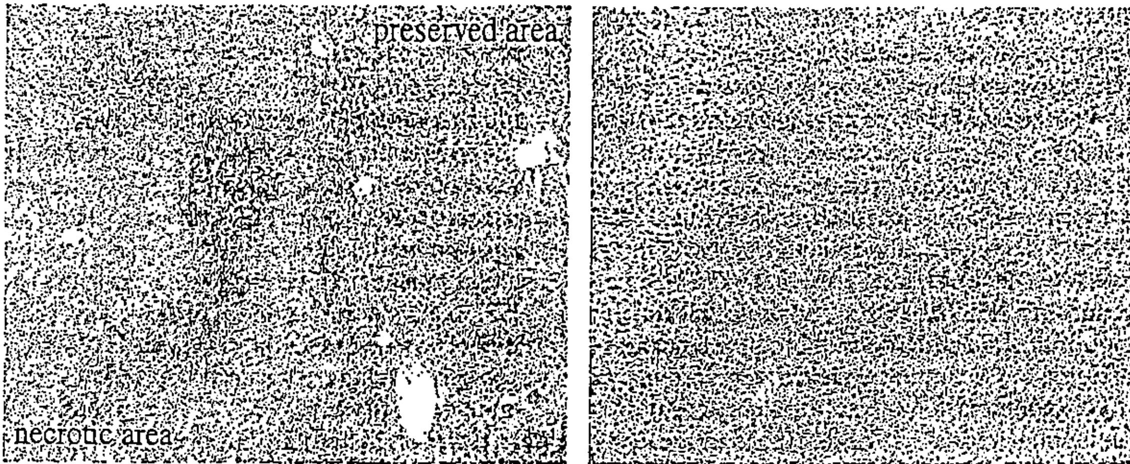

FIGURE 4 Hematoxylin and eosin staining of liver tissue. (A) After reperfusion in group C. Congestion and necrosis were noted. (B) After reperfusion in group HS. Almost normal histology was preserved. (Original magnification × 10).

(A)

(B)

(C)

FIGURE 5 Immunohistochemical staining of 8-OHdG. (A) After a 30 min ischemia and reperfusion in group C. (B) After a 30 min ischemia and reperfusion in group HS. (C) Untreated livers. (Original magnification × 20).

(A)

(B)

(C)

FIGURE 6 Immunohistochemical staining of HNE-modified proteins. (A) After a 30 min ischemia and reperfusion in group C (B) After a 30 min ischemia and reperfusion in group HS. (C) Untreated livers. (Original magnification × 20).

candidates. However, it is still difficult to nominate the proper proteins at present because the liver is a special organ producing numerous kinds of proteins. In spite of the difficulty in characterizing the nature of proteins, the present study demonstrates that these two key proteins were produced significantly less in group HS. As molecular chaperones, HSP72 may bind to denatured proteins and prevent their irreversible denaturation.[17] In light of the known functions of HSP72, other possible mechanisms behind reduction of HNE-modified proteins are: (1) suppression of the initiation and/or the propagation of ROS synthesis;[33] (2) suppression of the lipid peroxidation cascade;[34] and (3) degradation of HNE-modified proteins by proteasome.[35] Further studies are necessary to clarify the effect of HSPC on the decreased production of HNE-modified proteins.

Reperfusion of ischemic tissue causes an immediate increase in DNA damage, that includes base modifications and strand breaks. DNA is an important target for ROS attacks in cells. More than 100 different oxidative modifications have been observed in DNA after ROS attacks.[36,37] However, to date, only a few base modifications have been established as biomarkers. Among them, oxidative C-8 adduct of guanine has been the one most frequently studied. A sensitive and easy procedure for measurement using HPLC and electrochemical detector has been established;[38] and therefore, 8-OHdG has become the most popular indicator of oxidative DNA damage *in vivo* and *in vitro*.[39,40]

Our previous data have demonstrated that the production of 8-OHdG after reperfusion clearly reflects the intensity of an ischemic load to the liver and is a good marker in hepatic IR injury.[19] In this study, it was shown that a substantial level of 8-OHdG was produced in the nuclei even in untreated livers, and HSPC significantly suppressed the production of 8-OHdG during IR of the liver (Table II). Concerning the detection of 8-OHdG in untreated livers, artificial oxidation of DNA during sample preparation may be considered. However, it is very likely that to a certain extent normal metabolic pathways do generate ROS, resulting in oxidative DNA damage.

Immunohistochemical staining of liver tissue with HSP72 antibody revealed nuclear accumulation of HSP72 in hepatocytes (Fig. 2). However, it has not been determined what signals regulate this nuclear accumulation of HSP72, or why HSP72 moves to nuclei in response to a wide variety of stressors. Abe *et al.* suggested that HSP72 protein protected DNA from further damage or facilitated the repair of DNA through some unknown mechanism in nuclei.[41] In addition, HSP72 may act indirectly in reducing oxidative DNA damage. Actually, some current studies suggest that HSP72 may have the ability to decrease ROS injury by facilitating the translocation of nuclear factor-kappa B (NF-κB),[42] inhibiting the binding activity of NF-κB to DNA,[43] and attenuating the leukocyte-endothelial cell interaction.[44]

Our data do not allow us to ascribe the benefit of HSPC only to the induction of HSP72 because HSPC induces several other HSPs and free radical scavengers like superoxide dismutase[45] as well. However, other studies provide direct evidence for a significant role of HSP72 in cytoprotection. For example, Musche *et al.* demonstrated that the role of HSP72 in cellular protection using transfection of full-length complementary DNA of HSP72.[46] In addition, Feinstein *et al.* reported that antisense oligonucleotides against HSP72 blunted heat shock inhibition of nitric oxide synthase-2 activity in astroglial cells.[47]

In conclusion, the present study demonstrated that HSPC attenuated the formation of 8-OHdG in DNA, suppressed the production of HNE-modified proteins in cytosol, and improved liver-related enzyme release, energy metabolisms and survival rates after IR in rat livers.

Acknowledgments

This work was partly supported by the Grant-in-Aid of Japan Society for the Promotion of Science, Tokyo Japan (No 12470258, No 13557105).

References

[1] Fan, C., Zwacka, R.M. and Engelhardt, J.F. (1999) "Therapeutic approaches for ischemia/reperfusion injury in the liver", *J. Mol. Med.* 77, 577–592.

[2] Kurokawa, T., Nonami, T., Harada, A., Nakao, A. and Takagi, H. (1996) "Mechanism and prevention of ischemia-reperfusion injury of the liver", *Semin. Surg. Oncol.* 12, 179–182.

[3] Rauen, U., Viebahn, R., Lauchart, W. and de Groot, H. (1994) "The potential role of reactive oxygen species in liver ischemia/reperfusion injury following liver surgery", *Hepatogastroenterol.* 41, 333–336.

[4] Komatsu, H., Koo, A., Ghadishah, E., Zeng, H., Kuhlenkamp, J.F., Inoue, M., Guth, P.H. and Kaplowits, N. (1992) "Neutrophile accmulation in ischemic reperfused rat liver: evidence for a role for superoxide free radicals", *Am. J. Physiol.* 262, G669–G676.

[5] Vollmer, B., Menger, M.D., Glasz, J., Leiderer, R. and Messmer, K. (1994) "Impact of leukocyte–endothelial cell interaction in hepatic ischemia–reperfusion injury", *Am. J. Physiol.* 267, G786–G793.

[6] Sandstrom, P.A., Tebbey, P.W., Cleave, S.V. and Buttke, T.M. (1994) "Lipid hydroperoxides induce apoptosis in T cells displaying a HIV-associated glutathione peroxidase deficiency", *J. Biol. Chem.* 269, 798–801.

[7] Stadtman, E.R. (1992) "Protein oxidation and aging", *Science* 257, 1220–1224.

[8] Romani, F., Vertemati, M., Frangi, M., Aseni, P., Monti, R., Codeghini, A. and Belli, L. (1998) "Effect of superoxide dismutase on liver ischemia–reperfusion injury in the rat: a biochemical monitoring", *Eur. Surg. Res.* 20, 335–340.

[9] Mizoe, A., Kondo, S., Azuma, T., Fujioka, H., Tanaka, K., Hashida, M. and Kanematsu, T. (1997) "Preventive effects of superoxide dismutase derivatives modified with monosaccharides on reperfusion injury in rat liver transplantation", *J. Surg. Res.* 73, 160–165.

[10] Zwacka, R.M., Zhou, W., Zhang, Y., Darby, C.J., Dudus, L., Halldorson, J., Oberley, L. and Engelhardt, J.F. (1998) "Redox gene therapy for ischemia/reperfusion injury of the liver reduces AP1 and NF-κB activation", *Nat. Med.* 4, 698–704.

[11] Kondo, T., Terajima, H., Todoroki, T., Hirano, T., Ito, Y., Usia, T. and Messmer, K. (1999) "Prevention of hepatic ischemia–reperfusion injury by SOD-DIVEMA conjugate", *J. Surg. Res.* 85, 26–36.

[12] Yabe, Y., Koyama, Y., Nishikawa, M., Takakura, Y. and Hashida, M. (1999) "Hepatocyte-specific distribution of catalase and its inhibitory effect on hepatic ischemia/reperfusion injury in mice", *Free Radic. Res.* 30, 265–274.

[13] Marubayashi, S., Dohi, K., Ochi, K. and Kawasaki, T. (1986) "Role of free radicals in ischemic rat liver cell injury: prevention of damage by alfa-tocopherol administration", *Surgery* 99, 184–192.

[14] Saad, S., Kanai, M., Awane, M., Yamamoto, Y., Morimoto, T., Isselhard, W., Minor, T., Troidl, H., Ozawa, K. and Yamaoka, Y. (1995) "Protective effect of heat shock pretreatment with heat shock protein induction before hepatic warm ischemic injury caused by Pringle's maneuver", *Surgery* 118, 510–516.

[15] Shimabukurro, T., Yamamoto, Y., Kume, M., Kimoto, S., Okamoto, R., Morimoto, T. and Yamaoka, Y. (1998) "Induction of heat shock response: effect on the rat liver with carbon tetrachloride-induced fibrosis from ischemia–reperfusion injury", *World J. Surg.* 22, 464–469.

[16] Yamagami, K., Yamamoto, Y., Kume, M., Kimoto, S., Yamamoto, H., Ozaki, N., Yamamoto, M., Shimahara, Y., Toyokuni, S. and Yamaoka, Y. (1998) "Heat shock preconditioning ameliorates liver injury following normothermic ischemia–reperfusion in steatotic rat livers", *J. Surg. Res.* 79, 47–53.

[17] Lindquist, S. and Craig, E.A. (1988) "The heat shock proteins", *Annu. Rev. Genet.* 22, 631–677.

[18] Halliwell, B. and Gutteridge, J.M. (1984) "Oxygen toxicity, oxygen radicals, transition metals and disease", *Biochem. J.* 219, 1–14.

[19] Yamagami, K., Yamamoto, Y., Kume, M., Ishikawa, Y., Yamaoka, Y., Hiai, H. and Toyokuni, S. (2000) "Formation of 8-hydroxy-2′-deoxyguanosine and 4-hydroxy-2-nonenal-modified proteins in rat liver after ischemia–reperfusion: distinct localization of the two oxidatively modified products", *Antioxid. Redox Signal.* 2, 127–136.

[20] Esterbauer, H., Schaur, R.J. and Zollner, H. (1991) "Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes", *Free Radic. Biol. Med.* 11, 81–128.

[21] Toyokuni, S., Luo, X.P., Tanaka, T., Uchida, K., Hiai, H. and Lehotay, D.C. (1997) "Induction of a wide range of C2–12 aldehydes and a renal carcinogen, ferric nitrilotriacetate", *Free Radic. Biol. Med.* 22, 1019–1027.

[22] Kimoto, S., Yamamoto, Y., Yamagami, K., Ishikawa, Y., Kume, M., Yamamoto, H., Ozaki, N. and Yamaoka, Y. (2000) "The augmentative effect of repeated heat shock protein 72 and on ischemic tolerance in rat liver tissue", *Int. J. Hyperth.* 16, 247–261.

[23] Lamprecht, W. and Trautschold, I. (1974) "Adenosine-5′-triphosphate: determination with hexokinase and glucose-6-phosphate dehydrogenase", In: Bergmeyer, H.U., ed, Methods of Enzymatic Analysis (Academic Press, London and New York), pp 2101–2110.

[24] Jaworek, D., Gruber, W. and Bergmeyer, H.U. (1974) "Adenosine-5′-diphosphate and adenosine-5′-monophosphate", In: Bergmeyer, H.U., ed, Methods of Enzymatic Analysis (Academic Press, London and New York), pp 2127–2131.

[25] Nakae, D., Mizumoto, Y., Kobayashi, E., Noguchi, O. and Konishi, Y. (1995) "Improved genomic/nuclear DNA extraction for 8-hydroxydeoxy-guanosine analysis of small amounts of rat liver tissue", *Cancer Lett.* 97, 233–239.

[26] Toyokuni, S., Tanaka, T., Hattori, Y., Nishiyama, Y., Ochi, H., Hiai, H., Uchida, K. and Osawa, T. (1997) "Quantitative immunohistochemical determination of 8-hydroxy-2′-deoxyguanosine by a monoclonal antibody N45.1: its application to ferric nitrlotriacetate-induced renal carcinogenesis model", *Lab. Investig.* 76, 365–374.

[27] Luna, L.G. (1968) Manual of Histological Staining Methods of the Armed Forces Institute of Pathology (McGraw-Hill, New York), p 5.

[28] Toyokuni, S., Uchida, K., Okamoto, K., Hattori-Nakakuki, Y., Hiai, H. and Stadtman, E.R. (1994) "Formation of 4-hydroxy-2-nonnenal-modified proteins in the renal proximal tubules of rats treated with a renal carcinogen, ferric nitrilotriacetate", *Proc. Natl Acad. Sci. USA* 91, 2616–2620.

[29] Kume, M., Yamamoto, Y., Saad, S., Gomi, T., Kimoto, S., Shimabukuro, T., Yagi, T., Nakagami, M., Takada, Y., Morimoto, T. and Yamaoka, Y. (1996) "Ischemic preconditioning of the liver in rats: implications of heat shock protein induction to increase tolerance of ischemia–reperfusion injury", *J. Lab. Clin. Med.* 128, 251–258.

[30] Valen, G., Kawakami, T., Tahepold, P., Dumitrescu, A., Lowbeer, C. and Vaage, J. (2000) "Glucocoluticoid pretreatment protects cardiac function and induces cardiac heat shock protein 72", *Am. J. Physiol.-Heart Circul. Physiol.* 279, H836–H843.

[31] Javadpour, M., Kelly, C.J., Chen, G., Stokes, K., Leahy, A. and Bouchier-Hayes, D.J. (1998) "Thermotolerance induces heat shock protein 72 statement and protects against ischemia–reperfusion-induced lung injury", *Br. J. Surg.* 85, 943–946.

[32] Toyokuni, S. (1999) "Reactive oxygen species-induced molecular damage and its application in pathology", *Pathol. Int.* 49, 91–102.

[33] Callahan, T.E., Marins, J., Welch, W.J. and Horn, J.K. (1999) "Heat shock attenuates oxidation and accelerates apoptosis in human neutrophils", *J. Surg. Res.* 85, 317–322.

[34] Jacquier-Sarlin, M.R., Fuller, K., Dinh-Xuan, A.T., Richard, M.J. and Polla, B.S. (1994) "Protective effects of HSP70 in inflammation", *Experimentia* 50, 1031–1038.

[35] Beck, F.X., Neuhofer, W. and Muller, E. (2000) "Molecular chaperons in the kidney: distribution, putative roles, and regulation", *Am. J. Physiol.-Renal Physiol.* 279, F203–F215.

[36] Dizdaroglu, M. (1994) "Chemical determination of oxidative DNA damage by gas chromatography–mass spectrometry", *Meth. Enzymol.* 234, 3–16.

[37] Cadet, J., Ravanat, J.L., Buchko, G.W., Yeo, H.C. and Ames, B.N. (1994) "Singlet oxygen DNA damage: chromatographic and mass spectrometric analysis of damage products", *Meth. Enzymol.* 234, 79–88.

[38] Floyd, R.A., Watson, J.J., Wong, P.K., Altmiller, D.H. and Rickard, R.C. (1986) "Hydroxyl free radical adducts of deoxyguanosine: sensitive detection and mechanisms of formation", *Free Radic. Res. Commun.* 1, 163–172.

[39] Cao, W., Carney, J.M., Duchon, A., Floyd, A. and Chevion, M. (1988) "Oxygen free radical involvement in ischemia and reperfusion injury in post-ischemic tissues", *Neurosci. Lett.* 88, 233–238.

[40] Roy, D., Floyd, R. and Liehr, J. (1991) "Elevated 8-hydroxydeoxyguanosine levels in DNA of diethylstibestrol-treated Syrian hamsters: covalent DNA damage by free radicals generated by redox cycling of diethylstibestrol", *Cancer Res.* 51, 3882–3885.

[41] Abe, T., Konishi, T., Hirano, T., Kasai, H., Shimizu, K., Kashimura, M. and Higashi, K. (1995) "Possible correlation between DNA damage induced by hydrogen peroxide and translocation of heat shock 70 protein into the nucleus", *Biochem. Biophys. Res. Commun.* 206, 548–555.

[42] Yoo, C.G., Lee, S., Lee, C.K., Kim, Y.W., Han, S.K. and Shim, Y.S. (2000) "Anti-inflammatory effect of heat shock protein induction is related to stabilization of IkB* through preventing IkB kinase activation in respiratory epithelial cells", *J. Immunol.* 164, 5416–5423.

[43] Pritts, T.A., Wang, Q., Sun, X., Moon, M.R., Fischer, D.R., Fischer, J.E., Wong, H.R. and Hasselgren, P.O. (2000) "Induction of the stress response *in vivo* decreases nuclear factor-kappa B activation in jejunal mucosa of endotoxemic mice", *Arch. Surg.* 135, 860–866.

[44] Terajima, H., Kondo, T., Enders, G., Hammer, C., Thiery, J., Yamamoto, Y., Yamaoka, Y. and Messmer, K. (1999) "Reduction of hepatic microcirculatory failure caused by normothermic ischemia/reperfusion-induced injury by means of heat shock preconditioning", *Shock* 12, 329–334.

[45] Yamashita, N., Hoshida, S., Taniguchi, N., Kuzuya, T. and Hori, M. (1998) "Whole-body hyperthermia provides biphasic cardioprotection against ischemia/reperfusion injury in the rat", *Circulation* 98, 1412–1414.

[46] Musch, M.W., Cianco, M.J., Sarge, K. and Chang, E.B. (1997) "Induction of heat shock protein 70 protects intestinal epithelial IEC-18 cells from oxidant and thermal injury", *Am. J. Physiol.* 270, C429–C436.

[47] Feinstein, D.L., Gelea, E., Aquino, D.A., Li, G.C., Xu, H. and Reis, D.J. (1996) "Heat shock protein 70 suppresses astroglial-indusible nitric oxide synthase statement by decreasing NF-κB activation", *J. Biol. Chem.* 271, 17724–17732.

ELSEVIER

Journal of Hepatology 36 (2002) 653–659

Journal of Hepatology

www.elsevier.com/locate/jhep

Contribution of bone marrow cells to liver regeneration after partial hepatectomy in mice

Hideaki Fujii[1,*], Tetsuro Hirose[1,2], Shoshiro Oe[1], Kentaro Yasuchika[1], Hisaya Azuma[1], Takahisa Fujikawa[1], Masaya Nagao[3], Yoshio Yamaoka[1]

[1]*Department of Gastroenterological Surgery, Kyoto University Graduate School of Medicine, 54, Kawara-cho, Shogoin, Sakyo-ku, Kyoto 606-8507, Japan*
[2]*Department of Regenerative Medicine, Institute for Frontier Medical Sciences Kyoto University, Kyoto, Japan*
[3]*Department of Biosignals and Response, Kyoto University Graduate School of Biostudies, Kyoto, Japan*

See Editorial, pages 695–697

Background/Aims: **We examined whether bone marrow (BM) cells can commit to liver-consisting cells during liver regeneration after partial hepatectomy, using mice transplanted with green fluorescent protein (GFP) positive BM from GFP transgenic mice.**

Methods: **Partial hepatectomy or sham operation was performed. Lineage marker analysis of GFP positive liver cells was by immunostaining and flow cytometry. DiI-labeled acetylated low-density lipoprotein uptake or microsphere phagocytosis was examined in vitro. Lineage marker expression in BM and peripheral blood (PB) cells, and the vascular endothelial growth factor (VEGF) concentration in the liver were also examined.**

Results: **In hepatectomized mice, significantly more GFP positive cells participated in liver sinusoid than in sham-operated mice, expressing CD31 but not albumin. The percentage of cells that incorporated acetylated low-density lipoprotein but not microspheres was 69.5 ± 3.4%, while 28.3 ± 2.6% incorporated both, revealing sinusoidal endothelial and Kupffer cells, respectively. Increased expression of the CD31 and CD16/CD32 on GFP positive liver cells was also detected. The elevation of the VEGF concentration during liver regeneration and the increase in the CD34 and Flk-1 expression in the liver, BM, and PB cells suggested endothelial progenitor cell mobilization.**

Conclusions: **GFP cell-marking provided direct evidence of the BM cells participation in liver regeneration after hepatectomy, where the majority was committed to sinusoidal endothelial cells probably through endothelial progenitor cell mobilization.**

© 2002 European Association for the Study of the Liver. Published by Elsevier Science B.V. All rights reserved.

Keywords: **Liver regeneration; Bone marrow; Endothelial progenitor cell; Transplantation; Green fluorescent protein**

1. Introduction

Recent advances in stem cell research have revealed that bone marrow (BM) cells including hematopoietic stem cells can differentiate into cells in other lineages that consist of various tissues [1,2]. Related to the liver, a close relation between liver-consisting cells and hematopoietic cells has been reported. Fetal liver is a major site of hematopoiesis [3]. In addition, even in adults, the liver can support hematopoiesis in some situations [4]. In this respect, the possibility of BM cells committing to liver cells has been explored in several studies. One study reported that hepatic 'oval cells', which emerged in the injured liver, were of BM origin by in situ hybridization [5]. In another study, BM cells identified using the β-galactosidase gene-marking method were confirmed to become hepatocytes in fumarylacetoacetate hydrolase (FAH) deficient mice [6]. However, the behavior of BM cells after partial hepatectomy has not been investigated, although liver regeneration is an attractive biological problem long discussed and is often experienced clinically. To date, liver regeneration after partial hepatectomy has long been believed to depend only on

Received 24 May 2001; received in revised form 23 January 2002; accepted 28 January 2002

* Corresponding author. Tel.: +81-75-751-3242; fax: +81-75-751-4263.
E-mail address: hideaki@kuhp.kyoto-u.ac.jp (H. Fujii).

0168-8278/02/$20.00 © 2002 European Association for the Study of the Liver. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-8278(02)00043-0

replication of mature cells and not on stem characteristic cells [7]. Therefore, we intended to clarify if BM cells commit to liver cells during liver regeneration induced after partial hepatectomy. To directly evaluate and also to quantitatively analyze the BM cell commitment to liver cells, we employed a cell-marking method using green fluorescent protein (GFP) transgenic mice.

2. Materials and methods

2.1. Animals

C57BL/6J mice were provided from Nippon SLC (Hamamatsu, Japan). The syngenic GFP transgenic mice used were as described previously [8], which express GFP under CAG promoter. They were maintained according to the Animal Protection Guidelines of Kyoto University.

2.2. Reagents and antibodies

Phycoerythrin (PE)-conjugated anti-mouse platelet-endothelial cell adhesion molecule-1 (PECAM-1/CD31), CD16/CD32, and Flk-1, PE-conjugated rat IgG, and biotin-conjugated anti-rat IgG2a were obtained from BD PharMingen (San Diego, CA, USA). PE-conjugated anti-mouse CD34 was obtained from Caltag Lab. (Burlingame, CA, USA). Anti-mouse albumin was from Bethyl Lab. Inc., (Montgomery, TX, USA). Biotin-conjugated anti-goat IgG was from Chemicon International, Inc., (Temecula, CA, USA).

2.3. BM transplantation and 70% partial hepatectomy

GFP mice (8 weeks old, male) were sacrificed and their BM cells were obtained by flushing the tibiae and femora with RPMI 1640 medium. GFP positive BM cells were purified using Fluorescent Activated Cell Sorting (FACSVantage™, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA). A total of 1×10^5 sorted GFP positive BM cells were transplanted via a tail vein into age-matched recipient C57BL/6J mice that received whole body irradiation (12 Gy). Mice were then maintained with free access to food and water for 4 weeks until 70% partial hepatectomy ($n = 4$) or sham operation ($n = 4$) was performed under anesthesia with sodium pentobarbital.

2.4. Fluorescent microscopy and immunohistochemical staining

To evaluate cells included in the regenerating liver, passing hematopoietic cells were excluded by flushing with phosphate buffered saline (PBS) from the portal vein when liver specimens were collected 4 weeks after the operation. They were dissected to small pieces (2 or 3 mm thick), fixed with 4% paraformaldehyde for 7 h, and sectioned at 7-μm thickness. Either GFP expression or antigen stained by fluorescence-conjugated antibody was visualized by fluorescent microscopy Axiovert® 135 (Carl Zeiss, Oberkochen, Germany). In case of immunohistochemistry, liver tissues were incubated with anti-mouse PECAM-1 or albumin antibody for 16 h at 4°C after 2 h blocking followed by 1 h incubation with respective second antibodies at room temperature. Signals were amplified using streptavidin–horseradish peroxidase conjugates and tyramide–biotin (Life Science Products, Inc., Boston, MA, USA). Visualization was done using streptavidin conjugated Texas Red®-X (Molecular Probes, Inc., Eugene, OR, USA).

2.5. DiI-Ac LDL incorporation and phagocytosis

Four weeks after the operation, the livers of mice were perfused in situ via the inferior vena cava after ligation of the inferior vena cava above the diaphragm and dissection of the portal vein. Perfusion was performed using 50 ml Ca^{2+}-free Hanks' balanced solution containing 0.5 mM EGTA and 10 mM HEPES and then with 0.05% collagenase solution. After full digestion, cells were suspended in Hanks' balanced solution and filtered through a 71-μm nylon mesh to eliminate non-digested tissues. Parenchymal and non-parenchymal cells were separated using a low speed centrifuge method [9].

Freshly isolated non-parenchymal cells were cultured in Dulbecco's modified essential medium containing 10% fetal bovine serum on a collagen type I coated plate (Asahi Techno Glass Corp., Tokyo, Japan) at a density of 5×10^4 cells/cm^2. Two hours later, the cells were washed once to eliminate non-adherent cells. From 16 h after the beginning of culture, the cells were incubated with acetylated low-density lipoprotein labeled with dioctadecyl tetramethyl indocarbocyanine perchlorate (DiI-AcLDL) (Molecular Probes, Inc., Eugene, OR, USA) for another 8 h. In addition, 1.75-μm Bright Blue-latex microspheres (Fluoresbrite™ caboxylate microsphere, Polyscience, Inc., Warrington, PA, USA) were incubated for the last 1 h. DiI-AcLDL incorporation represents the character of the sinusoidal endothelial cells (SECs) or Kupffer cells, since DiI-AcLDL is incorporated in an LDL receptor-dependent manner, and the existence of LDL receptors is a marker for SECs or Kupffer cells [10]. In contrast, phagocytosis of latex microspheres solely represents Kupffer cell characterization [11]. Incorporation of DiI-AcLDL and latex microspheres among GFP positive cells were counted under fluorescent microscopy and analyzed.

2.6. Flow cytometry

After dissociation of the liver 4 weeks after the operation, 1×10^4 liver cells were analyzed by flow cytometry (FACSCalibur™, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) for PECAM-1, CD16/CD32, CD34, and Flk-1 expression. In addition, CD34 and Flk-1 expressions were examined in 1×10^4 BM and peripheral blood (PB) cells obtained from C57BL/6J mice 5 days after either partial hepatectomy or sham operation. The cells were washed twice and incubated at 4°C for 30 min with each monoclonal antibody. They were then rinsed with PBS twice and analyzed. Propidium iodide positive dead cells were excluded from the analysis. Negative controls were carried out using PE-conjugated rat IgG.

2.7. Vascular endothelial growth factor (VEGF) levels in liver tissue

Mouse VEGF was quantified using a commercially available immunoassay kit (AN'ALYZA™; Genzyme Techne, Minneapolis, MN, USA). Liver tissue lysates were prepared at the indicated times after partial hepatectomy or sham operation by homogenization in lysis buffer (150 mM NaCl, 10 mM Tris (pH 7.5) supplemented with proteinase inhibitors (0.11 TIU aprotinin, 1 mM phenylmethylsulfonyl fluoride). Tissue lysates were adjusted to contain similar protein concentrations (5 mg/ml). Detection of VEGF was performed according to the supplier's recommendation.

2.8. Statistical analysis

All experiments were performed in four replications except in VEGF enzyme linked immunosorbent assay (ELISA) experiments in which results were determined in three replications. Statistical analysis was performed by Student's t test. $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Fluorescent microscopic analysis of liver tissues

In 70% partial hepatectomized mice, a large number of GFP positive cells were detected in the liver surrounding the sinusoidal space (Fig. 1A). In contrast, only a few GFP

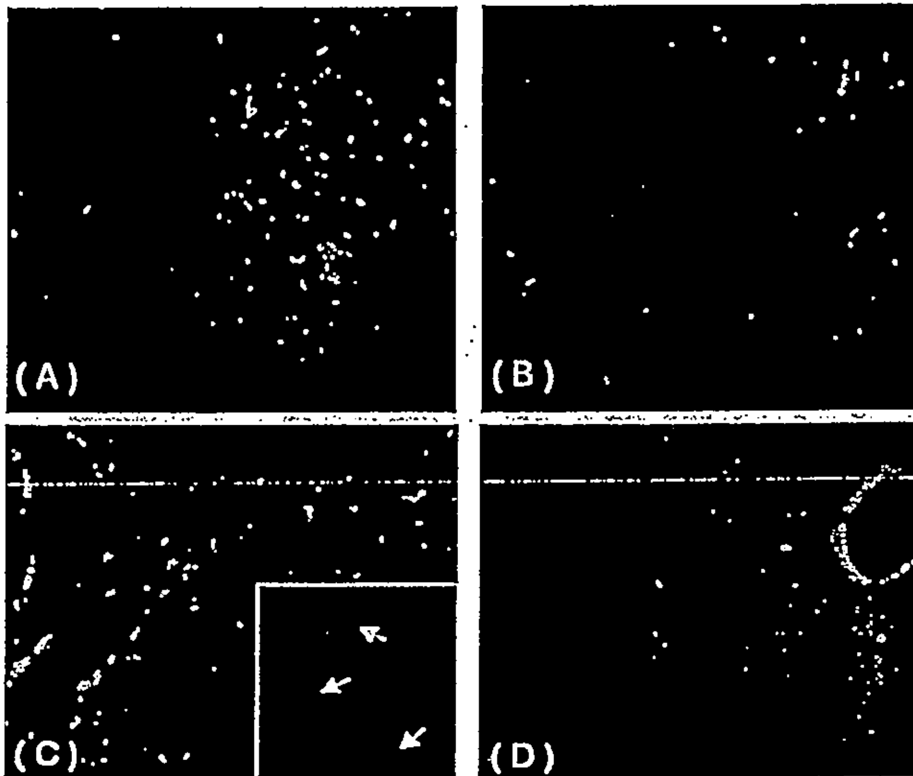

Fig. 1. Fluorescent microscopy of liver specimens after partial hepatectomy (A) or after sham operation (B). Fluorescent immunohistochemical staining against mouse PECAM-1 (C) and albumin (D) were merged with the GFP expression pattern. Arrows in the right lower quadrant square in C indicate both PECAM-1 and GFP positive cells. (A–D; original magnification ×200, the right lower quadrants square in C; original magnification ×400).

positive cells were detected in sham-operated mice (Fig. 1B). This localization of GFP positive cells suggested that these cells mainly consisted of non-parenchymal cells.

3.2. *Immunohistochemical detection of marker genes*

To further characterize GFP positive cells participating in regenerating liver, either PECAM-1 or albumin was immunostained (Fig. 1C, D). PECAM-1 is considered a marker for endothelial or Kupffer cells [4,12,13], while albumin is an endodermal marker [14]. Most GFP positive cells co-expressed PECAM-1 (Fig. 1C), but not albumin (Fig. 1D). This finding, together with the localization of GFP positive cells, suggested that the majority of GFP cells were either SECs or Kupffer cells.

3.3. *In vitro analysis for receptor mediated AcLDL uptake and phagocytosis*

To discriminate between SECs and Kupffer cells among GFP positive cells, we further confirmed their characterization by evaluating their incorporation of DiI-AcLDL and latex microspheres. When cultured in vitro, the majority of GFP positive cells in hepatectomized liver showed an endothelial morphology as shown in Fig. 2A. In addition, 94 ± 0.9% of GFP positive cells showed DiI-AcLDL incorporation, and 28.3 ± 2.6% of the cells showed phagocytosis of microspheres (Fig. 2B). Considering that DiI-AcLDL intake represents SEC or Kupffer cell characters and that phagocytosis solely represents Kupffer cell characters, at least about 70% of GFP cells can be considered SECs (Fig. 2C).

3.4. *FACS analysis of liver cells*

We confirmed the above results by evaluating surface marker expressions on GFP positive cells. Supporting the fluorescent microscopic observations of liver tissues, GFP positive cells in the liver appeared only in the non-parenchymal fraction (Fig. 3A), and 11.9 ± 2.3% of non-parenchymal cells become GFP positive in partial hepatectomized mice. The GFP positive cell number in partial hepatectomized mice was significantly higher than in sham-operated mice, again supporting the fluorescent microscopic results (Fig. 3B). Furthermore, we examined PECAM-1 and CD16/CD32 expression on GFP positive cells. CD16/CD32, a monocyte lineage marker was used for Kupffer cell characterization, since Kupffer cells were reported to differentiate from monocytes [15]. As expected, PECAM-1 and CD16/CD32 expression on GFP positive cells was significantly higher in partial hepatectomized mice compared with that in sham-operated mice (185.5 ± 32.9 vs. 986.5 ± 129.7, and 222.5 ± 17.8 vs. 326.8 ± 35.1 cells/1×10^4 cells,

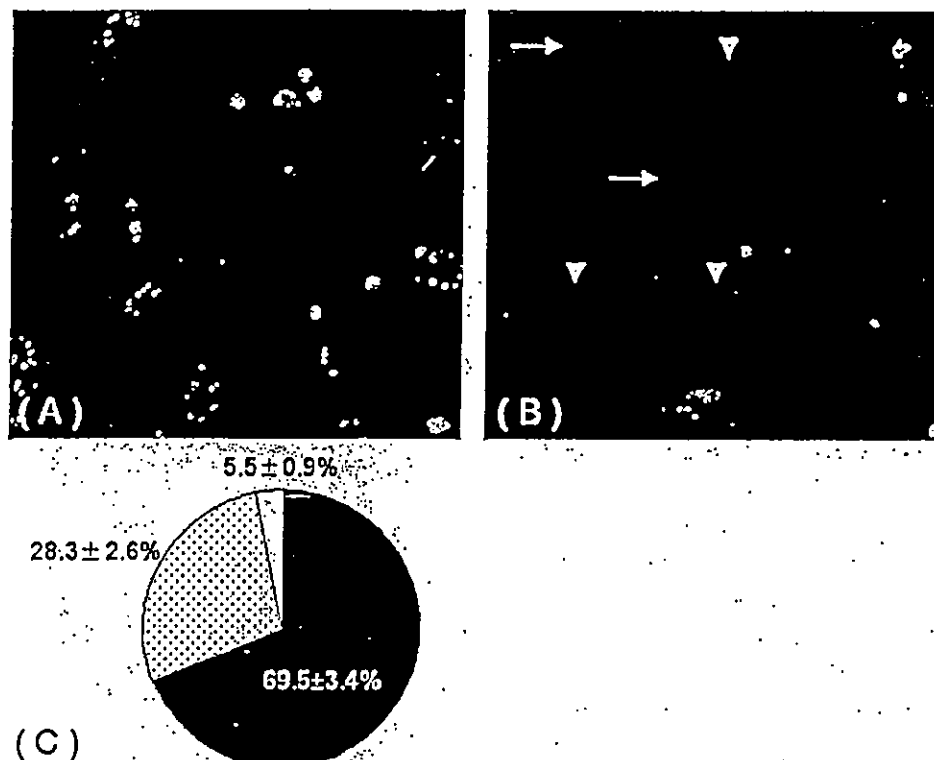

Fig. 2. (A) Phase contrast microscopy of isolated non-parenchymal cells from partial hepatectomized mice. (B) Merged image for GFP, DiI-AcLDL, and latex microspheres. Arrows indicate Kupffer cells positive for GFP incorporating both DiI-AcLDL and latex microspheres. Arrowheads indicate SECs positive for GFP incorporating DiI-AcLDL but not for latex microspheres. (A,B; original magnification ×200). These findings are summarized in C. Black, dotted, and white areas indicate the percentages of SECs, Kupffer cells, and the unidentified cell population among GFP positive cells, respectively ($n = 4$).

respectively, $P < 0.05$) (Fig. 3C). SECs became dominant in GFP positive cells after partial hepatectomy (48.2 ± 5.5 vs. 70.1 ± 4.9%, $P < 0.05$), which is compatible with the in vitro assay findings. Thus, the present findings suggest that BM cells can differentiate not only to Kupffer cells but also to hepatic SECs, and hepatic SECs differentiation is strongly induced after partial hepatectomy.

3.5. Expression of CD34 and Flk-1 in liver, BM, and PB cells

Since GFP positive cells were revealed to be SECs and Kupffer cells in regenerating liver, it is suggested that these cells have migrated from BM to the liver during regeneration. To support this hypothesis, we focused on the CD34, Flk-1 positive cell population since recent studies showed that CD34 positive, Flk-1 positive endothelial progenitor cells (EPCs) are mobilized from BM and incorporated into sites of vascular disorders for neovascularization [16–19]. In fact, CD34 and Flk-1 positive cells in the GFP positive population in regenerating livers were significantly higher compared with corresponding cells in the sham-operated livers (22.3 ± 7.4 vs. 119.3 ± 25.0, and 21.1 ± 11.7 vs. 88.3 ± 1.4 cells/1×10^4 cells, respectively, $P < 0.05$) (Fig. 4A). In partial hepatectomized mice, a significant increase in the monocyte fraction that was reported to include EPCs [20] was detected in BM cells as well as in PB cells (data not shown). In addition, the expressions of CD34 and Flk-1 in the monocyte fraction of BM cells as well as of PB cells after partial hepatectomy were significantly elevated (Fig. 4B, C).

3.6. Mouse VEGF concentration in liver tissue

From the above results, the EPC fraction of BM cells is suggested to migrate into the liver. Since VEGF is known to be one of the important stimuli for EPC activation [20], we next measured its concentration in the liver. After partial hepatectomy, VEGF protein concentrations in liver tissue were elevated to 268 ± 25 pg/ml at 7 days, although no significant change was seen in sham-operated mice (Fig. 5).

4. Discussion

Until recently, only Kupffer cells were suggested to be the cells in the liver derived from BM [15]. More recently, a new point of view has arisen. Petersen et al. showed that hepatic oval cells appearing in injured liver were of BM origin employing the in situ hybridization method. Furthermore, Lagasse et al. reported that BM cells can differentiate into hepatocytes in FAH deficient mice using the β-galactosidase gene-marking method. Although controversy

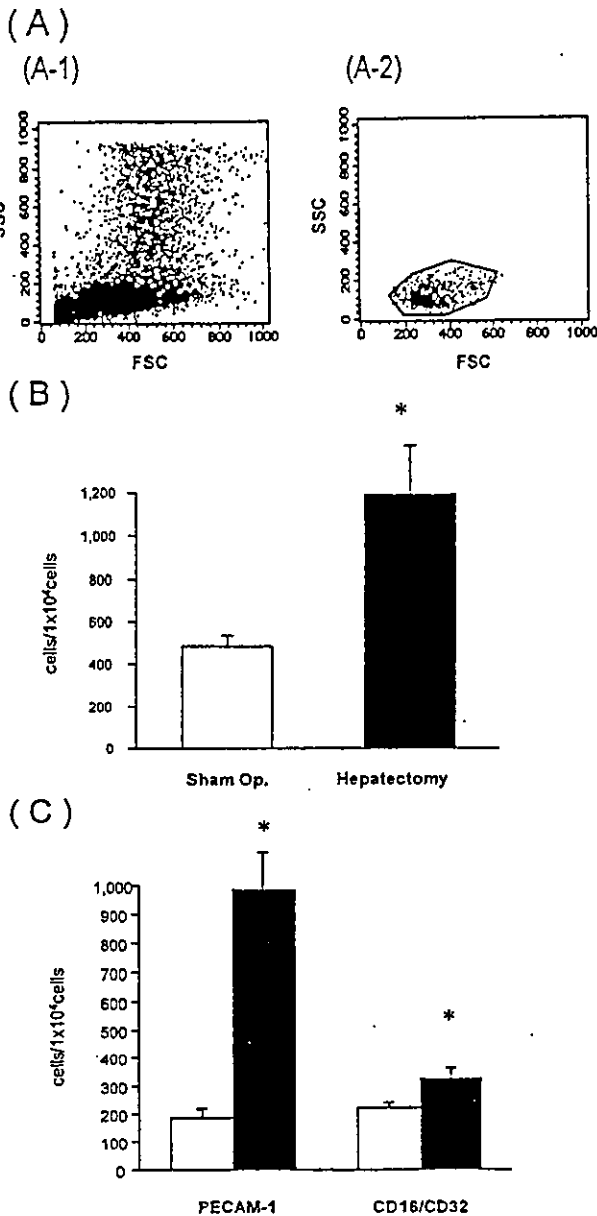

Fig. 3. Flow cytometric analysis of liver cells. (A-1) Dot blots of whole liver cell suspensions in partial hepatectomized mice. When GFP positive cells were gated, they were detected only in the non-parenchymal fraction depicted by the gate (A-2). (B) GFP positive cells in the liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test. (C) PECAM-1 and CD16/CD32 positive cells among GFP positive cells in liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

remains [21], these findings suggested that hepatic cells can be derived from BM. However, liver regeneration after partial hepatectomy is a long-discussed important biological response, and the behavior of BM cells in this situation has never been explored. To date, liver regeneration after partial hepatectomy was suggested to depend solely on the replication of mature cells in the liver. In the present study, to directly evaluate BM cells committing to liver cells after partial hepatectomy, we employed a GFP cell-marking system. As shown in the results, we could clearly show that BM cells migrated and contributed to the regenerating liver. To our knowledge, this is the first study directly showing that BM cells are involved in liver regeneration after partial hepatectomy.

In contrast to Lagasse's study using FAH deficient mice, GFP positive cells in the regenerating liver could only be detected in the liver surrounding the sinusoidal space. Further lineage marker studies together indicated that about 70% were SECs while 28% were Kupffer cells. In partial hepatectomy, SECs proliferate and migrate into avascular hepatic islands subsequent to the proliferation of parenchymal cells [22]. From the present findings, BM cells were shown to participate in this neovascularization by committing to SECs, which is also the first such evidence. Furthermore, from the detection of the EPC markers, CD34 and Flk-1, it was suggested that EPCs were mobilized from BM and differentiated to SECs during liver regeneration. This is compatible with the findings of Asahara et al. that showed EPC migration from BM to the site of vascular injury and their incorporation into neovascularization employing a different injury model [16,17,20]. In the present experiment, GFP positive SEC was also detected in non-hepatectomized liver. This finding is comparable with those of Gao et al.'s study [23] which showed replacement of liver venous endothelium by BM derived cells even in quiescent liver.

It is unclear what can be a trigger for EPC mobilization during liver regeneration. Shibuya and coworkers [24] reported that mRNA of VEGF is increased after hepatectomy and Taniguchi et al. [25] reported that VEGF expression after partial hepatectomy was mainly detected in periportal hepatocytes. Considering that VEGF is among the important stimuli for EPC activation [20], it should be a good candidate for stimulus inducing EPCs migration. As expected, the VEGF protein concentration in liver tissue was also increased at 5–7 days after partial hepatectomy, again supporting the proposed system of EPCs participation in the regenerating liver.

BM derived parenchymal cells were not apparent in the present study in contrast to previous studies [5,6,26]. To explain this discrepancy, three factors should be taken into consideration. One is the difference in the timing of observations. However, this explanation is not likely, since we could not detect BM cell differentiation to parenchymal cells even when we examined liver sections 2 months later, which is similar timing with Theise et al.'s study showing transdifferentiation of transplanted BM cells to hepatocytes [26]. Another factor is the difference in the detection system. Petersen et al. and Theise et al. showed their results by detecting BM derived Y-chromosome positive cells in the livers of female recipients using an in situ

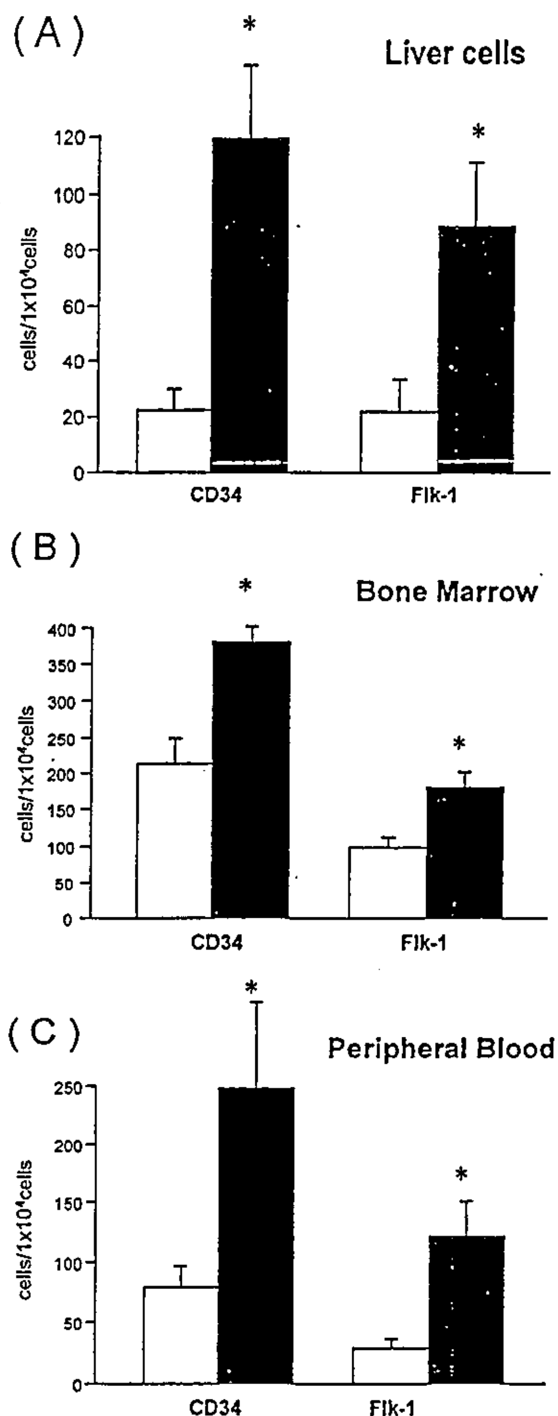

Fig. 4. Flow cytometric analysis of EPC markers in liver, BM, and PB cells. (A) CD34 and Flk-1 positive cells among GFP positive cells in liver after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test. Numbers of CD34 and Flk-1 positive cells in the monocyte fraction of BM (B) and PB (C) at 5 days after partial hepatectomy (closed column) and sham operation (open column). Values are expressed as means ± SEM. ($n = 4$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

hybridization technique [5,6,27]. The findings obtained from the detection of the Y-chromosome requires cautious interpretation, since it has been reported that a Y-chromosome can be identified in liver tissues by polymerase chain reaction in about 70% of women who have been pregnant with male children [28]. In contrast, the GFP cell-marking technique provides direct and quantitative evidence for cell fate. However, the differences in the results again appear not to stem from differences in the detection systems, since GFP positive cells were likely to be observed in parenchymal tissue in our other preliminary studies using liver cirrhosis mice. The last but most important factor likely to explain the discrepancy is that it stems from differences in differentiation stimuli. In Petersen et al.'s study, liver damage was induced by 2-AAF followed by hepatectomy that causes fulminant liver failure, in which situation the emergence of oval cells is indispensable to replace the massive cell death of mature hepatocytes. Similarly, liver damage was so severe in FAH deficient mice that loss of liver function caused their neonatal death. In contrast, partial hepatectomy does not induce such critical damage. The residual liver maintains a normal function and simultaneously hepatocytes can proliferate. In such a case, the participation of BM cells in the regeneration of parenchyma may not be necessary. Furthermore, stimuli inducing SECs differentiation that persists during liver regeneration may interfere with the differentiation of BM cells into the parenchymal lineage.

A more recent study revealed that BM derived cholangiocytes can be detected even in quiescent situations [29]. This may be compatible with the present findings since a few GFP positive cells, which did not express endothelial or monocytic markers, were detected in the present system. Growing evidence including that of the present study will clarify the differentiation ability of BM cells to liver composing cells in various situations, further evaluation

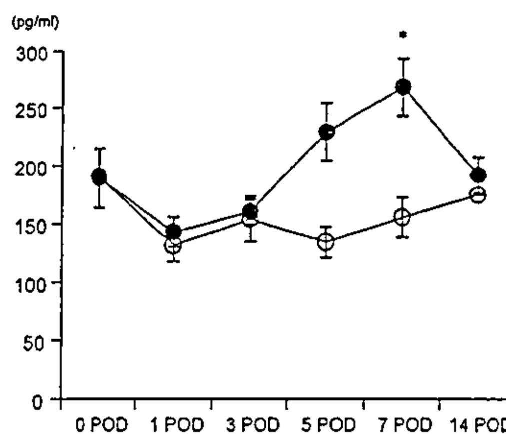

Fig. 5. Measurement of mouse VEGF protein concentrations in liver tissues. Closed circle (●) indicates partial hepatectomy and open circle (O) indicates sham operation. Values are expressed as means ± SEM. ($n = 3$) *: $P < 0.05$ vs. sham operation. Analysis was performed using Student's t test.

applying a direct system such as our GFP-marking-BM system in the case of critical liver damage would be ideal to clarify the mechanisms induced in transdifferentiation.

In conclusion, GFP cell-marking provided direct evidence of BM cells participation in regenerating liver after partial hepatectomy, the majority of cells committing to SECs. Not only helping clarification of aspects of liver regeneration but also of transdifferentiation, the present findings may provide a basis for therapy using BM cells as a vector for exogenous gene delivery, since BM cells may be isolated from patients themselves and be transfected ex vivo.

Acknowledgements

We thank Dr Masaru Okabe and Dr Norio Nakatsuji for generously donating GFP transgenic mice. This work was supported by Health Sciences Research Grants from Ministry of Health Labour and Welfare and by grants from the Ministry of Education, Science, Culture, and Sports of Japan (12470256) and the Japan Society for the Promotion of Science (RFTF96I00204).

References

[1] Ferrari G, Cusella-De Angelis G, Coletta M, Paolucci E, Stornaiuolo A, et al. Muscle regeneration by bone marrow-derived myogenic progenitors. Science 1998;279:1528–1530.

[2] Ono K, Takii T, Onozaki K, Ikawa M, Okabe M, Sawada M. Migration of exogenous immature hematopoietic cells into adult mouse brain parenchyma under GFP-expressing bone marrow chimera. Biochem Biophys Res Commun 1999;262(3):610–614.

[3] Kinoshita T, Sekiguchi T, Xu M, Ito Y, Kamiya A, Miyajima A, et al. Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. Proc Natl Acad Sci USA 1999;96(13):7265–7270.

[4] Cardier JE, Barbera-Guillem E. Extremedullary hematopoiesis in the adult mouse liver is associated with specific hepatic sinusoidal endothelial cells. Hepatology 1997;26:165–175.

[5] Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, et al. Bone marrow as a potential source of hepatic oval cells. Science 1999;284:1168–1170.

[6] Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, et al. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 2000;6:1229–1234.

[7] Sell S. Heterogeneity and plasticity of hepatocyte lineage cells. Hepatology 2001;33(3):738–750.

[8] Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y. 'Green mice' as a source of ubiquitous green cells. FEBS Lett 1997;407:313–319.

[9] Seglen PO. Preparation of isolated rat liver cells. Methods Cell Biol 1976;13:29–83.

[10] Kleinherenbrink-Stins MF, van de Boom JH, Schouten D, Roholl PJ, Niels van der Heyde M, Brouwer A, et al. Visualization of the interaction of native and modified lipoproteins with parenchymal, endothelial and Kupffer cells from human liver. Hepatology 1991;14:79–90.

[11] Shaw R, Johnson A, Schulz W, Zahlten R, Combes B. Sinusoidal endothelial cells from normal guinea pig liver: isolation, culture and characterization. Hepatology 1984;4(4):591–602.

[12] Neubauer K, Wilfling T, Ritzel A, Ramadori G. Platelet-endothelial cell adhesion molecule-1 gene expression in liver sinusoidal endothelial cells during liver injury and repair. J Hepatol 2000;32:921–932.

[13] Neubauer K, Ritzel A, Saile B, Ramadori G. Decrease of platelet-endothelial cell adhesion molecule 1-gene-expression in inflammatory cells and in endothelial cells in the rat liver following CCl (4)-administration and in vitro after treatment with TNFalpha. Immunol Lett 2000;74:153–164.

[14] Shiojiri N, Lemire JM, Fausto N. Cell lineages and oval cell progenitors in rat liver development. Cancer Res 1991;51(10):2611–2620.

[15] Takezawa R, Watanabe Y, Akaike T. Direct evidence of macrophage differentiation from bone marrow cells in the liver: a possible origin of Kupffer cells. J Biochem 1995;118:1175–1183.

[16] Asahara T, Murohara T, Sullivan A, Silver M, Schatteman G, Isner JM, et al. Isolation of putative progenitor endothelial cells for angiogenesis. Science 1997;275:946–967.

[17] Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Isner JM, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. Circ Res 1999;85(3):221–228.

[18] Bhattacharya V, McSweeney PA, Shi Q. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34 + bone marrow cells. Blood 2000;95(2):581–585.

[19] Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow-derived endothelial cells. Blood 1998;92(2):362–367.

[20] Asahara T, Takahashi T, Masuda H, Kalka C, Chen D, Iwaguro H, et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. EMBO J 1999;18(14):3964–3972.

[21] McDonnell WM. Liver stem cells from bone marrow. Hepatology 2000;32(5):1181.

[22] Martinez-Hernandez Aamenta PS. The extracellular matrix in hepatic regeneration. FASEB J 1995;9(4):1401–1410.

[23] Gao Z, McAlister V, Williams G. Repopulation of liver endothelium by bone-marrow-derived cells. Lancet 2001;357(9260):932–933.

[24] Mochida S, Ishikawa K, Inao M, Shibuya M, Fujiwara K. Increased expressions of vascular endothelial growth factor and its receptors, flt-1 and KDR/flk-1, in regenerating rat liver. Biochem Biophys Res Commun 1996;226:176–179.

[25] Taniguchi E, Sakisaka S, Matsuo K, Tanikawa K, Sata M. Expression and role of vascular endothelial growth factor in liver regeneration after partial hepatectomy in rats. J Histochem Cytochem 2001;49(1):121–129.

[26] Theise ND, Badve S, Saxena R, Henegariu O, Sell S, Crawford JM, et al. Derivation of hepatocytes from bone marrow cells in mice after radiation-induced myeloablation. Hepatology 2000;31:235–240.

[27] Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, et al. Liver from bone marrow in humans. Hepatology 2000;32:11–16.

[28] Tanaka A, Lindor K, Gish R, Batts K, Shiratori Y, Omata M, et al. Fetal microchimerism alone does not contribute to the induction of primary biliary cirrhosis. Hepatology 1999;30(4):833–838.

[29] Krause D, Theise N, Collector M, Henegariu O, Hwang S, Gardner R, et al. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. Cell 2001;105(3):369–377.

ELSEVIER

Journal of Hepatology 36 (2002) 590–599

Journal of Hepatology

www.elsevier.com/locate/jhep

Regulation of cultured rat hepatocyte proliferation by stellate cells

Naoki Uyama[1,*], Yasuyuki Shimahara[1], Norifumi Kawada[2], Shuichi Seki[2], Hiroaki Okuyama[1], Yuji Iimuro[1], Yoshio Yamaoka[1]

[1]*Department of Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, 54-Kawaracho, Shogoin, Sakyoku, Kyoto 606 8507, Japan*
[2]*Department of Hepatology, Graduate School of Medicine, Osaka City University, Osaka, Japan*

Background/Aims: This study using primary culture models was aimed to reveal the stellate cell-derived factors that regulate hepatocyte proliferation.

Methods: Rat hepatocytes and stellate cells were cultured in serum-free Williams-E medium. We prepared hepatocyte mono-culture and two different co-cultures of hepatocytes and stellate cells; (1) co-culture on the same surface (Co-mix.) and (2) co-culture without contact between hepatocytes and stellate cells using a culture insert (Co-sep.). The change in the number and the DNA synthesis of hepatocytes was evaluated.

Results: The number of hepatocytes decreased to 76% of the original number after 48 h of starting mono-culture, while it remained at 106% in mixed co-culture (Co-mix.) and increased to 135% in separated co-culture (Co-sep.). The hepatocyte DNA synthesis was enhanced by carbenoxolone in Co-mix. and reduced by NK1 in each co-culture. PD153035 had no effect. Heparitinase-I (20 mU/ml) and sodium chrolate (25 mM) reduced the hepatocyte DNA synthesis in Co-sep. to 71.8 and 61.6%, respectively. Activation of mitogen-activated protein kinase was induced in hepatocytes stimulated by conditioned mediums.

Conclusions: Hepatocyte proliferation was stimulated in the presence of stellate cells through hepatocyte growth factor, extracellular heparan sulfate (HS), and HS proteoglycan, and might be negatively regulated by gap junction-dependent mechanism.

© 2002 European Association for the Study of the Liver. Published by Elsevier Science B.V. All rights reserved.

Keywords: Hepatocytes; Stellate cells; Cell-to-cell interaction; Hepatocyte growth factor; Heparan sulfate; Gap junction

1. Introduction

After the liver injuries, residual hepatocytes undergo proliferation in a highly orchestrated manner. Non-parenchymal cell population seems to play important roles in this process. Recently, much attention has been focused on the role of hepatic stellate cells (HSC) which reside in the space of Disse, surround sinusoids with their dendritic processes, metabolize vitamin A, synthesize extracellular matrix materials (ECMs), such as collagens (types I, III, IV, V, VI and XIV), proteoglycans (heparan, dermatan and chondroitin sulfate proteoglycans) and glycoproteins (laminin and fibronectin), and secrete growth factors and inflammatory mediators [1,2]. HSC as well as Kupffer and sinusoidal endothelial cells are the source of several mitogens for hepatocytes. For instance, HSC generate hepatocyte growth factor (HGF), a most potent endogenous mitogen for hepatocytes [2–8]. Quiescent and activated HSC produce epidermal growth factor (EGF) and transforming growth factor-α (TGF-α), respectively [9,10].

ECMs and cell-to-cell contact may be additional factors

Received 31 July 2001; received in revised form 12 December 2001; accepted 6 January 2002

* Corresponding author. Tel.: +81-75-751-3606; fax: +81-75-751-3607.
E-mail address: uynk@f2.dion.ne.jp (N. Uyama).

Abbreviations: α-SMA, Alpha-smooth muscle actin; BrdU L.I., 5-Bromo-2′-deoxyuridine labeling index; CBX, carbenoxolone; CM, conditioned medium; CM of Co-mix., CM of mixed co-culture; CM of Co-sep., CM of separated co-culture; CM of Hc, CM of hepatocyte mono-culture; CM of HSC, CM of stellate cell mono-culture; Co-mix., mixed co-culture; Co-sep., separated co-culture; Co-sep (H), separated co-culture (hepatocytes were plated in high density); ECM, extracellular matrix material; EGF, epidermal growth factor; HGF, hepatocyte growth factor; HPR, heparin; HS, heparan sulfate; HSC, hepatic stellate cells; HSPG, heparan sulfate proteoglycan; Mono-Hc, mono-culture of hepatocytes; $NaClO_3$, sodium chlorate; TGF-α, transforming growth factor-α.

0168-8278/02/$20.00 © 2002 European Association for the Study of the Liver. Published by Elsevier Science B.V. All rights reserved.
PII: S0168-8278(02)00023-5

for growth and differentiation of hepatocytes [11–13]. Heparan sulfate (HS), which belongs to glycosaminoglycan, was reported to be a co-mitogen for HGF [14,15]. Laminin and collagens were reported to facilitate albumin production by hepatocytes [16,17]. Rojkind et al. reported that co-culture of hepatocytes with HSC induce the expression of connexin 43, a gap-junction protein, in HSC, which contributed to maintain the hepatocyte albumin mRNA expression [18]. Loreal et al. reported that their co-culture induced the deposition of various ECMs such as laminin, fibronectin, and collagens [19].

The current study was aimed at revealing the underlying mechanism of hepatocyte proliferation regulated by HSC, which has not yet been analyzed in detail. For this purpose, we have utilized experimental co-culture models.

2. Materials and methods

2.1. Animals

Pathogen-free male Wistar rats were obtained from SLC (Shizuoka, Japan). Animals were housed at a constant temperature and supplied with laboratory chow and water ad libitum.

2.2. Chemicals

Pronase-E was purchased from Merck (Darmstadt, FRG). DNase and ABTS were from Boehringer Mannheim (Mannheim, FRG). Collagenase, 3,3′-diaminobenzidine (DAB), sodium chlorate and soybean trypsin inhibitor were from Wako Pure Chemical Co. (Osaka, Japan). Nycodenz was from Nycomed Pharm AS (Oslo, Norway). 5-Bromo-2′-deoxyuridine (BrdU), heparan sulfate (HS), heparan sulfate proteoglycan (perlcan, HSPG) and carbenoxolone (CBX) were from Sigma (St. Louis, MO). Williams-E medium and fetal bovine serum (FBS) were from Gibco BRL (Gaithersburg, MD). Mouse anti-BrdU antibodies and rabbit anti-mouse immunoglobulins conjugated with horseradish peroxidase were from Dako (Hamburg, FRG). Rat albumin, goat anti-rat albumin antibodies, peroxidase-conjugated rabbit anti-albumin antibodies and heparin sodium were from Cappel (West Chester, PA). Rabbit polyclonal anti-human EGF receptor IgG and rabbit anti-mouse c-Met receptor IgG were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies for p42/44 mitogen-activated protein kinase (MAPK), phospho-p42/44 MAPK were from New England Biolabs Inc. (Beverly, MA). Block Ace and phosphate-buffered saline (PBS) were from Dainippon Pharm. Co. (Osaka, Japan). PD 153035, an EGF receptor-specific tyrosine kinase inhibitor, and PD98059, a MAPK-extracellular signal-regulated kinase inhibitor (MEK inhibitor), were from Calbiochem (La Jolla, CA). Recombinant rat HGF was from Toyobo Inc. (Osaka, Japan). Recombinant human TGF-α was from Pepro Teck Inc. (Rocky Hill, NJ). Heparitinase-I was from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). NK1 was kindly provided by Dr P. Bottaro (NIH, Maryland).

2.3. Isolation and culture of HSC and hepatocytes

HSC were isolated from male Wistar rats (300 g) as previously described [20]. Cell purity was around 95% as estimated by a typical star-like shape and by detecting vitamin A autofluorescence. Isolated HSC were suspended in Williams-E medium supplemented with 10% FBS, 70 mg/L penicillin, and 100 mg/L streptomycin, and plated at 4.0×10^4 cells/cm^2 on type I collagen-coated culture dishes (9.4 cm^2, Iwaki 4000-010, Flanklin Lakes, NJ). Hepatocytes were isolated from male Wistar rats (200 g) [21]. The purity of isolated hepatocytes was 99% and the viability of hepatocytes was approximately 95% as estimated by a trypan blue dye exclusion test. Hepatocytes were plated at the density of 1.5×10^4 cells/cm^2 in serum-free Williams-E medium. In some experiments, the cell density was increased to 5.5×10^4 cells/cm^2 (Fig. 1a). After 3 h, the culture medium was renewed and the culture was continued up to 4 days. The number of attached hepatocytes in the fixed area (1 mm^2) was scanned using a digital camera of FUJIX HC-2500 (Fuji film, Tokyo, Japan) under Olympus IX 70 microscope (Olympus, Tokyo, Japan) and counted.

2.4. Co-culture of hepatocytes and HSC

Freshly isolated hepatocytes in serum-free Williams-E medium were plated onto the culture dishes where isolated HSC had already been cultured for 24 h. After 3 h, the culture medium was replaced with serum-free Williams-E and the culture was continued up to 4 days (mixed co-culture (Co-mix.), Fig. 1b). For separated co-culture (Co-sep.), hepatocytes and HSC were co-cultured without any cell-to-cell contact by using a culture insert (4.2 cm^2, Millicell Culture Plate Inserts, pore size 0.4 μm, Millipore Co., Bedford, MA) coated with type I collagen (Cell matrix I-p, Nitta Zerachin Japan, Osaka) (Fig. 1c).

2.5. Preparation of conditioned culture mediums of stellate cell, hepatocyte and co-culture

Freshly isolated hepatocytes were cultured in serum-free Williams-E medium for 3 h. Then, the medium was renewed and the culture was continued for another 48 h. The culture supernatant was centrifuged at $400 \times g$ and 4°C for 7 min and used as conditioned medium (CM) of hepatocyte mono-culture (CM of Hc). Freshly isolated HSC were cultured in Williams-E medium supplemented with 10% FBS for 24 h. The culture medium was then replaced with serum-free Williams-E medium and the culture was continued for another 48 h. The culture supernatant was centrifuged and used as CM of HSC mono-culture (CM of HSC). CM of mixed co-culture (CM of Co-mix.) and CM of separated co-culture (CM of Co-sep.) were prepared in the same way. After hepatocytes were plated and cultured under serum-free Williams-E medium for 3 h, the culture medium was changed to a medium consisting of 90% of CM and 10% of Williams-

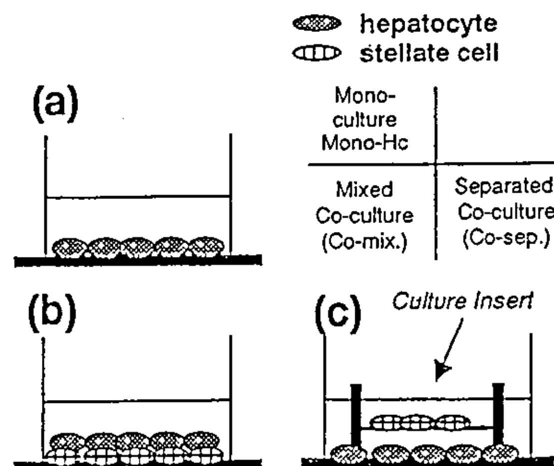

Fig. 1. Culture systems used in this experiment. (a) Hepatocyte mono-culture (Mono-Hc). Hepatocytes were plated on six-well plates coated with type I collagen. (b) Mixed co-culture (Co-mix.). Freshly isolated hepatocytes in serum-free Williams-E medium were plated on six-well plates coated with type I collagen where isolated HSC had already been cultured for 24 h. (c) Separated co-culture (Co-sep.). HSC were plated on culture inserts coated with type I collagen. After 24 h, hepatocytes were plated on six-well plates coated with type I collagen below the culture inserts in serum-free Williams-E medium.

E. The culture was continued for another 48 h. DNA synthesis of hepatocytes was evaluated by BrdU incorporation during the last 24 h.

2.6. Labeling of proliferating cell nuclei with BrdU

Hepatocytes cultured for the indicated days were treated with 100 μM BrdU for 24 h. Incorporated BrdU was immunocytochemically evaluated [22]. The number of cells with brown-colored nuclei was counted in four microscopic fields randomly selected in each well. BrdU labeling index (BrdU L.I.) was calculated as number of BrdU-positive cells/number of cells in the identical area × 100 (%).

2.7. Western blotting

Hepatocytes and HSC were homogenized in a lysis buffer consisting of 62.5 mM Tris, 0.1% glycerol, 2% SDS, 5% 2-β mercaptoethanol (pH 6.8). After heat-denaturation at 95°C for 5 min, the samples (10 μg protein) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto an Immobilon P membrane (Millipore). The membranes were subsequently treated with 5% skim milk and then with primary antibodies overnight at 4°C. After vigorous washing, the membranes were incubated with secondary horseradish peroxidase-conjugated anti-immunoglobulins for 1 h. Immunoreactive bands were visualized on Kodak XAR5 films by using ECL detection reagent (Amersham, Buckinghamshire, UK).

2.8. Quantification of HGF

The concentration of HGF in each CM was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Institute of Immunology, Tokyo, Japan) according to the manufacturer's instructions and as given in Ref. [23]. The absorbance of each well was monitored by an ELISA reader (Bio-rad, model 3550, CA) at 490 nm.

2.9. Measurement of albumin in the culture medium

We used the two-antibody sandwich method as described in detail by Harlow and Lane [24]. The absorbance of each well was monitored by an ELISA reader (Bio-rad, model 3550, CA) at 405 nm.

2.10. Statistical analysis

Values reported in Figs. 1–6 and Table 1 represent means ± SD of three or more independent samples. The results were analyzed by one-way analysis of variance (ANOVA) and Scheffe test using the StatView 5.0 program. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of co-culture on morphology and cell number of hepatocytes

Mono-cultured hepatocytes became flattened and some of them started detaching 48 h after plating (Fig. 2a,b). The number of cells attaching to the culture plate in the fixed area (1 mm^2) gradually decreased to 48.6 ± 0.7% of the original cell number 96 h after culture (Fig. 2j). When hepatocytes were cultured with HSC (Co-mix.), they came into contact with the processes of HSC 12 h after starting the culture (Fig. 2g), and thereafter, they were surrounded by activated HSC, which expressed α-smooth muscle actin (α-SMA) (Fig. 2i, lane 3), 48 h after starting the culture (Fig. 2h). Although hepatocytes became flattened as was the case with mono-cultured ones (Fig. 2c,d), their number was significantly maintained during the first 48 h (106 ± 4%, $P < 0.05$ vs mono-cultured hepatocytes) and then decreased to 49.7 ± 3.0% of the original cell number (Fig. 2j). When hepatocytes were co-cultured with HSC without cell-to-cell contact by using a culture insert (Co-sep.), there was considerable increase of cell density (Fig. 2e,f). The number of hepatocytes significantly increased to 135 ± 7% ($P < 0.05$ vs hepatocytes of mono-culture and Co-mix.) of the original cell number 48 h after the culture and decreased to 75.7 ± 3.8% 96 h after culture, maintaining a significantly high level in comparison with mono-cultured and Co-mix. hepatocytes (Fig. 2j).

3.2. Effect of co-culture on DNA synthesis of hepatocytes

In mono-culture, BrdU L.I. of hepatocytes at 48 and 72 h after plating was 2.41 ± 1.73 and 11.3 ± 2.8%, respectively (Fig. 3a,d). BrdU L.I. of hepatocytes in Co-mix. significantly increased to 25.8 ± 10.4% 48 h after plating ($P < 0.05$ vs mono-cultured hepatocytes), while no significant increase was observed 72 h after plating (Fig. 3b,d). BrdU L.I. of hepatocytes in Co-sep. was increased to 47.5 ± 5.8 and 48.5 ± 1.6%, respectively ($P < 0.05$ vs mono-cultured hepatocytes) (Fig. 3c,d). These results were also seen even when hepatocyte density in Co-sep. was increased to 5.5×10^4 cells/cm^2 [Co-sep. (H), Fig. 3d].

DNA synthesis of HSC showed no significant difference among co-cultures and mono-culture of HSC after 48 h while the DNA synthesis in Co-mix. was higher than that in mono-culture and Co-sep. after 72 h (data not shown).

3.3. Effect of a gap junction blocker on DNA synthesis of hepatocytes in Co-mix

Carbenoxolone (CBX), a gap junction blocker [25], significantly enhanced the DNA synthesis of hepatocytes in Co-mix. up to 125 ± 6 and 153 ± 27% at concentrations of 1 and 10 μg/ml, respectively. CBX had no effect on the DNA synthesis of hepatocytes in mono-culture (data not shown) and in Co-sep. (Fig. 3e).

3.4. Effect of conditioned mediums on DNA synthesis of hepatocytes

CMs of HSC, Co-mix., and Co-sep. significantly stimulated the BrdU L.I. of hepatocytes up to 10.2 ± 0.9, 11.9 ± 2.7 and 15.5 ± 1.8%, respectively. CM of Hc hardly stimulated the BrdU L.I. (Fig. 4a).

3.5. Is a hepatocyte mitogen derived from stellate cell identical to HGF?

ELISA analysis of each CM revealed that HGF concentration in CM of either Hc, HSC, Co-mix., or Co-sep. was 0.80 ± 0.42, 3.71 ± 0.11, 5.64 ± 0.25 or 2.37 ± 0.39 ng/ml, respectively (Fig. 4b).

Recombinant rat HGF dose-dependently stimulated DNA

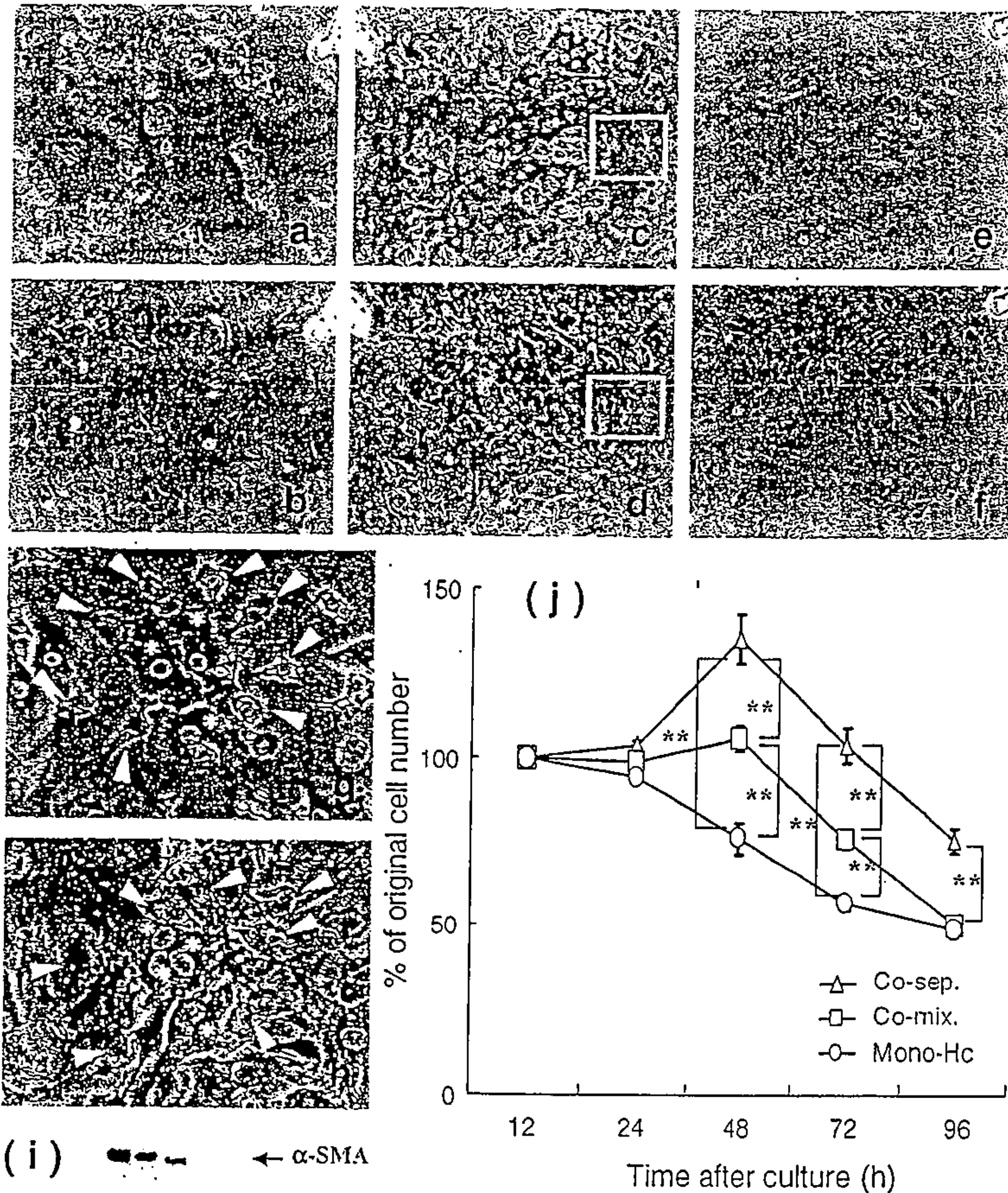

Fig. 2. Morphology and number of hepatocytes in culture. (a, b) Mono-cultured hepatocytes in the fixed area 12 h (a) and 48 h (b) after starting culture, respectively. Hepatocytes became flattened and some of them started detaching after 48 h. Magnification, 200 ×. (c, d) Hepatocytes cultured with stellate cells (Co-mix.) in the fixed area 12 h (c) and 48 h (d) after starting culture, respectively. Magnification, 200 ×. Enclosed parts of each picture were enlarged to 800 × in (g, h), respectively. Hepatocytes (*) made contact with the processes of HSC (arrow heads) 12 h after starting culture (g). Thereafter, HSC were getting myofibroblastic and surrounded the hepatocytes 48 h after starting culture (h). (e, f) Hepatocytes cultured with HSC (Co-sep.) in the fixed area 12 h (e) and 48 h (f) after starting culture, respectively. The number of hepatocytes in this area increased from 1.58×10^3 cells/mm^2 to 2.27×10^3 cells/mm^2. Magnification, 200 ×. (i) Western blot analysis of alpha-smooth muscle actin (α-SMA) expression. Lane 1, HSC mono-cultured for 1 day; lane 2, HSC mono-cultured for 3 days; lane 3, Co-mix. at day 2; lane 4, HSC cultured in Co-sep. for 2 days; lane 5, hepatopcytes mono-cultured for 2 days; lane 6, hepatopcytes cultured in Co-sep. for 2 days. Note that HSC in Co-mix. as well as in mono-culture expressed α-SMA. α-SMA was not expressed in hepatocytes. (j) Change in the number of hepatocytes mono-cultured and co-cultured with stellate cells. The number of hepatocytes in the fixed area (1 mm^2) was counted under phase-contrast microscope 12, 24, 48, 72, and 96 h after starting culture. Values of means ± SD in triplicate determinations are indicated. $^{**}P < 0.01$.

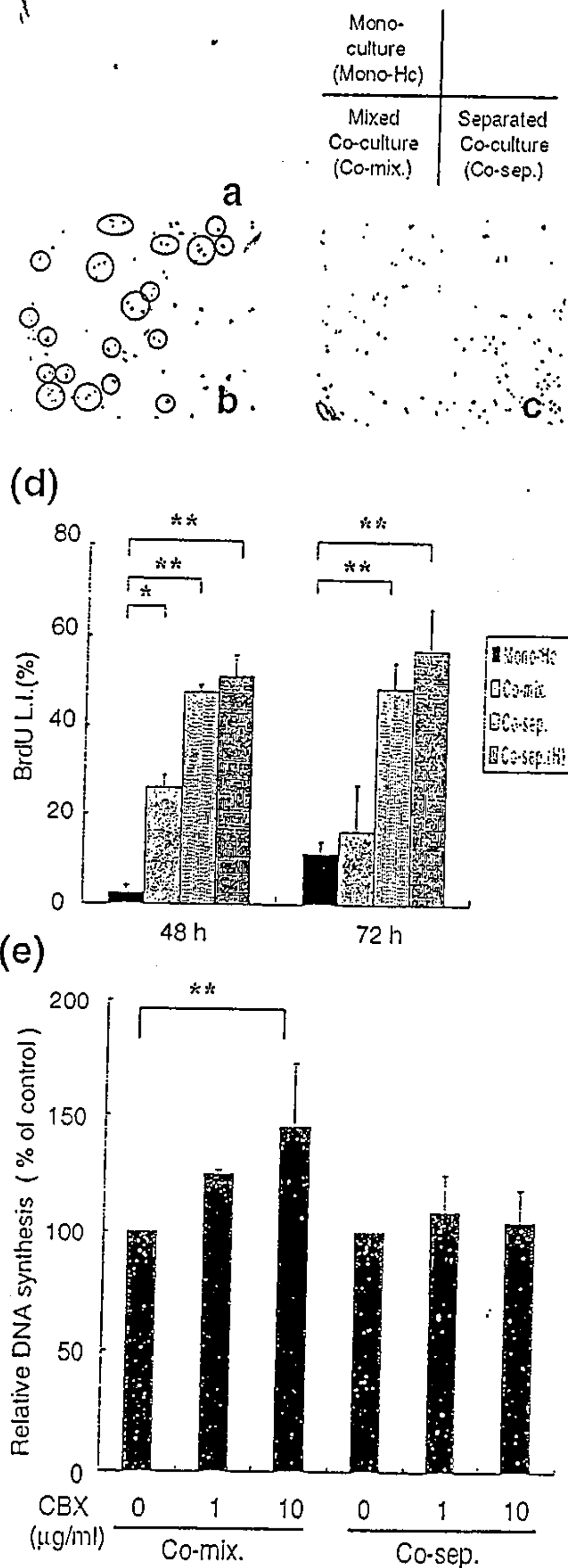

Fig. 3. Incorporation of BrdU by hepatocytes. (a–c) BrdU staining of hepatocytes in hepatocyte mono-culture [Mono-Hc (a)], Co-mix. (b) and Co-sep. (c) 48 h after starting culture. Magnification, 200×. In (b), encircled nuclei are of hepatocytes as judged by the cell appearance and the absence of vitamin A–lipid particles. (d) BrdU labeling index (BrdU L.I) of hepatocytes 48 and 72 h after starting culture. In Mono-Hc, hepatocytes were plated at the density of 1.5×10^4 cells/cm^2 on six-well plates coated with type I collagen. In Co-mix., hepatocytes and HSC were plated on the same surface at the density of 1.5×10^4 cells/cm^2 and 4.0×10^4 cells/cm^2, respectively, on six-well plates coated with type I collagen. In Co-sep., HSC were cultured on the culture inserts for 24 h at the density of 4.0×10^4 cells/cm^2, and thereafter hepatocytes were plated at the density of 1.5×10^4 cells/cm^2 on six-well plates coated with type I collagen below the culture inserts. In Co-sep. (H), hepatocytes were plated at the density of 5.5×10^4 cells/cm^2. All cultures were continued for another 72 h. DNA synthesis of hepatocytes was evaluated by BrdU incorporation during the last 24 h. Values of means ± SD of the triplicate determinations are indicated. $*P < 0.05$, $**P < 0.01$. (e) Effect of carbenoxolone (CBX) on DNA synthesis of hepatocytes in Co-mix. and Co-sep. Relative DNA synthesis of hepatocytes was evaluated by BrdU incorporation assay. Values of means ± SD of the triplicate determinations are indicated. $**P < 0.01$.

synthesis of hepatocytes as was consistent with previous reports (Fig. 4c) [26]. BrdU L.I. of hepatocytes increased to 14.2 ± 6.60 and 44.2 ± 10.2% by the addition of 5 and 50 ng/ml of recombinant HGF, respectively. NK1, a HGF antagonist [27], at a concentration of 100 nM, reduced the DNA synthesis of hepatocytes in Co-mix. and Co-sep. to 61.7 ± 3.9 and 69.3 ± 4.2%, respectively (Fig. 4d).

3.6. Effect of PD153035 on the DNA synthesis of hepatocytes stimulated by conditioned mediums and Co-sep

EGF, HB-EGF and TGF-α are known to share an identical receptor, EGF receptor, for their binding. Here, the effect of PD153035, a specific EGF receptor tyrosine kinase inhibitor [28,29], on DNA synthesis of hepatocytes was determined. In a control experiment, PD153035 was shown to hamper the hepatocyte DNA synthesis induced by TGF-α (Table 1). PD153035 (10 μM) reduced the DNA synthesis of hepatocytes stimulated by CM of either HSC, Co-mix. or Co-sep. to 66.9, 64.9 or 74.9%, respectively. However, PD153035 failed to affect the hepatocyte DNA synthesis in Co-sep. (Table 1).

3.7. Expression of c-Met and EGF receptors on hepatocytes and the activation of MAPK in hepatocytes stimulated by conditioned medium

Western blot analysis revealed that c-Met receptor was expressed in mono-cultured hepatocytes and those in Co-sep. at the same level (Fig. 5a). EGF receptor level was also unchanged (Fig. 5a). When hepatocytes were exposed to CM of either HSC, Co-mix., or Co-sep, phosphorylation of MAPK occurred within 5 min after stimulation without affecting the level of total MAPK (Fig. 5b). PD153035 (10 μM) failed to inhibit the phosphorylation of MAPK by CM of either HSC, Co-mix., or Co-sep. In the identical

condition, PD98059 (50 ng/ml), a MEK inhibitor, completely inhibited the activation of MAPK (Fig. 5c). TGFα-stimulated MAPK phosphorylation was hampered by both PD98059 and PD153035 (Fig. 5c).

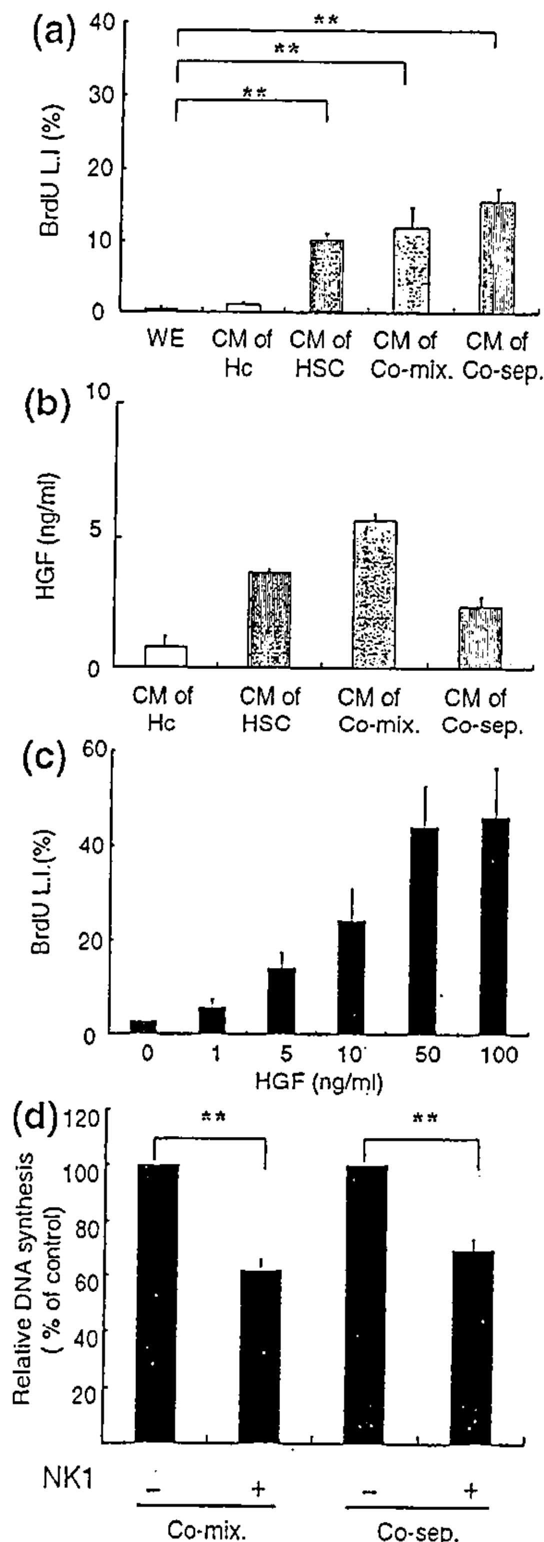

Fig. 4. Effect of conditioned mediums, recombinant rat HGF, and NK1 on BrdU L.I. of hepatocytes. (a) CMs were harvested as described in Section 2. Hepatocytes were plated on six-well plates at the density of 1.5×10^4 cells/cm^2 in serum-free Williams-E medium. After 3 h, culture medium was replaced with a medium consisting of 90% CM and 10% Williams-E. Cultures were continued for another 48 h. BrdU incorporation was evaluated during the last 24 h. Values of means ± SD of the triplicate determinations are indicated. $^{**}P < 0.01$. WE, Williams-E medium. (b) Concentration of immunoreactive HGF in the individual CM was measured by ELISA as described in Section 2. Values of means ± SD of triplicate determinations are indicated. (c) Effect of recombinant rat HGF on BrdU L.I. of mono-cultured hepatocytes. Values of means ± SD of triplicate determinations are indicated. (d) Effect of NK1, a HGF antagonist, on DNA synthesis of hepatocytes in co-culture. Relative DNA synthesis was evaluated. Values of means ± SD of the triplicate determinations are indicated. $^{**}P < 0.01$.

Table 1
Inhibitory effect of a specific EGF receptor tyrosine kinase inhibitor, PD153035, on DNA synthesis of hepatocytes[a]

	5-Bromo-2′-deoxyuridine labeling index (%)		Rate (%)
	Control	PD153035	
CM			
CM of HSC	14.90 ± 3.28	9.97 ± 3.61	66.90
CM of Co-mix.	17.40 ± 1.70	11.13 ± 2.73	64.90
CM of Co-sep.	21.07 ± 2.08	15.77 ± 0.45	74.90
Co-culture			
Co-sep.	58.67 ± 3.15	58.27 ± 1.18	99.32
TGF-α (30 ng/ml)	56.80 ± 5.09	21.63 ± 8.30	38.08

[a] Note: Inhibitory effect of PD153035 on DNA synthesis of hepatocytes. Hepatocytes in each culture were incubated with 10 μM PD153035 for 48 h. DNA synthesis of hepatocytes was measured by incorporation of BrdU during the last 24 h. Values of means ± SD of the triplicate determinations are indicated.

3.8. Effect of heparin, heparan sulfate and heparan sulfate proteoglycan on DNA synthesis of cultured hepatocytes

Although neither of HPR, HS nor HSPG at 1 μg/ml enhanced the DNA synthesis of hepatocytes (Fig. 6a, left), all of them were found to enhance significantly the HGF-induced DNA synthesis of hepatocytes (Fig. 6a, right).

Heparitinase-I (20 mU/ml), which catalyzes the eliminative cleavage of *N*-acetyl-D-glucosaminidic linkage in HS [30], reduced the BrdU L.I. of Co-sep. to 71.4 ± 2.2% (Fig. 6b), but did not reduce the DNA synthesis of hepatocytes in mono-culture with or without 10 ng/ml HGF (Fig. 6b).

Sodium chlorate ($NaClO_3$), which is a potent inhibitor for sulfate adenylyltransferase and reduces the production of proteoglycan [31], decreased the BrdU L.I. of Co-sep. to 82.0 ± 9.0 and 61.3 ± 7.5% at 10 and 25 mM, respectively (Fig. 6c, right), without affecting the DNA synthesis of hepatocyte in mono-culture in the presence (Fig. 6c, left) or absence (data not shown) of HGF. In these experiments, neither heparitinase-I nor sodium chlorate reduced HGF production of HSC in Co-sep. (data not shown).

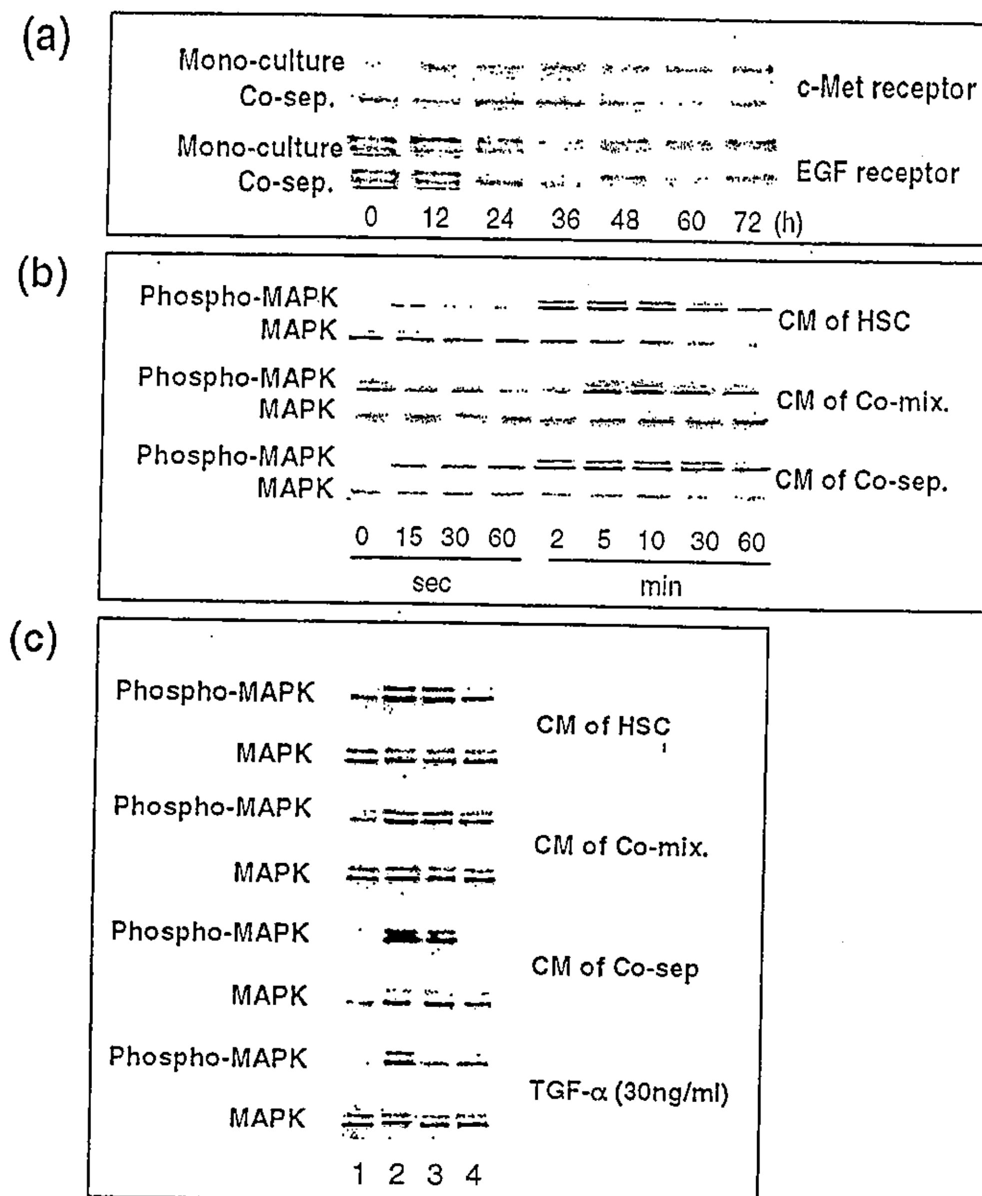

Fig. 5. Expression of c-Met and EGF receptors, and activation of MAPK and Akt in cultured hepatocytes. (a) Expression of c-Met receptor and EGF receptor on hepatocytes in mono-culture and in Co-sep. during 72 h after plating was determined by western blot. (b) Phosphorylation of p42/44 MAPK in hepatocytes stimulated by CM of either HSC, Co-mix., or Co-sep. CMs were harvested as described in Section 2. After plating for 3 h, the culture medium was replaced with CM of either HSC, Co-mix., or Co-sep. and the culture was continued up to 60 min. Phosphorylation of p42/44 MAPK was detected in combination with total of them by Western blot. (c) Effect of PD153035 (10 μM) and PD98059 (50 μM) on the phosphorylation of p42/44 MAPK in hepatocytes. CMs were harvested as described in Section 2. After plating for 2.5 h, the culture medium was renewed and hepatocytes were incubated with or without either PD153035 or PD98059, a specific MEK inhibitor, for 30 min. Then, the culture medium was replaced with CM of either HSC, Co-mix. or Co-sep. with or without either PD153035 or PD98059. The culture was continued up to 5 min. Phosphorylation of p42/44 MAPK was evaluated by immunoblot as in (b). Lane 1, no addition; lane 2, CM; lane 3, CM plus PD153035; lane 4, CM plus PD98059.

3.9. Albumin synthesis by hepatocytes

Total amount of albumin in the culture medium of mono-Hc, Co-mix., and Co-sep. for the first 24 h after starting culture was 2.92 ± 0.88, 2.96 ± 0.20 and 4.22 ± 1.12 μg/10^5 cells/day, respectively and that of the last 24 h after starting culture was 1.41 ± 0.18, 2.39 ± 1.27 and 2.66 ± 0.20 μg/10^5 cells/day, respectively. Thus, albumin production was not significantly modified in the presence of HSC.

4. Discussion

HSC were reported to express at least three mitogens for

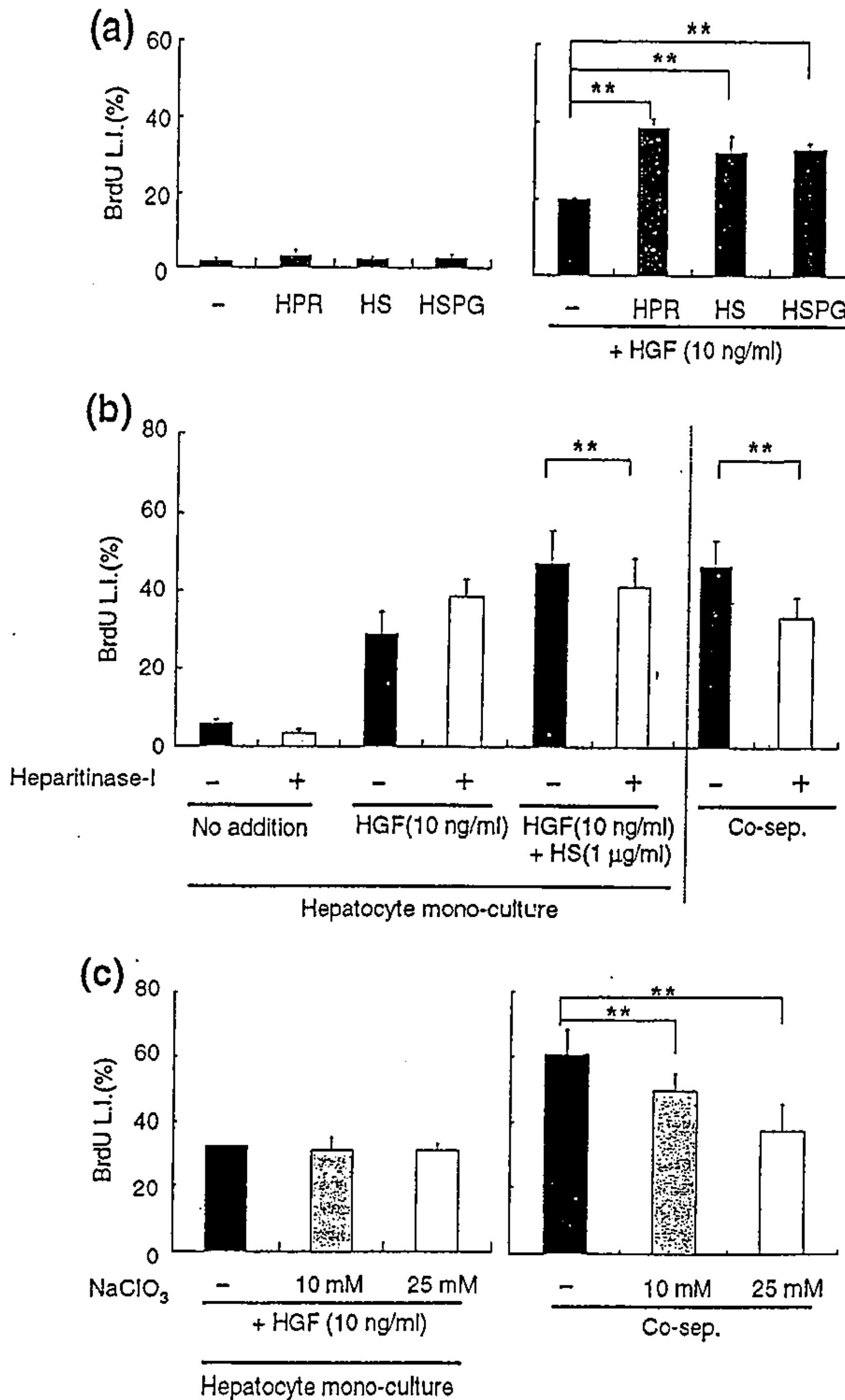

Fig. 6. Effect of HPR, HS and HSPG on DNA synthesis of hepatocytes. (a) Hepatocytes were mono-cultured on six-well plates at the density of 1.5×10^4 cells/cm^2 in serum-free Williams-E medium. After 3 h, HPR, HS and HSPG at a concentration of 1 μg/ml were added in the absence (left) or presence (right) of HGF (10 ng/ml). The culture was continued up to 2 days. HPR, heparin. HS, heparan sulfate. HSPG, heparan sulfate proteoglycan. $^{**}P < 0.01$. (b) Hepatocytes in mono-culture or in Co-sep. were prepared as described in Section 2. After plating for 3 h, heparitinase-I was added. In some experiments, HGF and/or HS were added to the mono-Hc. The culture was continued up to 2 days. $^{**}P < 0.01$. (c) Hepatocytes in mono-culture or in Co-sep. were prepared as described in Section 2. After plating for 3 h, $NaClO_3$ was added. HGF was added to the hepatocyte mono-culture. The culture was continued up to 2 days. In each experiment, BrdU incorporation of hepatocytes during the last 24 h was evaluated by immunostaining. Values of means ± SD of the triplicate determinations are indicated. $^{**}P < 0.01$.

hepatocytes, such as TGF-α, EGF and HGF [5–8]. However, whether their amount produced by HSC is enough to stimulate hepatocyte DNA synthesis has not been characterized in detail. NK1 and PD153035 reduced only partially the hepatocyte DNA synthesis induced either by co-culture or by each CM (Table 1 and Fig. 4d). Furthermore, MAPK activation by each CM was not inhibited by PD153035 (Fig. 5). Thus, although we believe that HGF, apart from EGF and TGF-α, is the most important mitogen for hepatocytes derived from HSC, we speculated that other factors and/or co-mitogens generated by HSC might be involved in the system.

Heparin was reported to promote dimerization of HGF and augment the response of hepatocytes to low dose of HGF [14,15]. HS in cell surface HSPG was reported to be involved in the binding of HGF to c-Met receptor [32]. Here, we demonstrated that exogenous HS and HSPG enhanced HGF-induced DNA synthesis of hepatocyte, and that heparitinase-I and sodium chlorate decreased it in Co-sep. Taken together, HS and HSPG derived from HSC may play important roles in the enhancement of hepatocyte proliferation. In fact, Gallai et al. reported that perlecan, one of HSPG, was produced and secreted by isolated HSC [33].

Because retinoids are known to affect the growth and differentiation of many types of cells [34], HSC-derived retinoids are possible candidates as a stimulator of hepatocyte DNA synthesis. However, we found here that neither *all-trans*-retinoic acid, 9-*cis*-retinoic acid, *all-trans*-retinol nor *all-trans*-retinol palmitate enhanced the DNA synthesis of hepatocytes in the presence or absence of HGF (date not shown).

The reason why hepatocyte DNA synthesis induced by CM of Co-sep. was one-third of that induced by Co-sep. is not known. We speculate that two hypotheses might be responsible for this mechanism; (1) the production of other growth factors whose mitogenic activity was lost immediately in the usual culture condition through degradation; and (2) the binding of growth stimulators secreted from HSC to ECMs or to the surface of culture plates, resulting in lowering of their concentrations in culture mediums.

We demonstrated here that DNA synthesis of hepatocytes in Co-mix. was much less than that in Co-sep. even if the total cell density was adjusted (Fig. 3d). CBX significantly enhanced the hepatocyte DNA synthesis in Co-mix. (Fig. 3e). Gap junction was formed between hepatocytes and HSC in co-culture [18]. Taken together, the cell-to-cell contact between hepatocytes and HSC might play a negative role in hepatocyte proliferation.

Albumin production was not maintained by co-culturing with HSC in the present study. This is in disagreement with that reported by Rojkind et al. [18]. This discrepancy may be caused by the different culture medium; they used hormonally defined medium containing insulin, glucagon, growth hormone, EGF, linoleic acid, etc. Therefore, these substances seem to be essential for albumin production by hepatocytes in culture.

In conclusion, the results described here indicate that HSC may stimulate the hepatocyte proliferation though the generation of HGF, HS and secreted HSPG. The cell contact between hepatocytes and HSC may disturb the DNA synthesis of hepatocytes via gap junctions.

References

[1] Blomhoff R, Wake K. Perisinusoidal stellate cells of the liver: important roles in retinol metabolism and fibrosis. FASEB J 1991;5:271–277.

[2] Pinzani M. Novel insights into the biology and physiology of the Ito cell. Pharmacol Ther 1995;66:387–412.

[3] Gohda E, Yamasaki T, Tsubouchi H. Biological and immunological properties of human hepatocyte growth factor from plasma of patients with fulminant hepatic failure. Biochim Biophys Acta 1990;1053:21–26.

[4] Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. J Biochem (Tokyo) 1996;119:591–600.

[5] Maher JJ. Cell-specific expression of hepatocyte growth factor in liver. Upregulation in sinusoidal endothelial cells after carbon tetrachloride. J Clin Invest 1993;91:2244–2252.

[6] Ramadori G, Neubauer K, Odenthal M. The gene of hepatocyte growth factor is expressed in fat-storing cells of rat liver and is downregulated during cell growth and by transforming growth factor-beta. Biochem Biophys Res Commun 1992;183:739–742.

[7] Schirmacher P, Geerts A, Pietrangelo A, Dienes HP, Rogler CE. Hepatocyte growth factor/hepatopoietin A is expressed in fat-storing cells from rat liver but not myofibroblast-like cells derived from fat-storing cells. Hepatology 1992;15:5–11.

[8] Schirmacher P, Geerts A, Jung W, Pietrangelo A, Rogler CE, Dienes HP. The role of Ito cells in the biosynthesis of HGF-SF in the liver. Exs 1993;65:285–299.

[9] Mullhaupt B, Feren A, Fodor E, Jones A. Liver expression of epidermal growth factor RNA. Rapid increases in immediate-early phase of liver regeneration. J Biol Chem 1994;269:19667–19670.

[10] Bachem MG, Meyer D, Melchior R, Sell KM, Gressner AM. Activation of rat liver perisinusoidal lipocytes by transforming growth factors derived from myofibroblast like cells. A potential mechanism of self perpetuation in liver fibrogenesis. J Clin Invest 1992;89:19–27.

[11] Iredale JP, Arthur MJ. Hepatocyte–matrix interactions. Gut 1994;35:729–732.

[12] Enat R, Jefferson DM, Ruiz-Opazo N, Gatmaitan Z, Leinwand LA, Reid LM. Hepatocyte proliferation in vitro: its dependence on the use of serum-free hormonally defined medium and substrata of extracellular matrix. Proc Natl Acad Sci USA 1984;81:1411–1415.

[13] Ben-Ze'ev A, Robinson GS, Bucher NL, Farmer SR. Cell–cell and cell–matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. Proc Natl Acad Sci USA 1988;85:2161–2165.

[14] Schwall RH, Chang LY, Godowski PJ. Heparin induces dimerization and confers proliferative activity onto the hepatocyte growth factor antagonists NK1 and NK2. J Cell Biol 1996;133:709–718.

[15] Zioncheck TF, Richardson L, Liu J. Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. J Biol Chem 1995;270:16871–16878.

[16] Bissell DM, Arenson DM, Maher JJ, Roll FJ. Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. J Clin Invest 1987;79:801–812.

[17] Bissell DM, Caron JM, Babiss LE, Friedman JM. Transcriptional regulation of the albumin gene in cultured rat hepatocytes. Role of basement-membrane matrix. Mol Biol Med 1990;7:187–197.

[18] Rojkind M, Novikoff PM, Greenwel P. Characterization and func-