Low-dose Recombinant Human Hepatocyte Growth Factor Enhances Effect of Hepatocyte Transplantation in Rats Treated with Retrorsine

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ABSTRACT

Background/Aim: The aim of this study was to regenerate transplanted hepatocytes selectively in a recipient using retrorsine and recombinant human hepatocyte growth factor (rhHGF).

Methodology: Nagase analbuminemic rats (NARs) received pretreatment with retrosine and were divided into three experimental groups. Group1: Hepatocyte transplantation (HcTx) + 50μg/kg/day rhHGF. Group2: HcTx + 250μg/kg/day rhHGF. Group3: HcTx + normal saline. The serum levels of albumin and the albumin-positive hepatocytes in the liver were investigated. The rat endogenous HGF of the rats given only retrorsine was measured.

Result: The serum albumin levels of Group1 were higher than those of Group2, while there was no significant difference between Group2 and Group3. Histological examination of Group1 and 3 showed the presence of a large number of albumin-positive hepatocytes, which frequently consisted of large clusters and occupied 53.90±2.31% and 31.25±5.36% of host liver, respectively. The liver sections of Group2 showed numerous albumin-positive hepatocytes, which were not seen as clusters. The rat endogenous HGF concentration was extremely high.

Conclusion: Low-dose rhHGF enhances the effect of HcTx under the suppressive state of proliferation of host hepatocytes. Because of the high endogenous HGF, the administration of a

high concentration of rhHGF suppressed the regenerative activity of the transplanted hepatocytes.

INTRODUCTION

Although the therapy for hepatic metabolic deficient disease still remains orthotopic liver transplantation (OLT), the donor organ shortage has been problematic. Hepatocyte transplantation (HcTx) has been proposed as an alternative therapy to OLT, but it is difficult to regenerate transplanted hepatocytes and to achieve the long term correction of a liver-related metabolic defect (1-3). Therefore, partial hepatectomy and portal branch ligation have been added as a growth stimulus for transplanted hepatocytes (4-8). However, these invasive methods are not suitable in a clinical setting for patients with congenital hepatic metabolic deficiency disease.

Human hepatocyte growth factor (HGF) was isolated and purified from the plasma of patients with fuluminant hepatic failure and was found to stimulate DNA synthesis even in adult rat hepatocytes (9, 10). A recombinant form of human hepatocyte growth factor (rhHGF) has also been developed and is as effective as the native HGF in terms of proliferative activities in rat and human hepatocytes (11). Furthermore, the cytoprotective effect of HGF through the immunoregulation action and antiapoptosis action have also been reported (12-15).

Recently, nearly total liver replacement by transplanted normal hepatocytes was reported

in rats treated with retrorsine (16-18). Retrorsine is a naturally occurring pyrrolizidine alkaloid that is taken up selectively by the liver and metabolized to bioactive compounds that alkylate DNA, which causes a long-lasting block of hepatocyte cell division (19-23). To selectively enhance the proliferation of transplanted hepatocytes, combined therapy with HGF and retrorsine seems to be an attractive strategy, although it has never been tested in vivo.

In the present study, we investigated the effect of rhHGF for HcTx in rats treated with retrorsine.

METHODOLOGY

Animals. All rats used were purchased from Japan SLC Inc.(Shizuoka, Japan) and were maintained at the Animal Center at Nagasaki University School of Medicine. Male Nagase analbuminemic rats (NARs) were used as the recipients, while male Sprague-Dawley (SD) rats were used as the hepatocyte donors. All animals were maintained in a climate-controlled (24℃) room with a 12-hour light-dark cycle and were provided tap water and standard laboratory chow *ad libitum*. All procedures were done in accordance with the guidelines of the University of Nagasaki Research Animal Resources.

Chemicals. Recombinant human hepatocyte growth factor (rhHGF) was manufactured and provided by Mitsubishi Pharma Corporation (Yokohama, Japan). Retrorsine, Collagenase typeIV, bovine serum albumin, and purified rat albumin were purchased from Sigma Chemical Co. (St. Louis, MO). Purified rabbit anti-rat albumin IgG antibodies and peroxidase conjugated rabbit anti-rat albumin were obtained from ICN Pharmaceuticals Inc.(Aurora, OH).

Hepatocyte Isolation and transplantation. Hepatocytes were isolated from SD rats according to a standard two-step collagenase perfusion technique (24). After enrichment through a Percoll gradient, hepatocytes viability was determined by the trypan blue exclusion test. The final viability of the purified hepatocytes suspension was always 90-95%. A suspension of 2×10^7 viable hepatocytes in one milliliter of saline was directly infused via the portal vein. The NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally, 2 weeks apart. Four weeks after the second injection, each animal received 2×10^7 viable hepatocytes via the portal vein. After the HcTx, the continuous systemic administration (c.s.a.) of rhHGF or normal saline was done using an osmotic pump as described below. All animals were given Cyclosporin A, at a dose of 15mg/kg, intramuscularly one day prior to HcTx and every other day until sacrifice to limit rejection of the transplanted cells.

Continuous systemic administration of rhHGF. Alzet osmotic pumps were purchased from ALZA Co.(Palo Alto, CA). The pump was connected to the catheter canulated into the jugular vein and placed in the subcutaneous layer of the anterior wall. Continuous infusion of rhHGF or normal saline was done using an osmotic pump.

Experimental designs. (Shown in Figure 1) The rats were divided into three experimental groups. The Group 1 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 50μg/kg/day of rhHGF for 7 days. The Group 2 (n=5) animals received retrorsine+HcTx followed by c.s.a. of 250μg/kg/day of rhHGF for 7 days. The Group 3 (n=5) animals received retrorsine+HcTx followed by c.s.a. of normal saline for 7 days. HGF concentration was set according to our previous study (25). Blood samples were obtained from the tail vein on days 0 (before HcTx), 3, 7, 14, 21, 28, 35, 42 and 56, respectively, for the determination of serum albumin. All animals were euthanized at the 56th day. At sacrifice, the liver specimens were fixed in 10% buffered formalin.

Serum Albumin determination Quantitative analysis of the serum levels of albumin was carried out by sandwich ELISA using rabbit anti-rat albumin IgG described previously (26).

Investigation of albumin positive hepatocytes Serial sections of the liver were immunostained

utilizing rabbit anti-rat albumin IgG antibody and an immunoperoxidase avidin-biotin peroxidase complex method. Albumin-positive area in the liver was quantitated with the help of a computer-assisted image analyzer.

Measurement of serum human HGF concentration

The human HGF concentration was measured by the Quantikine Human HGF EIA kit (R&D Systems, Inc., Minneapolis, MN) on day 3 to confirm the rise of the human HGF level for each administration of rhHGF.

Measurement of serum rat endogenous HGF concentration

The rat endogenous HGF concentration of rats given only retrorsine was measured by a rat HGF EIA kit (Institute of Immunology Co., Tokyo, Japan) on days 0 and 56 to check the host response against retrorsine (n=5).

Statistical Analysis

All data were expressed as a median and their range. Mann-Whitney's test was used for the data analysis. The differences were considered statistically significant when the p values were less than 0.05.

RESULTS

Serum albumin level

A significant increase in serum albumin levels were observed in each group following hepatocyte transplantation (v.s. base line levels; 1.26±0.04 ng/ml) (Figure 2). The serum albumin level of Group 1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference in the serum albumin level between Groups 2 and 3.

Albumin-positive hepatocytes in the liver

The liver sections obtained from Group 1 showed a markedly large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 53.90±2.31% of host liver (Figure 3a). The liver sections obtained from Group 2 showed sparse albumin-positive hepatocytes. They were uniformly distributed throughout the liver parenchyma, and were not observed as clusters (Figure 3b). The liver sections obtained from Group 3 showed a large number of cells that stained positive for albumin. They were frequently observed as large clusters and occupied 31.25±5.36% of host liver, although there were fewer clusters than in Group 1(Figure 3c). There was statistically difference in the albumin-positive hepatocyte area

between group 1 and 3($p<0.05$).

Serum human HGF concentration

The serum human HGF concentration through a c.s.a. of 50 and 250μg/kg/day of rhHGF were 5.59±1.20 and 8.44±0.51 ng/ml, respectively ($P<0.05$).

Serum endogenous rat HGF concentration

The endogenous rat HGF concentration on days 0 and 56 were 25.01±0.19 and 20.14±1.29 ng/ml, respectively, after retrorsine treatment. There was no statistically significant difference between the concentration on day 0 and that on day 56.

DISCUSSION

The present study demonstrated the enhanced effect of hepatocyte transplantation by the administration of low- dose rhHGF in rats treated with retrorsine as compared to that with high-dose rhHGF.

We transplanted hepatocytes under the host's hepatocyte proliferation potency control by retrorsine processing, and attempted selective proliferation of the transplanted hepatocytes. Pyrrolizidine alkaloids including retrorsine were studied originally because of their toxicity in

animals, particularly sheep and cattle, in which they cause both acute and chronic injury (19, 20). The administration of a high dose of pyrrolizidine alkaloid is lethal, but a low dose induces only chronic hepatic megalocytosis (27). In our study, there were no death in the rats that only received retrorsine. Pyrrolizidine alkaloids are established hepatocarcinogens (28-32). However, Laconi E et al. (16) and Laconi S et al. (18, 33) maintained that animals treated with retrorsine for a long time without neoplastic change in the host's hepatocytes and transplanted hepatocytes. To date, in order to inhibit the proliferation of endogenous hepatocytes, irradiation (34, 35) and anti-cancer drugs (36) have been used. Nevertheless, it is thought that those are not suitable in a clinical setting for co-effects to cells except hepatocytes. For the above reasons, we thought that retrorsine could likely be a drug which would the inhibit host's hepatocyte proliferation potency in clinical application.

In the present study, as a proliferation stimulus, instead of partial hepatectomy or portal branch ligation, rhHGF was administered, which was thought to be less invasive. In addition, carbon tetrachloride has been used as a proliferation stimulus for transplanted hepatocytes (37, 38), but it is not thought to be suitable for use in a clinical setting. Strain et al. (11) reported that 0.63ng/ml of rhHGF was the minimum concentration needed to stimulate hepatocyte

proliferation in a culture system. According to our previous study (25), when the rats were given a continuous systemic administration of rhHGF in a dose of 50μg/kg/day, the mean concentration of rhHGF in the portal blood was almost similar to the minimum concentration needed to stimulate hepatocyte proliferation in vitro. Based on this investigation, we considered that 50μg/kg/day should be given. We set the concentration of 250μg/kg/day as the high dose HGF, but could have set various concentrations of rhHGF between 50μg/kg/day and 250μg/kg/day, or lower concentration than 50μg/kg/day. Furthermore, we planned to administer rhHGF by continuous systemic infusion because the half-life of HGF in plasma was very short in vivo and vitro (39-42).

In the present study, the cell proliferation and function of the transplant hepatocytes of low-dose rhHGF were better than that with high-dose rhHGF. For that reason, since the rat endogenous HGF was already elevated by retrorsine treatment itself, high dose exogenous rhHGF administration was thought to counteract the proliferation of the transplanted hepatocytes. In fact, it was reported that there is a synergistic effect between rat HGF and rhHGF (40, 43). Furthermore, on the HGF concentration of 5-10ng/ml as growth stimuli, the DNA synthesis of the hepatocyte reaches a plateau, while the DNA synthesis is suppressed with

an HGF concentration of more than 10ng/ml (44). This is presumably the reason why the elevation of serum albumin was observed in Group 2 in the present study. In addition, cytoprotective effect rhHGF may be affecting the data. Whether infused rhHGF affect only transplanted hepatocytes, or only native hepatocytes injured by retrorsine, or both awaits further investigation.

In conclusion, low-dose rather than high-dose rhHGF enhances the therapeutic effect of hepatocyte transplantation under retrorsine treatment. Since we only investigated 50μg/kg/day and 250μg/kg/day, further investigation is needed using a more optimal concentration of rhHGF.

REFERENCES

1 Strom SC, Chowdhury JR, Fox IJ: Hepatocyte transplantation for the treatment of human disease. Seminars in Liver Disease 1999; 19:39-48.

2 Strom SC, Fisher RA, Rubinstein WS, et al: Transplantation of human hepatocytes. Transplant Proc 1997; 29:2103-2106.

3 Fox IJ, Roy Chowdhury JR, Kaufman SS, et al: Treatment of the Crigler-Najjar syndrome type Ⅰ with hepatocyte transplantation. N Engl J Med 1998; 338:1422-1426.

4 Raper SE, Wilson JM: Cell transplantation in the liver directed gene therapy. Cell Transplant 1993; 2:381-400.

5 Demetriou A: Experimental hepatocyte transplantation and liver gene therapy. Cell Transplant 1993; 2:401-403.

6 Eguchi S, Rozga J, Lebow LT, et al: Treatment of hypercholesterolemia in Watanabe rabbits using allogenic hepatocellular transplantation under a regeneration stimulus. Transplantation 1996; 62:588-593.

7 Moscioni AD, Rozga J, Chen SC, et al: Long-term correction of albumin levels in the Nagase analbuminemic rat: repopulation of the liver transported normal hepatocyte under a regeneration

response. Cell Transplant 1996; 5:499-503.

8 Rozga J, Jeppsson B, Bengmark S: Portal branch ligation. Reevalution of a model. Am J Patho 1986; 125:300-308.

9. Gohda E, Tsubouchi H, Nakayama H, et al: Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. Exp Cell Res 1986; 166:139-150.

10. Gohda E, Tsubouchi H, Nakayama H, et al: Purification and partial characterization of hepatocyte growth factor from plasma of a patient with fulminant hepatic failure. J Clin Invest 1988; 81:414-419.

11 Strain AJ, Ismail T, Tsubouchi H, et al: Native and recombinant human hepatocyte growth factor are highly potent promoters of DNA synthesis in both human and rat hepatocytes. J Clin Invest 1991; 87:1853-1857.

12 Ichiguchi O, Yamaguchi Y, Miyanari N, et al: Enhanced hepatocyte growth factor expression associated with prolonged rat hepatic allograft survival in recipients pretreated with donor-specific blood. Transplantation 1999; 67:115-123.

13 Uchiyama H, Yanaga K, Nishizaki T, et al: Effects of deletion variant of hepatocyte growth factor on reduced-size liver transplantation in rats. Transplantation 1999; 68:39-44.

14 Ikegami T, Nishizaki T, Uchiyama H, et al: Deletion variant of hepatocyte growth factor prolongs allograft survival after liver transplantation in rats. Surgery 1999; 125:602-607.

15 Sakakura Y, Kaibori M, Oda M, et al: Recombinant human hepatocyte growth factor protects the liver against hepatic ischemia and reperfusion injury in rats. J Surg Res 2000; 92:261-266.

16 Laconi E, Oren R, Mukhopadhyay DK, et al: Long-term, near-total liver replacement by transplantation of isolated hepatosytes in rats treated with retrorsine. Am J Patho 1998; 153:319-329.

17 Oren R, Dabeva MD, Petkov PM, et al: Restoration of serum albumin levels in Nagase analbuminemic rats by hepatocyte transplantation. Hepatology 1999; 29:75-81.

18 Laconi S, Pillai S, Porcu PP, et al: Massive liver replacement by transplanted hepatocytes in the absence of exogenous growth stimuli in rats treated with retrorsine. Am J Patho 2001; 158:771-777.

19 McLean EK: The toxic actions of pyrrolizidine (senecio) alkaloids. Pharmacol Rev 1970; 22:429-483.

20 Mattocks AR: Chemistry and toxicology of pyrrolizidine alkaloids. Orlando, FL, Academic Press, 1986.

21 Laconi S, Curreli F, Diana S, et al. Liver regeneration in response to partial hepatectomy in rats treated with retrorsine: a kinetic study. J Hepatol 1999; 31:1069-1074.

22 Picard C, Lambotte L, Starkel P, et al: Retrorsine: a kinetic study of its influence on rat liver regeneration in the portal branch ligation model. J Hepatol 2003; 39:99-105.

23 Pitzalis S, Doratiotto S, Greco M, et al. Cyclin D1 is up-regulated in hepatocytes in vivo following cell-cycle block induced by retrorsine. J Hepatol 2005; 43:485-490.

24 Seglen PO: Preparation of isolated rat liver cells. Methods Cell Biol 1976; 13:29-83.

25 Sugiyama N, Eguchi S, Kawazoe Y, et al: Intraportal administration of low-dose recombinant human hepatocyte growth factor enhances effects of hepatocellular transplantation. Hepato-Gastroenterology 2000; 47:1245-1249.

26 Holzman MD, Rozga J, Neuzil DF, et al: Selective intraportal hepatocyte transplantation in analbuminemic and Gunn rats. Transplantation 1993; 55:1213-1219.

27 Jago MV: The development of the hepatic megalocytosis of chronic pyrrolizidine alkaloid poisoning. Am J Pathol 1969; 56:405-422.

28 Svoboda DJ, Reddy JK: Malignant tumors in rats given lasiocarpine. Cancer Res 1972;

32:908-912.

29 Rao MS, Reddy JK: Malignant neoplasms in rats fed lasiocarpine. Br J Cancer 1987; 37:289-293.

30 Schoental R, Magee PN: Further observation on the subacute and chronic liver change in rats after a single dose of various pyrrolizidine (senecio) alkaloids. J Pathol Bacteriol 1959; 78:471-482.

31 Chou MW, Yan J, Nichols J, et al. Correlation of DNA adduct formation and riddelline-induced liver tumorigenesis in F344 rats and B6C3F1 mice. Cancer Lett 2003; 193:119-125.

32 Chan PC, Haseman JK, Prejean JD, et al: Toxicity and carcinogenicity of riddelline in rats and mice. Toxicol Lett 2003; 144:295-311.

33 Laconi S, Montisci S, Doratiotto S, et al: Liver repopulation by transplanted hepatocytes and risk of hepatocellular carcinoma. Transplantation 2006; 82:1319-1323.

34 Guha C, Sharma A, Gupta S, et al: Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res 1999; 59:5871-5874.

35. Malhi H, Gorla GR, Irani AN, et al: Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci USA 2002; 99:13114-13119.

36 Kim KS, Joseph B, Inada M, et al: Regulation of hepatocyte engraftment and proliferation after cytotoxic drug-induced perturbation. Transplantation 2005; 80: 653-659.

37 Dahlke MH, Popp FC, Bahlmann FH, et al: Liver regeneration in a retrorsine/CCl4-induced acute liver failure model: do bone marrow-derived cell contribute? J Hepatol 2003; 39:365-373.

38 Guo D, Fu T, Nelson JA, et al: Liver repopulation after cell transplantation in mice treated with retrorsine and carbon tetrachloride. Transplantation 2002; 73:1818-1824.

39 Naka D, Shimomura T, Yoshiyama Y, et al: Internalization and degradation of hepatocyte growth factor in hepatocytes with down-regulation of the receptor / c-Met. FEBS Lett 1993; 329:147-152.

40 Fujiwara K, Nagoshi S, Ohno A, et al: Stimulation of liver growth by exogenous human hepatocyte growth factor in normal and partially hepatectomized rats. Hepatology 1993; 18:1443-1449.

41 Ishii T, Sato M, Sudo K, et al: Hepatocyte growth factor stimulates liver regeneration and elevates blood protein level in normal and partially hepatectomized rats. J Biochem (Tokyo) 1995; 117:1105-1112.

42 Liu KX, Kato Y, Narukawa M, et al: Importance of the liver in plasma clearance of hepatocyte growth factor in rats. Am J Physiol 1992; 263:G642-649.

43 Ishiki Y, Ohnishi H, Muto Y, et al: Direct evidence that hepatocyte growth factor is a hepatototropic factor for liver regeneration and has a potent antihepatitis effect in vivo. Hepatology 1992; 16:1227-1235.

44 Gomez-Lechon MJ, Castelli J, Guillen I, et al: Effects of hepatocyte growth factor on the growth and metabolism of human hepatocytes in primary culture. Hepatology 1995; 21:1248-1254.

FIGURE LEGENDS

Figure 1

NARs were given two injections of retrorsine, 30mg/kg each, intraperitoneally (i.p.), 2 weeks apart. Four weeks after the second injection, each animal received HcTx. After the HcTx, c.s.a. of rhHGF or normal saline was done.

Figure 2

A significant increase in the serum albumin level was observed in each group following hepatocyte transplantation. The serum albumin level of Group 1 was statistically higher than that of Group 2 at each point except for day 14 ($p<0.05$). There was no statistically significant difference between Groups 2 and 3.

Figure 3

(a) A large number of albumin-positive hepatocytes can be seen in Group 1, frequently as large clusters, which occupied 53.90±2.31% of host liver.

(b) There are numerous albumin-positive hepatocytes in Group 2, which are distributed

throrough the liver parenchyma, but not seen as clusters.

(c) A large number of albumin-positive hepatocytes can be seen in Group 3. They are frequently seen as large clusters, which occupied 31.25±5.36% of host liver.

Figure 1

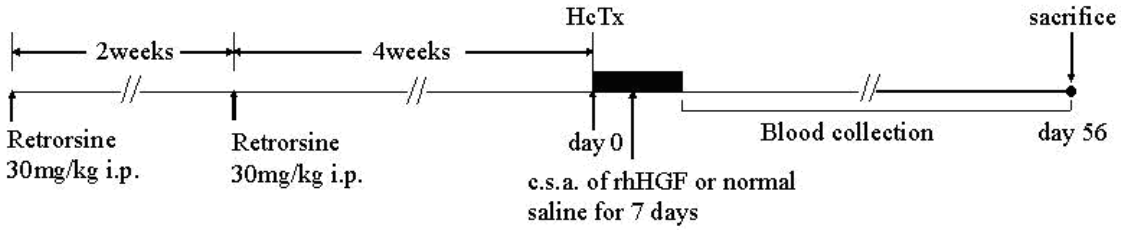

Figure 2

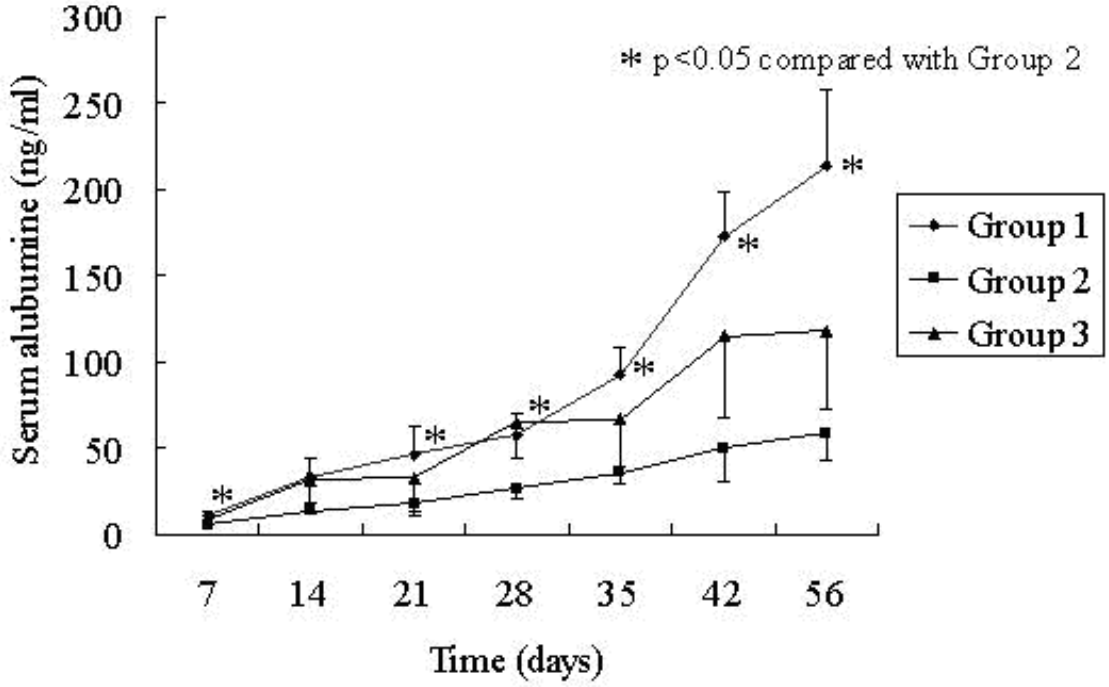

Figure 3

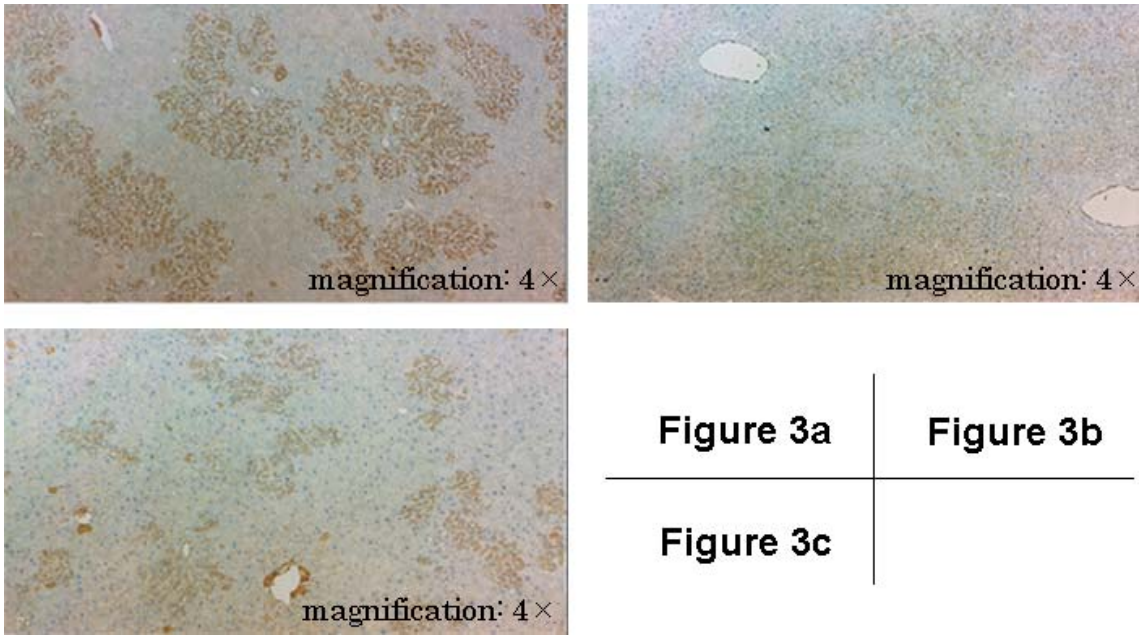
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Figure 3a
Figure 3b
Figure 3c