibility, we prepared liver cell extract from mice with 5-day myocardial ischemia, administered the liver cell extract or PBS to recipient mice starting at 24 hrs before the ligation of the LAD coronary artery, and analyzed the degree of myocardial infarction. As shown in Figure 5 and 6, administration of liver cell extract significantly reduced the volume fraction of myocardial infarcts compared to PBS administration at 2, 6, 12, and 24 hrs as tested by the TTC assay. It should noted that, while mice with PBS administration exhibited TTC-negative infarction across the entire left ventricular wall in the apical region at 24 hrs of myocardial ischemia, mice with administration of liver cell extract exhibited an endocardial layer of TTC-positive myocardium, which was defined as the protected endocardial layer of myocardium (Figure 5). This layer was significantly thicker in mice with administration of myocardial ischemia-conditioned liver cell extract compare to that with PBS administration.

As liver cell mobilization and disintegration occurred in myocardial ischemia, we sought to investigate whether myocardial ischemic preconditioning was required to induce the cardioprotective effect of liver cell extract. We tested the differential effects of liver cell extract derived from myocardial ischemic and sham control mice on the degree of myocardial infarction at 24 hrs. As shown in Figure 7, administration of myocardial ischemia-conditioned liver cell extract resulted in a significant reduction in myocardial infarction (TTC-negative volume fraction) compared to administration of liver cell extract from sham control mice. These observations suggested that myocardial ischemia stimulated expression of cardioprotective factors in liver cells.

We further tested the effect of liver cell extract from myocardial ischemic mice on the relative density of TUNEL-positive cells in the myocardial tissue at 24 hrs of myocardial ischemia. As show in Figure 8, the relative density of TUNEL-positive cells in the myocardial tissue of mice with administration of liver cell extract was significantly lower than that of mice with PBS administration, suggesting that administration of liver cell extract protected cells from injury in ischemic myocardium. While the level of TUNEL-positive cells was comparable between the endocardial and epicardial regions of the left ventricle in mice with PBS administration, the level in the endocardial region was significantly lower than that in the epicardial region in mice with administration of liver cell extract. These results were consistent with the observations based on the TTC assay.

4.5. Gene upregulation in myocardial ischemia-conditioned liver cells

As liver cell extract derived from myocardial ischemic mice, but not sham control mice, exerted beneficial effects on cardioprotection, myocardial ischemia possibly stimulated upregulation of cardioprotective factors. Identifying these factors has significant clinical implications. We tested differential gene expression in liver cells of myocardial ischemic and sham control mice. While there were a large number of genes that are significantly upregulated or downregulated in myocardial ischemia-conditioned liver cells, we focused on only liver cell-secreted factors for identifying cardioprotective factors as secreted factors can act on other cell types via cell membrane receptors. Gene profiling tests demonstrated that myocardial ischemia induced >3-fold upregulation of 9 genes encoding liver cell-secreted factors, including alpha-1-acid glycoprotein type 2, bone morphogenetic protein binding endothelial regulator, small-inducible cytokine B13

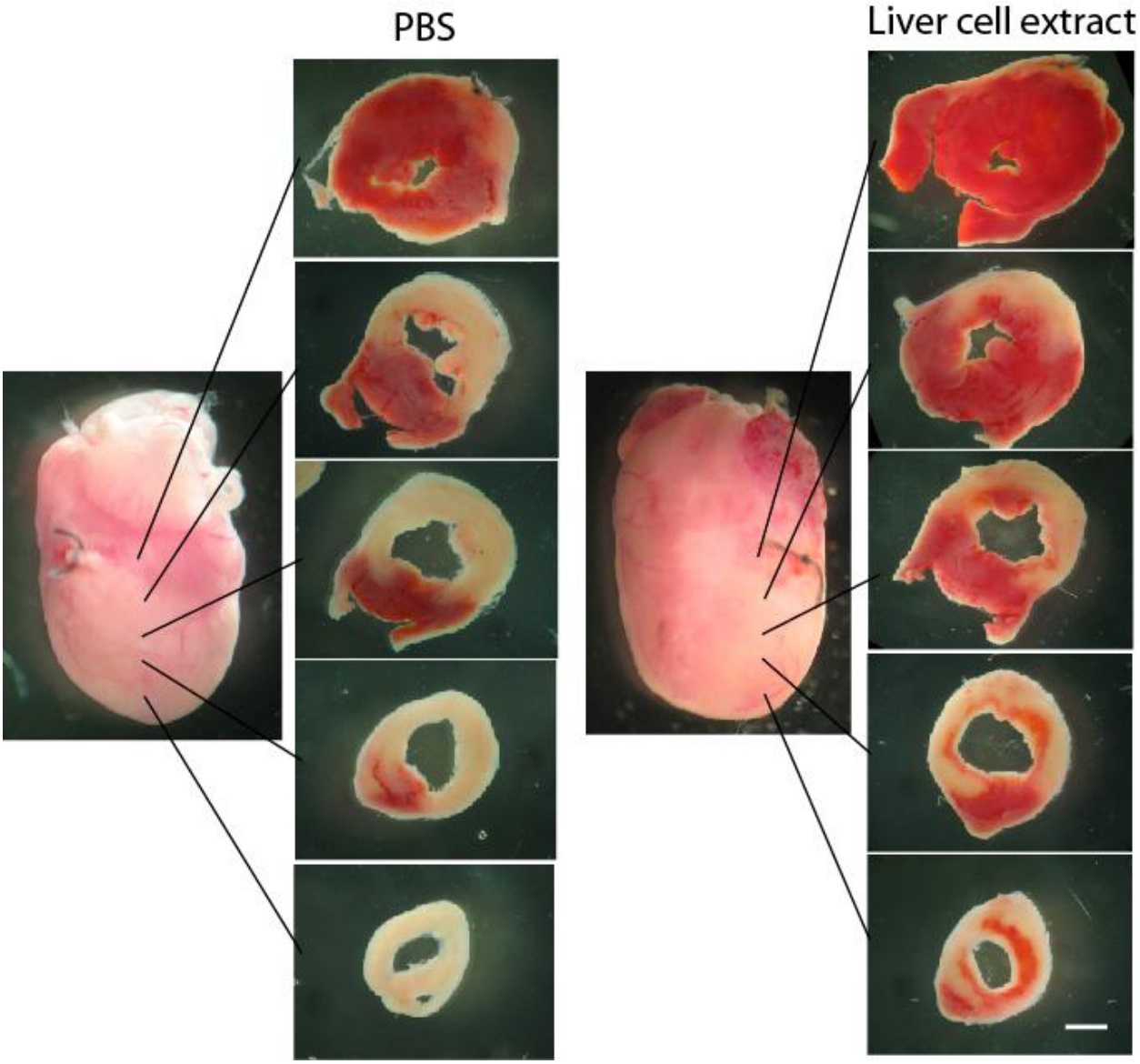

Figure 5. Alleviation of acute myocardial infarction in response to administration of myocardial ischemia-conditioned liver cell extract. Left ventricular slices (~1 mm in thickness) were prepared at 24 hrs of myocardial ischemia, and immediately incubated in 1% TTC/PBS at 37^{o} C for 30 min. The red color represents TTC-positive intact myocardium, and the white color represents myocardial infarcts. The ventricular location of each slice is indicated. Note that the area of myocardial infarcts was considerably smaller in mice with administration of liver cell extract compared to that with PBS administration. An endocardial layer of TTC-positive myocardium was found in the apical region (where maximal infarction was found) of the left ventricle with administration of liver cell extract, but not in mice with PBS administration. Scale: 1 mm for all panels.

or chemokine (C-X-C motif) ligand 13, fibroblast growth factor 21, megakaryocyte-stimulating factor, neuregulin 4, serum amyloid A (A1 and A2), and trefoil factor 3. As shown in Figure 9, the majority of these genes were upregulated during the early phase of myocardial ischemia from 0.5 to 5 days. Tff3 was the only gene that exhibited a high expression level from day 5 to 30. The expression of the beta actin gene did not change noticeably from 0.5 to 30 days in myocardial ischemia (Figure 9). The upregulated genes may include those encoding secreted cardioprotective factors.

5. DISCUSSION

5.1. Liver responses to myocardial ischemia

The liver has long been considered an organ responsible for metabolism, detoxification, bile secretion, and production of serum proteins. As a vital organ for controlling homeostasis, the liver has evolved with a unique self-protective function – complete mass regeneration in response to chemical-induced liver injury and partial hepatectomy. The liver contains a large reserve of hepatocytes and biliary epithelial cells, which are

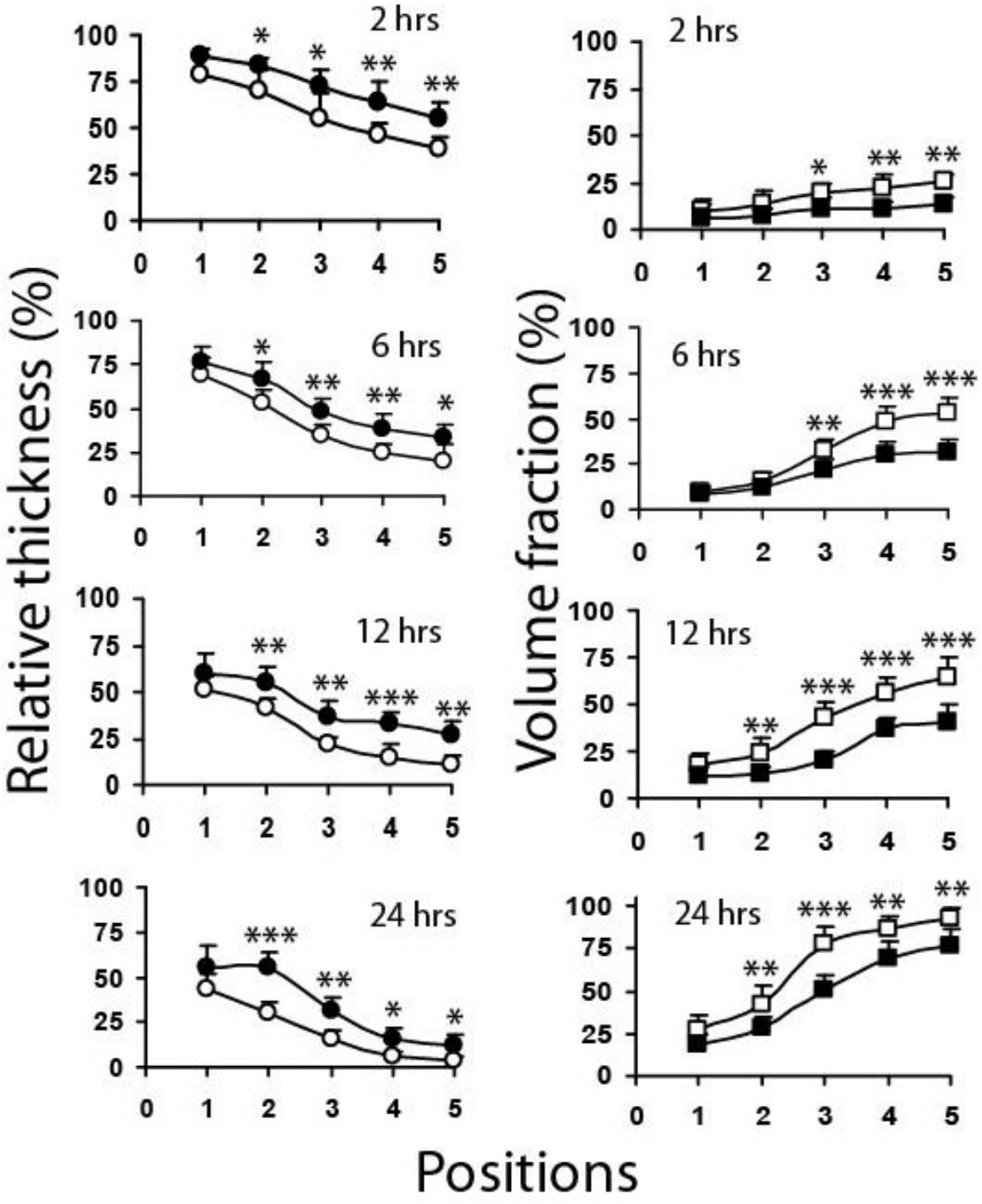

Figure 6. Graphic demonstration of alleviation of acute myocardial infarction in response to administration of myocardial ischemia-conditioned liver cell extract. The left and right columns represent changes in the thickness of the endocardial layer of TTC-positive myocardium and the infarct volume fraction, respectively, from the ligation site of the LAD coronary artery (position 1) to the apex (position 5) of the left ventricle at 2, 6, 12, and 24 hrs of myocardial ischemia. Data were measured from left ventricular specimens (examples are shown in Figure 5). The open symbols represent data from myocardial ischemic mice with PBS administration, and the solid symbols represents data from ischemic mice with administration of liver cell extract. Means and SDs are presented with a sample size 6 at each time point. * $p < 0.0.5$, ** $p < 0.0.1$, *** $p < 0.0.01$.

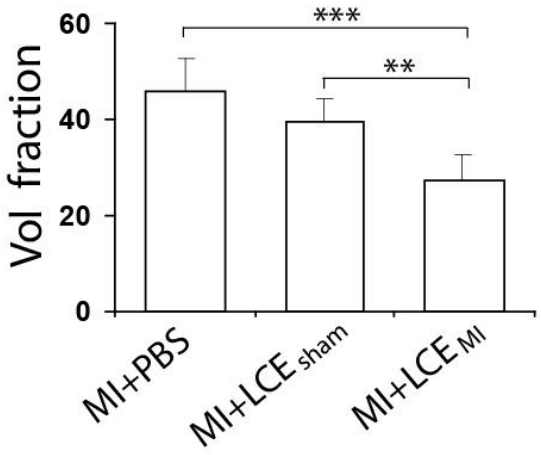

Figure 7. Differential effects of liver cell extract prepared from mice with 5-day myocardial ischemia (MI) and sham-operation on the volume (vol) fraction of myocardial infarcts at 24 hrs. MI+PBS: MI with administration of PBS. $MI+LCE_{sham}$: MI with administration of liver cell extract from mice with 5-day sham-operation. $MI+LCE_{MI}$: MI with administration of liver cell extract from mice with 5-day myocardial ischemia. Means and SDs are presented (n = 6). ** $p < 0.0.1$, *** $p < 0.0.01$.

quiescent under physiological conditions. In response to liver injury, more than 90% of these cells can be activated to proliferate, resulting in rapid liver regeneration (29-33). In contrast to the liver, the adult heart possesses a limited capacity of self-protection and regeneration in myocardial injury. Various cardioprotective mechanisms, such as activation of resident cardiac stem and progenitor cells (1-5), mobilization of bone marrow cells (6-11), and infiltration of leukocytes to ischemic myocardium (20, 21), may be involved in myocardial protection from ischemic injury. In the present investigation, we demonstrated that liver cells were able to mobilize to the circulatory system in response to myocardial ischemia. While a fraction of mobilized liver cells engrafted to the lesion of ischemic myocardium (Liu et al., unpublished data), the remaining circulating liver cells were disintegrated rapidly to release cell contents. The liver cell contents may exert beneficial effects on myocardial protection from ischemic injury, which is supported by the observation that administration of liver cell extract from 5-day myocardial ischemic mice resulted in a significant reduction in myocardial infarction. These observations suggest that, in an integral physiological system, cells within an organ as well as from different organs can work coordinately for maintaining or achieving homeostasis under injury conditions.

The liver contains several cell types, including hepatocytes, biliary epithelial cells, vascular endothelial and smooth muscle cells, bone marrow-derived cells, Kupffer cells, and Ito cells. All these cell types may be mobilized in response to myocardial ischemia. As hepatocytes and biliary epithelial cells make up about 84% of total liver cells, we focused primarily on these two cell types in this investigation. Other cell types were not examined because of the lack of identification markers.

5.2. The fate of mobilized liver cells

There are two possible fates for mobilized liver cells, including disintegration and engraftment to the peripheral tissues. In another study, we demonstrated that a fraction of mobilized liver cells engrafted to the lesion of ischemic myocardium, exerting beneficial effects on the survival and performance of ischemic myocardium (Liu et al., unpublished data). In this investigation, we tested the fate of mobilized liver cells remaining in the circulatory system. As demonstrated in the Results section, the population of circulating EYFP+ liver cells differed considerably among blood samples collected from the right and left ventricular chambers and the femoral artery and vein. A striking observation was that the population of EYFP+ liver cells in the left ventricular chamber (0.65 +/- 0.18% with reference to the total nucleated blood cells) reduced to 0.16 +/-0.09% in the femoral artery ($p < 0.0.01$) in the same individuals with 24-hr myocardial ischemia. The sharp drop of the circulating liver cell population from the left ventricle to the femoral artery suggested rapid disintegration of liver cells in the arterial system. While it remains poorly understood how circulating liver cells were disintegrated, oscillatory fluid shear stress in the arterial system may contribute to this process.

Fluid shear stress is a frictional force component that influences the structure and integrity of circulating cells. When mobilized liver cells circulate through the thoracic vena cava and the pulmonary circulation, the liver cells may maintain their integrity without significant disintegration because of the presence of relatively low and stable fluid shear stress in these vascular systems. This notion was supported by the observation that the population of EYFP+ liver cells was not significantly reduced from the right ventricle to the left ventricle. When mobilized liver cells were subject to systemic arterial blood flow, disintegration possibly took place in response to large oscillatory fluid shear stress. The sharp drop of circulating EYFP+ liver cells from the left ventricle to the femoral artery supported the role of arterial blood shear stress in liver cell disintegration.

5.3. Potential cardioprotective therapies based on liver cells

As liver cell mobilization and disintegration for cell content release are naturally occurring processes possibly contributing to myocardial protection from ischemic injury, it is conceivable that cardioprotective therapies may be developed based on myocardial ischemia-conditioned liver cells. One possible therapeutic approach is to transplant myocardial ischemia-conditioned liver cells to the circulatory system of individuals prior to or immediately following the onset of myocardial ischemia. However, it is difficult to prepare autologous or allogeneic myocardial ischemia-conditioned liver cells for clinical applications. An alternative approach is to identify and administer liver cell-derive cardioprotective factors to individuals with myocardial ischemia. As shown in this investigation, administration of liver cell extract from 5-day

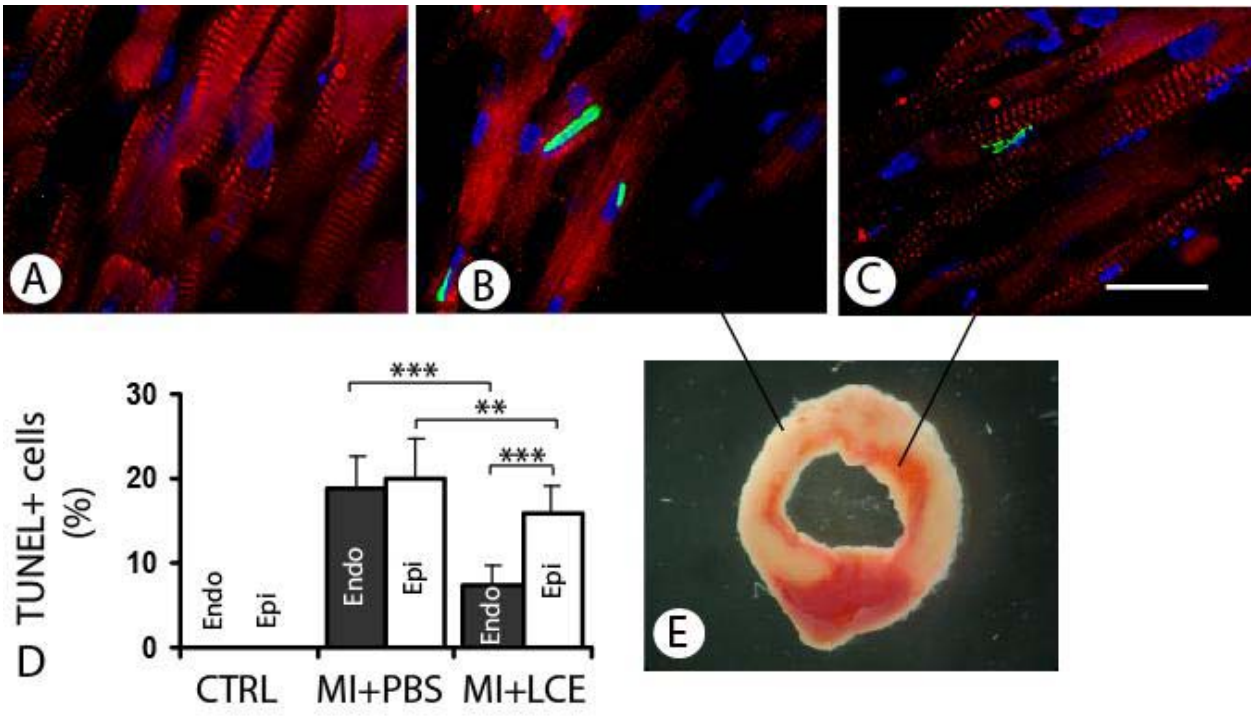

Figure 8. Reduction in the density of TUNEL+ cells in the myocardial tissue in response to administration of myocardial ischemia-conditioned liver cell extract. (A) Fluorescence micrograph showing left ventricular myocardium from a sham control mouse. (B, C) Left ventricular specimens from a mouse with 24 hr myocardial ischemia with administration of myocardial ischemia-conditioned liver cell extract, showing TUNEL+ cells (green) in a TTC-negative infarcted epicardial region (panel B) and a TTC-positive endocardial region (panel C) as indicated in panel E. For panel A through C, the red color represents cardiac troponin I, a cardiomyocyte marker, the blue color represents cell nuclei, and the scale is 10 microns. (D) Graphic demonstration of the distribution of TUNEL+ cells in the left ventricular wall with myocardial infarction at 24 hrs. Note that, while the distribution of TUNEL+ cells was relatively uniform in the left ventricular wall of myocardial ischemic mice with PBS administration, the left ventricular wall of myocardial ischemic mice with administration of liver cell extract exhibited a significantly lower density of TUNEL+ cells in the endocardial region compared to that in the epicardial region (see Discussion for interpretation). Means and SDs are presented with a sample size 6. ** $p < .001$, *** $p < 0.0.01$. (E) Micrograph showing a TTC-stained left ventricular slice, indicating locations for TUNEL tests. Endo: endocardial region. Epi: epicardial region.

myocardial ischemic mice, but not sham control mice, resulted in a significant reduction in myocardial infarction and the density of TUNEL+ cells in the myocardial tissue during the early phase of myocardial ischemia. These observations suggested that myocardial ischemia-conditioned liver cell extract possibly included factors effective for myocardial protection.

An interesting phenomenon found in this investigation was that liver cell extract protected the endocardial myocardium more effectively than the epicardial myocardium in infarcted regions of the left ventricular wall (Figure 5). This non-uniform cardioprotective effect across the left ventricular wall was possibly dependent on the oxygen gradient established from the endocardial surface (oxygenated blood in the ventricular chamber) to the epicardial surface of the ventricular wall following the ligation of the LAD coronary artery. Whereas the left ventricular myocardium of mice without administration of liver cell extract did not survive the reduced oxygen level across the entire ventricular wall in the ischemic regions, administration of liver cell extract protected the endocardial myocardium with reduced oxygen level, resulting in the survival of the endocardial layer of myocardium as shown in Figure 5. However, the cardioprotective effect of liver cell extract was no longer observed in deep regions away from the endocardial surface, likely because oxygen was reduced to a level below the limit for myocardial survival. These observations suggested that myocardial ischemia-conditioned liver cell extract was able to protect myocardium from infarction at a certain level of hypoxia, but not myocardium with severe or complete oxygen deficiency.

5.4. Potential cardioprotective factors from myocardial ischemia-conditioned liver cells

Although administration of liver cell extract represents an effective therapeutic strategy, it is desired to identify and isolate from myocardial ischemia-conditioned liver cells the factors effective for myocardial protection, which may provide essential information for understanding the mechanisms underlying liver cell-based cardioprotection and establishing cardioprotective therapies with reduced adverse effects. In this investigation, we

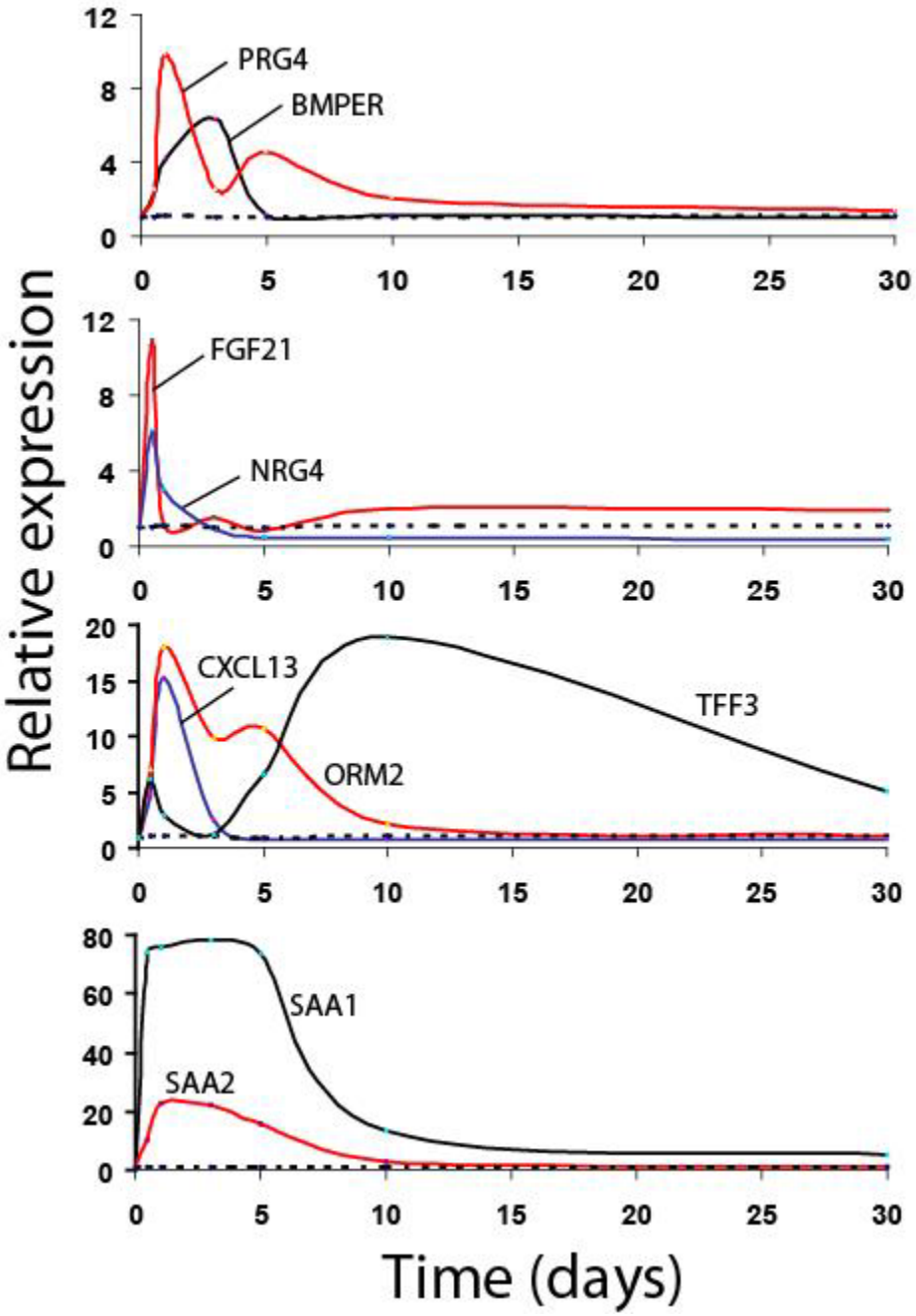

Figure 9. Time-dependent expression of genes encoding liver cell-secreted proteins in the liver cells of mice with myocardial ischemia. Data at time zero were measured from sham control mice. "Relative expression" is defined as the ratio of gene expression in myocardial ischemia to that in sham controls. The beta actin gene was used as a control gene (dotted line).

tested differential gene expression in the liver cells of myocardial ischemic and sham control mice and identified 9 upregulated genes encoding liver cell-secreted factors in response to myocardial ischemia, including alpha-1-acid glycoprotein type 2 (AGP2), bone morphogenetic protein binding endothelial regulator (BMPER), small-inducible cytokine B13 or chemokine (C-X-C motif) ligand 13 (CXCL13), fibroblast growth factor 21 (FGF21), megakaryocyte-stimulating factor (MSF), neuregulin 4, serum amyloid A (A1 and A2), and trefoil factor 3 (TFF3). While these genes have been cloned and characterized, the cardioprotective involvement of these genes have not been investigated.

Among the upregulated genes encoding liver cell-secreted factors, the AGP2 gene encodes an acute-phase

plasma protein known to mediate the activity of the immune system during acute-phase inflammatory responses (34). The BMPER gene encodes a protein reported to regulate bone morphogenetic protein responsiveness of osteoblasts and chondrocytes (35). The CXCL13 gene encodes a protein that stimulates B lymphocyte migration (36). The FGF21 gene encodes a protein reported to mediate glucose metabolism (37). The MSF gene encodes a protein that forms a large proteoglycan containing both chondroitin sulfate and keratan sulfate glycosaminoglycans synthesized by chondrocytes, functions as a boundary lubricant at the cartilage surface, and also plays a role as a growth factor acting on the primitive cells of hematopoietic and endothelial cell lineages (38). The neuregulin 4 gene encodes a protein that serves as a ligand for tyrosine kinase-type cell surface receptor HER4 and mediates cell growth (39). The serum amyloid A1 and A2 genes encode proteins that are major acute phase reactants upregulated in inflammatory disorders and are apolipoproteins for the HDL complex (40, 41). The TFF3 gene encodes a protein that protects the intestinal mucosa from injury, stabilizes the intestinal mucus layer, and regulates the healing process of intestinal epithelial cells (42). We are currently investigating the potential cardioprotective effects of the liver cell-secreted factors encoded by these genes.

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