Transplanted Fibroblast Cell Sheets Promote Migration of Hepatic Progenitor Cells in the Incised Host Liver in Allogeneic Rat Model

Short Title: Fibroblast Cell Sheets Promote Migration of Hepatic Progenitor Cells

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List of Abbreviations:

DF: Dermal Fibroblasts

H&E: Hematoxylin-Eosin

CK: Cytokeratin

AFP: α -fetoprotein

POD: Postoperative Days

pEYFP-Mito:

Plasmid Encoding Enhanced Yellow Fluorescent Protein Target to Mitochondria

PBS: phosphate-buffered saline

BD: Bile Duct

VEGF: Vascular Endothelial Growth Factor

HGF: Hepatocyte Growth Factor

Abstract

'Cell sheet engineering' has been noted as a new and valuable approach in the tissue engineering field. The objective of this study is to explore procedure to induce hepatic progenitor cells and biliary duct structures in the liver. Sprague-Dawley rat dermal fibroblast (DF) sheets were transplanted into the incised surface of the liver of F344 nude rats. In the control group, an incision was made without transplantation of the DF sheets. Bile duct (BD)-like structures and immature hepatocyte-like cells were observed in the DF sheet transplant site. These BD-like structures were cytokeratin-8 positive, while the hepatocyte-like cells were both OV-6 positive and α-fetoprotein positive as well. These proliferation and differentiation of liver progenitor cells were not influenced by hepatectomy. We also transplanted DF sheets transfected with a plasmid encoding the enhanced yellow fluorescent protein target to mitochondria (pEYFP-Mito) by electroporation, and found that the new structures were pEYFP-Mito negative. We observed new BD-like structures and immature hepatocytes after transplantation of DF sheets onto incised liver surfaces, and clarified that the origin of these BD-like structures and hepatocyte like cells was the recipient liver. The present study described an aspect of the hepatic differentiation process induced at the site of

liver injury.

1. Introduction

Orthotropic liver transplantation is the only established treatment for end-stage liver disease. The shortage of donors is a universal problem affecting this treatment, and hepatocyte transplantation or bio-artificial liver devices have been developed to address this problem (Behbahan et al., 2011; Puppi et al., 2011). However, there are still many challenges to obtain satisfying results with these therapies, because the utilization of primary hepatocytes in therapy has been hindered by their slow growth, loss of function and de-differentiation *in vitro* (Ohashi et al., 2001).

Owing to the recent development of tissue engineering technology, the liver tissue engineering field has also been expected to provide a new therapeutic modality for several kinds of liver diseases (Ohashi et al., 2007). We (co-author K.O.) previously reported that hepatic tissue sheets using isolated primary hepatocytes cultured on temperature-responsive surfaces could be transplanted into the subcutaneous space and stably persist for longer than 200 days in mice (Ohashi et al., 2007; 2011). These reports demonstrated that transplanted hepatocytes can keep functioning by interacting with interstitial cells in the subcutaneous space. These results indicated that interstitial cells may therefore have the potential to support the liver function by

being transplanted as sheets onto a dysfunctional liver.

Recently, Miyagawa et al. (Miyagawa et al., 2010) demonstrated that skeletal muscle cell sheets produced histologically normal cells, and functionally prevented the deterioration of the impaired myocardium in a swine infarction model. They showed that the smooth muscle cells existed in the lesion where they transplanted the skeletal muscle cell sheet, and the function of the ischemic heart was improved by the transplant. These results indicate that transplanted cell sheets have the potential not only for functional expression, but also for induction of organ-specific progenitor cells. However, the mechanisms underlying why smooth muscle cells grew at the site where the skeletal muscle cell sheet was transplanted are still unclear.

'Cell sheet engineering' using temperature-responsive dishes has been noted as a new approach in the tissue engineering field. Using temperature-responsive dishes, cells can be harvested by lowering the culture temperature from 37 to 20℃, thereby avoiding the use of digestive enzymes and chelating agents (Nagase et al., 2009). This technique has already been used in some clinical fields; for example, corneal epithelial sheets have been applied for the treatment of patients with unilateral or bilateral total corneal stem cell deficiencies arising from alkali burns or Stevens-Johnson syndrome (Nishida et al., 2004), layered autologous skeletal myoblast sheets have been used in the

treatment of dilated cardiomyopathy (Memon et al., 2005) and autologous mucosal epithelial cell sheets have been used for the treatment of esophageal ulceration after endoscopic submucosal dissection (Ohki et al., 2006; Takagi et al., 2010). The usefulness of cell sheet engineering has also been expected in the hepatic tissue engineering field.

Based upon these previous studies, we hypothesized that cell sheets have the potential to heal injured tissues or accelerate cell regeneration under certain circumstances. To observe the influence of transplanted cell sheets on the cut surface of the liver, we transplanted rat dermal fibroblast (DF) allograft sheets onto the liver where incision was made in recipient rats. We chose to use DFs as the donor cells, because fibroblast are easily obtained, which makes them easier to apply as a clinical cell source in the future.

2. Materials and methods

2.1. Rat dermal fibroblast sheets

Rat DFs (Cell Applications, Inc., CA, USA) were seeded at a density of $1x10^6$cells/dish onto 35mm temperature-responsive culture dishes (Cellseed, Tokyo, Japan). RDF

growth medium (Cell Applications, Inc., CA, USA) with 1% penicillin and streptomycin (Sigma- Aldrich, Inc.,MO, USA) was used to culture the cells, and the medium was replaced with fresh medium every 48-72 hours. After 10 days of culture at 37℃ in a humidified 5% CO_2 atmosphere, confluent fibroblasts on the temperature-responsive dishes were transferred to another CO_2 incubator set at 20℃ for about 30 minutes, upon which the fibroblast sheets detached spontaneously (Kobayashi et al., 2008).

2.2. Rats, transplantation of DF sheets, and surgical hepatic injury

All rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health (NIH publication 86-23 revised 1985). The animal protocol was approved by the Animal Experimentation Committee of Nagasaki University. Eight- to 10-week-old male F344 nude rats (F344/NJcl-rnu/rnu, CLEA Japan, Tokyo, Japan) were used for the experiments. While under pentobarbital (1ml/kg) anesthesia by intraperitoneal injection, the rats were laparotomized, and a 1cm long incision was made on the edge of the anterior lobe of the liver. The rats were randomly divided into two experimental groups. In the DF group, DF sheets were

transplanted onto the cut surface of the liver using a Cell Shifter (Cellseed, Tokyo, Japan) and 1 suture was made on the edge of the incision. In the control group, the 1cm long incision and 1suture were made in the same way, but no cells were transplanted. The rats were sacrificed on days 7 (DF group n=5, Control group n=5), 14 (DF group n=5, Control group n=5), and 28 (DF group n=5, Control group n=5) after the laparotomy. Five rats per group were examined at each time point. We also examined the impact of the hepatectomies in one additional groups. In the DF with hepatectomy group (DF-H, n=3), the classical Higgins-Anderson 70% hepatectomy (Melo et al., 2010) was performed on postoperative days (POD) 7 of the initial DF sheet transplantation. The rats were then sacrificed on day 7 after the hepatectomy.

2.3. Histology and immunohistochemistry

Formalin-fixed paraffin embedded (4 μm) sections were used for hematoxylin-eosin (H&E) staining, Azan-Mallory stain as a marker of collagen fibers, and immunohistochemical staining with cytokeratin (CK) 8 as a marker of rat bile duct epithelial cells (Mitaka et al. 1999), OV-6 as a marker of oval cells (rat liver progenitor cells) (Chen et al., 2012; Dunsford et al., 1989; Sell et al., 1989) and α-fetoprotein (AFP) as a marker of immature hepatocytes (Cascio et al.,1991). For CK8 and OV-6

immunostaining, a high temperature antigen unmasking technique was used. The sections were boiled in pH 9.0 EDTA buffer solution at 95℃ for 20 minutes. After 10 minutes of blocking with 0.1% H_2O_2/methanol, the sections were incubated for 30 minutes at room temperature in a humidified chamber with primary antibodies. For primary antibodies, anti-CK8 rabbit polyclonal antibodies (dilution 1:300, Abcam, MA, USA), anti-human/rabbit OV-6 mouse monoclonal antibodies (dilution 1:100, R&D Systems, Inc, MN, USA) and anti-α-fetoprotein rabbit polyclonal antibodies (dilution 1:50, EpitomicsInc, CA, USA) were used. After a 30 minute reaction with primary antibodies, slides were reacted with Histofine® Simple Stain RAT MAX PO (MULTI) (Nichirei Bioscience, Tokyo, Japan) at room temperature for 30 minutes. Slides were then visualized with H_2O_2 and DAB solution (Tamaru et al., 2004) at room temperature for 5 minutes and stained in hematoxylin for 1.5 minutes. The coverslips were mounted with 90% glycerol containing 1 mg/ml p-phenylenediamine and the slides were reviewed by an expert histologist (co-author T.K.).

2.4. Statistical analysis

To evaluate the influence of the 70% hepatectomy on the DF sheet transplantation site, we compared the total number of OV-6 positive cells, which appeared at the site in the

non-hepatectomy group (POD 14, n=3) and the hepatectomy group (n=3). Five different fields at 200x magnification were randomly selected on each slide of OV-6 immunostaining, and the total number of positive cells was counted. We performed a post-test analysis using the Mann-Whitney *U* test. We considered *P* values < 0.05 to be statistically significant.

2.5. Transfection of the plasmid encoding enhanced yellow fluorescent protein targeted to the mitochondria (pEYFP-Mito) into DF sheets

To make clarify the origin of the new structures which appeared in the site of the DF sheet transplantation, we used a plasmid encoding enhanced yellow fluorescent protein targeted to the mitochondria (pEYFP-Mito) as a marker of DF cells. We used the pEYFP-Mito vector, which encodes a fusion of the enhanced yellow fluorescent protein and the mitochondrial targeting sequence from subunit VIII of human cytochrome c oxidase (BD Biosciences, Palo Alto, CA). Plasmid DNA was prepared, and purified using a Qiagen Maxi kit (Qiagen, Hilden, Germany), and dissolved in phosphate-buffered saline (PBS) (pH 7.2) at a final concentration of 1 mg/ml. By using an electroporation method, current was applied via a NEPA21 electroporator (NEPA GENE, Chiba, Japan. Setting: 20V, 50 pulses 50ms wide in 5s interval),

pEYFP-Mito was transfected into the DFs resulting in 40-50% transfection efficiency and 90% of cell viability, which were then used to make cell sheets (YFP-DF sheets). The generation of the cell sheets and the sheet transplantation process were the same as in the previous studies, and rats were sacrificed on POD 7 (n=3). Liver tissues were fixed in OCT compound (TissueTek, Tokyo, Japan) and kept -80°C until use.

2.6. Observation of pEYFP-Mito expression

The 5μm-thick serial sections were obtained using a cryostat and fixed with 4% paraformaldehyde in PBS (pH 7.4) for 20 minutes at room temperature. After 1 minute of staining with DAPI, the slides were observed, and imaging was done using a Biorevo BZ-9000 (Keyence Co., Osaka, Japan). H&E staining and CK8 immunostaining were also performed.

3. Results and discussion

3.1. Collagen production after the DF sheet transplantation

Macroscopically, the incision site became linear and indistinguishable from the recipient original liver tissue in the control group, especially by POD 28 (Figure 1a). On the

other hand, thickened yellow-whitish granulation tissue was observed on the incision site in the DF group at POD 7, 14 and 28 (Figure 1b). Azan staining showed higher volumes collagen fiber on the thickened tissue in the DF group compared to the control group at each time point (Figure 1c, d).

3.2. Histological assessment; H&E staining

In the control group, necrotic tissue or inflammatory cell infiltration was observed on POD7, and these were gradually absorbed. On POD 28, the incision site had become linear and almost could not be distinguished from the normal liver (Figure 2a, c). On the other hand, in the DF group, the site where the DF sheets were transplanted became thicker (Figure 2b, d). Under high magnification, bile duct (BD)-like structures were observed near the recipient liver on POD 7 (Figure 3a). On POD 14, these BD-like structures more intensely formed and some immature hepatocyte-like cells were observed (Figure 3b). On POD 28, the BD-like structures had formed nearly normal BD structures (Figure 3c, d). In addition, hepatocyte-like cells were found to be more prevalent and had formed cell aggregates (Figure 3c, e). The BD-like structures and hepatocyte like cells seemed to have arisen from the recipient liver site and grew towards the thickened tissues where the DF sheets had been transplanted. This

phenomenon was observed in all individuals in the DF group.

3.3. Histological assessment; immunohistochemistry

The BD-like structures which appeared in the DF groups were CK8 positive (Figure 4a). Interestingly, around the bile duct-like structures, OV-6 positive oval shape cells were observed (Figure 4b). We suggest that the OV-6 positive oval shape cells were hepatocyte progenitor cells, which have the ability to differentiate into hepatocytes or bile duct epithelial cells. Some of the hepatocyte-like cells which formed aggregates in the site of the DF sheet transplantation demonstrated AFP positivity (Figure 4c, d).

Oval cells, which were first described by Farber in 1956, have the capacity to proliferate and differentiate into hepatocytes or biliary epithelial cells, and their existence has led to the hypothesis that there is a liver stem cell compartment that proliferates in response to severe injury (Coleman et al., 1998). Although there are no definitive markers for oval cells or liver progenitor cells, the epitope identified by the monoclonal antibody OV-6 has been found to be particularly useful in identifying early proliferation of oval cells in a rat model (Dunsford et al., 1989). In the rat liver, bile ducts, oval cells and nodular hepatocytes, as well as transitional hepatocytes, which are believed to be cells differentiating from oval cells to hepatocytes, are positive to OV-6

(Crosby et al., 1998). In our study, the BD-like structures and small cell aggregates around the BD-like structures, which appeared near the thickened tissue where the DF sheets were transplanted, were also clearly positive to OV-6. Some of the OV-6 positive BD-like structures showed CK-8 positivity. CK expression has been well studied in liver development, and they are often used as lineage markers. CK-7, 8, 18 and 19 are expressed in bile duct cells but CK7 and 19 are rarely in hepatocytes (Melo et al., 2010), thus CK-7 or 19 are usually used as a BD epithelium marker. The BD-like structures showed CK-7, 8 and 19 positive, however CK-8 staining marker was the strongest. Several previous reports confirmed that hepatoblasts express genes characteristics of hepatocytes and biliary epithelial cells, including AFP, albumin, laminin, and CK-7, 8, 18, and 19. As development proceeds, hepatoblasts become committed to either lineage and develop into mature cells with the appropriate morphology and gene expression (Marcean et al., 1994; Melo et al., 2010; Shiojiri et al., 1994). We considered the BD-like structures which appeared in the DF sheet transplanted site to still be in the developmental process and to possibly have characteristics of both BD epithelium cells and hepatocytes, however farther investigations will be needed to confirm this. We presumed the presence of these BD-like structures to demonstrate cell differentiation from hepatic progenitor cells to

bile ducts or hepatocytes.

We also considered this phenomenon equivalent to the ductular reaction which is observed in the damaged especially in the cirrhotic liver. Ductular reactions are primarily composed of oval cells which are known as “intermediate hepatobiliary cells” (Zhou et al.,2007). The mechanisms underlying these reactions are still unclear, but the proliferation of liver progenitor cells may be stimulated by damage to the liver, such as cirrhosis. This may be a reaction by the body to improve the function of the damaged liver. In our series, however, even though the recipient rats had normal liver functions, we observed ductular reactions in the DF sheet transplanted groups. There is a possibility that the transplanted DF sheets stimulated the proliferation and differentiation of liver progenitor cells.

3.4. Influence of 70% hepatectomy

The total number of OV-6 positive cells in the 5 randomly selected fields of the DF sheet transplantation site of the non-hepatectomy group (n=3) and hepatectomy group (n=3) were 361.7 and 372.3 respectively (Figure 5). The number of OV-6 positive cells in the site where the DF sheets were transplanted was not significantly different between hepatectomy group and the non-hepatectomy group, and although we expected

that stimulation from hepatic regeneration, such as that which occurs after a partial hepatectomy, would activate the proliferation and differentiation of hepatic progenitor cells, there was no statistically significant difference between the two groups. Some reports mentioned that hepatocytes are the replicating cells responsible for liver regeneration after partial hepatectomy, and that progenitor cell activation leading to lineage generation is not observed during this process (Fausto et al., 2003), although there was no experimental evidence for this inference. In the present study, we used rats which had normal liver function as recipients. It could be speculated that the proliferation and differentiation of hepatic progenitor cells may be activated by partial hepatectomy in rats which already have a dysfunctional liver, as would be the case in cirrhosis, because these livers have defects in regeneration due to their pre-existing damage (Gu et al., 2011).

3.5. Origin of the BD-like structures and hepatocyte like cells

We observed a proliferation of the pEYFP-Mito positive cells in the YFP-DFs sheet transplanted site, however, the cells that were recognized by H&E staining and CK8 immunostaining as BD-like structures and hepatocyte like cells were pEYFP-Mito negative (Figure 6a-e). These results indicate that the origin of the BD-like structures

and hepatocyte like cells was the recipient liver.

Sekiya et al. (Sekiya et al., 2011) concluded that adult mouse dermal fibroblasts converted into hepatocyte-like cells that can mature to functional hepatocytes *in vivo* under certain conditions. In this study, however, we used pEYFP-Mito transfected DF sheets as a donor and clarified that the new structures had developed from the recipient liver. Dermal fibroblasts have been shown to have the potential to produce a heterogeneous population of cytokines and growth factors, including vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor-β_1 and angiopoetin-1 (Mansbridge et al., 1999; Pinney et al., 2000). We hypothesized that the secretion of these factors from transplanted DF sheets might lead to the migration of hepatic progenitor cells from the recipient liver and accelerate their differentiation.

4. Conclusions

We demonstrated the migration of liver structures in the site where the DF sheets were transplanted onto the incised surface of the liver in rats. This is the first report indicating that viable dermal fibroblast sheets induce the migration of hepatic

progenitor cells and cause their differentiation into hepatic structures *in vivo*. Although further investigations will be needed to confirm our results and optimize the procedures, this tissue engineering technology may become a useful therapeutic method for liver diseases.

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Figure legends

Figure 1: Macroscopic changes, Azan-Mallory staining on day 28 and the effects of the DF sheet transplantation. (H&E staining) (a) A representative liver from the control group on day 28 (n=5). The incision site is indicated by a white arrow. (b) A representative liver from the DF group on day 28 (n=5). The DF sheet transplantation site is indicated by a gray arrow. (c) Azan-Mallory staining of a liver from the control group on day 28 (n=5). Collagen was stained blue. (d) Azan-Mallory staining of a liver from the DF group on day 28 (n=5). Scale bars, 100μm

Figure 2: The effects of the DF sheet transplantation (H&E staining) (a) Day 7 for the control group (n=5). (b) Day 7 for the DF group (n=5). (c) Day 28 for the control group (n=5). The incision site is indicated by a black arrow. (d) Day 28 for the DF group (n=5). Scale bars, 100μm RL; recipient liver

Figure 3: Chronological changes at the site where the DF sheet was transplanted. (H&E stains, each group n=5) (a) Bile duct (BD)-like structures are indicated with black arrows. (b) New structures are surrounded by a black line, and immature

hepatocyte-like cells are indicated by a gray arrow. (c) BD-like structures are indicated with black arrows, and immature hepatocyte-like cells are surrounded by a black dotted line. (d) Day 28 (n=5). The proliferation of BD-like structures. (e) Day 28 (n=5). The aggregation of immature hepatocyte-like cells. The immature hepatocyte-like cells are surrounded by a black dotted line. A-C; Scale bars, 80μm D, E; Scale bars, 20μm RL; recipient liver

Figure 4: The immunohistochemical findings of the site where the DF sheets were transplanted (each group n=5). (a) BD-like structures were shown to be CK8 positive. (b) Oval shape cells which existed around the BD showed OV-6 positivity (black arrow). (c, d) Some of the immature hepatocyte-like cells were shown to be AFP positive (black arrow). A; Scale bars, 20μm B; Scale bars, 10μm C, D; Scale bars, 5μm

Figure 5: A bar graph of number of OV-6 positive cells in the DF-non HT group (n=5) and DF-HT group (n=5). A P value < 0.05 was considered to be statistically significant (Mann-Whitney U test). NS; not significant.

Figure 6: The origin of the new structures. (a) H&E staining of the DF group on day 7 (n=3). (b) CK8 immunostaining of the DF group on day 7 (n=3). (c) pEYFP-Mito positive fibroblasts proliferated around the new BD-like structures. The cells which composed BD-like structures were pEYFP-Mito negative (n=3). (d) DAPI staining (n=3). Nuclei were stained blue. (e) A synthesized picture of the staining in (c) and (d). Scale bars, 80μm (the pEYFP-Mito negative area in the new structures is surrounded by a white dotted line).

Figure 1

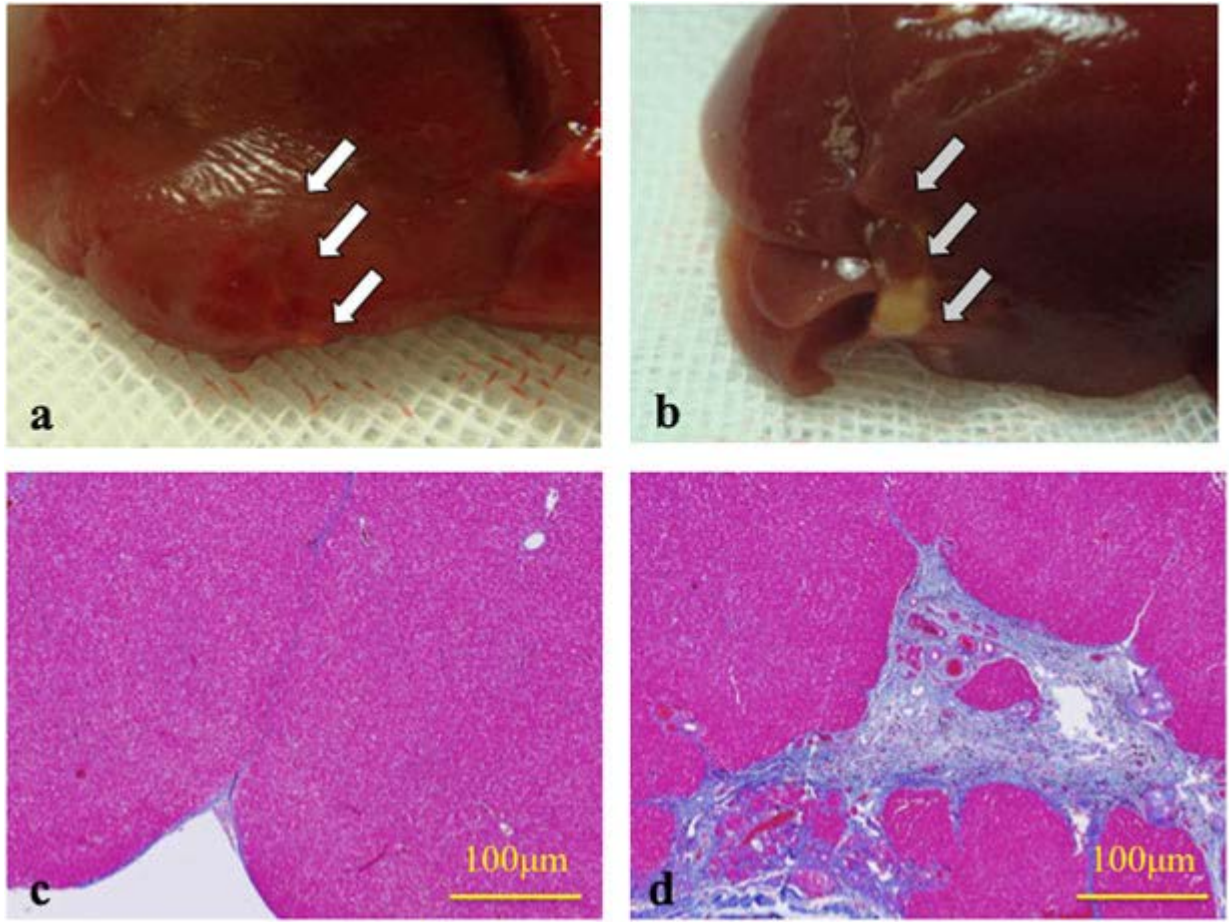

Figure 2

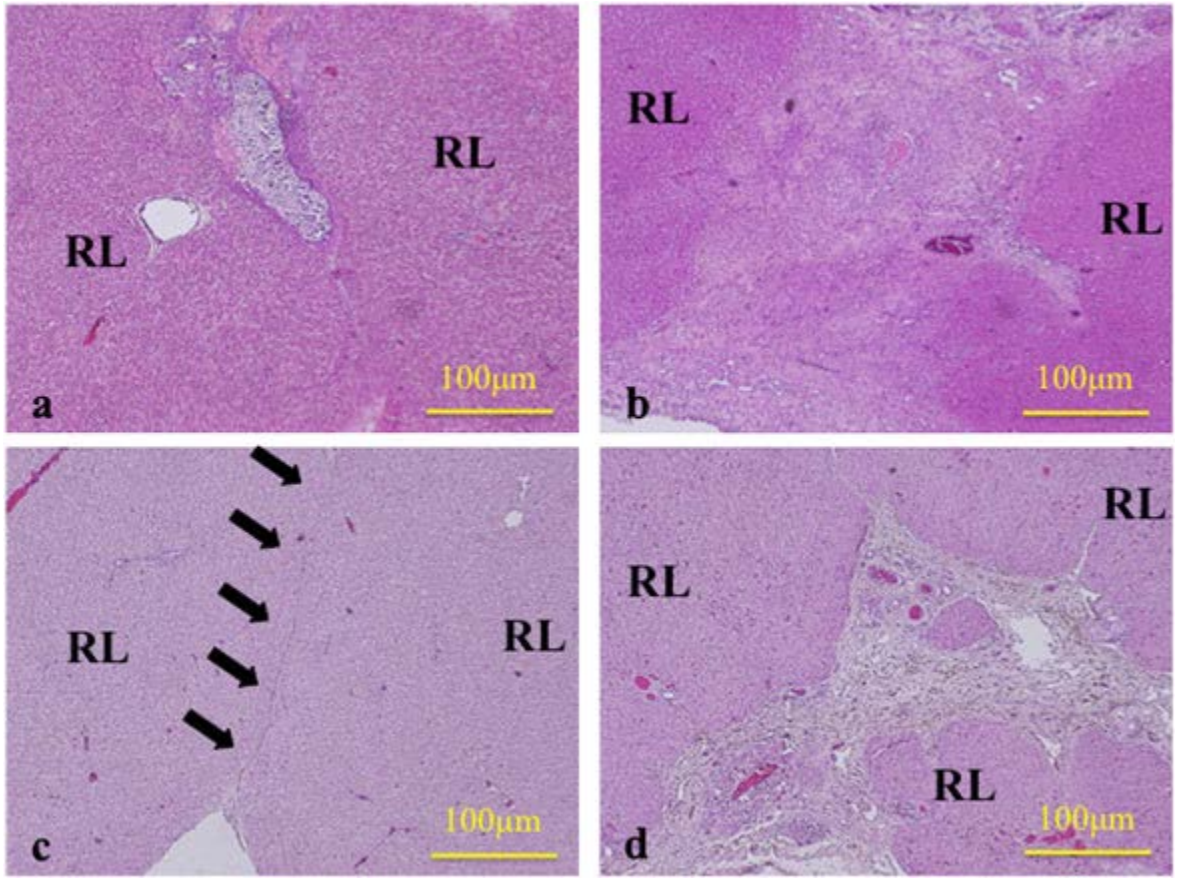

Figure 3

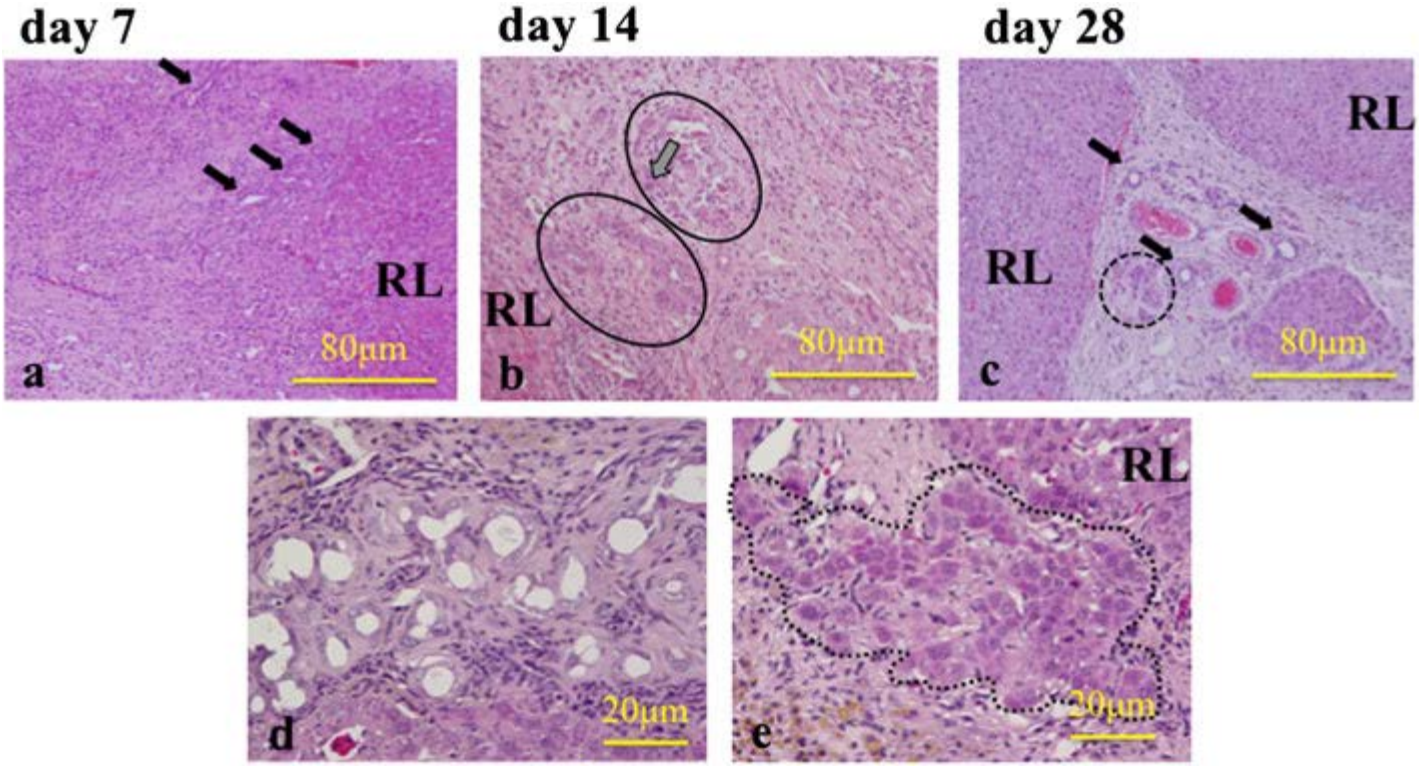

Figure 4

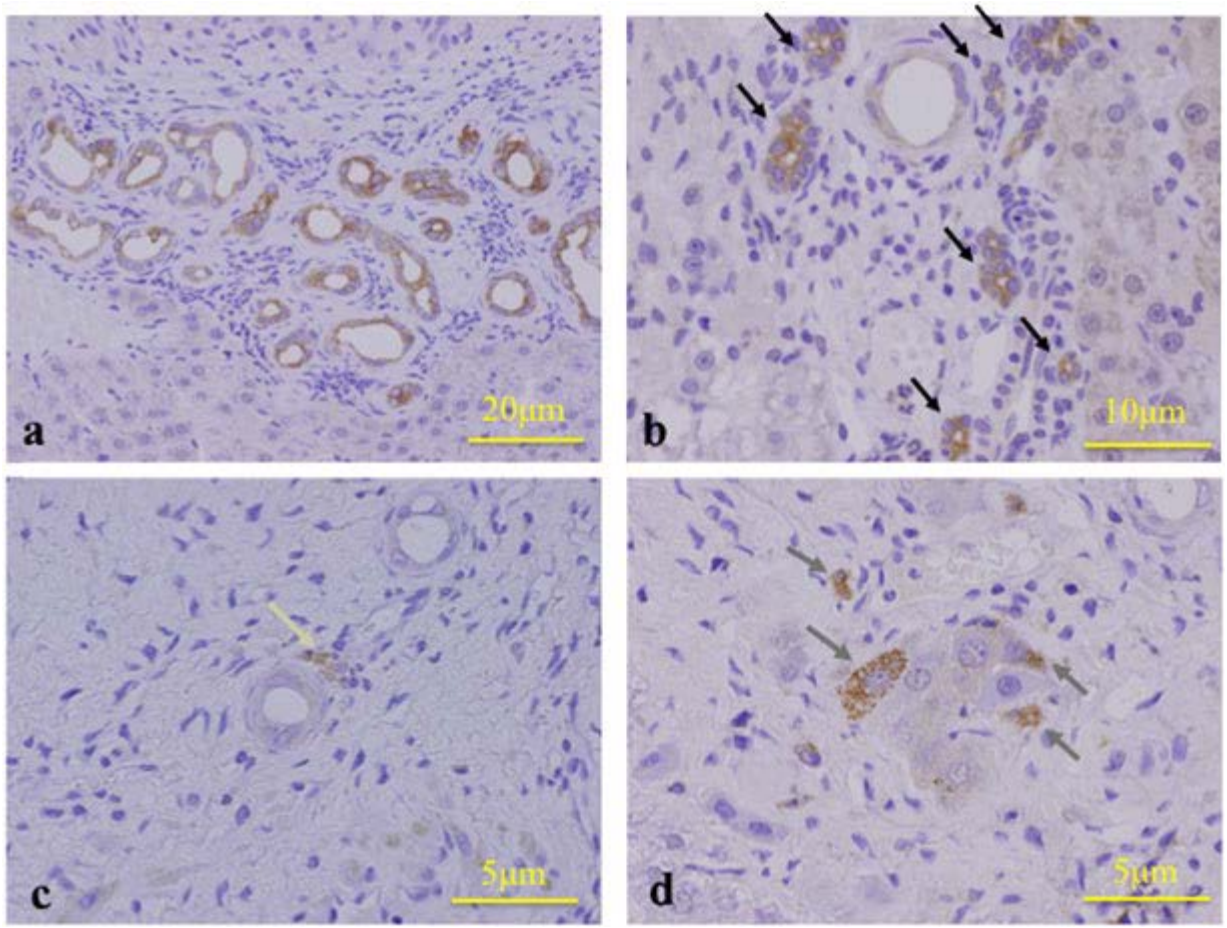

Figure 5

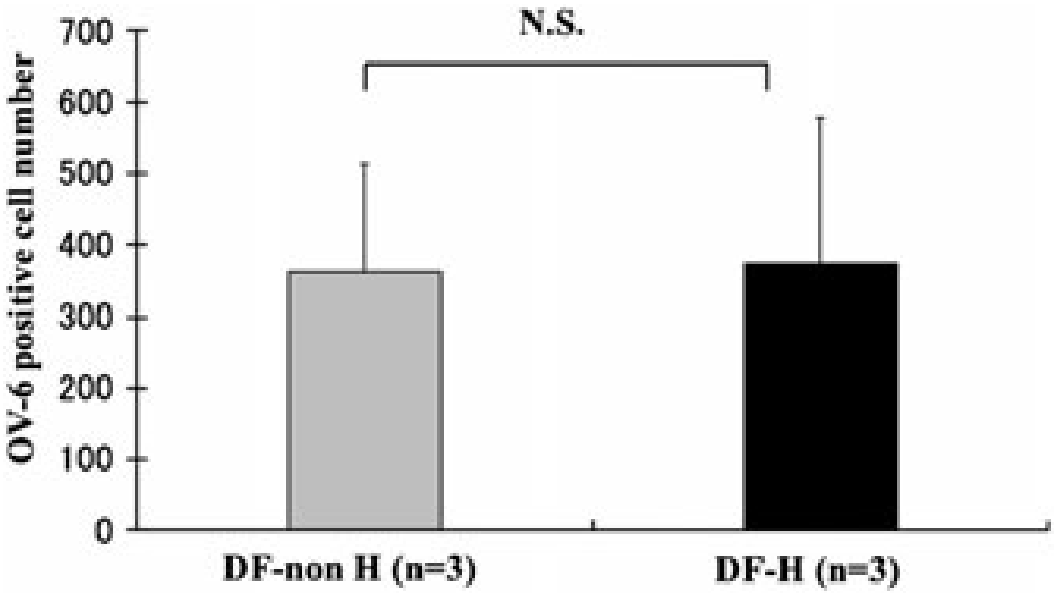
N.S.
OV-6 positive cell number
700
600
500
400
300
200
100
0
DF-non H (n=3)
DF-H (n=3)

Figure 6