VEGFR-1mAb and VEGFR-2mAb, both antibodies significantly attenuated the VEGF-mediated tumor development in a dose dependent manner, with associated reduction in tumor angiogenesis. The inhibitory effect of VEGFR-2mAb was more potent than that of VEGFR-1mAb, and the combination treatment with both mAbs almost completely attenuated VEGF-mediated tumor development. Immunohistochemical analysis revealed that apoptosis increased markedly in the tumor. The inhibitory effects of both antibodies were achieved even on established tumors and orthotopic transplantation. Taken together, in addition to VEGFR-2, VEGFR-1 also participates in the signal transduction pathway by which VEGF augments tumor development and angiogenesis not only at the initial stages but also in established tumors [43].

Regarding the intracellular signaling cascade, several reports have shown that protein kinase-C (PKC), which is composed of a family of serine-threonine kinases, plays an important role in the VEGF-initiated angiogenesis [44, 45]. In VEGFR-2 transfected NIH3T3 cells and ECs, the mitogen-activated protein kinase (MAPK) was activated upon VEGF stimulation. This MAPK activation was mainly mediated by PKC, especially the β-isoform [46]. Another report showed that a PKC-β inhibitor strongly suppressed the VEGF-dependent growth of ECs *in vitro* [47]. We previously employed a combination of the Retro-Tet system and the specific the PKC inhibitor, especially the β-isoform to elucidate the role of PKC-β in tumor development and angiogenesis [48]. In the HCC syngeneic xenograft study, tumor augmentation induced by VEGF-overexpression was markedly suppressed by means of oral PKC-β inhibitor treatment, with concomitant inhibition of neovascularization and p44/42 MAPK activation. This inhibitory effect was achieved even after the tumor was fully established. Immunohistochemical analysis revealed that apoptosis markedly increased in the tumor by PKC-β inhibitor treatment, whereas the tumor cell proliferation itself had not changed. Furthermore, with orthotopical transplantation, the PKC-β inhibitor suppressed HCC development in the liver. These results suggested that PKC-β is a major regulator of VEGF-mediated tumor development and angiogenesis not only at the initial stage, but also after the tumor has been fully established. Treatment with VEGFR-2mAb significantly suppressed intracellular PKC activity, whereas VEGFR-1mAb did not. These results suggested that, similar to the *in vitro* data [46, 47], VEGFR-2 utilized mainly the PKC pathway as an intracellular cascade. On the other hand, VEGFR-1 utilized a different pathway other than PKC. *In vitro*, VEGFR-1 regulates cell migration through p38 activation and actin reorganization [49]. Further studies to identify the respective *in vivo* cascades for VEGFR-1 and VEGFR-2 are required in the future.

VEGF (VEGF-A) was originally identified as a VPF with a potent ability to permeate capillaries at a level 50,000-fold higher than histamine. It induces extravasation of plasma proteins, such as fibrinogen; and when it is deposited in the extracellular matrix (ECM), it may serve as a foundation for the formation of the tumor stroma and new capillaries. A high VEGF-A level has been found in human and mouse malignant ascites, and a high concentration of VEGF-A in the leaky capillaries lining the peritoneal cavity was found in the ascitic fluid of tumor-bearing animals [50-52]. The VEGF-A specific neutralizing antibody (VEGF-A mAb) showed a significant inhibitory effect against the experimental malignant ascites accumulation [51, 53]. Invasion of tumor cells into the peritoneum causes malignant ascites, which is manifested frequently in the patients with advanced neoplasms. The patient's quality of life with this condition is seriously decreased due to abdominal distension and pressure on the chest cavity. Although various alternative therapies, such as diuretic medications, and ascites drainage, have been employed, there are still no satisfactory therapeutic modalities for the malignant hepatic ascites. We previously reported the role of VEGFR-2 in the murine MH134 hepatic malignant ascites formation by means of the VEGF-A mAb and VEGFR-2 mAb [54]. The mean volume of ascites, the number of tumor cells in the ascitic fluid, and the peritoneal capillary permeability were significantly suppressed by VEGF-A mAb and VEGFR-2 mAb treatment. These inhibitory effects of VEGFR-2 mAb were more potent than those of VEGF-A mAb. The autophosphorylation of VEGFR-2 in the peritoneal wall was almost completely abolished by VEGFR-2 mAb, whereas a certain level of activation was still shown by the VEGF-A mAb treatment. Another member of the VEGF-family; namely, VEGF-C, which also binds to VEGFR-2, was detected in the ascitic fluid. Furthermore, in the therapeutic experiments, although both VEGF-A mAb and VEGFR-2 mAb prolonged the survival rate of ascites-bearing mice, the latter showed a more significant impact on the survival of animals. These results suggest that VEGFR-2 is a major regulator of malignant hepatic ascites formation, and that in addition to VEGF-A, VEGF-C may also be involved in malignant ascites formation *via* VEGFR-2 activation.

Until recently, it was believed that angiogenesis starts at a relatively late stage when the tumor attains a size of several hundred microns to 1 mm in diameter or when the tumor contains roughly 10^5-10^6 cells. Recently, it has been demonstrated that angiogenesis begins at a very early stage even when the tumor contains only 100-300 cells [55]. The recent studies have revealed that angiogenesis can also be induced at the early stages of tumor formation, and carcinogenic processes in several types of experimental models, such as the RIP1-Tag2 pancreatic β-cell islet carcinoma in transgenic mice [56, 57]. In this model, treatment with a small molecule inhibitor of the VEGF receptors, resulted in a significant reduction in the number of the angiogenic islets and in a substantial reduction of tumor growth [58]. A recent study on EC markers in dysplastic lesions of the liver has suggested that alterations in the hepatic microcirculation already occur at a very early stage of liver carcinogenesis [59]. The other clinical report showed that angiogenesis in the liver gradually increased from low-grade dysplastic nodules during hepatocarcinogenesis, before emergence of morphologically identifiable HCC [60]. In an experimental study, a semisynthetic derivative of fumagilin, TNP-470, which possesses anti-angiogenic activity, suppressed the progression of HCC [61]. In agreement with these studies, we found that neovascularization and VEGF expression increased stepwise during hepatocarcinogenesis. Moreover, the inhibition of either VEGFR-1 or VEGFR-2 significantly attenuated liver carcinogenesis along with angiogenesis suppression, and that the treatment with

VEGFR-2mAb was more potent than that with VEGFR-1mAb. The combination treatment with both mAbs almost completely attenuated liver carcinogenesis and even spontaneous lung metastasis [62]. These results indicated that, in addition to VEGFR-2, VEGFR-1 also played an important role in the process of carcinogenesis, tumor growth, and distant organ metastasis (Fig. (1)).

Although VEGF is known as one of the important angiogenic factors for tumor growth, it is now recognized that the *in vivo* angiogenesis status is determined by a balance of several angiogenic factors, rather than by a single regulator alone. In addition to VEGF, bFGF is also known as a representative potent angiogenic factor. bFGF is a prototype member of 13 structurally related, heparin-binding growth factors, and is a known mitogen for several types of cells, including vascular ECs and fibroblasts [63, 64]. Using a transgenic mouse model, it has been clarified that the angiogenic switch in the bovine papilloma virus-induced fibrosarcoma correlates with the export of bFGF from the tumor cells [65]. Several *in* vitro studies have shown that VEGF and bFGF possessed a synergistic effect in the induction of angiogenesis, and that bFGF increased the VEGF and VEGFR-2 expressions in ECs and several other types of cells [66, 67]. It has been shown that bFGF-enhanced angiogenesis is significantly inhibited by VEGF suppression, e.g. by VEGF neutralizing antibody and antisense oligonucleotide [67, 68]. The combined *in vivo* administration of VEGF and bFGF exerted a synergistic effect in an animal model of hind limb ischemia and in the mouse cornea angiogenesis [67, 69]. In the animal experiments, single gene overexpression of bFGF and VEGF significantly augmented tumor growth and angiogenesis, whereas suppression of each factor inhibited the tumor growth in several types of tumors [17, 70-72]. We have recently found that bFGF and VEGF synergistically increased tumor growth and angiogenesis in murine HCC cells [73]. This synergistic effect was also found in established tumors. The VEGF mRNA expression in the tumor was increased 3.1-fold by bFGF overexpression, and the bFGF-induced tumor development was significantly attenuated by treatment with VEGFR-2 neutralizing mAb. These results suggested that bFGF synergistically augmented the VEGF-mediated HCC development and angiogenesis, at least partly, *via* induction of VEGF activity through VEGFR-2.

THE RENIN-ANGIOTENSIN SYSTEM (RAS) AND CANCER

The RAS normally regulates renal blood flow and fluid homeostasis, and also plays a key role in blood pressure control [74]. Angiotensin (AT)-II, which is an octapeptide

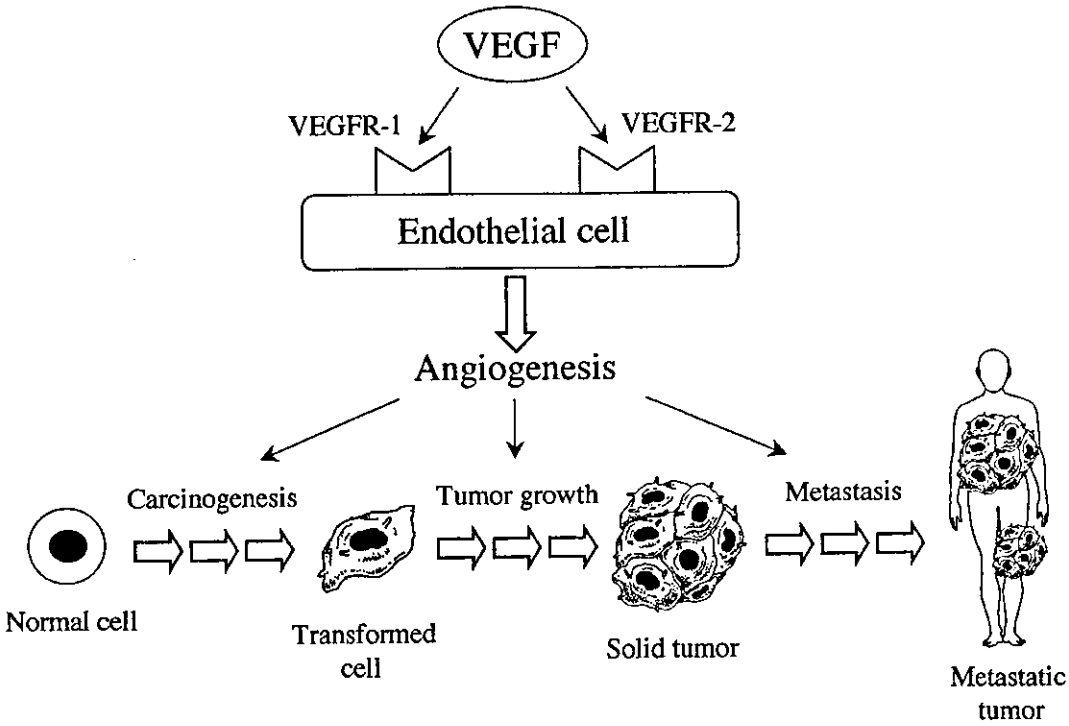

Fig. (1). VEGF-mediated angiogenesis is a prerequisite in tumor progression; e.g. carcinogenesis, tumor growth, and distant organ metastasis. VEGF utilizes the VEGFR-1 and VEGFR-2 in the angiogenesis process. VEGFR-1 and VEGFR-2 serve different roles in the angiogenesis and signal transduction pathways. VEGFR-2 is believed to be a major regulator of angiogenesis. VEGFR-1 has a dual function in angiogenesis, acting in a positive or negative manner under different conditions. VEGFR-1 is an important mediator in tumor angiogenesis, arthritis, and arteriosclerosis *via* bone marrow-derived hematopoietic stem-cell recruitment, and mobilization. VEGFR-1 also plays an important role in lung metastasis through induction of MMP-9.

produced mainly by proteolytic cleavage of its precursor AT-I *via* angiotensin-converting enzyme (ACE), has many physiological effects, including vascular hormonal secretion, tissue growth and neuronal activities through several types of receptors (ATRs). ACE is a metallopeptidase that contains *zinc* in the catalytic site, like MMPs [75]. ACE-Is competitively bind to ACE to inhibit the conversion of AT-I to AT-II by binding to zinc in the catalytic site of ACE. Among the ATRs, AT1-R and AT2-R are known to be physiologically important. Most of the biological activities of AT-II are mediated by AT1-R. However, there is increasing evidence to suggest that AT2-R also plays an important role, especially under pathological conditions [76]. A recent study has showed that VEGF induction by AT-II is mediated by both AT1-R and AT2-R [77]. In addition to these two receptors, it has been suggested that other types of receptors; namely, AT3-R and AT4-R, mediate the biological activities of AT-II, although the cloning of the cDNAs of these receptors has not yet been reported (Fig. (2)).

Recently, a retrospective cohort study on 5,207 patients receiving ACE-Is or other anti-hypertensive drugs with a 10-year follow-up has shown that ACE-Is decreased incident cancer and fetal cancer (Glasgow study) [78]. The other anti-hypertensive drugs, e.g. calcium channels blockers, diuretics, and β–blockers have no apparent effects on the risk of cancer development. AT-II has been shown to induce neovascularization and enhance vessel density in experimental systems [79-81]. It has been shown that AT-II selectively increased blood flow, and ACE-Is decreased intratumoral blood flow without affecting blood flow in healthy organs. In the experimental models, ACE-Is reduced the tumor cell growth rate and modulated gene expression *in vitro*. *In vivo*, captopril, an ACE-I, inhibited tumor growth and angiogenesis [80, 82, 83]. Other than cancer, angiogenesis has been shown to play an important role in several pathological processes, such as ocular neovascularization, arterial plaque formation, psoriasis, gastrointestinal ulcers, and rheumatoid arthritis [25]. The results of the EUCID study have highlighted the importance of the RAS in the pathogenesis of diabetic retinopathy [84]. This study suggested that lisinopril, an ACE-I, might slow the progression of diabetic retinopathy in type-2 diabetic patients. Several clinical and experimental studies have also suggested a strong correlation between RAS and diabetic retinopathy. The VEGF concentrations in the vitreous humor

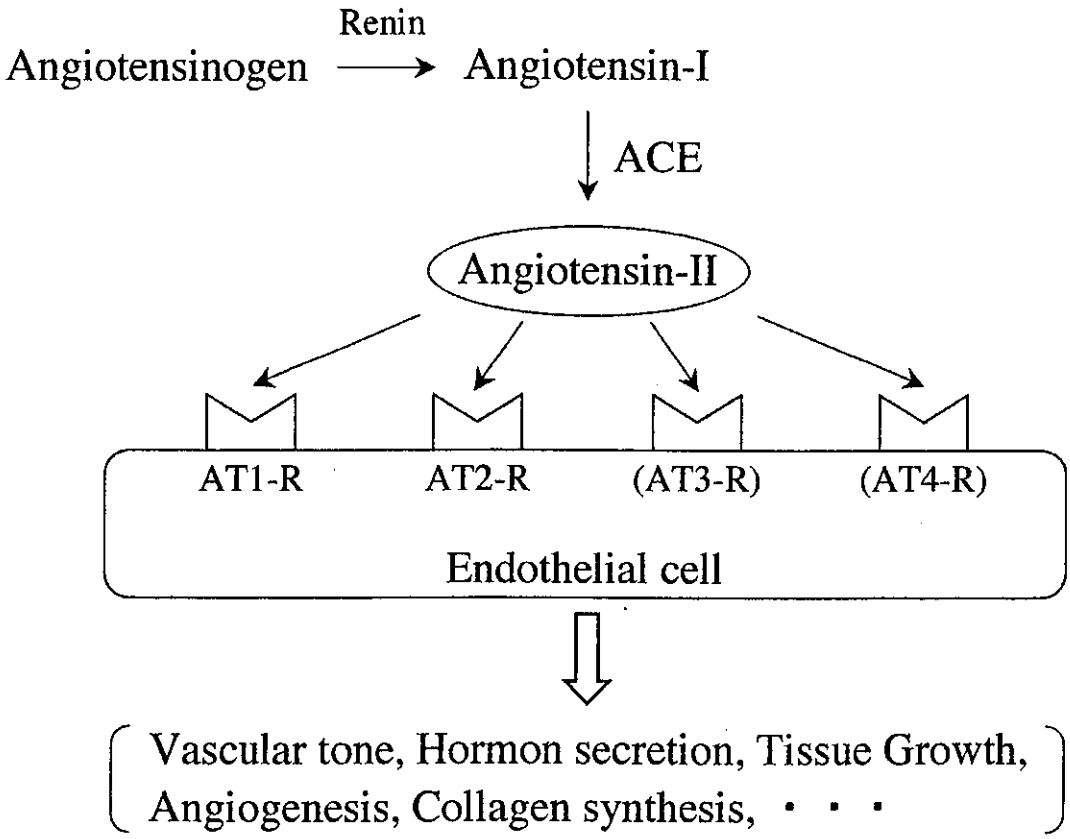

Fig. (2). A schema of the renin-angiotensin system. Angiotensin (AT)-II, which is an octapeptide produced mainly by proteolytic cleavage of its precursor AT-I by angiotensin-converting enzyme (ACE), has many physiological effects, including vascular hormonal secretion, cell growth, cell differentiation, collagen synthesis, and angiogenesis. To date, several types of AT-II receptors have been identified. The AT-II type 1 receptor (AT1-R) mediates most of the biological effects of AT-II. Other than AT1-R, AT2-R has been shown to exert biological functions different from those of AT1-R. In addition to these two receptors, it has been suggested that other types of receptors; e.g., AT3-R and AT4-R, mediate the biological activity of AT-II.

were higher in patients with diabetic retinopathy, and the expression level of the retinal VEGF mRNA was increased in streptzotocin-induced diabetic rats [85, 86]. Furthermore, treatment with ramipril and perindopril (PE), both ACE-Is, significantly reduced the diabetes-associated changes in VEGF gene expression and vascular permeability [85].

ACE-Is are currently used in more than 100 countries for the treatment of hypertension and congestive heart failure without causing serious side effects, such as myelosuppression. If these drugs really do exert an anti-angiogenic activity at clinically comparable low doses, they may become useful therapeutic modalities as anti-angiogenesis agents. It has been reported that serum ACE activity was a marker for the patients with certain types of cancer, and that the expression of ACE correlated with disease progression of prostate cancer [87, 88]. We have reported that the clinically used ACE-Is; namely, captopril, temocapril, and PE, significantly suppressed tumor growth in the murine HCC experimental model, associated with inhibition of neovascularization in the tumor [81]. Among these ACE-Is, PE showed a more potent inhibitory effect than the other two compounds. To date, several other clinically available agents have also been shown to inhibit tumor development and angiogenesis in animal experiments, but, most of these drugs have been tested at very high doses when compared to the clinical dose range. Therefore, the clinical use of these agents does not appear feasible now. Noteworthy was the finding that PE showed a tumor growth inhibitory effect at a low dose comparable to the human clinical dose. PE also exerted a significant inhibitory effect on tumor growth even after the tumor was fully established. It was found that perindopriat, which is an active metabolite of PE, did not influence the *in vitro* proliferation of tumor cells or ECs, suggesting that the inhibitory effect of PE on tumor development was not related to cytotoxicity. VEGF gene expression is induced by several types of cytokines, and some recent studies have shown that AT-II also induces VEGF in several types of cells, including tumor cells in a

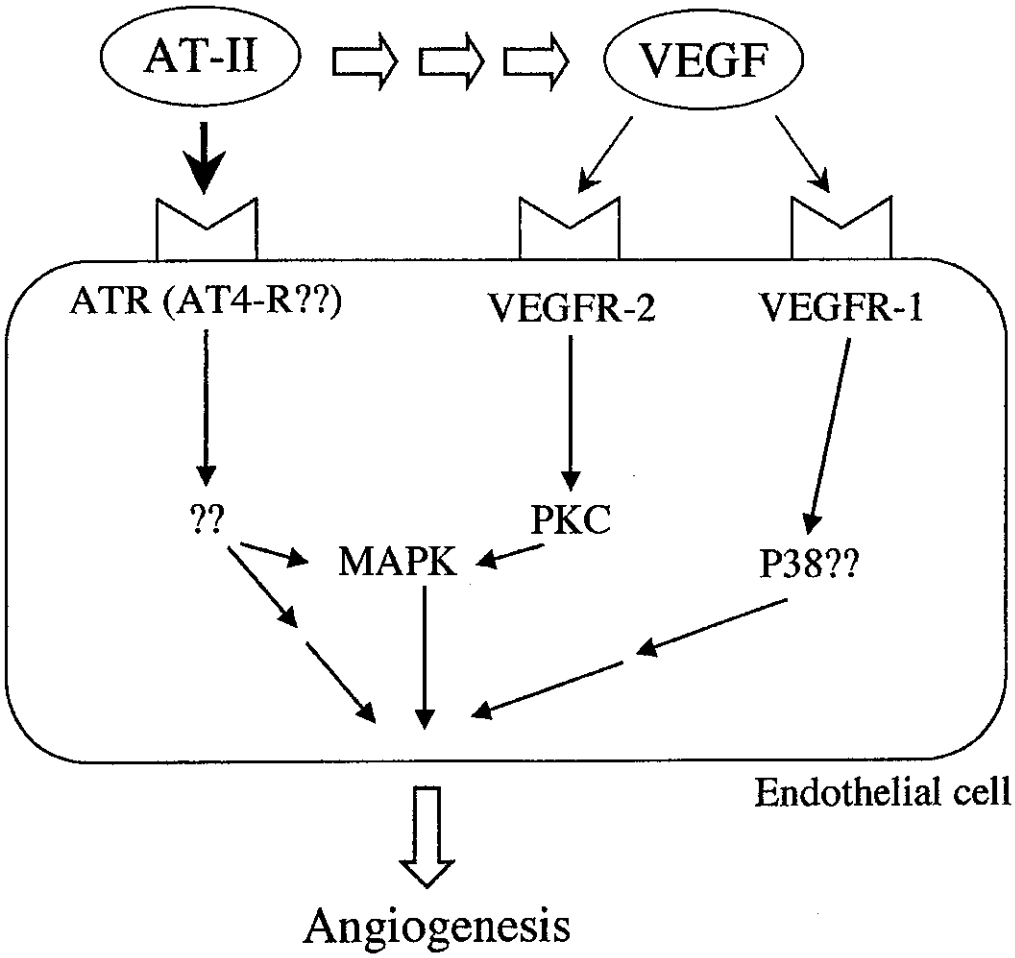

Fig. (3). Intracellular signal transduction pathway of AT-II- and VEGF-mediated angiogenesis in endothelial cells (ECs). AT-II seems to mediate the angiogenic signal by means other than AT1-R; e.g., *via* AT4-R, for proliferation of EC through mitogen-activated protein kinase (MAPK). AT-II also induces VEGF expression in a dose dependent manner. VEGF utilizes both VEGFR-1 and VEGFR-2. The protein kinase-C (PKC) and MAPK lie in the signaling pathway of VEGFR-2, whereas a different cascade, such as p38, mediates the signaling from VEGFR-1.

dose-dependent fashion. AT-II also induces the proliferation of ECs [89]. PE significantly suppressed VEGF mRNA expression in tumor cells and ECs *in vitro*. It has been shown that PE at a low clinically comparable dose markedly suppressed the VEGF-induced augmentation of tumor growth [90]. Interestingly, we did not find any inhibitory effects of the angiotensin-type I receptor blocker (ARBs), losartan and candesartan comparable to those of ACE-Is in our experiment. The most striking biological difference between ACE-Is and ARBs treatment is the AT-II level, which has been shown to stimulate angiogenesis. The AT-II level is decreased by ACE-Is, whereas the level does not change with the ARB. Other than AT1-R, AT2-R has been shown to exert a biological function different from that of AT1-R [91]. It has been also demonstrated that AT4-R induces DNA synthesis of ECs [92]. It is possible that AT-II utilizes other types of receptors besides AT1-R in tumor development (Fig. (3)). Alternatively, the surrounding stroma plays an important role in producing VEGF in addition to the tumor cell-derived VEGF, since a strong VEGF promoter activity has been found in the stroma [93]. ACE-Is, including PE, reportedly inhibit the synthesis of stromal components such as type IV collagen and transforming growth factor (TGF)-β [94]. This inhibitory effect is not mediated by AT1-R activation [95]. Since we found that the relative stromal volume was decreased in the PE-treated tumors, it is possible that there is some interaction between PE and the stromal VEGF, which cannot be evaluated by the *in vitro* methods. Contrary to our results, several studies have recently demonstrated that ARBs exerted a marked suppression of tumor growth [96]. Further studies are required to determine whether the ineffectiveness of ARB in our study was only due to a cell type-specific action or it is a common phenomenon in HCC.

It has been shown that captopril, temocapril, and PE all inhibit liver tumor development and angiogenesis. Among these agents, PE has the most potent inhibitory activity and was even effective at a clinical dose. PE is a hydrophilic compound, whereas the other two ACE-Is are more lipophilic. The liver has a specific organic anion transporter that allows the hydrophilic compounds to cross the cell membrane more efficiently than by simple diffusion, which is the only route for the lipophilic compounds. PE is metabolized to an organic anion after administration and may show a higher affinity for the liver cells than the other two drugs, thereby, producing higher concentrations in the HCC tumors. This needs to be investigated.

ACE-Is may also have other mechanisms of the anti-tumor activity. It has been reported that captopril inhibited the activities of MMP-2 and MMP-9 in a dose dependent manner, which could be neutralized by zinc [97]. Another group also proved that captopril inhibited the gelatinolytic activities of MMP-2 and MMP-9, and this inhibition was reversed by zinc [98]. Several ACE-Is, such as captopril and zofenopril, have a free-sulfhydryl donor (FSD) in their

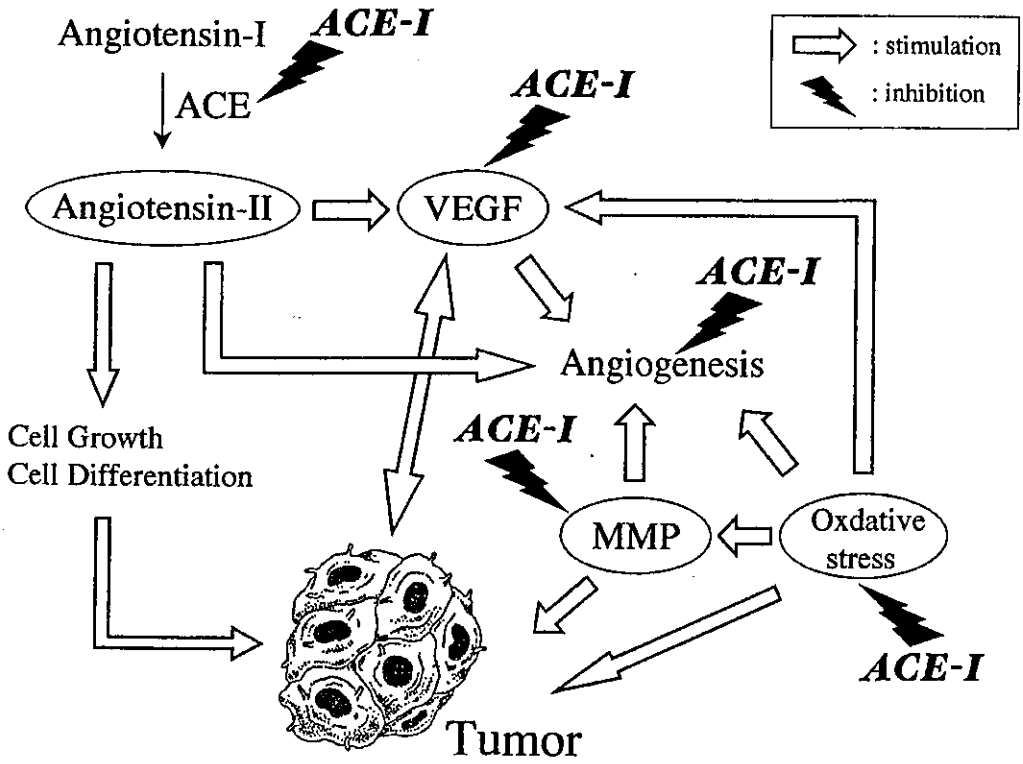

Fig. (4). A possible interaction between AT-II and other molecules in tumor growth. AT-II directly stimulates tumor cell growth and differentiation, and it also induces VEGF for angiogenesis augmentation. ACE inhibitors (ACE-Is) inhibit the conversion of AT-I to AT-II, and VEGF expression in tumor cells. Oxidative stress up-regulates the expression matrix metalloproteinase (MMP) and VEGF. The MMP and oxidative stress stimulate angiogenesis, and are inhibited by ACE-Is.

structure. It is known that a FSD acts as a free-radical scavenger, and that it also produces angiostatin [99, 100]. Reactive oxygen species have been shown to up-regulate MMP-2, MMP-9, and VEGF expression [101], and angiostatin is known to possess potent endogenous angiostatic properties [102]. In addition to the anti-angiogenic activity, these biological effects of ACE-Is should also be involved in the anti-tumor effect (Fig. (4)).

It has been reported that the use of anti-angiogenic agents as monotherapy in treating patients with advanced cancer has not yet shown significant efficacy [5]. Therefore, monotherapy with ACE-Is would not exert a sufficient effect on advanced tumors. However, tt has been demonstrated that a combination of anti-angiogenic therapy with cytotoxic therapy, such as chemotherapy or radiotherapy, increases the curative therapeutic effect in tumor-bearing animals, for which either agent alone showed only an inhibitory effect. The limitations of monotherapy with an anti-angiogenic agent in this setting were in fact predicted by preclinical studies using the angiogenesis inhibitors endostatin and angiostatin. It has been reported that the combination treatment of anti-angiogenic agents, such as endostatin and angiostatin, exerted a synergistic inhibitory effect on tumor development and angiogenesis [5, 103, 104]. It has also been reported that the combination of TNP-470, which is one of the anti-angiogenic agents under clinical trials, and interferon (IFN) inhibited angiogenesis synergistically [105]. We also observed that the combination of PE and IFN-β showed a greater suppressive effect on tumor growth than PE alone [106]. Other agents, such as 5-fluorouracil also exerted a synergistic effect with PE (Yanase *et al.*, personal communication). For the future clinical applications of PE in cancer therapy, it will be preferable to employ combination with other modalitities achieve therapeutic efficacy.

As described, it has been suggested that angiogenesis is involved in the early carcinogenesis step [56, 57, 107]. We examined the effects of PE on the exogenous and endogenous models of rat liver carcinogenesis, using diethylnitrosamine and a choline-deficient, L-amino acid-defined (CDAA) diet, respectively, to determine whether or not PE also affects the carcinogenesis step. The CDAA-model exhibits pathological sequences similar to those of the human liver disease; namely, hepatocellular necrosis, fibrotic change, cirrhosis, and finally HCC development [108]. In both models, PE significantly suppressed not only HCC tumor growth, but also hepatocarcinogenesis at a low clinically comparable dose [109]. It is now believed that a primary or secondary chemoprevention against cancer is a promising approach to improve overall survival. Since the high-risk group of HCC development seems to be more defined than other types of tumor, it is likely that a chemopreventive agent would be beneficial in improving the prognosis of HCC. Several agents, such as IFN and acyclic retinoid, have been shown to prevent secondary HCC recurrence [110, 111], but, there are still problems concerning their common clinical application; e.g. its high cost and long term-toxicity. Some of the clinically available compounds, such as thalidomide and penicillamine, have been shown to possess anti-angiogenic activity, and are currently under clinical trials [5]. Long-term application, however, of these agents sometimes leads to severe side-effects, such as bone marrow suppression. Taken together, it is possible that the ACE-I, PE, can be utilized as a chemopreventive agent against HCC in the future.

RAS AND EXTRACELLULAR MATRIX REMODELING

In addition to transformed cells, ECM remodeling has been shown to play an important role in cancer development [112]. Recent studies have suggested that AT-II also plays an important role in ECM remodeling. It has been demonstrated that ECM remodeling is not a simple structural component, but it exerts an active role in the tumor development. For example, ECM provides the vascular supply that any tumor requires for satisfactory gas exchange, obtaining nutrients, and removal of waste products. In human HCC, it has been recently shown that an increased ECM remodeling was associated with tumor progression [113]. In an experimental study, it has been shown that ECM remodeling accelerates the development of preneoplastic lesions in the liver [114]. AT-II increased the TGF-β and collagen-I gene expression levels in the fibroblasts *in vitro* [115]. Extensive remodeling of the ECM has been shown to play a pivotal role during the development of organ fibrosis, including that of the liver [116]. We have recently reported that PE, and the ARB, candesartan, exerted a significant inhibitory effect on experimental pig serum-induced liver fibrosis development, associated with suppression of the hepatic stellate cells (HSCs), which play a pivotal role in liver fibrogenesis, *via* activation of AT1-R [117]. It has been revealed that AT-II induced HSC contraction and proliferation which play a important role in liver fibrogenesis [118]. We also employed an experimental model that feeds rats on the CDAA-diet [119]. We found a marked suppressive effect of PE against the liver fibrosis development associated with inhibition of the activated HSC in CDAA-induced liver fibrosis [109]. It should be noted that, in both models, a significant suppressive effect was achieved at clinically comparable low doses.

Gross remodeling of ECM is likely to be regulated by the net balance between synthesis and degradation of the ECM. The balance between MMP and tissue inhibitors of metalloproteinases (TIMP) has been shown to play a key role in maintaining the balance between the ECM deposition and degradation. TIMP-1 is a broad spectrum inhibitor of MMPs and functions by forming direct non-covalent 1:1 complexes with MMPs. During ECM remodeling, TIMP-1 expression and the serum TIMP-1 level were markedly up-regulated both in humans and murine fibrosis models [120]. We reported that TIMP-1 significantly promoted the development of liver fibrosis in the transgenic mouse model [121]. In the rat model of reversible liver fibrosis, the ECM remodeling and resolution of liver fibrosis were closely associated with a marked decrease in TIMP-1 expression [122]. Moreover, it has recently been reported that the spontaneous liver fibrosis resolution was markedly attenuated by TIMP-1 overexpression [123]. It has been reported that factors such as phobol esters, IL-1β and TGF-β, up-regulated TIMP-1 expression. It has been shown that AT-II also stimulates TIMP-1 production *via* a PKC-dependent pathway in ECs *in vitro* [124]. TIMP-1 expression was also

significantly increased by AT-II in activated HSCs in time- and dose-dependent manners *via* the PKC pathway; and the suppression of AT-II by PE significantly attenuated liver fibrosis development in association with TIMP-1 inhibition and HSC activation. Furthermore, ACE-Is inhibited the MMP activity in a dose dependent manner. The coordination of these biological activities results in inhibition of the ECM remodeling by ACE-Is.

Although the previous studies conducted to determine the molecular process associated with the ECM remodeling, such as liver fibrosis and angiogenesis were performed independently, recent studies have revealed that both biological phenomena emerged synergistically [125]. It was proven that neovascularization significantly increased during the development of liver fibrosis both in the human and animal experimental studies. [126-128] Furthermore, TNP-470, a semisynthetic analogue of fumagilin, which possesses anti-angiogenic activity, suppressed experimental liver fibrosis development [129]. Recently, it has been reported that VEGF and its receptor expression significantly increased during the course of development of the liver fibrosis [127, 128]. It has been shown that the expressions of VEGFR-1 and VEGFR-2 were induced during the activation of HSC [130]. In the experimental liver fibrogenesis model, it has been found that VEGFR-1 expression increased in the liver, although the expression level was lower than that of VEGFR-2. Treatment with both VEGFR-1 mAb and VEGFR-2 mAb significantly attenuated the development of fibrosis associated with suppression of neovascularization in the liver. The suppressive effects of VEGFR-1 mAb and VEGFR-2 mAb against angiogenesis were of similar magnitude to inhibition of the fibrosis areas, indicating that angiogenesis mediated by interaction between VEGF and its receptor is a prerequisite for progression of the liver fibrosis [131]. It has been reported that TIMP-1 overexpression augmented VEGF expression associated with an increase in ECM remodeling [132]. Taken together, these findings suggest that AT-II also plays a pivotal role in ECM remodeling through the complex interaction with TIMP, MMP, and VEGF.

CONCLUSION AND FUTURE PERSPECTIVE

Angiogenesis plays a pivotal role not only in the tumor growth, but also in carcinogenesis and distant metastasis. A potent angiogenic factor, VEGF, regulates tumor growth and development. Recently, a mAb against VEGF has been clinically approved for colon cancer in the United States. This agent may be also beneficial for other types of cancers, but a considerable time is needed for this agent to become available for other types of cancer, such as HCC. Until these new agents become widely available, ACE-Is may be an alternative strategy as anti-angiogenic agents. It should be noted, however, that ACE-Is including PE also show pro-angiogenic activity under certain conditions Quanaprilat, an ACE-I, promotes angiogenesis in rabbits with hind-limb ischemia [133]. PE also has been shown to promote angiogenesis in the experimental model of ischemia reperfusion [134]. A clinical study showed that treatment with ACE-Is restores hepatocyte growth factor (HGF), which is one of the potent angiogenic factors [135], produced in patients with congestive heart failure [136]. It is difficult to explain precisely why ACE-Is exert such diverse effects on angiogenesis. It has been reported that the tumor neovascular ECs showed a different phenotype from the normal ECs [137]. It is possible that the physiological angiogenesis of the cardiovascular system and the pathological angiogenesis of the tumor may have different molecular mechanisms. Further studies are required to elucidate these issues before clinically prescribing an ACE-I as an anti-angiogenic agent. It has also been shown that there is a significant relationship between AT-II polymorphism and the progression of chronic hepatitis, and that the high ACE expression gene polymorphism significantly correlates with the poor prognosis in certain types of cancer [88, 138]. This suggests that the effectiveness of RAS inhibition by ACE-Is may markedly vary in each clinical case. It would be a better approach to examine the genotype of the ACE gene in each patient before starting treatment with ACE-Is. This approach can predict the effectiveness of ACE-Is.

Also, the results of the epidemiological studies on the effect of ACE-Is on the development of cancer are still controversial. Contrary to the Lever's data, another report did not find any significantly inhibitory effect for ACE-I on cancer incidence and mortality [139]. In this report, the standardized cancer incidence ratio (SIR) was quite variable according to the type of cancer. SIR of liver cancer was low (0.5), whereas that of renal cancer was high (1.6). This result indicates that the effectiveness of ACE-Is may differ according to the type of cancer. Condition matched prospective studies on large numbers of patients will be required in the future.

In conclusion, therapies aiming at the destruction of the tumor vasculature should be a promising approach for the treatment of cancer. Therefore, targeting the key molecules, such as VEGF and its receptor VEGFR-2, is an essential objective. However, it appears that more time is still required before these compounds under current clinical trials can be applied widely in clinical practice. Until these new drugs become available, the clinically used compounds with proven anti-angiogenic activity, such as ACE-Is, would be an alternative approach. ACE-Is are already used widely in the clinical practice without serious side-effects as compared to the conventional chemotherapeutic drugs, which cause adverse effects such as bone marrow suppression. Furthermore, it has been reported that ACE-Is reduced the risk of stroke among the hypertensive as well as the non-hypertensive patients with a history of stroke or transient ischemic attack (PROGRESS study) [140]. Since a considerable time is needed to develop new anti-angiogenesis drugs for the widespread clinical use and since the safety of ACE-Is has been demonstrated, ACE-Is such as PE may provide a new strategy for cancer therapy.

ABBREVIATIONS

AT-II	=	Angiotensin-II
ATR	=	Angiotensin receptor
AT1-R	=	Angiotensin-I receptor
ACE	=	Angiotensin converting enzyme
ACE-I	=	Angiotensin-converting enzyme inhibitor
ARB	=	Angiotensin-I receptor blocker

EC	=	Endothelial cell
ECM	=	Extracellular matrix
FSD	=	Free-sulfhydryl donor
HCC	=	Hepatocellular carcinoma
HSC	=	Hepatic stellate cell
IFN	=	Interferon
mAb	=	Monoclonal antibody
MAPK	=	Mitogen-activated protein kinase
MMP	=	Matrix metalloproteinase
PE	=	Perindopril
PKC	=	Protein kinase-C
PLGF	=	Placental growth factor
RAS	=	Renin-angiotensin system
TGF	=	Transforming growth factor
TIMP	=	Tissue inhibitors of metalloproteinases
VEGF	=	Vascular endothelial growth factor
VEGFR	=	Vascular endothelial growth factor receptor

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Efficacy of combination therapies of percutaneous or laparoscopic ethanol-lipiodol injection and radiofrequency ablation

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Abstract. Percutaneous radiofrequency ablation (RFA) is able to destroy hepatocellular carcinoma (HCC) in a few sessions without major complications. We have previously shown that not only the combined use of percutaneous ethanol injection and RFA (PEI-RFA) but also injection of mixture of ethanol and lipiodol (PELIT) was useful for the treatment of HCC. In the present study, we further developed the combined use of PELIT and RFA through percutaneous or laparoscopic approach (PELI-RFA or LELI-RFA) and evaluated its usefulness. Nineteen nodules in 18 cases were treated with PELI-RFA or LELI-RFA. In the cases treated with LELI-RFA, no bleeding and no spilling milky fluid containing tumor cells were observed from the surface of ablated tumors. In the cases sufficiently treated with PELI-RFA or LELI-RFA, the mixture of ethanol and lipiodol was accumulated in the entire region of the tumor and low-density area was observed around the lipiodol deposit by computed tomography (CT). These delineations of coagulated area were helpful to evaluate the precise area of safety margin around the tumor treated with PELI-RFA or LELI-RFA. Furthermore, the total volume of coagulated necrosis significantly and positively correlated with the product of energy requirement for ablation and the volume of ethanol injected by PELI-RFA or LELI-RFA. Among the cases treated with PELI-RFA or LELI-RFA, local recurrence emerged only in one case in whom enough safety margin could not be achieved by PELI-RFA. Therefore, it is critical to evaluate whether enough safety margin could be obtained with RFA therapy, and PELI-RFA and LELI-RFA are helpful in visualizing the safety margin area.

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Key words: percutaneous radiofrequency ablation, hepatocellular carcinoma, percutaneous ethanol injection

Introduction

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. There has been extensive research on new non-surgical modalities for HCC. Among them, radiofrequency ablation (RFA) (1-3) and interventional radiology have been highly developed and clinically tested. The treatments are selected on the basis of types of HCC and levels of hepatic reserve. In particular, extensive efforts have been made for the development of image-guided percutaneous ablation techniques. Consequently, strategies for the treatment of HCC have been drastically shifting from surgical to non-surgical modalities by the introduction of RFA therapy in clinical settings. One reason for this shift is that RFA is able to achieve certain therapeutic effects on HCC or metastatic liver tumor (4,5) in a few sessions of treatment without serious side effects. A randomized control trial showed that RFA was superior to percutaneous ethanol injection (PEI) (6). Although the RFA technique serves a central role in the treatment of HCC, we further developed the combination therapy of RFA and ethanol injection (PEI-RFA) to improve the therapeutic effects and showed that PEI-RFA could significantly enlarge coagulated areas equally to the horizontal and vertical directions (7-9). In contrast to the fact that RFA is able to achieve wide coagulated necrosis, local recurrence after the therapy often emerged around the tumor lesion. Thus, the importance of reaching the therapeutic ablation beyond the margin of the tumor; namely obtaining the safety margin, is pointed out for preventing the local recurrence after treatment (10). Recently, we further developed the combination of percutaneous ethanol and lipiodol injection therapy (PELIT) and confirmed that this novel therapy was effective for the treatment of HCCs, especially for those lacking vascularity, invisible by ultrasonography (US) or CT, or for patients with severe hepatic reserve (11). In the present study, we used the mixture of ethanol and lipiodol instead of ethanol alone in the treatment of PEI-RFA (percutaneous or laparoscopic ethanol and lipiodol injection and RFA: PELI-RFA or LELI-RFA) and evaluated the usefulness in the treatment of HCC.

Patients and methods

Patients and study design. PELI-RFA or LELI-RFA was performed against 19 nodules in 18 cases (12 males and 6 females; mean age of 61 years) with HCC (range: 1.5-5 cm in diameter). LELI-RFA was performed against 3 nodules in 3 patients and PELI-RFA was performed against other 16 nodules in 15 patients. These studies were conducted with informed-consent at the time of enrollment for this study in all patients.

Treatments. PELI-RFA was performed under the real-time US guidance with a 3.5-MHz sector probe (Power Vision 5000; Toshiba Medical, Tokyo, Japan). RFA was performed by Cool-tip RF System (Radionics, Burlington, USA) (12) according to the method described in our previous reports (7,8). Briefly, a 17-gauge RFA needle with electrode of 3 cm in length was firstly inserted into the center of tumor, and then a 21-gauge PEI needle was inserted into the tumor in the liver through the same hole of the attachment beside the echo probe. The mixture of ethanol (99.8%) and lipiodol (Japan Shering, Osaka, Japan), a lipid-based contrast medium, at a ratio of 20:3 was slowly injected into the tumor. The volume of injected ethanol-lipiodol was always kept below the double volume of the estimated tumor volume. The ablation was performed under impedance control. For LELI-RFA, a laparoscope was inserted into the abdominal cavity from the left-upper portion of the navel. RFA electrode and PEI needle were percutaneouly inserted from other portions of the abdomen according to the location of the tumor. For lifting up the left lobe of the liver, a sonde was inserted and kept under the reverse surface of the liver during the ablation.

Evaluation of therapeutic efficacy. Five to seven days after the treatment, plain or contrast enhanced CT was performed to evaluate the response to PELI-RFA or LELI-RFA. Tumor necrosis was considered to be complete when no foci of early enhancement were seen around the original regions.

Calculation of energy requirement for ablation and the volume of safety margin. Energy requirement for ablation was calculated as follows: Energy (Joule) = Watt (W) x Duration of ablation (Second). The length of coagulated necrosis of the lesion was measured from the CT. The approximation volume of whole coagulated necrosis area including lipiodol deposit and the area of lipiodol deposit were calculated as follows: whole coagulated volume including lipiodol deposit (V1 cm^3) = $4/3\pi$ x r1(cm) x r2(cm) x r3(cm); (r1 = longest diameter/2; r2 = shortest diameter/2; r3 = height/2), volume of lipiodol deposit (V2 cm^3) = $4/3\pi$ x r4(cm) x r5(cm) x r6(cm)/2; (r4 = longest diameter/2; r5 = shortest diameter/2; r6 = height/2). The volume of safety margin was calculated by subtracting V2 from V1. The scheme of each diameter of coagulated necrosis and lipiodol deposit is depicted in Fig. 1.

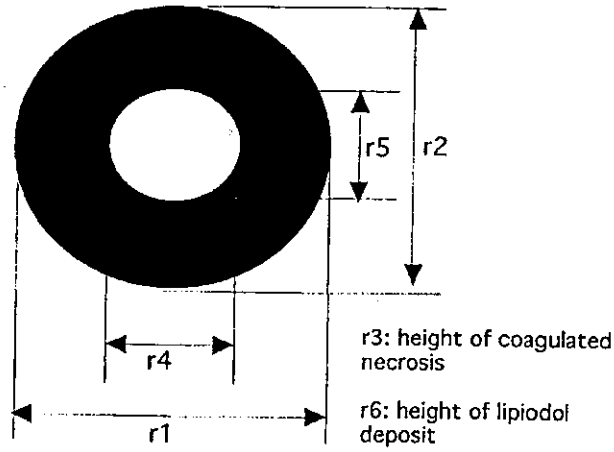

Figure 1. Schematic diagram for calculating the volume of the safety margin area is depicted. White circle shows the lipiodol deposit and black area shows the low density area around the lipiodol deposit in the late phase of dynamic CT after PELI-RFA or LELI-RFA. The equation for calculating the volume of safety margin area is indicated in Patients and methods.

Results

Treatment of LELI-RFA. In the first case, HCC (2 cm in diameter) was located in S2 region of the liver. The tumor showed enhancement in the early phase and defect in the late phase of dynamic CT (Fig. 2A and B). Because the tumor grew outside the reverse surface of the hepatic left lobe and was located close to the heart, ablation with a percutaneous approach was likely to have a risk of leading to bleeding into the abdominal cavity or puncturing the heart. Laparoscopic surgery has been shown to be milder depression of the immune system than open surgery (13). Therefore, LELI-RFA was chosen as a treatment. After inserting the laparoscope into the abdominal cavity, the tumor was observed as a slight elevation on the upper surface of the hepatic left lobe (Fig. 3A). After inserting the RFA electrode into the tumor, PEI needle was inserted beside the electrode, and then the mixture of ethanol-lipiodol (7 ml) was slowly injected into the tumor. RFA was initiated at 40 W. The power output was increased stepwise to 70 W and kept for 12 min. In the process of tumor ablation, the elevated tumor was gradually atrophied by vaporizing the water in the tumor (Fig. 3B). As a next step, the left lobe of liver was turned to upper direction by a sonde, and then a round-shaped HCC was observed there (Fig. 3C). After inserting the RFA electrode into the center of the tumor, the second ablation was performed in the same way at 40 W for 7 min. During the ablation, neither bleeding nor spill of milky fluid containing tumor cells from the coagulated tumor was observed. Color of the tumor surface changed to white (Fig. 3D and E). Final temperature of the ablated tissue was confirmed to be 78°C. Contrast enhanced CT taken 5 days after the treatment showed the lipiodol deposit in the center of the tumor and the low-density area around the lipiodol deposit (Fig. 2C). The region of the lipiodol deposit corresponded to the area of tumor lesion before the treatment (11) and the safety margin area was clearly visualized.

Treatment of PELI-RFA. In the second case, the HCC (2 cm in diameter) was located in S6 region of the liver close to the right kidney. The tumor was visualized as a vague high echoic space-occupying lesion (SOL) without halo (Fig. 4A)

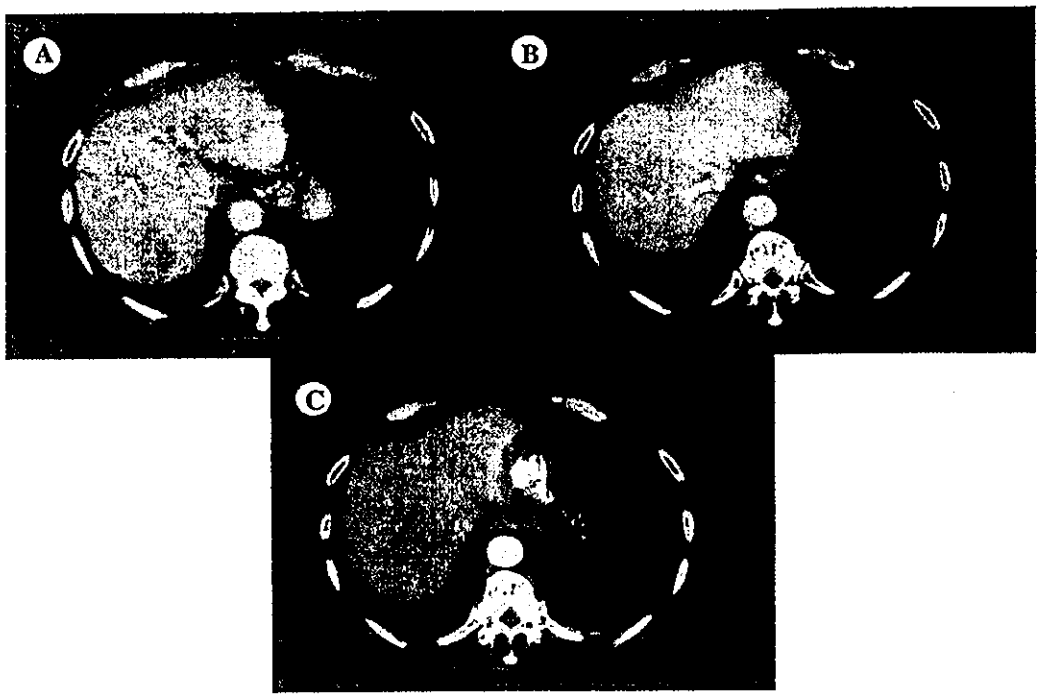

Figure 2. Contrast-enhanced CT before (A, early phase; B, late phase) and after LELI-RFA (C) for HCC (3 cm) located in the S2 region of the liver. The HCC was located close to the heart, which made it difficult to insert the RFA electrode through a percutaneous approach even under the US guidance. Therefore, LELI-RFA was chosen for the treatment. The lipiodol-deposit corresponded to the entire tumor region and the low-density area is seen around the lipiodol-deposit.

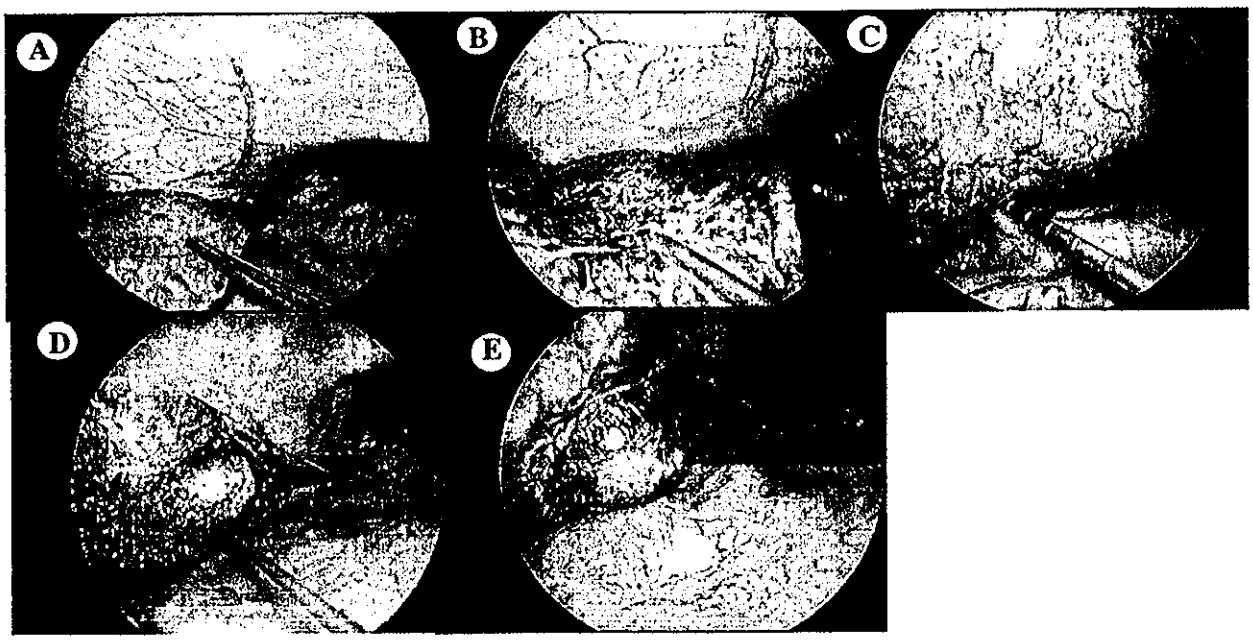

Figure 3. Laparoscopic findings of case no. 2 treated with LELI-RFA. HCC (3 cm) was located in the S2 region of the liver. The shape of the tumor on the upper surface of the liver (A) and the reverse surface of the liver (B) are shown. The tumor was visualized as a slightly elevated lesion on the upper surface and as a round globe on the reverse surface of the liver. The shape of the tumor lesion during the ablation is shown in C and D and that after the treatment in E.

and showed early enhancement in dynamic CT (Fig. 4B). Although this tumor appeared to be non-encapsulated tumor, the mixture of ethanol-lipiodol gradually accumulated in the center of tumor and its flow was more easily detected by US than the cases injected with ethanol alone. Because the kidney was located close to the tumor, RFA was performed

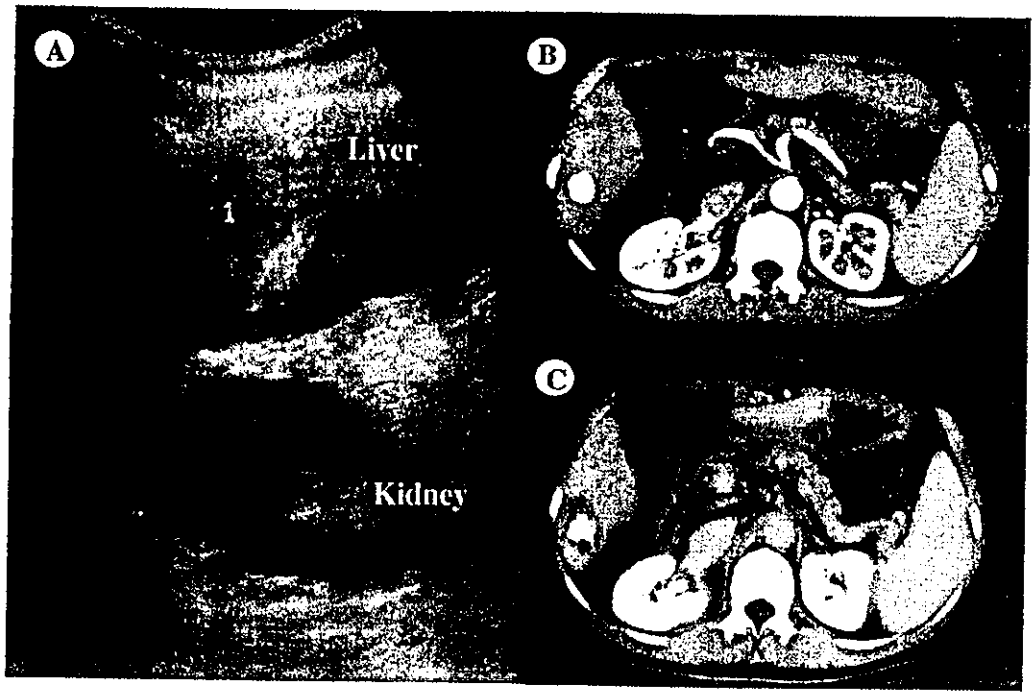

Figure 4. US image of HCC (2 cm) located in the S6 region close to right kidney (A). Contrast-enhanced dynamic CT before (B, early phase) and after (C, late phase) PELI-RFA is shown. The tumor showed clear early enhancement. PELI-RFA was performed at 70 W for 12 min after injecting 1.4 ml of the ethanol and lipiodol mixture. Although the amount of ethanol injected was small, accumulation of lipiodol was observed in the entire tumor and it was surrounded by the low-density area.

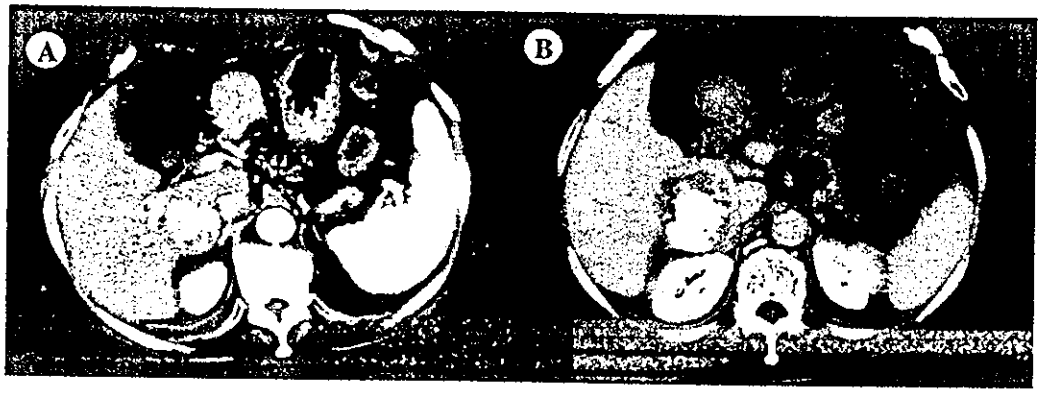

Figure 5. Contrast-enhanced dynamic CT before (A, early phase) and after (B, late phase) for HCC (4 cm) located in the S5 region of the liver of case no. 1. HCC was located close to the right kidney. The tumor showed the clear early enhancement. PELI-RFA was performed at 50 W for 4 min and 70 W for 20 min after injecting 15 ml of the mixture of ethanol and lipiodol. The safety margin area around the tumor region was visible after the treatment.

with a relatively low power output at 70 W for 12 min after injecting 2.0 ml of the ethanol-lipiodol mixture. High echoic area spread around the RFA electrode. The final temperature of the ablated tissue was confirmed to be 70°C. Although the amount of injected ethanol was small, lipiodol deposit covered the entire region of the tumor and a low density area was detected around its lipiodol deposit (Fig. 4C).

In the third case, the HCC was also located in S6 region close to the right kidney. It was a large-sized tumor (5 cm in diameter) and the edge of the tumor lesion was facing to the right kidney (Fig. 5A). Because of the tumor size, relatively large amount of ethanol and lipiodol was injected into the tumor lesion. The PELI-RFA was performed at 50-70 W for 24 min in total after injecting 15 ml of the ethanol-lipiodol mixture. As shown in the first and second cases, the lipiodol deposit corresponded to the entire region of the tumor and a low-density area was observed around the lipiodol deposit (Fig. 5B). In this case, no local recurrence around the treated tumor has occurred for one year.

Relationship between the total volume of coagulated necrosis and the product of energy requirement for ablation and the amount of ethanol-lipiodol injected. It turned out that the contrast of lipiodol deposit and low-density area around

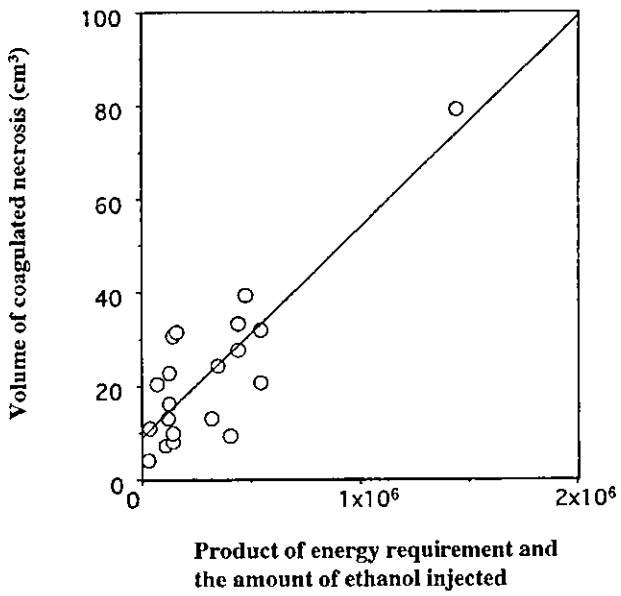

Figure 6. Relationship between the total volume of coagulated necrosis and the product of energy requirement for ablation and the amount of ethanol-lipiodol injected. PELI-RFA and LELI-RFA were performed against 19 nodules in 18 patients after injecting 1.2-15 ml of ethanol into the tumor. The ablation was done by using the Cool-tip RF system. The total volume of coagulated necrosis was significantly and positively correlated with the product of energy requirement for ablation and the amount of ethanol injected with a high correlation coefficient (r) ($r=0.85$, $p<0.0001$).

lipiodol deposit in dynamic CT made the safety margin area easier. However, the significance of the volume of safety margin area should be always considered with the size of tumor. Thus, prior to the evaluation of the volume of safety margin, relationship between the total volume of coagulated necrosis and the energy requirement and the amount of ethanol injected was evaluated. In the treatment of PELI-RRA and LELI-RFA, both of the energy requirement for ablation and the amount of ethanol injected affects the total volume of coagulated necrosis. Therefore, relationship between product of the energy requirement for ablation and the amount of ethanol injected and the total volume of coagulated necrosis was evalulated. The total volume of coagulated necrosis was shown to be positively correlated with the product of the energy requirement and the amount ethanol injected with high correlation coefficient (r) ($r=0.85$, $p<0.0001$) (Fig. 6). This result implies that the wider coagulated necrosis can be obtained even under a low power control by increasing the amount of ethanol-lipiodol injected into the tumor in PELI-RFA or LELI-RFA treatment.

Summary of PELI-RFA and LELI-RFA treatment. As shown in the above-mentioned cases treated with PELI-RFA or LELI-RFA, area of lipiodol accumulation was shown to be corresponding to the tumor lesion surrounded by the low-density area in dynamic CT. This CT finding made it easier to calculate the volume of the safety margin area obtained by PELI-RFA or LELI-RFA. The approximation volume of the safety margin area was calculated by the equation described in Patients and methods. Sizes of tumors treated with PELI-RFA or LELI-RFA were similar, ranging from 1.5 to 3 cm, except one large-sized tumor with 5 cm in diameter. In all the patients treated with PEI-RFA or LELI-RFA, there were no postoperative complications. As shown in Table I, enough safety margin areas could be obtained even in cases with tumors of 3-5 cm in diameter (cases no. 1 and 2, 6, 7, 13, 16) under a high power control and relatively high amount of ethanol injected. There were two cases in which enough safety margin areas could not be obtained after the treatment (cases no. 9 and 11) due to the low power control or lack of the amount of ethanol injected into the tumor. Both cases were treated with PELI-RFA but not with LELI-RFA, and local recurrence occurred in one patient within 6 months. Recurrence in short-term was not detected in other 18 nodules. These observations indicated the importance of achieving safety margin around the tumor region for preventing the local recurrence after RFA therapy.

Discussion

Soon after we innovated the RFA technology in 1999 in our department, we developed a novel combination therapy of PEI and RFA (PEI-RFA) and confirmed that PEI-RFA could induce much better therapeutic effects on HCC (7-9). We have shown that the combined use of ethanol injection on RFA could expand coagulated necrosis areas equally in 3 dimensions and the amount of injected ethanol and the volume of coagulated necrosis were positively correlated (7). Even though PEI-RFA made it possible to achieve larger coagulated necrosis, we still experienced local tumor recurrence after the therapy especially in cases with large-sized HCCs. Therefore, it is important to ablate the tumor including the safety margin area for preventing local recurrence (10). However, in cases treated with PEI-RFA using ethanol alone or RFA alone, it is sometimes difficult to evaluate whether we obtained the safety margin after treatment. Therefore, one of the advantageous events of PELI-RFA is the easy detection of the safety margin that is clearly visible by CT. This confirmation should be useful to consider whether additional treatment is necessary. In the present study, typical images of lipidol-deposit surrounded by the low-density area was clearly detected in contrast-enhanced CT and the lipidol deposit was shown to be mainly in the entire tumor lesion in the cases sufficiently treated with PELI-RFA or LELI-RFA. It has also been demonstrated that the safety margin area was equally expanded to horizontal and vertical directions beyond the lipiodol deposit (Figs. 2 and 4). These findings suggest that lipiodol injected percutaneously into the tumor has affinity to HCC cells, but not to normal liver cells, in PELI-RFA or LELI-RFA as shown in our previous report (11).

In previous studies, combined use of some other treatment modalities with RFA has been shown to enhance the therapeutic effects on HCC. Saline injection prior to RFA has been shown to enlarge the coagulated necrosis (14,15) and the use of transarterial chemembolization (TAE) has also been shown to enhance the effect (16). We have reported that the combined use of ethanol injection with RFA was very effective in enlarging the coagulated region (7-9). In the combination therapy of TAE with RFA, lipiodol is usually

Table I. Summary of patients with HCC treated with PELI-RFA or LELI-RFA.

Patients	Tumor site	Tumor size (cm)	Energy (Joule)	Ethanol (ml)	Approach	Safety margin (cm^3)	Recurrence
No. 1	S5	5	96000	15	P	38.2	No
No. 2	S2	3	67200	7	L	29.6	No
No. 3	S3	2	40000	8	L	9.0	No
No. 4	S7	2.5	84000	1.5	P	10.9	No
No. 5	S6	2.5	50400	1.4	P	14.5	No
No. 6	S5	3	63300	2	P	15.8	No
No. 7	S7	3.1	38400	9	P	13.1	No
No. 8	S5	1.3	54000	2	P	6.4	No
No. 9	S5	2.8	24000	1.2	P	0	Yes
No. 10	S5	1.5	72000	2	P	9.2	No
No. 11	S2	2.5	36000	4	P	0	No
No. 12	S3	2	29000	14	P	6.9	No
No. 13	S2	3	78000	2	P	18.5	No
No. 14	S8	2.5	56400	9.5	P	24.1	No
No. 14	S3	2	99600	4.4	L	29.0	No
No. 15	S7	1.5	46800	2.5	P	11.4	No
No. 16	S6	3	47700	3	P	18.7	No
No. 17	S8	2	16800	2	P	6.7	No
No. 18	S6	2.5	54000	10	P	14.2	No

Summary of 19 nodules in 18 patients is listed. Presence or absence of recurrence within 6 months is shown. Among the nodules listed, the recurrence in a short term was detected only in one nodule in which enough safety margin could not be obtained by the initial treatment. Approaches of the treatment were as follows: P, percutaneous; L, laparoscopic.

injected transarterially into the tumor lesion with an anti-cancer drug. Contrast-enhanced CT taken thereafter shows the accumulation of lipiodol in the tumor lesion (16). However, lipiodol is deposited in the hypervascular tumors, but not in the hypovascular tumors, in this approach, implying that the combined use of TAE with RFA is not necessarily effective for all types of HCCs, especially for hypovascular HCCs. Whereas, percutaneous injection of lipiodol and ethanol shown in the present study is considered to treat hypovascular tumors as well as hypervascular ones. This is another advantageous event of PELI-RFA or LELI-RFA.

Microwave coagulation therapy (MCT) is another ongoing therapy for ablation of HCC. Because the frequency of microwave is higher than that of radiowave, microwave is appropriate to ablate a relatively small area in short time. Therefore, the power of energy tends to accumulate in a small area in MCT compared with RFA therapy. As a result, several adverse effects such as biloma, bleeding, hepatic failure and dissemination of cancer cells have been reported in MCT (17-19). Among the cases treated with PELI-RFA or LELI-RFA in the present study, no adverse effects occurred during or after the treatment even in the cases with a large-sized tumor and the cases in whom enough safety margin area could be obtained, indicating these therapies are safe. In the cases treated with LELI-RFA in the present study, changes of the surface of tumors during the ablation was visible and it is noteworthy that no spill of milky fluid including viable cells was seen. Also from this standpoint, PELI-RFA and LELI-RFA may be a safe treatment for patients with HCC. Furthermore, no local recurrence around the treated tumor has been observed for one year even in a large-sized tumor as shown in case no. 1. From this observation, additional use of lipiodol on PEI-RFA treatment may play critical roles for preventing local recurrence around the treated tumor. The effect of PELI-RFA or LELI-RFA on preventing the local recurrence awaits further elucidation.

In conclusion, PELI-RFA or LELI-RFA, additional treatment modalities on PEI-RFA, may be safe treatment modalities and useful to evaluate precisely the presence of safety margin after the treatment. These treatments on HCC may have critical roles in preventing local recurrence around the treated tumor.

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Adenovirus-mediated gene transfer into rat livers: Comparative study of retrograde intrabiliary and antegrade intraportal administration

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Abstract. To examine the feasibility of liver-directed *in vivo* gene therapy, we administered recombinant adenoviruses carrying a reporter *lacZ* gene retrogradely into the common bile duct of rats, as well as antegradely into the portal vein. Transduction efficiency of the *lacZ* gene in the liver was estimated not only histochemically by X-gal staining, but also quantitatively by a chemiluminescent reporter gene assay. Retrograde infusion of adenoviruses into the common bile duct was shown to successfully induce transgene expression in the liver. Transduction efficiency induced by intrabiliary adenoviral administration was not significantly different from that induced by intraportal adenoviral administration. Although transgene expression induced not only by intraportal, but also by intrabiliary adenoviral administration was observed predominantly at periportal areas, a considerable number of cells expressing the transgene were detectable even in lobular and centrilobular areas. Mild infiltration of inflammatory cells into the liver and mild hyperplastic changes of hepatocytes were observed after intrabiliary and intraportal adenoviral administration. However, hepatic damage estimated pathologically was not substantial. Furthermore, although intrabiliary and intraportal adenoviral administration resulted in very mild elevation of liver-related serum biochemical parameters, apparent complications were not observed in any rats. Our results demonstrated in the present study suggest that retrograde administration of adenoviruses into the common bile duct can induce efficient transgene expression in the liver without causing severe adverse effects, supporting the feasibility of adenovirus-mediated gene transfer into the liver in clinical settings by means of endoscopic retrograde cholangiography.

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Introduction

The effectiveness of gene therapy for various diseases has been examined intensively not only in animal models, but also in humans. Recombinant adenoviruses have emerged as a promising technology for *in vivo* gene therapy because of their ability to transduce many tissues *in vivo* with relatively high efficiency. Adenovirus serotypes 2 and 5, which cause respiratory diseases in humans, have been developed for use in gene therapy; both belong to the subgroup C adenoviruses that are not associated with human malignancies (1). The natural tropism of adenoviruses for airway epithelia has been exploited in the development of gene therapy for cystic fibrosis (2-4). Another application of recombinant adenoviruses, not expected by the usual spectrum of naturally-acquired infections, has been for liver-directed gene therapy. Recombinant adenoviruses infused directly into the circulation primarily target hepatocytes (5-15).

Liver-directed gene therapy employing replication-deficient adenoviral vectors promises to become an important form of gene therapy not only for a variety of liver diseases, but also for a variety of genetic disorders, because many genetic diseases arise from a lack of proteins or enzymes that are produced in hepatocytes. Numerous studies have demonstrated that systemic administration of recombinant adenoviral vectors led to therapeutic levels of gene expression in the liver, resulting in complete amelioration of the clinical phenotype in a number of animal models for an inherited metabolic disease (8,9,16). Thus, the liver represents an important organ for gene therapy.

In the present study, we retrogradely administered adenoviruses carrying a reporter *lacZ* gene into the common bile duct. We also antegradely administered the same adenoviral construct into the portal vein. We histochemically

and quantitatively estimated transgene expression in the liver induced by the intrabiliary and intraportal adenoviral administration. Furthermore, we pathologically and biochemically evaluated the safety of retrograde administration of adenoviruses into the common bile duct.

Materials and methods

Animals. Female 10-week-old Sprague-Dawley rats were purchased from Japan SLC (Hamamatsu, Japan). Rats were kept under a specific pathogen-free condition at 24±2°C and in a 12-h day/night light cycle with food and water available *ad libitum* throughout the experimental period. Animal experiments were performed with the approved protocols and in accordance with the institutional recommendations for the proper care and use of laboratory animals.

Adenoviral vector. Adex1CAlacZ adenovirus was generously provided by Dr Izumu Saito (Institute of Medical Science, University of Tokyo, Tokyo, Japan), and details of the construction procedures have been described elsewhere (17). This adenoviral vector carries an adenovirus serotype-5 genome lacking the early gene region 1A (E1A), E1B and E3 regions to prevent virus replication, and contains the *Escherichia coli* ß-galactosidase gene, *lacZ* gene, as a reporter gene between the CAG promoter (18) and the rabbit ß-globin polyadenylation signal in the place of the E1A and E1B regions. The recombinant adenovirus was propagated and isolated in 293 cells, as described previously (19). Viral solutions were stored at -150°C until use. To titer the viral solutions, an aliquot of virus was serially diluted and assayed for ability to form plaques on 293 cell monolayers, as described previously (20). Briefly, a total of 50 µl of Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% heat-inactivated fetal calf serum (FCS) was dispensed into each well of a collagen-coated 96-well tissue culture plate, and then 8 rows of 3-fold serial dilution of the test virus starting from 10^{-4} dilution were prepared. A total of $3x10^5$ of 293 cells in 50 µl of DMEM supplemented with 5% FCS were added to each well. The plate was incubated at 37°C in 5% CO_2 in air, and 50 µl of DMEM supplemented with 10% FCS was added to each well every 3 days. Twelve days later, the end point of the cytopathic effect was determined by microscopy, and a 50% tissue culture infectious dose was calculated as described previously (21). A single batch of high-titer adenovirus stock [$1x10^9$ plaque-forming units (pfu)/ml] was used throughout the subsequent experiments.

Adenoviral administration into the portal vein. For intraportal adenoviral administration, 5 rats were anesthetized with ether and 500 µl of the adenoviral solution ($1x10^9$ pfu/ml) was infused into the portal vein. Briefly, a 26-gauge needle connected to a 1-ml syringe was inserted into the portal vein through the greater omentum, and 500 µl of adenoviral solution was injected slowly into the liver through the portal vein followed by oppression for 3 min for hemostasis. Animals were sacrificed 4 days after adenoviral infusion. When animals were sacrificed, approximately 5 ml of blood were collected from the portal vein and 50 ml of phosphate-buffered saline (PBS) was perfused from the portal vein. Their livers were then removed for analysis of transgene expression.

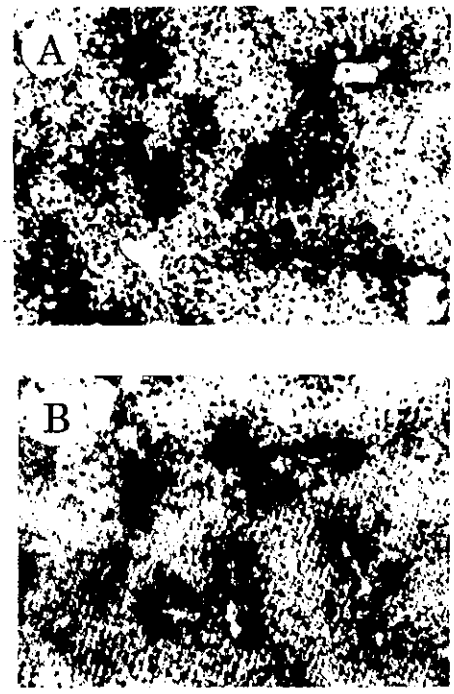

Figure 1. *LacZ* gene expression in rat livers induced by intrabiliary and intraportal adenoviral administration. Recombinant adenoviruses ($1x10^8$ pfu/500 µl) carrying the *lacZ* gene were infused retrogradely into the liver via the common bile duct (A) or antegradely via the portal vein (B). Animals were sacrificed 4 days after adenoviral administration. Their livers were removed, sliced into 50-µm-thick sections and subjected to X-gal staining. Each group consisted of 5 animals. A representative picture is shown. Original magnification x40.

Adenoviral administration into the biliary tract. To allow adenoviral administration into the liver via the biliary tract, adenoviruses were infused directly into the common bile duct. Briefly, 5 rats were anesthetized with ether and a midline abdominal incision was made. The intestinal duct was displaced to expose the liver and common bile duct. After clamping the distal site of the common bile duct to avoid antegrade outflow of the virus, a 30-gauge needle (Becton-Dickinson, Franklin Lakes, NJ, USA) connected to a 1-ml syringe was inserted directly into the common bile duct. Adenovirus solutions ($5x10^8$ pfu contained in 500-µl inoculum volume) were infused retrogradely into the biliary tract over 3 min. Upon completion of the infusion, the needle was removed and pressure was gently applied over the puncture site of the common bile duct for another 3 min. After removing the clamp from the common bile duct, the skin and fascia were closed in one layer with interrupted sutures, and the animal was allowed to recover. Animals were sacrificed 4 days after adenoviral infusion. When animals were sacrificed, approximately 5 ml of blood were collected from the portal vein and 50 ml of PBS was perfused from the portal vein. Their livers were then removed for analysis of transgene expression.

Histochemical evaluation of ß-galactosidase activity in the liver. For histochemical evaluation of the ß-galactosidase