previous results on rats (*22*). The difference in our findings may depend on the amount of DNA administered to each pig, or due to species specificity of the sialic receptor for sendai viral transfection. However, this gene transduction ratio may be sufficient for the treatment of certain liver diseases including inherited liver enzyme deficiencies, and the application of immunogene therapies. For example, 5% of the normal enzyme activity was reported to be sufficient for the treatment of Crigler Najjar syndrome (*37*). In addition, the repeated transfections proved to be possible probably because of the low immunogenicity of the HVJ-liposome vector system. Collectively, HVJ-liposome mediated gene transfer using the TVE technique of the liver may be one promising approach in the field of hepatic gene therapy.

The advantages of using HVJ-liposome vector is its low immunogenicity, and its ability to be used for repetitive transfections (*29*). Using this vector, Bcl2 transfection into the rat liver graft was able to inhibit apoptosis after ischemia reperfusion injury (*38*, *39*), and a FasL transfected liver graft could eliminate the infiltrated cytotoxic lymphocytes efficiently (*40*). These previous results suggested that the HVJ-liposome vector mediated gene delivery system might therefore be a suitable treatment modality in the transplant setting.

In conclusion, our data shows the potential usefulness of HVJ-liposome as a gene transfer vector for liver directed-gene therapy in a pig model. This method may be applied to either liver transplantation or the treatment of the various liver diseases including inherited liver enzyme deficiencies.

ACKNOWLEDGMENTS

We wish to thank Dr. H. Satoh for approving the animal experimental protocol in the laboratory of the Animal Center for Biomedical Research, Nagasaki University School of Medicine, Japan.

REFERENCES

1. Gojo S, Cooper DK, Iacomini J, LeGuern C. Gene therapy and transplantation. *Transplantation* 2000; 69 (10): 1995.
2. Muraca M, Gerunda G, Neri D, et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet* 2002; 359 (9303): 317.
3. Wang X, Willenbring H, Akkari Y, et al. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003; 422 (6934): 897.
4. Vassilopoulos G, Wang PR, Russell DW. Transplanted bone marrow regenerates liver by cell fusion. *Nature* 2003; 422 (6934): 901.
5. Nguyen TH, Ferry N. Liver gene therapy: advances and hurdles. *Gene Ther* 2004; 11 Suppl 1: S76.
6. Ramirez P, Chavez R, Majado M, et al. Life-supporting human complement regulator decay accelerating factor transgenic pig liver xenograft maintains the metabolic function and coagulation in the nonhuman primate for up to 8 days. *Transplantation* 2000; 70 (7): 989.
7. Kren BT, Chowdhury NR, Chowdhury JR, Steer CJ. Gene therapy as an alternative to liver transplantation. *Liver Transpl* 2002; 8 (12): 1089.
8. Kren BT, Parashar B, Bandyopadhyay P, et al. Correction of the UDP-glucuronosyltransferase gene defect in the gunn rat model of crigler-najjar syndrome type I with a chimeric oligonucleotide. *Proc Natl Acad Sci U S A* 1999; 96 (18): 10349.
9. Yant SR, Meuse L, Chiu W, et al. Somatic integration and long-term transgene expression in normal and haemophilic mice using a DNA transposon system. *Nat Genet* 2000; 25 (1): 35.
10. Sauter BV, Parashar B, Chowdhury NR, et al. A replication-deficient rSV40 mediates liver-directed gene transfer and a long-term amelioration of jaundice in gunn rats. *Gastroenterology* 2000; 119 (5): 1348.
11. Olivares EC, Hollis RP, Chalberg TW, et al. Site-specific genomic integration produces therapeutic Factor IX levels in mice. *Nat Biotechnol* 2002; 20 (11): 1124.
12. Guo L, Li X, Enosawa S, et al. Prolongation of liver xenograft survival by adenovirus vector-mediated CTLA-4Ig gene transfer. *Transpl Immunol* 2003; 11 (2): 155.
13. Hofling AA, Devine S, Vogler C, Sands MS. Human CD34+ hematopoietic progenitor cell-directed lentiviral-mediated gene therapy in a xenotransplantation model of lysosomal storage disease. *Mol Ther* 2004; 9 (6): 856.
14. Breyer B, Jiang W, Cheng H, et al. Adenoviral vector-mediated gene transfer for human gene therapy. *Curr Gene Ther* 2001; 1 (2): 149.
15. Chia SH, Geller DA, Kibbe MR, et al. Adenovirus-mediated gene transfer to liver grafts: an improved method to maximize infectivity. *Transplantation* 1998; 66 (11): 1545.
16. Yei S, Mittereder N, Wert S, et al. In vivo evaluation of the safety of adenovirus-mediated transfer of the human cystic fibrosis transmembrane conductance regulator cDNA to the lung. *Hum Gene Ther* 1994; 5 (6): 731.
17. Raper SE, Chirmule N, Lee FS, et al. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab.* 2003; 80 (1-2): 148.
18. Andrews JL, Kadan MJ, Gorziglia MI, et al. Generation and characterization of E1/E2a/E3/E4-deficient adenoviral vectors encoding human factor VIII. *Mol Ther* 2001; 3 (3): 329.
19. Muruve DA, Cotter MJ, Zaiss AK, et al. Helper-dependent adenovirus vectors elicit intact innate but attenuated adaptive host immune responses in vivo. *J Virol* 2004; 78 (11): 5966.
20. Kaneda Y, Iwai K, Uchida T. Increased expression of DNA cointroduced with nuclear protein in adult rat liver. *Science* 1989; 243 (4889): 375.
21. Kato K, Nakanishi M, Kaneda Y, et al. Expression of hepatitis B virus surface antigen in adult rat liver. Co-introduction of DNA and nuclear protein by a simplified liposome method. *J Biol Chem* 1991; 266 (6): 3361.
22. Hirano T, Fujimoto J, Ueki T, et al. HVJ-liposome mediated gene transfer into hepatocytes in vivo. *J Hepatol* 1998; 29 (6): 910.
23. Calne RY, White HJ, Herbertson BM, et al. Pig-to-baboon liver xenografts. *Lancet* 1968; 1 (7553): 1176.
24. Tector AJ, Berho M, Fridell JA, et al. Rejection of pig liver xenografts in patients with liver failure: implications for xenotransplantation. *Liver Transpl* 2001; 7 (2): 82.
25. Kaneda Y. Virus (Sendai virus envelopes)-mediated gene transfer. In: Cell Biology: A Laboratory Handbook. Celis JE, ed. San Diego: Academic Press, 1994.
26. Berry MN, Friend DS. High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. *J Cell Biol* 1969; 43 (3): 506.
27. Alves A, Charre L, Panis Y, et al. Total vascular exclusion of the liver enhances the efficacy of retroviral-mediated associated thymidine kinase and interleukin-2 genes transfer against multiple hepatic tumors in rats. *Surgery* 2003; 133 (6): 669.
28. Alves A, Vibert E, Trajcevski S, et al. Adjuvant interleukin-12 gene therapy for the management of colorectal liver metastases. *Cancer Gene Ther* 2004; 11 (12): 782.
29. Hirano T, Fujimoto J, Ueki T, et al. Persistent gene expression in rat liver in vivo by repetitive transfections using HVJ-liposome. *Gene Ther* 1998; 5 (4): 459.
30. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162 (1): 156.
31. Tsukazaki T, Ohtsuru A, Namba H, et al. Parathyroid hormone-related protein (PTHrP) action in rat articular chondrocytes: comparison of PTH(1-34), PTHrP(1-34), PTHrP(1-141), PTHrP(100-114) and antisense oligonucleotides against PTHrP. *J Endocrinol* 1996; 150 (3): 359.
32. Anderson WF. Human gene therapy. *Nature* 1998; 392 (6679 Suppl): 25.
33. Kimura Y, Aoki H, Shimokata K, et al. Characterization of altered BHK cells resistant to HVJ (Sendai virus) infection. *J Gen Virol* 1980; 47 (1): 193.
34. Oku N, Nojima S, Inoue K. Studies on the interaction of Sendai virus with liposomal membranes. Sendai virus-induced agglutination of liposomes containing glycophorin. *Biochim Biophys Acta* 1981; 646 (1): 36.

35. Umeda M, Nojima S, Inoue K. Activity of human erythrocyte gangliosides as a receptor to HVJ. *Virology* 1984; 133 (1): 172.
36. Kawashita Y, Ohtsuru A, Fujioka H, et al. Safe and efficient gene transfer into porcine hepatocytes using Sendai virus-cationic liposomes for bioartificial liver support. *Artif Organs* 2000; 24 (12): 932.
37. Fox IJ, Chowdhury JR, Kaufman SS, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998; 338 (20): 1422.
38. Yamabe K, Shimizu S, Kamiike W, et al. Prevention of hypoxic liver cell necrosis by in vivo human bcl-2 gene transfection. *Biochem Biophys Res Commun* 1998; 243 (1): 217.
39. Kita J, Kobayashi E, Hishinuma A, Kaneda Y. Genetic modification of cold-preserved renal grafts using HSP70 or bcl-2 HVJ-liposome method. *Transpl Immunol* 2003; 11 (1): 7.
40. Li XK, Okuyama T, Tamura A, et al. Prolonged survival of rat liver allografts transfected with Fas ligand-expressing plasmid. *Transplantation* 1998; 66 (11): 1416.

Gene Therapy (2005) 12, 1633–1639

www.nature.com/gt

RESEARCH ARTICLE

Eradication of hepatocellular carcinoma xenografts by radiolabelled, lipiodol-inducible gene therapy

Y Kawashita[1], A Ohtsuru[2,3], F Miki[2], H Kuroda[1], M Morishita[2,3], Y Kaneda[4], K Hatsushiba[5], T Kanematsu[1] and S Yamashita[2]

[1]*Department of Transplantation and Digestive Surgery, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;* [2]*Department of Molecular Medicine, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan;* [3]*Takashi Nagai Memorial International Hibakusha Medical Center, Nagasaki University Hospital, Nagasaki, Japan;* [4]*Division of Gene Therapy Science, Graduate School of Medicine, Osaka University, Osaka, Japan; and* [5]*Pharmaceutical Chemistry & Technology Research Laboratories Research Center, Daiichi Radioisotope Laboratories, Ltd, Japan*

The promoter region of the early-growth response-1(Egr-1) gene has been shown to be activated by external radiation, thus making a selective tumoricidal effect possible. A previous experiment showed that the Egr-1 promoter can be activated by internal radiation using radioisotopes as well as external radiation. Internal radiation using I-131 lipiodol (I-131-Lip) has been established as one of the most useful therapeutic strategies against hepatoma. We herein linked the Egr-1 promoter to the herpes simplex virus-thymidine kinase (HSV-TK) gene, and investigated its efficacy in hepatoma gene therapy in combination with I-131-Lip. A luciferase assay showed the Egr-1-promoter activity to be markedly increased in hepatoma tissue specimens in an I-131-dose-dependent manner, whereas a less than two-fold increase in this activity was observed in other organs. In addition, the radioactivity derived from I-131 was selectively accumulated in the tumor tissue specimens. To examine the efficacy of EgrTK/ganciclovir (GCV) gene therapy in vivo, subcutaneous hepatoma xenografts in nude mice were transfected using a hemagglutinating virus of Japan (HVJ)-liposome vector. Complete tumor regression was observed in all the EgrTK-transfected tumors following combination treatment with I-131-Lip and GCV 42 days after treatment without any side effects (n = 8). In contrast, the tumors continued to grow in all control mice (n = 10). Furthermore, the serum α-fetoprotein levels decreased in the combination therapy group, while they increased in the controls. In conclusion, these data indicate that Egr-1 promoter-based gene therapy combined with internal radiation has a selective effect on hepatoma tumors while also showing an improved in vivo efficacy. This combination therapy might, therefore, be an effective human hepatoma gene therapy, even in advanced multiple cases.
Gene Therapy (2005) **12,** 1633–1639. doi:10.1038/sj.gt.3302531; published online 4 August 2005

Keywords: *Egr-1 promoter; hepatoma; HVJ-liposome; internal radiation; mouse model*

Introduction

Hepatocellular carcinoma (HCC) is a common human malignancy with an extremely poor prognosis.[1,2] Gene therapy represents a promising treatment for HCC. Among the potential suicide-gene therapies for cancer, the herpes simplex virus (HSV)-thymidine kinase (TK)/ganciclovir (GCV) system has been widely investigated.[3–5] HSV-TK converts the nontoxic agent GCV into a highly toxic phosphorylated GCV, which acts as a chain terminator of DNA synthesis and an inhibitor of DNA polymerase.[6–8] It is not yet possible to transduce therapeutic genes into all of the target tumor cells. However, one considerable advantage of the HSV-TK system is the 'bystander effect', in which HSV-TK-negative tumor cells are destroyed as a result of their proximity to any HSV-TK-positive tumor cells that have been exposed to phosphorylated GCV.[9] The tumor-specific activation of the therapeutic gene is crucial in order to minimize the side effects of treatment, and suicide-gene therapy controlled by the α-fetoprotein (*AFP*) promoter has proved satisfactory in this respect in both *in vitro* and *in vivo* models.[10–14] However, the level of AFP expression varies among cases, and nonmalignant tissue – particularly, chronic inflammatory tissue – can also express *AFP*.[2,15] In order to overcome the difficulties of tumor-specific activation, radiation-inducible promoters can provide spatial control of irradiation, and might therefore be useful in the treatment of hepatomas.[16]

We previously reported a novel technique for hepatoma-specific suicide-gene therapy, using a radio-inducible early-growth response-1 (*Egr-1*)-promoter containing six CArG boxes corresponding to the radiosensitive and serum-responsive elements.[17] The *Egr-1* gene was preferentially expressed in hepatoma cells compared with the surrounding nontumor tissue, and its promoter was significantly activated after radiation exposure. However, this approach might not be suitable for multiple

Correspondence: Dr A Ohtsuru, Takashi Nagai Memorial International Hibakusha Medical Center, Nagasaki University Hospital, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
Received 17 September 2004; accepted 19 February 2005; published online 4 August 2005

hepatomas, because whole-liver irradiation could be fatal to patients: firstly, as a result of direct injury from irradiation, which produces free radicals that cause DNA-strand breaks; and secondly, through the non-specific activation of suicide genes. Recently, the *Egr-1* promoter was shown to be activated not only by external radiation but also by internal radiation from isotopes such as Tc-99m, Ga-67 and I-131.[18] Importantly, I-131-labelled lipiodol (I-131-Lip) concentrates preferentially within hepatomas, where it is retained for significantly longer periods than in non-neoplastic tissues.[19–22] In addition, I-131 generates 606-MeV β-rays that produce a strong effect over short distances, which could therefore enhance the bystander effect through increased intracellular communication.[23]

In the present study, we investigated whether combined therapy comprising transduction of the *EgrTK* gene and internal radiation with I-131 was able to reduce tumor development in HCC.

Results

Biodistribution of I-131-Lip in tumor-bearing mice analyzed using autoradiography after injection into the tumor xenograft

Autoradiography was carried out after I-131-Lip injection in order to examine whether I-131-Lip accumulated preferentially in the tumor. These mice did not receive iodide-uptake inhibitors such as NaI. As shown in Figure 1, I-131-Lip accumulated preferentially at the tumor site and remained there for up to 7 days, which is consistent with the observed physiological half-life of I-131. In contrast, no I-131-Lip accumulation was detected in any other organs, including the thyroid and gut. A densitometric analysis using Bio-imaging Analyzer System (BAS5000, Fuji) showed the diphasic accumulation of radioisotopes.

Softex roentgenograms revealed the iodide uptake, which was visualized as white dots, only in tumors treated with I-131-Lip. No accumulation in any other organs was observed (data not shown).

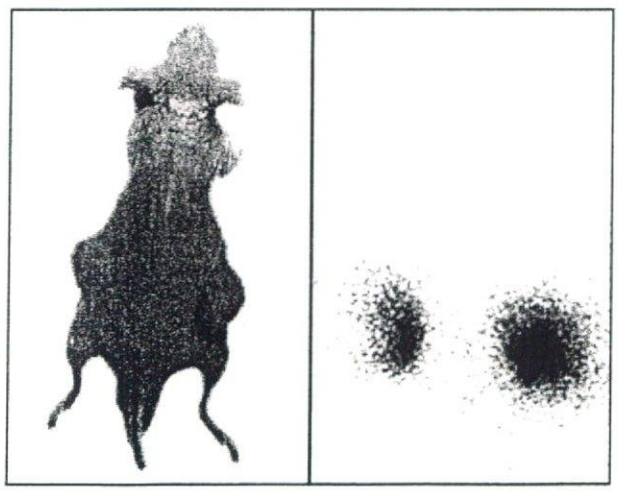

Figure 1 Biodistribution of I-131-lipiodol. I-131 accumulation was detected in mice using autoradiography. I-131-lipiodol (I-131-Lip) accumulated preferentially in the tumor. The iodide uptake was not observed in other organs, such as the thyroid and the gut.

Egr-1-promoter activity in vivo

An *in vivo* luciferase assay was performed to examine whether the *Egr-1* promoter could be activated by I-131-Lip in tumor xenografts. *EgrLuc*-HVJ-liposome was introduced into tumors when they reached 8–10 mm in size, along with 0–20 μCi of I-131-Lip, which was injected at 16 h post-transfection. The tumor tissue specimens were excised and examined to determine their luciferase activity 40 h after transfection. Radiation-induced promoter activity was expressed as relative light units, after correction using nonirradiated cell lines. The differences in transfection efficiencies between tumors were negligible. Internal radiation therapy using I-131-Lip significantly increased the *Egr-1*-promoter activity in the hepatoma tissue specimens (4.4-fold at 2 μCi and 28.8-fold at 20 μCi), whereas no increase was observed in the normal liver and lung tissue specimens (Figure 2).

Egr-1 protein expression in tumor tissue after I-131-Lip treatment

Egr-1 immunohistochemistry was carried out in tumors after treatment in order to examine the level of *Egr-1* gene expression. Faint staining was detected in the tumor cell clusters of untreated hepatoma tissue (Figure 3a), but not in the absence of the primary antibody (Figure 3b). In contrast, a significant degree of *Egr-1* activation was observed in the tissue specimens treated with I-131-Lip (Figure 3c). These results clearly show that *Egr-1* gene expression can be induced by internal irradiation using I-131, as well as by external radiation exposure.

In vivo EgrTK/GCV treatment of tumor xenografts in mice

The whole-body effective half-life of the therapeutic activity, which was measured daily using a radiation-survey meter, showed an exponential decrease with a mean of 8 days (data not shown). To further investigate

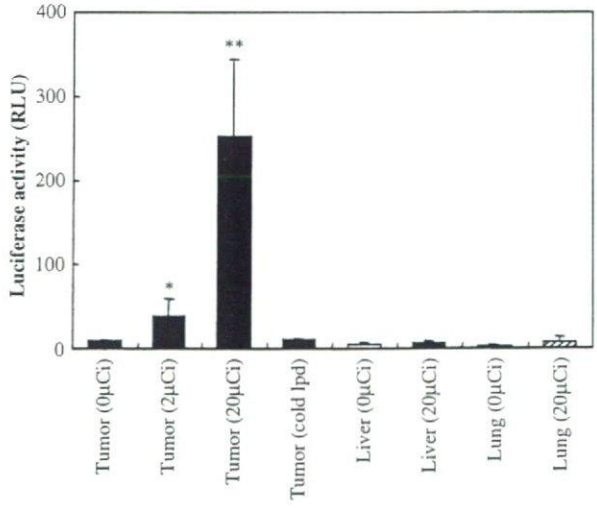

*Figure 2 Egr-1-promoter activity in vivo. Egr-1-promoter activity was significantly increased by internal irradiation to tumors that were transfected with the Egr-Luc gene. However, no increase in the activity was detected in either the liver or lung tissue specimens. *$P<0.05$, **$P<0.01$ versus control groups.*

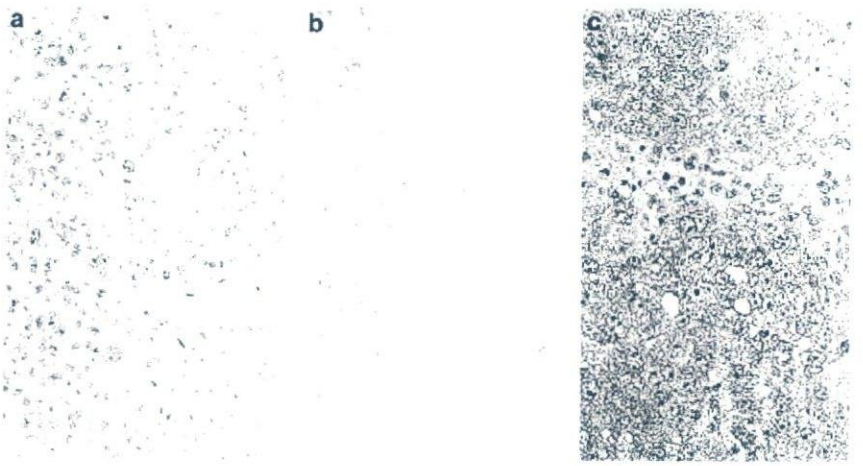

Figure 3 Egr-1 immunohistochemistry of the tumor xenograft. Low levels of Egr-1 gene expression were observed in nonirradiated hepatoma tissue (a), but not in the absence of the primary antibody (b). Internal irradiation greatly increased Egr-1 gene expression in hepatoma tissue (c).

the cytotoxic effects of *EgrTK*/GCV *in vivo*, Huh7 cells (1×10^7) were injected subcutaneously into nude mice. Gene therapy was initiated when the tumor volume reached approximately 200 mm³. No spontaneous tumor regression was observed in the untreated tumors during the course of treatment. The data for each treatment group were calculated as a percentage of the initial (day 0) volume, and were plotted as the mean fractional volume ± standard error. I-131-Lip alone showed a minor dose-responsive growth-suppressive effect (Figure 4a). In contrast, the *EgrTK*-transfected animals ($n = 8$) that received irradiation without GCV exhibited no suppression of the tumor volume compared to the untreated animals ($n = 10$). A retardation of tumor growth was seen in the *EgrTK*-transfected group that received GCV without irradiation ($n = 8$). Complete tumor regression was observed in all the *EgrTK*-transfected tumors that received I-131-Lip and GCV from 24 days to 42 days after gene therapy (Figure 4b). Thereafter, seven of eight tumors continued to show regression at 80 days post gene therapy, although one remaining tumor slowly regrew.

Serum AFP measurement

AFP is commonly used as a diagnostic marker for HCC because of its high sensitivity, and human Huh7 cells produce human AFP. Therefore, in order to estimate the viable volume of implanted hepatoma tumors, the serum AFP levels were measured at the time points indicated in Figure 5. The serum AFP values progressively increased in the untreated controls, whereas a strong suppression was observed in the *EgrTK* plus I-131-Lip group, and these findings were consistent with those from the *in vivo* gene-therapy data.

Histological findings

Hematoxylin–eosin staining revealed massive apoptotic and partially necrotic cells with intratumoral lymphocyte-infiltration lesions in the *EgrTK*/GCV plus I-131-Lip group (Figure 6d) compared to the untreated tumor (Figure 6a). In contrast, slight tumor cell damage with necrosis, but without any inflammatory cell infiltration, was observed in the I-131-Lip-injected group (Figure 6c).

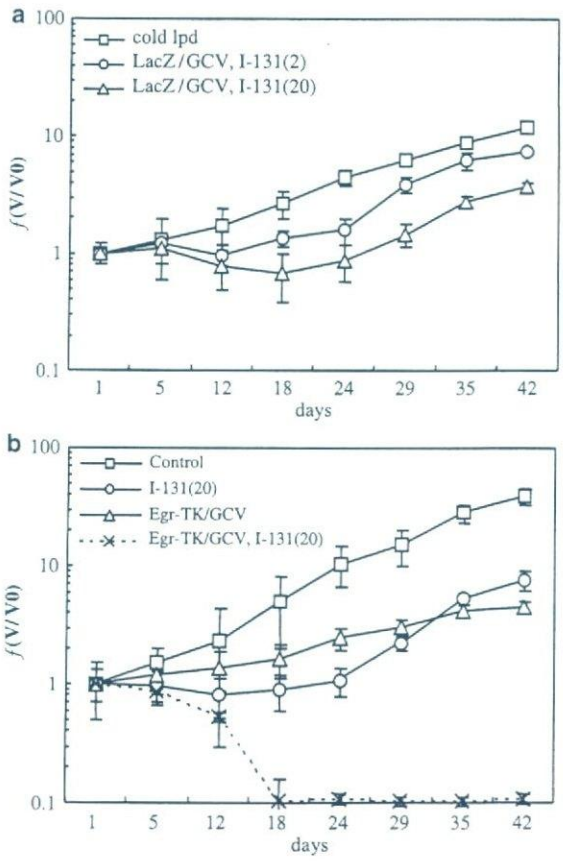

Figure 4 In vivo gene-therapy experiments in mice with hepatoma tumor xenografts. The injection of cold lipiodol did not cause tumor retardation. However, slight transient retardation was observed in the I-131-treated group in a radiation dose-dependent manner (a). Minor growth retardation was also observed in the groups treated with EgrTK/GCV or I-131-Lip alone. In contrast, complete tumor retardation was observed in the group treated with EgrTK/GCV plus I-131-Lip (b).

EgrTK/GCV treatment resulted in an increased number of apoptotic tumor cells, but it did not cause any necrotic tumor tissue damage (Figure 6b).

Discussion

The lymphographic agent lipiodol (also known as iodized oil) has been shown to specifically localize and persist within hepatoma tissue.[24–27] Although lipiodol itself does not seem to have any significant anticancer effects, it allows anticancer drugs to be delivered selectively to tumor tissue when applied as an emulsion.[28] Lipiodol contains sterile iodine 127, so the labelling of lipiodol with radioactive I-131 can be achieved through a simple radioisotopic exchange method.[29] The therapeutic effectiveness is primarily limited to areas in which lipiodol accumulates, as I-131 generates 606-MeV β-rays that produce strong effects over distances of less than 2.0 mm.[30] These findings indicate that I-131-Lip has potential advantages for use in hepatoma therapy.[31–33] The selective delivery and retention of lipiodol are thought to result from the developed neovasculature, enhanced permeability and the poor reticuloendothelial systems observed in hepatoma tissues. Despite this capacity for the selective accumulation at the tumor site, clinical studies have shown the effectiveness of such I-131-Lip therapy alone to only be temporary, and early HCC recurrence was observed in many cases. In contrast, recent reports have shown that I-131 therapy following a curative resection significantly improved both the disease-free interval and the survival rates.[22,34] Furthermore, no significant side effects – such as liver toxicity, renal disorders, bone-marrow suppression, thyroid dysfunction or respiratory dysfunction – have been reported after I-131-Lip administration.

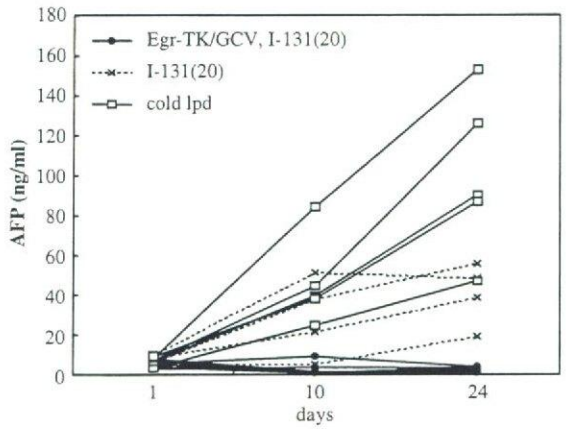

Figure 5 Measurements of serum AFP in tumor-bearing mice during treatment. The serum AFP values significantly decreased after treatment in the EgrTK/GCV plus I-131-Lip group, which was consistent with the data on tumor size. In addition, a slight decrease in serum AFP levels was observed in the group treated with I-131-Lip alone.

We previously reported that a total dose of 20 Gy of external ionizing radiation could selectively and effectively activate the *Egr-1* promoter, thus resulting in tumor regression.[17] However, it is not possible to focus specifically on multiple tumor lesions, even when using conformal irradiation. Furthermore, high doses (50 Gy or above) of whole-liver irradiation are known to be toxic to normal livers,[15] and livers with hepatitis or cirrhosis are thought to be even more highly radiosensitive. These factors have clear implications regarding the treatment of HCC.

A recent report demonstrated that the *Egr-1* promoter can be activated by both external and internal radiation, which thus prompted our investigation of I-131-Lip as both an antitumor agent and a molecular switch for radio-inducible promoter *Egr-1*-based gene therapy. Our results revealed a 28.8-fold upregulation of the *Egr-1* promoter in tumors treated with 20 μCi of I-131-Lip compared to the controls, thus resulting in the complete regression of treated tumor xenografts. Immunohistochemical analyses also showed diffuse *Egr-1* activation in the HCC tissue. Clinical trials have used between 50 and 70 mCi of I-131-Lip administered via a tumor-supplying

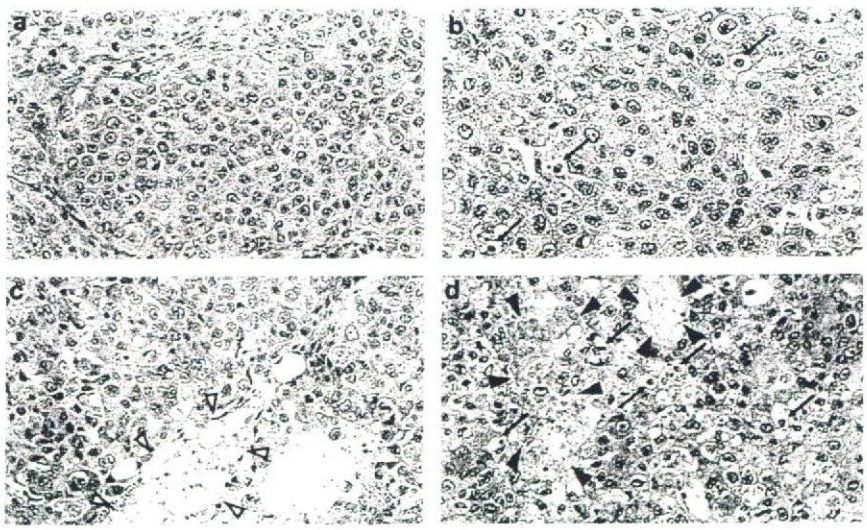

Figure 6 Histological findings in tumor tissue after treatment. Hematoxylin–eosin staining revealed apoptotic shrinkage in tumor cells (arrows) in the EgrTK/GCV group after treatment (b) compared to the normal control (a). In addition, severe steatotic changes (open triangle) were observed in the cytoplasm of the tumor cells of the I-131-Lip group (c). Both apoptotic cells (arrow) and necrotic area (arrow heads) were found in the group treated with EgrTK/GCV plus I-131-Lip (d), which supported the growth-suppressive effect of the combination therapy reported in the in vivo gene-therapy experiments.

artery. The local radioactivity of the 20 μCi dose is therefore estimated to be 10 times lower than that used in clinical I-131-Lip therapy. The selective uptake of I-131-Lip into tumor cells was also confirmed by autoradiography.

Many different genes can be activated after irradiation, including *c-fos*, *c-jun*, *TNF-α* and *NF-κB*. However, the advantage of the *Egr-1* promoter is that it is tightly regulated by irradiation and its basal activity is relatively low in nonirradiated tumors. Indeed, our *in vivo* luciferase assay data showed the leakage of the transduced suicide gene to be extremely low, thus suggesting that I-131-Lip could efficiently regulate therapeutic gene expression. Although, we have not examined whether restimulation of the *Egr-1* promoter is possible after multiple treatments with internal radiation, our *in vivo* gene-therapy data suggested that *HSV-TK* gene expression might be prolonged. We have previously shown that the *Egr-1* promoter was relatively hepatoma tissue specific.[17] More interestingly, the *Egr-1* gene has recently been shown to be overexpressed in prostate cancer and to be closely associated with its carcinogenesis.[35]

Regarding the optimal method for administering I-131-Lip to patients, arterial chemoembolization is generally performed to treat multiple HCC. However, repeated embolization is limited by ischemic damage to normal livers and/or occlusion of the main feeding artery. Importantly, I-131-Lip therapy without embolization can be repeated in patients with hepatoma, thus making internal radiation-controlled suicide-gene therapy suitable for use even in regions of tumor recurrence.

Regarding future human clinical trials, a subcutaneous HCC implantation model may not be an ideal situation. If adequate animal models are developed, further studies will be necessary to establish an adequate treatment method for intrahepatic tumors with this strategy with an ultimate goal of future clinical application.

Up to now, adenoviruses have proven to be the most efficient gene-transfer vectors. However, the clinical findings from gene therapies using these vectors directed at the liver have also revealed fatal side effects that occur as a result of systemic inflammatory responses.[36] Safer gene-transfer methods, such as nonviral vectors, must therefore be considered for liver-related gene therapy. In the present study, we used an HVJ-liposome vector for the transfer of genes, which proved to be efficient, safe and simple to use *in vivo*. This vector was constructed from inactivated HVJ envelope proteins and anionic liposomes. It therefore had a low immunogenicity, which allowed for repeated administrations without any significant tissue damage.[37–39] The gene-transfer efficiency might thus be further improved by using other lipid formations or cointroduced proteins. Single applications of HVJ-liposome-mediated gene transfer are transient, as the transferred gene remains episomal in the nucleus, similar to adenoviruses and other physical methods. However, repeated gene transfer might result in a long-term gene expression. Moreover, previous studies have detected no obvious functional or histological liver damage after gene transfer using the HVJ-liposome vector.[39] These preliminary results suggest that the HVJ-liposome method has several advantages compared with other gene-delivery systems for the *in situ* transduction of cancer cells, even though the vector itself does not have any specificity for tumor cells.

In conclusion, radio-inducible suicide-gene therapy in combination with I-131-Lip treatment was found to enhance the efficacy of gene therapy for hepatomas. This approach might therefore represent a potentially effective treatment modality for clinically unresectable multiple HCC.

Materials and methods

Plasmid construction

EgrLuc and EgrTK plasmids were constructed according to the method reported previously.[17] Briefly, a region of the murine *Egr-1* promoter (−425 to +0) was amplified using the polymerase chain reaction with specific primers from the pE425 plasmid,[40] under the kind guidance of Dr DW Kufe of the Dana-Farber Cancer Institute, Harvard Medical School, USA. The polymerase chain reaction fragment was inserted upstream of firefly luciferase or the *HSV-TK* gene, producing the EgrLuc or EgrTK plasmid, respectively.

Cell lines

The human hepatoma cell line Huh7, which was kindly provided by Dr H Nakabayashi of the Department of Biochemistry, Hokkaido University School of Medicine, Japan, was cultured in RPMI 1640 medium (Gibco, Long Island, NY, USA) supplemented with 5% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

Preparation of the HVJ-liposome vector

The HVJ-liposome vector was prepared according to the method described previously. Briefly, phosphatidylserine, phosphatidylcholine and cholesterol were mixed at a weight ratio of 1:4.8:2. The lipid mixture (10 mg) was dried by the removal of chloroform, and then hydrated in 200 μl balanced salt solution containing DNA–high-mobility group I complex (200:64 μg), which had been incubated previously at 20°C for 1 h. Liposomes were prepared by shaking and sonication. The liposome suspension (10 mg lipids) was mixed with HVJ (30 000 HAU) in a total volume of 1 ml balanced salt solution. The mixture was incubated at 4°C for 10 min, and then for 1 h with shaking at 37°C. Free HVJ was removed from the HVJ-liposome solution using sucrose density gradient centrifugation, and the second layer of sucrose containing the HVJ-liposome vector was collected.

Labelling of lipiodol with I-131

I-131-labelled lipiodol, which was produced by replacing the iodine residue of lipiodol with I-131-C104 under aseptic conditions, was supplied by The First Radioisotope Co. Ltd (Tokyo, Japan). The labelling index was consistently more than 99% and the radioactivity of the I-131-labelled lipiodol was 131 MBq/ml.

In vivo luciferase assay

To correct for transfection efficiencies, the cytomegalovirus–renilla (sea pansy) luciferase vector was cotransfected in each experiment. EgrLuc (196 μg) and cytomegalovirus–Luc (4 μg) plasmids were encapsulated in the HVJ-liposome and transfected into established tumors in nude mice. Based on the findings of previous reports, we administered 2–20 μCi of I-131-labelled

lipiodol to each animal in a total volume of 100 µl, diluted with cold lipiodol 24 h after transfection.[41] Subsequently, 48 and 72 h after transfection, the tumors, livers and lungs of the animals were excised and immersed in 1200 µl of 3× reporter lysis buffer, and then were cut with sterile scissors. The mixture was transferred to a 15 ml falcon tube and homogenized using a sonicator for 5–10 min, followed by centrifugation at 3000 r.p.m. for 5 min. The supernatant was then transferred into a 1.5 ml Eppendorf tube. Aliquots were assayed for luciferase activity in a Lumat LB9501 luminometer (Berthold Systems, Aliquippa, PA, USA) using 20 µl of supernatant and 100 µl of reconstituted luciferase-assay reagent (Promega, Madison, WI, USA). The light units produced were measured for 20 s, 3–5 min after mixing. The reagents used in this step were obtained from the Dual-Luciferase® Reporter Assay System (Promega).

In vivo imaging and quantitation of accumulated radionuclides in tumor xenografts in Balb/c nu/nu mice

Autoradiography was carried out in order to visualize I-131 accumulation in the tumors. The mice were placed under general anesthesia by the intraperitoneal injection of a sterile 2.5% solution of 2-2-2-tribromoethanol (Aldrich chemicals, Milwaukee, WI, USA). The anaesthetized animals were then placed on an imaging plate (Fuji, Tokyo, Japan) in a supine position in a dark room for 5 min, followed by a densitometric analysis using a Fujix Bioimaging Analyzer BAS2000 (Fuji).

An *in vivo* imaging study was also performed in order to examine the distribution of I-131 in established tumors in nude mice. After 3–5 weeks, when the tumor diameter had reached 8–10 mm, 200 µl of *EgrLuc*-HVJ-liposome solution was injected into the tumor.

In vivo gene therapy

Female nude mice (Charles-River Japan, Tokyo, Japan) aged 4–6 weeks were injected subcutaneously on both flanks with 200 µl of an Huh7 cell suspension (1×10^7 cells) in PBS. A transduction efficiency to the subcutaneous tumor of more than 50% was attained using the HVJ-liposome method, as reported previously.[17] After 3–5 weeks, when the tumor diameter had reached 8–10 mm, 200 µl of the *EgrTK*-HVJ-liposome was injected (on days 1 and 8). For the *in vivo* luciferase assay experiments, 0–20 µCi I-131-lipiodol was injected into the tumor xenografts on day 2. The doses were selected on the basis of the clinical data and our pilot study. GCV administration (20 mg/kg per day injected intraperitoneally) was initiated 3 h after transfection and then continued for 2 weeks.

The mice were divided into four groups as follows: untreated animals (Group A; $n=10$); those receiving *EgrTK*-vector transfection and irradiation (Group B; $n=8$); those receiving *EgrTK*-vector transfection and GCV (Group C; $n=8$) and those receiving *EgrTK*-vector transfection, irradiation and GCV (Group D; $n=8$). None of the mice showed signs of wasting or any other indications of toxicity. The tumor volume was calculated using the formula $1/2 \times (ab^2)$, where '*a*' represents the longer diameter and '*b*' represents the shorter diameter. The care and treatment of the animals were in accordance with the guidelines of the Nagasaki University Institutional Ethics Committee, Japan.

Measurement of serum AFP

In parallel with the measurement of tumor size, blood samples were collected from representative mice at various intervals by retro-orbital bleeding. Serum AFP levels were analyzed using a radioimmunoassay.[42]

Hematoxylin–eosin staining

To evaluate the mechanism causing the synergistic effects of *HSV-TK* gene therapy and internal radiation by I-131-Lip, samples of subcutaneous tumors were carefully dissected and removed at various intervals during therapy. The tumor tissues were fixed with 10% neutral formalin, embedded in paraffin and histologically examined.

Egr-1 immunohistochemistry

Immunohistochemical testing was performed in the hepatoma xenografts in order to evaluate the activation of the *Egr-1* gene after internal radiation. The samples were fixed with 4% paraformaldehyde and embedded in paraffin. The tissues were then cut into 3 µm sections, deparaffinized in xylene and rehydrated. The sections were preincubated with normal bovine serum to prevent nonspecific binding, and then were incubated overnight at 4°C with an optimal dilution (5 µg/ml) of the primary mouse monoclonal antibody against human Egr-1 (Oncogene Science, Uniondale, NY, USA). The slides were sequentially incubated with the secondary antibody and the reaction products were visualized by DAB using a SIMPLE STAIN MAX-PO (MULTI) kit (Nichirei, Tokyo, Japan). Negative control samples were prepared by replacing the primary antibody with nonimmune murine serum.

Statistical analysis

All data are presented as the mean ± standard deviation. Statistical analyses were performed using the Mann–Whitney *U*-test. Probability (*P*) values <0.05 were considered to be statistically significant.

References

1 Askari F, Wilson J. Provocative gene therapy strategy for the treatment of hepatocellular carcinoma. *Hepatology* 1992; **16**: 273–274.
2 Colombo M. Hepatocellular carcinoma. *J Hepatol* 1992; **15**: 225–236.
3 Caruso M *et al*. Regression of established macroscopic liver metastases after *in situ* transduction of a suicide gene. *Proc Natl Acad Sci USA* 1993; **90**: 7024–7028.
4 Barba D, Hardin J, Sadelain M, Gage FH. Development of antitumor immunity following thymidine kinase-mediated killing of experimental brain tumors. *Proc Natl Acad Sci USA* 1994; **91**: 4348–4352.
5 Chen SH *et al*. Gene therapy for brain tumors: regression of experimental gliomas by adenovirus-mediated gene transfer *in vivo*. *Proc Natl Acad Sci USA* 1994; **91**: 3054–3057.
6 Moolten FL. Tumor chemosensitivity conferred by inserted herpes thymidine kinase genes: paradigm for a prospective cancer control strategy. *Cancer Res* 1986; **46**: 5276–5281.

7 Moolten FL, Wells JM. Curability of tumors bearing herpes thymidine kinase genes transferred by retroviral vectors. *J Natl Cancer Inst* 1990; **82**: 297–300.
8 Moolten FL. Drug sensitivity ('suicide') genes for selective cancer chemotherapy. *Cancer Gene Ther* 1994; **1**: 279–287.
9 Freeman SM *et al*. The 'bystander effect': tumor regression when a fraction of the tumor mass is genetically modified. *Cancer Res* 1993; **53**: 5274–5283.
10 Ido A *et al*. Gene therapy for hepatoma cells using a retrovirus vector carrying herpes simplex virus thymidine kinase gene under the control of human alpha-fetoprotein gene promoter. *Cancer Res* 1995; **55**: 3105–3109.
11 Kaneko S *et al*. Adenovirus-mediated gene therapy of hepatocellular carcinoma using cancer-specific gene expression. *Cancer Res* 1995; **55**: 5283–5287.
12 Wills KN *et al*. Gene therapy for hepatocellular carcinoma: chemosensitivity conferred by adenovirus-mediated transfer of the HSV-1 thymidine kinase gene. *Cancer Gene Ther* 1995; **2**: 191–197.
13 Su H, Chang JC, Xu SM, Kan YW. Selective killing of AFP-positive hepatocellular carcinoma cells by adeno-associated virus transfer of the herpes simplex virus thymidine kinase gene. *Hum Gene Ther* 1996; **7**: 463–470.
14 Kanai F *et al*. Gene therapy for alpha-fetoprotein-producing human hepatoma cells by adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. *Hepatology* 1996; **23**: 1359–1368.
15 Ohguchi S *et al*. Expression of alpha-fetoprotein and albumin genes in human hepatocellular carcinomas: limitations in the application of the genes for targeting human hepatocellular carcinoma in gene therapy. *Hepatology* 1998; **27**: 599–607.
16 Hallahan DE *et al*. Spatial and temporal control of gene therapy using ionizing radiation. *Nat Med* 1995; **1**: 786–791.
17 Kawashita Y *et al*. Regression of hepatocellular carcinoma *in vitro* and *in vivo* by radiosensitizing suicide gene therapy under the inducible and spatial control of radiation. *Hum Gene Ther* 1999; **10**: 1509–1519.
18 Takahashi T, Namiki Y, Ohno T. Induction of the suicide HSV-TK gene by activation of the Egr-1 promoter with radioisotopes. *Hum Gene Ther* 1997; **8**: 827–833.
19 Bretagne JF *et al*. Hepatic artery injection of I-131-labeled lipiodol. Part II. Preliminary results of therapeutic use in patients with hepatocellular carcinoma and liver metastases. *Radiology* 1988; **168**: 547–550.
20 Raoul JL *et al*. Hepatic artery injection of I-131-labeled lipiodol. Part I. Biodistribution study results in patients with hepatocellular carcinoma and liver metastases. *Radiology* 1988; **168**: 541–545.
21 Raoul JL *et al*. Preoperative treatment of hepatocellular carcinoma with intra-arterial injection of 131I-labelled lipiodol. *Br J Surg* 2003; **90**: 1379–1383.
22 Boucher E *et al*. Adjuvant intra-arterial injection of iodine-131-labeled lipiodol after resection of hepatocellular carcinoma. *Hepatology* 2003; **38**: 1237–1241.
23 Glover D, Little JB, Lavin MF, Gueven N. Low dose ionizing radiation-induced activation of connexin 43 expression. *Int J Radiat Biol* 2003; **79**: 955–964.
24 Nakakuma K *et al*. Studies on anticancer treatment with an oily anticancer drug injected into the ligated feeding hepatic artery for liver cancer. *Cancer* 1983; **52**: 2193–2200.
25 Iwai K, Maeda H, Konno T. Use of oily contrast medium for selective drug targeting to tumor: enhanced therapeutic effect and X-ray image. *Cancer Res* 1984; **44**: 2115–2121.
26 Kanematsu T *et al*. Selective effects of lipiodolized antitumor agents. *J Surg Oncol* 1984; **25**: 218–226.
27 Madsen MT, Park CH, Thakur ML. Dosimetry of iodine-131 ethiodol in the treatment of hepatoma. *J Nucl Med* 1988; **29**: 1038–1044.
28 Chou FI, Lui WY, Chi CW, Chan WK. I-131-lipiodol cytotoxicity in hepatoma cells. *Proc Natl Sci Counc Repub China B* 1994; **18**: 154–160.
29 Park CH *et al*. Evaluation of intrahepatic I-131 ethiodol on a patient with hepatocellular carcinoma. Therapeutic feasibility study. *Clin Nucl Med* 1986; **11**: 514–517.
30 Kajiya Y, Kobayashi H, Nakajo M. Transarterial internal radiation therapy with I-131 lipiodol for multifocal hepatocellular carcinoma: immediate and long-term results. *Cardiovasc Intervent Radiol* 1993; **16**: 150–157.
31 Raoul JI *et al*. Internal radiation therapy for hepatocellular carcinoma. Results of a French multicenter phase II trial of transarterial injection of iodine 131-labeled Lipiodol. *Cancer* 1992; **69**: 346–352.
32 Partensky C *et al*. Intra-arterial iodine 131-labeled lipiodol as adjuvant therapy after curative liver resection for hepatocellular carcinoma: a phase 2 clinical study. *Arch Surg* 2000; **135**: 1298–1300.
33 Buscombe JR. Interventional nuclear medicine in hepatocellular carcinoma and other tumours. *Nucl Med Commun* 2002; **23**: 837–841.
34 Lau WY *et al*. Adjuvant intra-arterial iodine-131-labelled lipiodol for resectable hepatocellular carcinoma: a prospective randomised trial. *Lancet* 1999; **353**: 797–801.
35 Abdulkadir SA *et al*. Impaired prostate tumorigenesis in Egr1-deficient mice. *Nat Med* 2001; **7**: 101–107.
36 Raper SE *et al*. Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer. *Mol Genet Metab* 2003; **80**: 148–158.
37 Morishita R *et al*. Novel *in vitro* gene transfer method for study of local modulators in vascular smooth muscle cells. *Hypertension* 1993; **21**: 894–899.
38 Kato K *et al*. Expression of hepatitis B virus surface antigen in adult rat liver. Co-introduction of DNA and nuclear protein by a simplified liposome method. *J Biol Chem* 1991; **266**: 3361–3364.
39 Hirano T *et al*. Persistent gene expression in rat liver *in vivo* by repetitive transfections using HVJ-liposome. *Gene Therapy* 1998; **5**: 459–464.
40 Seung LP *et al*. Genetic radiotherapy overcomes tumor resistance to cytotoxic agents. *Cancer Res* 1995; **55**: 5561–5565.
41 Ho S, Lau WY, Leung TW, Johnson PJ. Internal radiation therapy for patients with primary or metastatic hepatic cancer: a review. *Cancer* 1998; **83**: 1894–1907.
42 Bellet DH, Wands JR, Isselbacher KJ, Bohuon C. Serum alpha-fetoprotein levels in human disease: perspective from a highly specific monoclonal radioimmunoassay. *Proc Natl Acad Sci USA* 1984; **81**: 3869–3873.

Surg Today (2005) 35:705–710
DOI 10.1007/s00595-005-3024-5

SURGERY TODAY

Review Article

Liver Repopulation: A New Concept of Hepatocyte Transplantation

YUJO KAWASHITA[1], CHANDAN GUHA[2], KOSHO YAMANOUCHI[2], YUICHIRO ITO[1], YUKIO KAMOHARA[1], and TAKASHI KANEMATSU[1]

[1]Department of Transplantation, Digestive Surgery, Nagasaki University Graduate School of Biomedical Science, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan
[2]Department of Radiation Oncology, Albert Einstein College of Medicine, New York, NY, USA

Abstract
Hepatocyte transplantation has been recognized as an alternative strategy for organ transplantation because the supply of donor livers is limited. However, in conventional hepatocyte transplantation, only 1%–10% of the liver replaced with transplanted hepatocytes. Recently a novel concept termed "liver repopulation" has been established, where the whole recipient liver can be replaced by a small number of donor hepatocytes. To induce liver repopulation, growth advantage of the donor hepatocytes against the host liver seems to be required according to the data of previous studies. Additionally, various cell sources, including bone marrow cells and other stem cells, could potentially be used as donor cells for liver repopulation. In this article, we discuss recent progress and future perspectives of this emerging technology.

Key words Hepatocyte transplantation · Liver repopulation

Introduction

Many patients with inherited liver disorders and liver failure have been successfully treated by orthotopic liver transplantation (OLT). However, this procedure is associated with several problems; primarily, donor scarcity, the lifelong use of immunosuppressive drugs, and extraordinary expenses. Thus, much interest is now focused on hepatocyte transplantation as part of regenerative medicine. The advantages of hepatocyte transplantation include:

1. It is less invasive
2. It costs much less
3. Cells from a single donor can be used for multiple recipients
4. Cells can be cryopreserved
5. Autologous hepatocytes can be isolated from the small piece of resected tissue and genetically modified as ex vivo gene therapy

The wider use of hepatocytes to treat liver disease will not be possible until hepatocytes for cell transplantation become more readily available. Liver tissue obtained from patients without any liver disease, but whose liver is unsuitable for OLT, is the usual source of hepatocytes. However, the availability of viable hepatocytes is limited, and as yet there are very few medical centers performing hepatocyte transplantation worldwide. To address this issue, strategies to amplify the transplanted hepatocytes are being vigorously investigated. In particular, the new concept of "liver repopulation" has great potential for future clinical trials of hepatocyte transplantation.

Hepatocyte Transplantation for Liver Failure

Experimental studies have shown that acute liver failure induced by the administration of liver toxic drugs such as D-galactosamine or by 90% hepatectomy can be treated by hepatocyte transplantation in various animals.[1,2] In rats with hepatic encephalopathy caused by an end-to-side portacaval shunt, which reflects fulminant hepatic failure (FHF), the intrasplenic transplantation of hepatocytes resulted in improved survival with recovery from hepatic coma.[3] Hepatocyte transplantation in rats with chronic decompensated liver cirrhosis stabilized the total bilirubin level and prothrombin time, and improved serum albumin and encephalopathy, thereby increasing survival.[4–6]

Reprint requests to: T. Kanematsu
Received: May 28, 2004 / Accepted: November 16, 2004

Table 1. Clinical trial of hepatocyte transplantation for liver failure

First author[Ref.] (year)	Target disease	No. of cells transplanted	Site of transplantation	Outcome
Mito[7] (1992)	LC, CH 10 cases	$1–60 \times 10^7$	Spleen	One patient recovered Clinically applicable
Habibullah[8] (1994)	FHF 7 cases	6×10^7/kg BW	Abdominal cavity	Survival and encephalopathy improved
Strom[25] (1997)	FHF 5 cases	$2.8–29 \times 10^7$	Spleen	Ammonemia improved and 3 patients were bridged to OLT
Bilir[26] (2000)	FHF 5 cases	$1–3.9 \times 10^{10}$	Spleen	Ammonemia improved Brain edema improved

LC, liver cirrhosis; CH, chronic hepatitis; FHF, fulminant hepatic failure; OLT, orthotopic liver transplantation; BW, body weight

The transplantation of hepatocytes in patients with acute liver failure may replace lost hepatocytes and serve as a bridge to OLT, or extend survival long enough to allow recovery and regeneration of the injured liver. Table 1 summarizes the clinical trials of hepatocyte transplantation against liver failure which have been performed to date. The first clinical trial was performed in 1991 by Mito et al.,[7] who transplanted autologous hepatocytes into the spleen of ten patients with liver cirrhosis or chronic hepatitis. The transplanted hepatocytes could be seen in the spleens for several months. Although the results were not conclusive, probably because of the insufficient number of hepatocytes isolated, it is noteworthy that hepatocyte clusters were found in the spleens. This pioneering study paved the way for subsequent clinical trials of hepatocyte transplantation. In 1994, Habibullah et al.[8] reported injecting 5×10^9 human fetal hepatocytes into the peritoneal cavity of patients with FHF, which decreased the plasma levels of ammonia and serum bilirubin and improved survival slightly. So far, hepatocyte transplantation has only been performed clinically as a "bridge to OLT" for FHF or chronic liver failure.

Hepatocyte Transplantation for Inherited Metabolic Diseases

Hepatocyte transplantation does not require replacement of the entire organ for many liver diseases, particularly the single gene defect-derived liver disorders listed in Table 2. Patients with ornithine transcarbamylase (OTC) deficiency, α1-antitrypsin deficiency, and Crigler–Najjar syndrome type 1 have all been treated with hepatocyte transplantation. One infant with OTC deficiency received more than 4×10^9 hepatocytes, which resulted in temporary relief of hyperammonemia and protein intolerance.[9] The metabolic stability achieved was lost 11 days after transplantation, presumably because the transplanted cells were rejected as a result of inappropriate immunosuppression. However, this infant underwent successful OLT at 6 months of age.

The long-term therapeutic efficacy of hepatocyte transplantation was demonstrated in a patient with Crigler–Najjar syndrome type 1, who received 7.5×10^9 hepatocytes via a percutaneous portal vein catheter.[10] High-pressure liquid chromatography analysis of bile samples showed the excretion of conjugated bilirubin, reflecting the presence of functioning hepatocytes in the host liver. The serum bilirubin levels declined remarkably, allowing the time of phototherapy to be reduced from 12 h/day to 6 h/day.

Hereditary hypercholesterolemia is a fatal disease caused by the deficiency of a single-gene low-density lipoprotein (LDL) receptor. Chowdhury et al.[11] developed a method of harvesting primary hepatocytes from Watanabe rabbits, a model of LDL-receptor deficiency, and transducing them with a recombinant retrovirus expressing the LDL receptor gene. The transduced hepatocytes were then transplanted back into the rabbits. Long-term transgene expression and a 20%–30% reduction in serum cholesterol levels were confirmed during several months of observation. A clinical trial in patients with familial hypercholesterolemia resulted in a slight reduction of serum LDL cholesterol levels, but because the liver cell mass replaced was estimated to be

Table 2. Clinical trials of hepatocyte transplantation for inherited liver enzyme deficiencies

First author[Ref.] (year)	Diseases	No. of cells transplanted	Site of transplantation	Outcome
Grossman[27] (1995)	LDL receptor deficiency 5 cases	1×10^8/kg BW	Portal vein	Cholesterol value dropped
Strom[25] (1997)	α1-AT deficiency	2.2×10^7	Splenic artery	Bridged to OLT 3 days later
Fox[10] (1998)	Crigler–Najjar syndrome type I	7.5×10^9	Portal vein	50%–60% decrease in total bilirubin
Muraca[28] (2002)	Glycogen storage disease type Ia	2×10^9	Portal vein	Food intake was normalized for 9 months
Horslen[9] (2003)	OTC deficiency	4×10^9	Portal vein	Temporary relief of hyperammonemia and protein intolerance
Sokal[29] (2003)	Refsum's disease	2×10^6	Portal vein	Total bile acids and abnormal DHCA decreased for 1 year

LDL, low-density lipoprotein; OTC, ornithine transcarbamylase; α1-AT, α1-antitrypsin; BW, body weight; OLT, orthotopic liver transplantation; DHCA, dihydroxycoprostanoic acid

less than 1%, the serum cholesterol reduction was not therapeutically beneficial.[12]

Limitations of Hepatocyte Transplantation

Although hepatocyte transplantation has been performed safely, sometimes with long-term benefits, in humans, its application is still limited. Because of the critical shortage of donor organs, there are insufficient isolated hepatocytes for hepatocyte transplantation. Furthermore, transplanted hepatocytes cannot proliferate in the host liver without adequate growth stimulus. Unfortunately, the use of partial hepatectomy or cell growth factor treatment to stimulate liver growth and increase the proportion of transplanted hepatocytes in the liver has generally failed to show a significant benefit. Because the host hepatocytes divide during liver regeneration after hepatectomy, both the host and the transplanted hepatocytes should respond similarly to this regenerative stimulus, but repeated cell transplantations have been performed without success.[13] Thus, interest is now focused on finding a way to provide selective growth pressure to the transplanted hepatocytes.

A New Concept: Repopulation of Transplanted Hepatocytes

Data from clinical trials suggest that the efficacy of hepatocyte transplantation has been hampered by the fact that only a small percentage of the host hepatocytes can be replaced with transplanted hepatocytes. Experimental studies have shown that mature hepatocytes have the potential to repopulate diseased livers in vivo under regenerative stimulus directed at the transplanted cells. In other words, replacement of the host liver cells with transplanted hepatocytes requires a combination of proliferative stimuli to the hepatocytes and suppression of the division of host hepatocytes. An example of this concept has been found in urokinase plasminogen activator transgenic mice (uPA), in which the life span of the host hepatocytes is restricted, resulting in preferential proliferation of the transplanted normal hepatocytes until the whole liver is replaced.[14] Similar data were obtained by using fumarylacetoacetate hydrolase (FAH)-deficient mice, a model of hereditary tyrosinemia.[15] To popularize the concept of "liver repopulation," chemical and physical devices to inhibit the division of host hepatocytes are being explored. Using one approach, a recipient was treated with a DNA-kilating alkaloid, retrorsine, followed by partial hepatectomy and hepatocyte transplantation.[16] This resulted in the transplanted cells progressively repopulating the host liver. Instead of retrorsine, we used hepatic irradiation to arrest the cell cycle of the host hepatocytes, which resulted in near total liver replacement by the transplanted hepatocytes[17,18] (Fig. 1). We also showed that partial hepatectomy can be replaced by adenovirus-mediated Fas ligand gene transfer, which induces strong mitotic stimuli by causing apoptotic cell killing of endogenous hepatocytes. Table 3 lists the various methods used to achieve massive liver repopulation.

Table 3. Repopulation of transplanted hepatocytes in experimental animals

First author[Ref.] (year)	Recipient animal	Preconditioning of the host	Character of transplanted hepatocytes
Moscioni[30] (1996)	NAR rat	PBL	Normal
Ilan[31] (1997)	Gunn rat	PBL	Normal
Laconi[16] (1998)	DPPIV– /– rat	PH + retrorsine	DPPIV+/+
Mignon[32] (1998)	Normal mouse	Fas antibody treatment (Jo2)	Bcl2 transduced
Overturf[15] (1998)	FAH deficient mouse	NTBC administration	FAH transduced
Guha[33] (1999)	DPPIV–/– rat	PH + RT	DPPIV+/+
Oren[34] (1999)	DPPIV–/– rat	T3 and retrorsine	DPPIV+/+
Laconi[16] (2001)	DPPIV–/– rat	Retrorsine alone	DPPIV+/+
Guha[17] (2002)	Gunn rat	PH + RT	Normal
Guo[35] (2002)	C57BL/6J mouse	CCl_4, retrorsine	X-gal transduced
Malhi[36] (2002)	DPPIV–/– rat	IR injury; RT	DPPIV+/+
Mallet[37] (2002)	Normal rat	Fas antibody treatment (Jo2)	Bcl2 transduced
Mitchell[38] (2002)	Normal rat	Fas antibody treatment (Jo2)	BclXL transduced
Takahashi[18] (2003)	Gunn rat	Fas-Ligand gene transfer + RT	Normal
Yuan[39] (2003)	DPPIV, Rag2–/– rat	CCl_4	p27–/–
Suzuki[40] (2004)	Normal rat	PH + retrorsine	c-Met+ CD45– sorted

PH, partial hepatectomy; DPPIV, dipeptidyl-peptidase IV; RT, radiation treatment; IR, ischemia–reperfusion injury; FAH, fumarylacetoacetate hydrolase; NTBC, 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione; PBL, portal branch ligation; SD, Sprague-Dawley

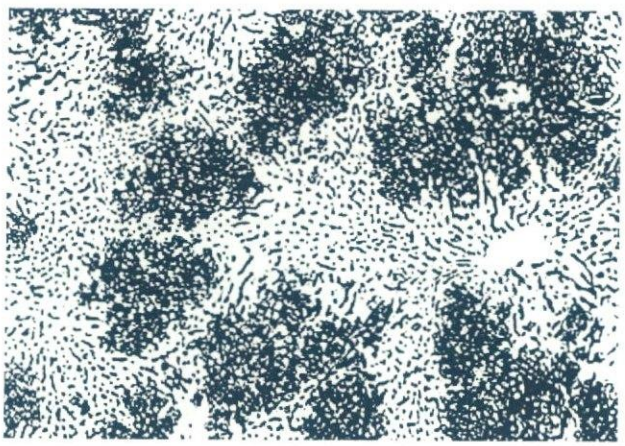

Fig. 1. Hepatocyte repopulation in F344 rats that received hepatic irradiation. Dipeptidyl-peptidase IV (DPPIV) +F344 hepatocytes (stained *red*) were transplanted intrasplenically into congenic, DPPIV-deficient F344 rats subjected to whole liver irradiation (50 Gy)

Novel Sources of Hepatocytes

The success of hepatocelluar transplantation depends greatly on the eventual number of viable hepatocytes transplanted. Autologous hepatocytes are an ideal source because immunosuppression is not required; however, realistically, when cells are obtained from allogenic donors, immunosuppressant therapy is needed. To address this issue, a novel source of hepatocytes should be investigated. Immortalizing isolated hepatocytes is one approach.[19,20] Although successful results have been reported, complete suppression of tumorigenic potential is a major concern when transplanting these immortalized cells to humans. Xenogeneic cells such as porcine hepatocytes have also been considered as an unlimited source of mature hepatocytes. These cells were originally used as a bioreactor in artificial liver support systems with encouraging results,[21,22] but reports of porcine endogenous retrovirus, which can infect human cells, halted the use of porcine hepatocytes.[23] Stem/progenitor cells are the most promising source of hepatocytes. Recent advances in stem cell biology have changed the dogma concerning lineage relationships. Hepatocytes derived from bone marrow cells were proven capable of repopulating the host liver in a murine model of tyrosinemia, although it is not clear what signals are implicated in the differentiation of the bone marrow cells into mature hepatocytes.[24]

Future Perspectives

The idea that isolated cells can be transplanted into the body and directed to repair damaged organs is gaining much attention. As described in this review, hepatocyte transplantation is technically feasible and clinically beneficial; however, the number of transplanted hepatocytes that survive in the host liver limits the efficiency of hepatocyte transplantation. We think that liver repopulation technology will enable the expansion of a few transplanted hepatocytes, which can be obtained from small pieces of liver from the patient (autologous) or healthy donors (allogenic). In our mind, preparative liver irradiation of the host liver can be translated in the clinical settings as follows. A partial hepatectomy is performed in patients with liver dis-

eases, and hepatocytes are isolated from the resected liver tissue. Then, after irradiation to the remnant liver, the isolated hepatocytes (with or without genetic modification) are transplanted back into the patient. If the host liver is not suitable for cell isolation or transplantation, the allogenic hepatocytes obtained from the healthy donors can be used instead, although this requires immunosuppression.

In conclusion, hepatocyte transplantation to treat human liver disorders is still in the early stages of development. Further research and clinical trials are needed, but hepatocyte transplantation shows the promise of becoming a therapeutic option for patients with end-stage liver diseases, particularly those awaiting OLT.

Acknowledgment. This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research (B), 2003, 15390381.

References

1. Demetriou AA, Reisner A, Sanchez J, Levenson SM, Moscioni AD, Chowdhury JR. Transplantation of microcarrier-attached hepatocytes into 90% partially hepatectomized rats. Hepatology 1988;8:1006–9.
2. Baumgartner D, LaPlante-O'Neill PM, Sutherland DE, Najarian JS. Effects of intrasplenic injection of hepatocytes, hepatocyte fragments and hepatocyte culture supernatants on D-galactosamine-induced liver failure in rats. Eur Surg Res 1983; 15:129–35.
3. Ribeiro J, Nordlinger B, Ballet F, Cynober L, Coudray-Lucas C, Baudrimont M, et al. Intrasplenic hepatocellular transplantation corrects hepatic encephalopathy in portacaval-shunted rats. Hepatology 1992;15:12–8.
4. Gupta S, Yerneni PR, Vemuru RP, Lee CD, Yellin EL, Bhargava KK. Studies on the safety of intrasplenic hepatocyte transplantation: relevance to ex vivo gene therapy and liver repopulation in acute hepatic failure. Hum Gene Ther 1993;4:249–57.
5. Kobayashi N, Ito M, Nakamura J, Cai J, Gao C, Hammel JM, et al. Hepatocyte transplantation in rats with decompensated cirrhosis. Hepatology 2000;31:851–7.
6. Ahmad TA, Eguchi S, Yanaga K, Miyamoto S, Kamohara Y, Fujioka H, et al. Role of intrasplenic hepatocyte transplantation in improving survival and liver regeneration after hepatic resection in cirrhotic rats. Cell Transplant 2002;11:399–402.
7. Mito M, Kusano M, Kawaura Y. Hepatocyte transplantation in man. Transplant Proc 1992;24:3052–3.
8. Habibullah CM, Syed IH, Qamar A, Taher-Uz Z. Human fetal hepatocyte transplantation in patients with fulminant hepatic failure. Transplantation 1994;58:951–2.
9. Horslen SP, McCowan TC, Goertzen TC, Warkentin PI, Cai HB, Strom SC, et al. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. Pediatrics 2003;111:1262–7.
10. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, et al. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 1998; 338:1422–6.
11. Chowdhury JR, Grossman M, Gupta S, Chowdhury NR, Baker JR Jr, Wilson JM. Long-term improvement of hypercholesterolemia after ex vivo gene therapy in LDLR-deficient rabbits. Science 1991;254:1802–5.
12. Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D, et al. Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat Genet 1994; 6:335–41.
13. Rozga J, Holzman M, Moscioni AD, Fujioka H, Morsiani E, Demetriou AA. Repeated intraportal hepatocyte transplantation in analbuminemic rats. Cell Transplant 1995;4:237–43.
14. Petersen J, Dandri M, Gupta S, Rogler CE. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc Natl Acad Sci USA 1998;95:310–5.
15. Overturf K, Al-Dhalimy M, Manning K, Ou CN, Finegold M, Grompe M. Ex vivo hepatic gene therapy of a mouse model of hereditary tyrosinemia type I. Hum Gene Ther 1998;9:295–304.
16. Laconi E, Oren R, Mukhopadhyay DK, Hurston E, Laconi S, Pani P, et al. Long-term, near-total liver replacement by transplantation of isolated hepatocytes in rats treated with retrorsine. Am J Pathol 1998;153:319–29.
17. Guha C, Parashar B, Deb NJ, Garg M, Gorla GR, Singh A, et al. Normal hepatocytes correct serum bilirubin after repopulation of Gunn rat liver subjected to irradiation/partial resection. Hepatology 2002;36;354–62.
18. Takahashi M, Deb NJ, Kawashita Y, Lee SW, Furgueil J, Okuyama T, et al. A novel strategy for in vivo expansion of transplanted hepatocytes using preparative hepatic irradiation and FasL-induced hepatocellular apoptosis. Gene Ther 2003;10: 304–13.
19. Fox IJ, Chowdhury NR, Gupta S, Kondapalli R, Schilsky ML, Stockert RJ, et al. Conditional immortalization of Gunn rat hepatocytes: an ex vivo model for evaluating methods for bilirubin-UDP-glucuronosyltransferase gene transfer. Hepatology 1995;21: 837–46.
20. Kobayashi N, Fujiwara T, Westerman KA, Inoue Y, Sakaguchi M, Noguchi H, et al. Prevention of acute liver failure in rats with reversibly immortalized human hepatocytes. Science 2000;287: 1258–62.
21. Watanabe FD, Mullon CJ, Hewitt WR, Arkadopoulos N, Kahaku E, Eguchi S, et al. Clinical experience with a bioartificial liver in the treatment of severe liver failure. A phase I clinical trial. Ann Surg 1997;225:484–91; discussion 91–4.
22. Kanematsu T, Eguchi S. Hepatocyte-based bioartificial liver support: past, present and future. Surg Today 1998;28:483–6.
23. van der Laan LJ, Lockey C, Griffeth BC, Frasier FS, Wilson CA, Onions DE, et al. Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice. Nature 2000;407:90–4.
24. 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;6:1229–34.
25. Strom SC, Fisher RA, Thompson MT, Sanyal AJ, Cole PE, Ham JM, et al. Hepatocyte transplantation as a bridge to orthotopic liver transplantation in terminal liver failure. Transplantation 1997;63:559–69.
26. Bilir BM, Guinette D, Karrer F, Kumpe DA, Krysl J, Stephens J, et al. Hepatocyte transplantation in acute liver failure. Liver Transpl 2000;6:32–40.
27. Grossman M, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ 3rd, et al. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. Nat Med 1995;1: 1148–54.
28. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, et al. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 2002;359:317–8.
29. Sokal EM, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R, et al. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. Transplantation 2003;76:735–8.
30. Moscioni AD, Rozga J, Chen S, Naim A, Scott HS, Demetriou AA. Long-term correction of albumin levels in the Nagase

analbuminemic rat: repopulation of the liver by transplanted normal hepatocytes under a regeneration response. Cell Transplant 1996;5:499–503.
31. Ilan Y, Roy-Chowdhury N, Prakash R, Jona V, Attavar P, Guha C, et al. Massive repopulation of rat liver by transplantation of hepatocytes into specific lobes of the liver and ligation of portal vein branches to other lobes. Transplantation 1997;64:8–13.
32. Mignon A, Guidotti JE, Mitchell C, Fabre M, Werent A, De La Coste A, et al. Selective repopulation of normal mouse liver by Fas/CD95-resistant hepatocytes. Nat Med 1998;4:1185–8.
33. Guha C, Sharma A, Gupta S, Alfieri A, Gorla GR, Gagandeep S, et al. Amelioration of radiation-induced liver damage in partially hepatectomized rats by hepatocyte transplantation. Cancer Res 1999;59:5871–4.
34. Oren R, Dabeva MD, Karnezis AN, Petkov PM, Rosencrantz R, Sandhu JP, et al. Role of thyroid hormone in stimulating liver repopulation in the rat by transplanted hepatocytes. Hepatology 1999;30:903–13.
35. Guo D, Fu T, Nelson JA, Superina RA, Soriano HE. Liver repopulation after cell transplantation in mice treated with retrorsine and carbon tetrachloride. Transplantation 2002;73:1818–24.
36. Malhi H, Gorla GR, Irani AN, Annamaneni P, Gupta S. Cell transplantation after oxidative hepatic preconditioning with radiation and ischemia-reperfusion leads to extensive liver repopulation. Proc Natl Acad Sci USA 2002;99:13114–9.
37. Mallet VO, Mitchell C, Mezey E, Fabre M, Guidotti JE, Renia L, et al. Bone marrow transplantation in mice leads to a minor population of hepatocytes that can be selectively amplified in vivo. Hepatology 2002;35:799–804.
38. Mitchell C, Mallet VO, Guidotti JE, Goulenok C, Kahn A, Gilgenkrantz H. Liver repopulation by Bcl-x(L) transgenic hepatocytes. Am J Pathol 2002;160:31–5.
39. Yuan RH, Ogawa A, Ogawa E, Neufeld D, Zhu L, Shafritz DA. p27Kip1 inactivation provides a proliferative advantage to transplanted hepatocytes in DPPIV/Rag2 double knockout mice after repeated host liver injury. Cell Transplant 2003;12:907–19.
40. Suzuki A, Zheng YW, Fukao K, Nakauchi H, Taniguchi H. Liver repopulation by c-Met-positive stem/progenitor cells isolated from the developing rat liver. Hepatogastroenterology 2004;51:423–6.

成人生体肝移植におけるネオーラル投与の実際

島津元秀・若林 剛・田辺 稔・河地茂行・星野 健・北島政樹[*1)]，
早川智久・三原 潔・谷川原祐介[*2)]

Optinal application of Neoral in adult living donor liver transplantation

key words : 成人生体肝移植，ネオーラル，AUC

近年，生体肝移植において，免疫抑制剤の血中濃度が脳死肝移植に比して相対的に高値を示すこと[1)]，タクロリムス(FK506)の至適投与量がグラフト重量に規定されること[2)]などが報告され，免疫抑制剤の薬物動態が生体肝移植と脳死肝移植とで異なることが示されている．

そこで，生体肝グラフトの再生がシクロスポリン(ネオーラル)の代謝に及ぼす影響について検討し，生体肝移植における therapeutic drug monitoring(TDM)に基づいたネオーラルの至適使用法の確立を目指した[3)]．

対象と方法

2001年4月から現在までネオーラル経口投与をベースとする免疫抑制療法を施行し，薬物動態を解析できた10例の成人生体肝移植症例を対象とした．

術前日，術後3, 7, 21病日(POD)に経時的(ネオーラル投与後1, 2, 4, 6, 12時間後)に末梢静脈血および門脈血を採取し，area under the time concentration curve(AUC)を測定し，7POD，30PODに施行したCT volumetryによりグラフト肝容量を測定した．

免疫抑制はネオーラルとステロイドの2剤で行い，ネオーラル投与量は術前日5 mg/kg/dayで開始し，術後4週間はトラフ値200～300 ng/mLを目標とした．

また，Level/Dose比(L/D比)＝トラフ値/投与量，Δ AUC＝門脈血AUC－末梢血AUC，肝除去率＝Δ AUC/門脈血AUC，などについても検討した．

結 果

(1) 周術期のAUC

術前はいずれの症例においてもC_2がピーク

表1 ネオーラル使用例の臨床成績：FK506との対比

(Mean follow up)	Neoral-based (24.3 months)	FK506-based (41.5 months)
Total cases(Male/Female)	10(6/4)	15(5/10)
Acute rejection	3(30.0%)	6(40.0%)
Infection	2(20.0%) CMV enterocolitis, HCV acute hepatitis	3(20%) CMV enterocolitis, sepsis, pulmonary aspergillosis
Side effects(others)	2(20.0%) seizure, renal failure	2(13.3%) renal failure
Graft or patient loss	1(10.0%) PSC recurrence(reALDLT)	2(13.3%) idiopathic cardiac failure, pulmonary aspergillosis

[*1)]慶應義塾大学医学部外科学教室，[*2)]同 病院薬剤部

値で，C_2，AUCは脳死肝移植で報告されているデータとほぼ同様であった(C_2 = 1,013 ± 216 ng/mL，$AUC_{0\text{-}12}$ = 6,952 ± 1,143 ng・hr/mL)．一方，3PODではピークが明らかでない症例や遅れる症例が多く，C_{max} 484 ± 263 ng/mLと低かった．7PODでは術前のAUCに戻る傾向であったが，脳死肝移植での報告[4]とくらべてC_0，C_2，AUCともに低値であった．

術後1週間以内の$AUC_{0\text{-}12}$はC_2のみならずC_0ともよく相関し(r = 0.773，p = 0.0001)，トラフ値でモニターしても支障のないことが示唆された．

（2）肝再生に伴うシクロスポリンの代謝変動

シクロスポリンの肝除去率は7PODでは3PODにくらべ有意に増加し，グラフト容積の増加率と相関したが，プロトロンビン時間やASTなどの肝機能とは相関しなかった．30PODのL/D比はグラフト容積/標準肝容積(GV/SLV)と有意な負の相関(r = − 0.699，p < 0.05)を示し，肝再生に伴うグラフト容積の増大がシクロスポリンの代謝亢進に関与することが示唆された．

生体肝移植では，グラフトサイズがシクロスポリンの代謝に影響を与えることを考慮してネオーラルの投与量を加減する必要がある．

（3）臨床成績

ネオーラルを*de novo*で使用した症例の成績は**表1**のごとくで，FK506をベースにした症例と拒絶率，合併症率，生存率などに差はなく，良好であった．

おわりに

ネオーラルは，生体肝移植においても安全・有効に使用できたが，脳死肝移植にくらべて吸収・代謝に違いがあり，生体肝移植独自の投与プロトコールが必要である．

文　献

1) Trotter JF et al.: Living donor liver transplant recipients achieve relatively higher immunosuppressant blood levels than cadaveric recipients.Liver Transpl　8 : 212-218, 2002.
2) Sugawara Y et al.: Correlation between optimal tacrolimus doses and the graft weight in living donor liver transplantation. Clin Transpl　16 : 102-106, 2002.
3) Kawachi S et al.: Optimal application and monitoring of Neoral in adult living donor liver transplantation.Am J Transplant　8(Suppl) : 212-213, 2004.
4) Levy GA: C2 monitoring strategy for optimizing cyclosporine immunosuppression from the Neoral formulation. Bio Drugs　15 : 279-290, 2001.

Journal of Medical Virology 77:399–413 (2005)

Identification of Novel HCV Subgenome Replicating Persistently in Chronic Active Hepatitis C Patients

Shintaro Yagi,[1] Kenich Mori,[1] Eiji Tanaka,[2] Akihiro Matsumoto,[2] Fumiko Sunaga,[1] Kendo Kiyosawa,[2] and Kenjiro Yamaguchi[1]*

[1]*R&D Group, Advanced Life Science Institute, Inc., Saitama, Japan*
[2]*2nd Department of Internal Medicine, Shinshu University School of Medicine, Nagano, Japan*

In an effort to clarify the life cycle of HCV, the HCV genome in liver biopsies taken from chronic active hepatitis C patients undergoing interferon treatment was investigated. Molecular cloning by long distance reverse-transcription polymerase chain reaction (RT-PCR) revealed that the HCV genome in two patients with high viral loads in the liver had in-frame deletions of approximately 2 kb between E1 and NS2, which encode the E1–NS2 fusion protein and six other HCV proteins: core, NS3, NS4A, NS4B, NS5A, and NS5B. Among the remaining 21 chronic active hepatitis C patients, these types of deletion were found in another two patients and in two hepatocellular carcinoma patients. Out-of-frame deletions in the structural region were isolated from the other five patients, but the dominant RT-PCR products were non-truncated genomes. Retrospective analysis of a series of serum samples taken from a patient carrying the subgenome with the in-frame deletion revealed that both the subgenome and the full genome persisted through the 2-year period of investigation, with the subgenome being predominant during this period. Sequence analysis of the isolated cDNA suggested that both the subgenome and the full genome evolved independently. Western blotting analysis of HCV proteins from the HCV subgenome indicated that they were processed in the same way as those from the full genome. HCV subgenomes thus appear to be involved in the HCV life cycle. ***J. Med. Virol. 77:399–413, 2005.*** © 2005 Wiley-Liss, Inc.

KEY WORDS: HCV; deletion; replication; biopsy

INTRODUCTION

Hepatitis C virus (HCV) is primarily transmitted via blood and blood-derived materials [Alter et al., 1989] and often causes chronic hepatic diseases that progressively worsen to chronic active hepatitis, cirrhosis, and finally to hepatocellular carcinoma (HCC) [Kiyosawa et al., 1990, 1994, 2004; Alter and Seeff, 2000]. Interferon (IFN) and interferon with ribavirin treatment are effective in eradicating HCV from patients [Iino et al., 1994; McHutchison and Fried, 2003], improving liver histological findings, and in prolonging life in patients with hepatitis C [Yoshida et al., 1999; Kasahara et al., 2004]; however, their efficacy is limited.

HCV was first identified as cDNA clones, and was characterized molecularly using cDNA isolates [Choo et al., 1989; Kuo et al., 1989]. The HCV genome is single-stranded RNA of about 9,600 nucleotides with an untranslated region (UTR) at each end, and encodes a polyprotein of about 3,010 amino acids [Choo et al., 1989; Kato et al., 1990], which is processed into 10 proteins by a host peptidase and two HCV proteases [Hijikata et al., 1991, 1993; Grakoui et al., 1993a]; Core, E1, and E2 are structural proteins for virion formation, and NS3, NS4A, NS4B, NS5A, and NS5B are components of the replication machinery for the RNA genome [Houghton et al., 1994]. However, isolation of virion particles has been difficult owing to a lack of in vitro culture systems for HCV.

HCV replication in chimpanzee following intrahepatic injection of an RNA transcript from HCV genomic cDNA proved that a molecular clone could represent a functional HCV genome [Yanagi et al., 1997]. Lohmann

Shintaro Yagi's present address is Laboratory of Cellular Biochemistry, Animal Resource Science, Graduate School of Agricultural and Life Science, The University of Tokyo, Tokyo, Japan.

*Correspondence to: Kenjiro Yamaguchi, Advanced Life Science Institute, Inc., R&D Group, 2-10-23 Maruyamadai, Wako, Saitama, 351-0112, Japan. E-mail: kmori@alsi-i.co.jp

Accepted 20 July 2005
DOI 10.1002/jmv.20469
Published online in Wiley InterScience (www.interscience.wiley.com)

et al. [1999] established a dicistronic subgenomic RNA that replicates in a hepatoma cell line (Huh7) and consists of the HCV NS protein coding region, the 5′- and 3′-UTR of HCV and a selective marker gene. HCV subgenomic RNA replicon systems are vital to the study of the mechanisms of HCV RNA replication, but there remain problems with regard to viral replication. Information obtained from liver biopsies of hepatitis C patients thus remains important in clarifying the life cycle of HCV.

Histological grading for diagnosis [Perrillo, 1997], immunohistochemical analysis, immuno-staining [Infantolino et al., 1990; Hiramatsu et al., 1992], electron microscopic analysis [Fagan et al., 1992], in-situ hybridization and in-situ reverse transcription polymerase chain reaction (RT-PCR) [Lau et al., 1996; Dries et al., 1999], and quantitation of HCV RNA in liver biopsy specimens [Sakamoto et al., 1994; Nuovo et al., 2002] have all been used to demonstrate HCV replication in liver. However, molecularly characterized data have been limited; HCV RNA isolated from liver was found to be equivalent in size to the well-characterized RNA seen in circulating HCV [Nielsen et al., 2004], thus confirming HCV replication in liver.

In order to obtain data to elucidate the nature of HCV in liver, viral loads and the structure of the HCV genome in patient liver biopsy specimens were examined. A highly sensitive ELISA for quantitation of the HCV core antigen [Aoyagi et al., 1999; Tanaka et al., 2000] and a quantitative RT-PCR system were applied to estimate viral loads in serum and liver biopsies. For structural analysis of the whole genome in specimens, molecular clones were used. Surprisingly, it was found that novel HCV subgenomes were predominant in several patients and, in one patient, these persisted for several years. The nature of these subgenomes are described and discussed in this paper.

MATERIALS AND METHODS

Samples and Antibodies

Serum and liver biopsy specimens were taken from patients undergoing IFN-α treatment at Shinshu University Hospital. Informed consent was obtained from all patients from whom samples were taken. A 7.2 mega-unit dose of IFN-α was administered daily for 2 weeks, followed by three times per week for 22 weeks. Serum samples were collected 1 or 2 months before administration of IFN-α. A series of serum specimens was collected from one patient from before IFN treatment until 1 year after treatment. Liver biopsy specimens from two HCC patients were obtained from surgically removed cancerous liver tissues.

An anti-core monoclonal antibody (5E3) has been described previously [Kashiwakuma et al., 1996]. Anti-E1 monoclonal antibody was raised against recombinant E1 and E1/E2 proteins expressed in Sf-9 cells infected with recombinant baculoviruses (Yamaguchi unpublished). Anti-NS3 rabbit polyclonal antibody was purified from the serum of rabbits immunized with the recombinant NS3 antigen expressed in *E. coli* [Saito et al., 1992]. Anti-mouse and rabbit immunoglobulin antibody conjugated with HRP were purchased from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD) and Bio-Rad Laboratories (Tokyo, Japan), respectively. All primers were purchased from Sigma Genosys (Tokyo, Japan) and Texas Genomics Japan (Tokyo, Japan), and sequences are available on request.

Quantitation of HCV Core Antigen

Quantities of HCV core antigen were measured by EIA as described previously [Aoyagi et al., 1999; Kato et al., 2003]. The concentration of core antigen was expressed in fmol/L, and the cut-off value of the assay was set at 7.5 fmol/L. For quantitation of the core antigen in liver, extracts were diluted to 100 μg of liver protein per milliliter with negative control serum before pretreatment of the samples. Samples were heated at 56°C for 30 min with pretreatment solution containing SDS, CHAPS, and Triton X-100, and were then added to wells pre-coated with anti-HCV core antibodies, and reaction buffer was used to fill the wells. Captured core antigen after 1-hr incubation was reacted for 30 min with anti-HCV antibodies conjugated with horseradish peroxidase after stringent washing. Bound enzyme activities were measured using a Fusion plate reader (PerkinElmer, Tokyo, Japan) with a chemiluminescent reagent (SuperSinal Pico ELISA, Pierce, Rockford, IL)

Real-Time PCR Assay for HCV RNA

HCV RNA was recovered from samples by using the QIAamp viral RNA kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's instructions. HCV RNA was reverse-transcribed and amplified using QuantiTect One-Step RT-PCR kit (QIAGEN) with primers. For quantitation of the 5′-UTR, the forward primer, chiba-s (5′-TAGTGGTCTGCGGAACCGGT-3′), and reverse primer, chiba-as (5′-TGCACGGTCTACGAGACCT-3′), yielded fragments corresponding to nucleotides 141–339 of HCV RNA. In the case of the E2 region, HC1986S (5′-TGGTTCGGCTGYACATGGATGAA-3′) and HC2199AS (5′-GGRTAGTGCCARAGCCTGTATGGGTA-3′) primers were used. Reactions were performed with a LightCycler system (Roche Diagnostics K.K., Tokyo, Japan), and fluorescence by SYBR green was monitored after each elongation reaction for real-time monitoring of DNA products during PCR. The amount of HCV RNA was calculated according to the calibration curve produced with serial dilutions of standard RNA synthesized by T7 RNA polymerase (Ambion, Inc., Austin, TX) from plasmids carrying the HCV cDNA isolate (genotype 1b). To examine the specificity of PCR, the melting point of DNA products was analyzed by melting curve analysis using LCDA software (Roche Diagnostics).

Cloning and Analysis of HCV cDNA

HCV cDNA was amplified by long distance RT-PCR (LD-RT-PCR) as described previously [Tellier et al., 1996;

TABLE I. Viral Data of Patients

Patient No.	HCV genotype	Viral loads: Serum: Core antigen (fmol/L)	Viral loads: Serum: HCV RNA (copies/ml)	Viral loads: Liver biopsy: Core antigen (fmol/g protein)	Viral loads: Liver biopsy: HCV RNA (copies/g protein)	PCR primer sets for positive results: Non-truncated genome	PCR primer sets for positive results: Truncated genome	PCR primer sets for positive results: Test primer sets
368	1b	17,108.5	1.73×10^5	5,462.4	4.71×10^8	—	j	j
207	1b	12,695.2	1.44×10^5	30,792.3	1.43×10^{10}	NT	NT	NT
204	1b	5,082.4	5.74×10^4	8,779.7	2.22×10^9	i	—	i
274	1b	1,034.4	4.24×10^3	2,651.7	3.56×10^7	a, b, c, d	e	a–e
193	1b	988.8	3.09×10^4	14,519.9	1.07×10^9	a, b, c, d, e	—	a–e
331	1b	922.2	2.03×10^3	2,387.1	2.84×10^8	a, c, d	b, e	a–e
325	1b	623.5	3.82×10^3	10,127.9	7.28×10^7	a, b, c, d, e	d	a–e
288	1b	254.5	1.00×10^1	4,037.9	9.50×10^6	a, b, c, d, e	d, e	a–e
299	1b	166.6	1.14×10^3	1,287.8	5.35×10^7	c, d	—	a–e
295	1b	1.0	5.11×10^1	261.5	2.62×10^7	a, b, c, d, e	b	a–e
171	1b	1,077.3	6.42×10^3	3,781.8	6.91×10^6	c, d	b	a–e
257	1b	12.7	1.06×10^2	568.5	2.78×10^7	d	—	a–e
372	1b	723.7	2.28×10^4	1,784.1	3.35×10^8	a, b, c, d, e	—	a–e
373	1b	597.0	8.31×10^3	33,919.0	2.65×10^9	—	a, c, d	a–e
248	2a	209.3	2.58×10^2	4,417.1	3.70×10^8	—	—	a–e
235	2a	3,616.2	3.66×10^2	7,462.1	1.55×10^9	c	—	a–e
203	2b	95.1	1.46×10^2	5,590.9	1.82×10^9	—	b, d	a–e
178	2b	34.5	8.08×10^1	609.1	4.51×10^7	—	—	a–e
297	2	3,112.7	8.35×10^3	2,883.6	1.14×10^8	—	—	a–e
298	2a	180.0	8.09×10^2	3,015.0	1.76×10^9	b	—	a–e
305	2a	173.6	1.12×10^3	1,782.8	5.96×10^7	b	—	a–e
201	2a	127.6	2.40×10^3	497.6	1.87×10^7	—	—	a–e
357	2	227.2	3.11×10^3	321.9	2.29×10^7	—	—	a–e

NT: not tested.

Yanagi et al., 1998]. HCV cDNA was synthesized using Superscript II reverse transcriptase (Invitrogen K.K., Tokyo, Japan) with HC1b9405R primer (5′-GCCTA-TTGGCCTGGAGTGTTTAGCTC-3′). After RNase H (Invitrogen) treatment at 37°C, a cDNA mixture was subjected to PCR with KlenTaq DNA polymerase (BD Biosciences Clontech, Tokyo, Japan), HClong A1 primer (5′-GCCAGCCCCCTGATGGGGGCGACA-

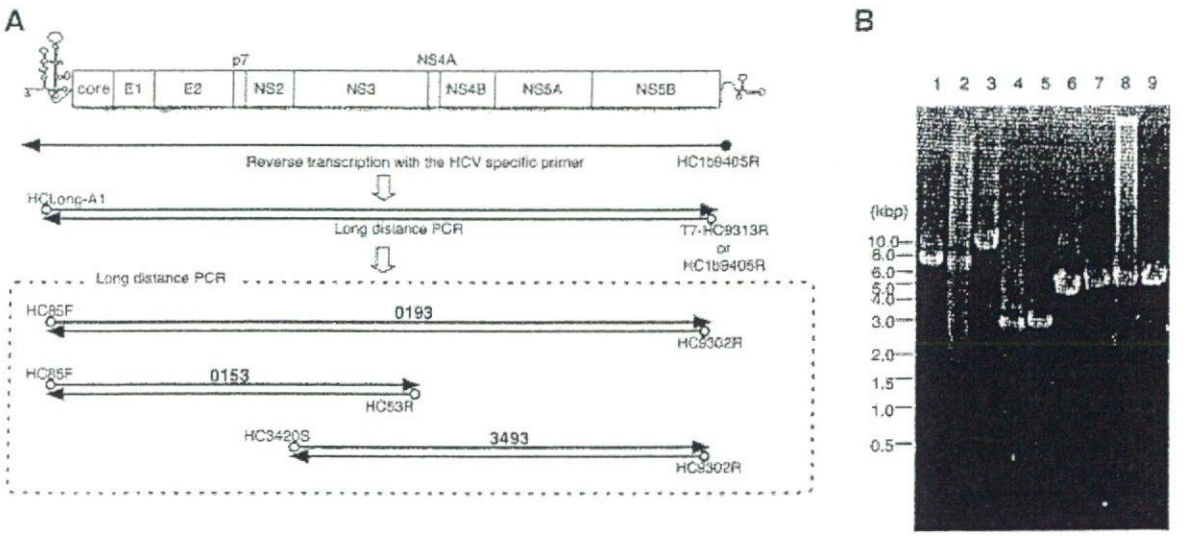

Fig. 1. Molecular cloning of HCV genome by long distance reverse-transcription PCR. **A**: Schematic view of HCV RNA is shown at the top of the figure. HCV cDNA, which was synthesized from total RNA from liver using reverse transcriptase with HCV-specific primers for the 3′-UTR, was amplified by nested PCR with HCV-specific primers. The longest LD-RT-PCR product, 0193, covered 99% (amino acids 1–2,987) of the HCV polyprotein coding sequence of genotype 1b HCV (length: 3,011 amino acids). Fragments obtained by LD-RT-PCR using HC85F and HC9302R, HC85F, and HC53R; and HC3420S and HC9302R were designated 0193, 0153, and 3493, respectively. After agarose gel electrophoresis, LD-RT-PCR products from liver biopsy samples were stained with ethidium bromide. **B**: **Lanes 1–3**, **lanes 4–6**, and **lanes 7–9** represent 0193, 0153, and 3493 fragments from Patient 207 (lanes 1, 4, and 7), 373 (lanes 2, 5, and 8), and control HCV cDNA (lanes 3, 6, and 9), respectively. The positions of markers are indicated at the left side of the image.