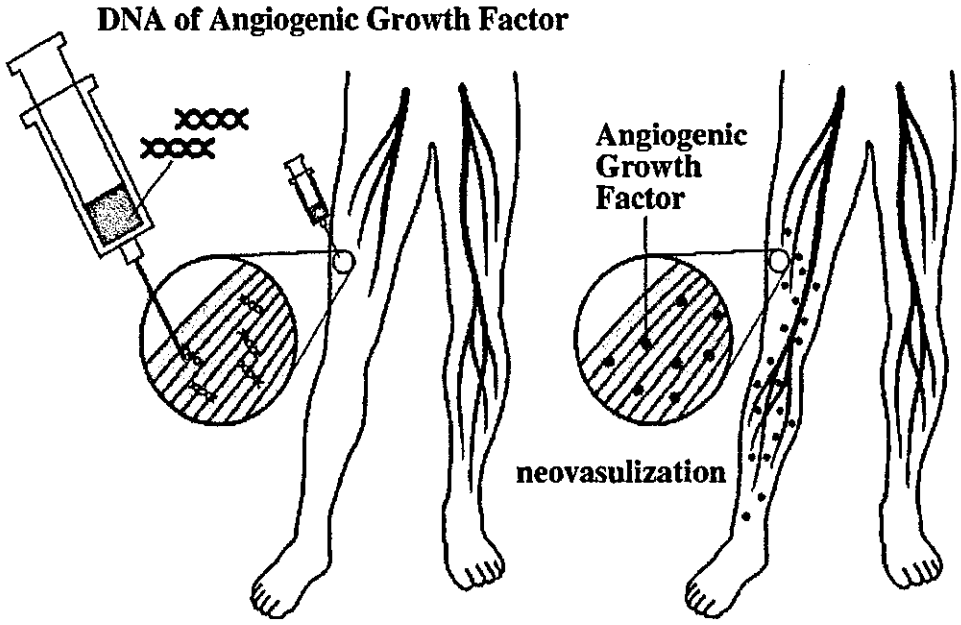

Fig. 1. Concept of therapeutic angiogenesis using DNA of angiogenic growth factors.

ful *(7)*. Follow-up digital subtraction angiography revealed increased vascularity in the VEGF-treated groups distally to the gene transfer site and the region of the most clinically severe ischemia *(7)*. A recent report, using adenovirus-encoding $VEGF_{121}$, demonstrated the improvement of endothelial dysfunction in response to acetylcholine or nitroglycerine *(8)*. However, a high incidence of edema has been reported as side effect in the VEGF trial. The recent results from the Regional Angiogenesis with Vascular Endothelial growth factor (RAVE) trial demonstrated that adenoviral $VEGF_{121}$ gene transfer was not successful in subjects with intermittent claudication *(9)*. The selection of the agent ($VEGF_{121}$ vs $_{165}$), patient population (intermittent claudication vs critical limb ischemia), and outcome measures (peak walking time vs ulcer size) should be considered in the quest for optimal angiogenic strategies that result in the growth of functional blood vessels and improvement in clinical symptoms.

The safety and efficacy of increasing single and repeated doses of intramuscular naked plasmid DNA encoding for *FGF* type 1 administered to patients with unreconstructible end-stage PAD was also reported *(6)*. A significant reduction in pain and aggregate ulcer size was detected after *FGF* gene transfer associated with an increased transcutaneous oxygen pressure and Ankle Pressure Index (ABI) as compared with baseline pretreatment values *(6)*. We also identified HGF as a novel candidate for therapeutic angiogenesis. HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells, and is thus considered a humoral mediator of epithelial–mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis. We and others previously reported that HGF stimulated angiogenesis in a rabbit ischemic hindlimb model, a rat ischemia model, and mouse ischemia models *(11–18)*. In addition, the angiogenic activity of HGF is more potent than VEGF or bFGF in vitro as well as in vivo *(11,19)*. Moreover, transfection of the human *HGF* gene by naked plasmid DNA or HVJ-liposome method resulted in a significant increase in blood flow *(14)*. The angiogenic property of transfection of the *HGF* gene was also proven in a diabetic and a high lipoprotein (a) models *(15,16)*.

Based upon these findings, we planned a human clinical trial using intramuscular injection of naked human HGF plasmid (0.5 mg × 4 sites) two times. Currently, *HGF* gene transfer has been performed in six patients with PAD ($n = 3$) or Buerger disease ($n = 3$) of Fontaine grade III or IV who had failed conventional therapy. Reduction of pain scale (1 cm in visual analog scale) was observed in five of six patients (efficacy rate approx 80%). Increase in ABI to greater than 0.1 was observed in five of five patients (efficacy rate 100%), whereas in one patient we failed to measure ABI before gene therapy because of severe calcification. Importantly, the serum level of human HGF protein did not change during gene therapy. No acute severe complications or allergic events were observed in any patient. Two-month follow-up studies showed no evidence of the development of neoplasm or hemangioma. Although these results are still preliminary, gene therapy using *HGF* may have therapeutic value to treat PAD. It is noteworthy that there was no evidence of edema in patients that were transfected with the human *HGF* gene. This finding is in marked contrast to the VEGF trial in which 60% of patients developed moderate or severe edema in a phase I/IIa trial. Currently, there is a phase III trial to treat PAD underway in Japan and a phase II trial in the United States.

We believe that one of the distinguishing features of HGF is that it stimulates the migration of vascular smooth muscle cells (VSMC) without the replication of VSMC, whereas VEGF does not stimulate either the migration or proliferation of VSMC because of the lack of its receptors in VSMC *(20)*. As shown in Fig. 2A, the initial event in angiogenesis induced by VEGF is the migration of endothelial cells, leading to the sprouting of blood vessels. Later, the migration of VSMC occurs as a result of the release of platelet-derived growth factor, followed by the migration of endothelial cells. However, a delay in the maturation of blood vessels might exist in the case of angiogenesis induced by VEGF. In contrast, HGF simultaneously stimulated the migration of both endothelial cells and VSMC (Fig. 2B). Thus, the blood vessels may maturate at an earlier time point, thereby avoiding the release of blood-derived cells into the extracellular space, although further studies might be necessary to examine the angiogenic properties among various angiogenic growth factors including HGF, VEGF, and FGF. Although these trials are not complete, the feasibility of gene therapy using angiogenic growth factors to treat peripheral arterial disease seems obtainable in near future. Based upon these properties, it is assumed that the first gene therapy drug may be commercial available in 2005.

Gene Therapy to Treat Myocardial Ischemic Disease Using Therapeutic Angiogenesis

Similar ideas have been applied to treat coronary artery disease. A human gene therapy trial to treat coronary artery disease using $VEGF_{165}$ gene has been started by Professor Isner and colleagues *(21,22)*. His group performed intramuscular injection of naked plasmid encoding *VEGF* gene into ischemic myocardium through mini-operation. Similar to human trials in PAD, transfection of *VEGF* gene resulted in a marked increase in blood flow and improved clinical symptoms without apparent toxicity *(21)*. More recently, the results from 13 consecutive patients with chronic stable angina have been reported *(22)*. Although all of them had failed conventional therapy (drugs, percutaneous transluminal coronary angioplasty, and/or coronary artery bypass graft), reduction in the size of the defects documented by serial single-photon emission computed tomography imaging was observed after direct myocardial injection of $phVEGF_{165}$ via a minithoraco-tomy *(22)*.

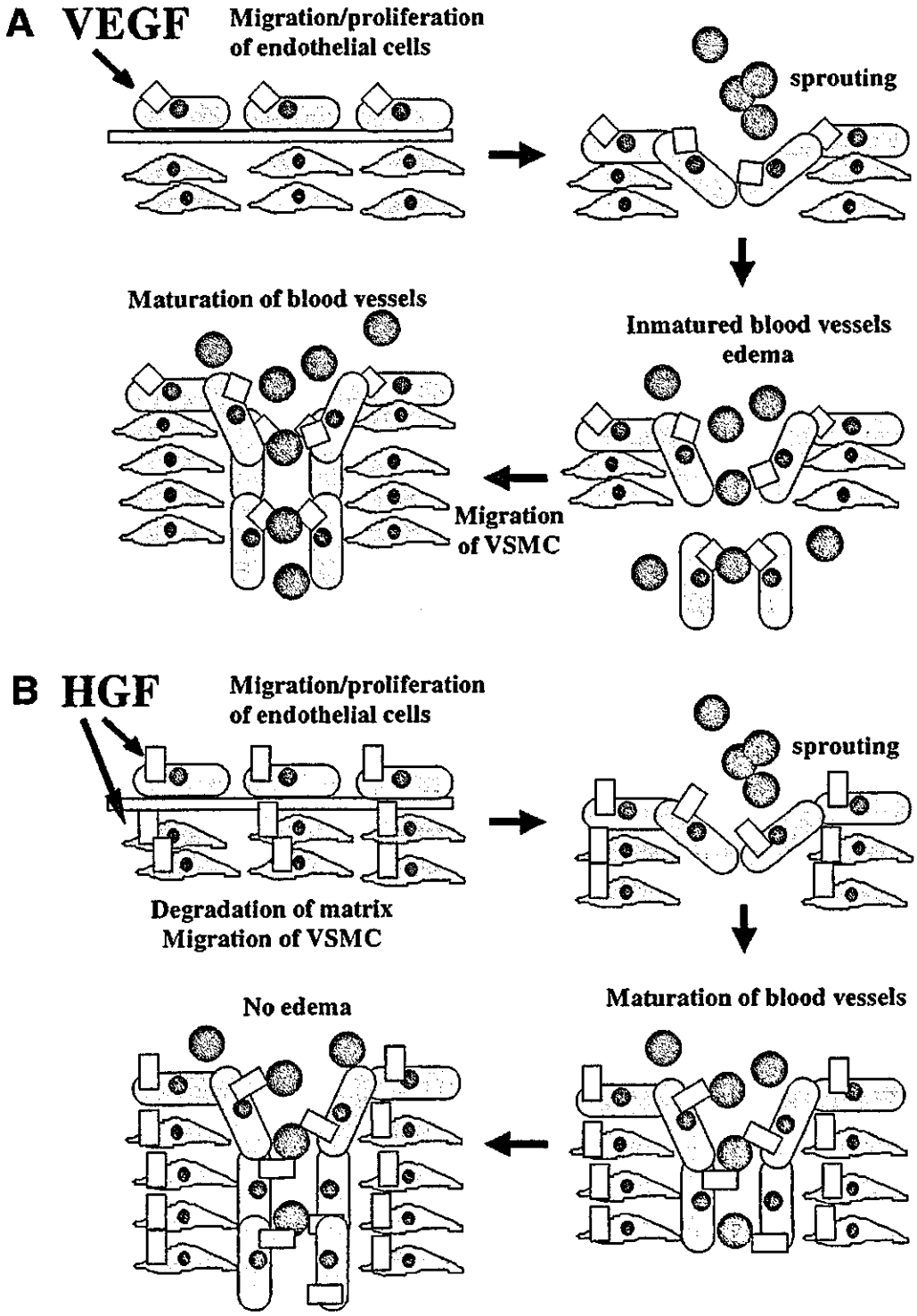

Fig. 2. Model of collateral formation induced by vascular endothelial growth factor (VEGF) (A) and hepatocyte growth factor (HGF) (B). HGF stimulated the growth and migration of endothelial cells together with the migration, but not proliferation, of vascular smooth muscle cells (VSMC) through c-met. In contrast, VEGF only stimulated the growth and migration of endothelial cells without the migration or proliferation of VSMC, because of the lack of receptors in VSMC.

These data clearly suggest that *phVEGF*$_{165}$ gene therapy may successfully rescue foci of hibernating myocardium. In addition, the recent report summarized the anesthetic management of 30 patients with class 3 or 4 angina, enrolled in a phase 1 clinical trial of direct

myocardial gene transfer of naked DNA-encoding VEGF165, as sole therapy for refractory angina. Twenty-nine of 30 patients experienced reduced angina (56.2 ± 4.1 episodes/week preoperatively vs 3.8 ± 1.6 postoperatively) and reduced sublingual nitroglycerin consumption (60.1 ± 4.4 tablets/week preoperatively vs 2.9 ± 1.1 postoperatively) *(23)*. Even at 1-yr follow-up, the average number of angina episodes per week and average number of nitroglycerin tablets used per week significantly improved at all measured time points after gene transfer *(24)*. This observation persisted at a 12-mo follow-up, when the average number of anginal episodes was 10 ± 19 and average weekly nitroglycerin tablet usage was 3 ± 8 ($p < 0.05$ vs baseline for both). Following this success, gene therapy using $VEGF_{121}$ gene was performed by intramuscular injection of adenoviral vector *(25)*. A phase I study using adenovirus-mediated transfection of $VEGF_{121}$ gene demonstrated clinical safety *(25)*. It is noteworthy that no evidence of systemic or cardiac-related adverse events related to vector administration was observed up to 6 mo after therapy *(26)*. Intracoronary gene transfer of $VEGF_{165}$ resulted in a significant increase in myocardial perfusion, although no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-mo follow-up *(27)*. More recently, intracoronary infusion of adenovirus encoding *FGF* gene was performed in a multicenter trial as phase I/IIa. The report documented that intracoronary infusion of *FGF* gene improved cardiac dysfunction without severe toxicity *(28)*. Seventy-nine patients with chronic stable angina Canadian Cardiovascular Society class 2 or 3 underwent double-blind randomization (1:3) to placebo ($n = 19$) or Ad5-FGF4 ($n = 60$). Excitingly, a protocol-specified, subgroup analysis showed the greatest improvement in patients with baseline exercise tolerance test ≤ 10 min (1.6 vs 0.6 min, $p = 0.01$, $n = 50$). In addition, the report documented that treatment of 52 patients with stable angina and reversible ischemia with FGF4 adenoviral injection resulted in a significant reduction of ischemic defect size *(29)*. Currently, the phase IIb/III trials using adenoviral delivery of FGF4 are now underway.

In addition to these angiogenic growth factors, overexpression of HGF was also reported to stimulate angiogenesis and collateral formation in a rat myocardial infarction model *(30)*. Moreover, it was reported that intramuscular injection of HGF gene into the ischemic myocardium resulted in a significant increase in blood flow and prevention of cardiac dysfunction in a canine model *(31)*. The molecular mechanisms of the angiogenic activity of HGF seem to be largely dependent on the ets pathway (an essential transcription factor for angiogenesis), because members of the ets family play important roles in regulating gene expression in response to the multiple developmental and mitogenic signals. The ets family of transcription factors has a DNA-binding domain in common that binds to a core GGA (A/T) DNA sequence. *In situ* hybridization studies have revealed that the proto-oncogene c-ets 1 is expressed in endothelial cells at the start of blood vessel formation, under normal and pathological conditions. Thus, the ets family may activate the transcription of genes encoding collagenase 1, stromelysine 1, and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation. It is believed that the ets family takes part in regulating angiogenesis by controlling the transcription of these genes, whose activity is necessary for the migration of endothelial cells from pre-existing capillaries. Our previous study demonstrated that HGF upregulated ets activity and ets-1 protein in a myocardial infarction model *(30)*. In addition, exogenously expressed HGF also stimulated endogenous HGF expression through induction of ets activity *(32)* (Fig. 3), because the promoter region of the *HGF* gene contains a number of putative regulatory elements, such as a B cell- and a macrophage-specific transcription factor binding site (PU.1/ETS),

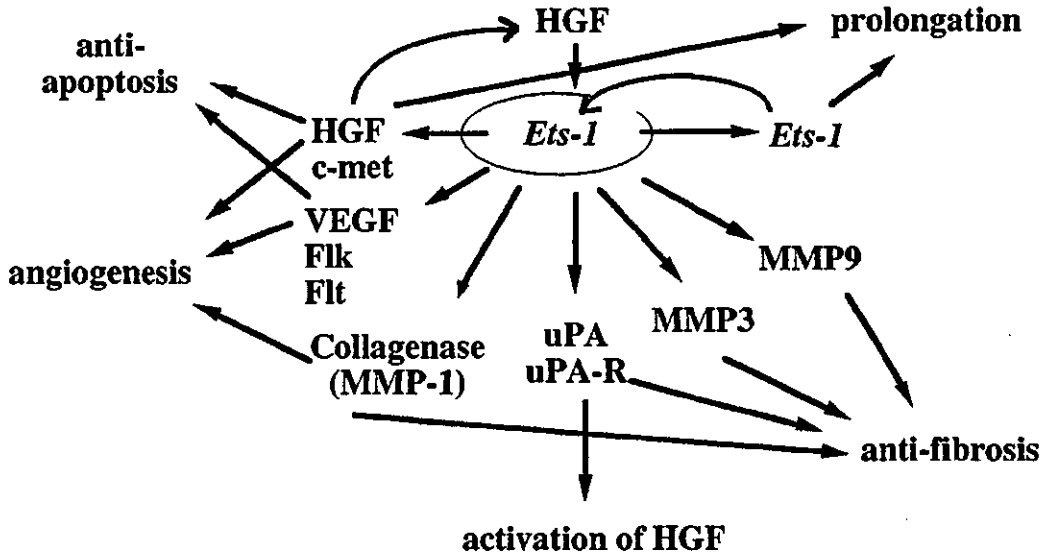

Fig. 3. Molecular mechanisms of angiogenesis induced by hepatocyte growth factor (HGF) through ets-1. HGF stimulated various actions on collateral formation through ets-1, revealing that HGF plays a pivotal role as a master gene in the cascade of angiogenesis.

as well as an interleukin (IL)-6 response element (IL-6 RE), a transforming growth factor (TGF)-β inhibitory element, and a cAMP response element *(33)*. Severe ischemic heart disease may also be curable using therapeutic angiogenesis by HGF, as a result of the autoinduction of the endogenous HGF system.

Recently, an antifibrotic action of HGF has been identified, as HGF inhibited collagen synthesis through TGF-β and stimulated collagen degradation through upregulation of matrix metalloproteinase (MMP-1) and urinary plasminogen activator (uPA) *(34)*. Although the mechanisms through which HGF inhibited TGF-β synthesis are not clear, HGF stimulated various metallo-proteases, such as MMP-1 through induction of ets-1 activity *(35)*. Prevention of fibrosis by HGF was confirmed by previous studies in which administration of human rHGF or gene transfer of human HGF prevented and/or regressed fibrosis in liver and pulmonary injury models *(36,37)*. Thus, HGF may also provide a new therapeutic strategy to treat fibrotic cardiovascular disease, e.g., cardiomyopathy. Our group has also applied to start a human gene therapy protocol using intracardiac muscular injection of HGF plasmid DNA through surgical operation. Overall, the treatment for coronary artery disease may also be curable using therapeutic angiogenesis by gene therapy.

Gene Therapy Into the Brain: A New Era for Cardiovascular Gene Therapy

In addition, gene therapy may be used to treat cerebrovascular disease. Cerebral occlusive disease caused by atherosclerosis of the cerebral arteries or Moyamoya disease often causes chronic hypoperfusion of the brain. Such a condition leads to not only cerebral ischemic events, but also neuropathological changes including dementia. Currently, an effective treatment to improve hypoperfusion has not yet been established. It is known that ischemic stroke induces active angiogenesis, particularly in the ischemic penumbra, which correlates with longer survival in humans. However, the natural course of angiogenesis is not sufficient to compensate for the hypoperfusion state. In the pathophysiology of the disease, in the presence of the obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous devel-

opment of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, residual ischemia occurs. From this viewpoint, therapeutic angiogenesis must be an effective therapy for cerebral ischemia, resulting in the prevention of future stroke. Angiogenesis can be promoted in the rat brain using adenoviral vectors containing cDNA from bFGF *(38)*. After intraventricular administration of the viral vector, bFGF gene transfer induced angiogenesis in normal rat brain accompanied by an extremely high concentration of bFGF in the brain. In addition to bFGF and VEGF, HGF might be useful to treat ischemic cerebrovascular disease. Our preliminary data revealed that transfection of the HGF gene into the subarachnoid space immediately after occlusion of the bilateral carotid arteries induced angiogenesis on the brain surface and had a significant protective effect against the impairment of cerebral blood flow by carotid occlusion. Moreover, HGF has been the center of interest in neuroprotective substances, because HGF is both a chemoattractant and a survival factor for embryonic motor neurons *(39)*. Sensory and sympathetic neurons and their precursors also respond to HGF with increased differentiation, survival, and axonal outgrowth *(39)*. The broad spectrum of HGF activities suggests that the major role of HGF is to potentiate the response of developing neurons to specific signals. Our recent study demonstrated that gene transfer of HGF into the subarachnoid space has a profound neuroprotective effect against postischemic delayed neuronal death in the hippocampus *(40)*. Stimulation of new vessel formation and prevention of neuronal death by HGF is likely to create new therapeutic options in angiogenesis-dependent conditions, such as stroke, Moyamoya disease, and dementia, although a number of important issues, such as safety and side effects, have not yet been addressed. Our recent report demonstrated that gene transfer into the brain by injection of human *HGF* gene with HVJ-envelope vector into the cerebrospinal fluid via the cisterna magna resulted in a significant decrease in the infarcted brain area without cerebral edema or destruction of the blood–brain barrier *(41)*. Reduction of brain injury by HGF may provide a new therapeutic option to treat cerebrovascular disease.

Gene Therapy for Restenosis After Angioplasty

Another important cardiovascular disease potentially amenable to gene therapy is restenosis after angioplasty, because the long-term effectiveness of this procedure is limited by the development of restenosis in over 40% of patients. Balloon angioplasty is one of the major therapeutic approaches to coronary artery stenosis. However, restenosis occurs in 30–40% of patients after angioplasty. Intimal hyperplasia develops in large part as a result of VSMC proliferation and migration induced by a complex interaction of multiple growth factors that are activated by vascular "injury." The process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Therefore, inhibition of the cell cycle using nonphosphorylated retinoblastoma *(Rb)* gene or antioncogenes, such as *p53* and *p21* has been reported in several animal models *(42–45)*. Recently, overexpression of inducible nitric oxide synthase gene has been tested in human subjects, although the results are not yet published.

Alternatively, it has been hypothesized that rapid regeneration of endothelial cells without replication of VSMC may also modulate vascular growth, because multiple antiproliferative endothelium-derived substances (PGI_2, NO, CNP) are secreted from endothelial cells. This concept was first tested by overexpression of $VEGF_{165}$ gene *(46)*. Asahara et al. reported a significant inhibition of neointimal formation by acceleration of endothelial cells replication by *VEGF* gene transfer *(46)*. Based on this finding, a

human trial using $VEGF_{165}$ gene by hydrogel catheter delivery of naked $VEGF_{165}$ plasmid DNA has been started for restenosis after angioplasty in a peripheral artery *(47)*. Although the final results have not yet been reported, the preliminary results documented the successful inhibition of restenosis after angioplasty *(48)*. A similar trial using $VEGF_{165}$ gene has been started in Finland. In this trial, *VEGF* gene was transfected by cationic liposome or adenovirus with a catheter into the coronary artery *(49)*. A recent report demonstrated the clinical safety of *VEGF* gene transfer with cationic liposome or adenovirus *(49)*. Although gene transfer with *VEGF* using adenovirus during PTCA and stenting shows that intracoronary gene transfer can be performed safely (no major gene transfer-related adverse effects were detected), no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-mo follow-up *(27)*. Nevertheless, a significant increase was detected in myocardial perfusion in the VEGF-treated patients *(27)*. Further studies are necessary to prove the efficacy of re-endothelialization strategy to treat restenosis. In addition, we also reported preclinical experiments in which overexpression of *HGF* gene in balloon-injured arteries could accelerate re-endothelialization, thereby attenuating intimal hyperplasia *(50)*. In this study, we also found that re-endothelialized balloon-injured arteries showed impairment of endothelial dysfunction *(50)*. Further studies are necessary to clarify the utility of gene therapy to treat restenosis after angioplasty.

GENE THERAPY FOR CARDIOVASCULAR DISEASE USING OLIGONUCLEOTIDE-BASED STRATEGY

General Concept

Recent progress in molecular biology has provided new techniques to inhibit target gene expression. The application of DNA technology, such as antisense strategy to regulate the transcription of disease-related genes in vivo has especially important therapeutic potential. Antisense oligodeoxynucleotides (ODN) are widely used as inhibitors of specific gene expression, because they offer the exciting possibility of blocking the expression of a particular gene without any change in function of other genes (*see* Fig. 4). Therefore, antisense ODN are useful tools in the study of gene function and may be potential therapeutic agents. The second approach is the use of ribozymes, a unique class of RNA molecules that not only store information but also process catalytic activity. Ribozymes are known to catalytically cleave specific target RNA, leading to degradation, whereas antisense ODN inhibit translation by binding to mRNA sequences on a stoichiometric basis. Theoretically, ribozymes are more effective to inhibit target gene expression. Conversely, we recently have found a novel molecular strategy in which synthetic double-stranded DNA with high affinity for a target transcription factor may be introduced into target cells as a "decoy" *cis* element to bind the transcription factor and alter gene transcription *(51)*.

Antisense or Ribozyme-Based Gene Therapy

As discussed earlier, angioplasty is limited by the development of restenosis in over 40% of patients. Intimal hyperplasia develops in large part as a result of VSMC proliferation and migration induced by the complex interaction of multiple growth factors. First, the effectiveness of antisense ODN against a proto-oncogene, c-myb, was reported for the treatment of restenosis *(52)*. Accordingly, inhibition of other proto-oncogenes,

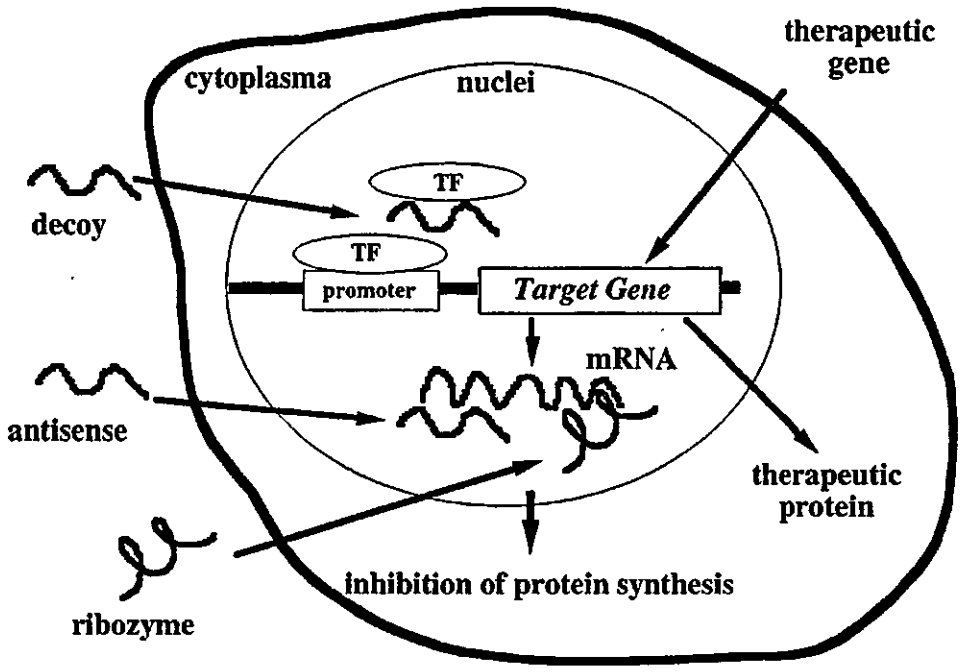

Fig. 4. Target sites for antisense, ribozyme and decoy strategies. Antisense, antisense ODN; ribozyme, ribozyme ON; decoy, decoy ODN; TF, transcription factor.

such as c-myc by antisense ODN, was also reported to inhibit neointimal formation in several animal models *(53)*. Currently, a phase II trial using antisense c-myc to treat restenosis is underway, although its results have not been reported. However, as this trial utilized intracoronary infusion of antisense c-myc ODN, several issues, such as low transfection efficiency may limit the efficacy of this strategy.

On the other hand, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Our previous data revealed that a single administration of antisense ODN against proliferating cell nuclear antigen (PCNA) and cdc 2 kinase genes inhibited neointimal formation after angioplasty for up to 8 wk after transfection *(54)*. In addition, a single administration of cyclin B_1/cdc 2 antisense ODN combination significantly inhibited the extent of neointimal formation for a period of 8 wk after transfection *(55,56)*. Similar trials have been employed for other vascular diseases, e.g., restenosis after vein grafting and vasculopathy in transplanted heart. This proliferative vascular disease may also be an ideal target for antisense ODN-based strategy. Mann et al. reported that transfection of antisense ODN against proliferating cell nuclear antigen (PCNA) and cdc 2 kinase resulted in the inhibition of hyperplasia at 2 wk after transfection in a vein graft model *(52)*. Moreover, the prevention of neointimal formation in the balloon injury model by the cell cycle inhibition strategy was sustained long-term over the period of antisense survival *(54–57)*. This may relate to the vascular remodeling induced by the inhibition of cell cycle progression. Indeed, administration of antisense PCNA and cdc 2 kinase ODN into a vein graft model improved the resistance to diet-induced atherogenesis *(58)*. In addition to the prevention of restenosis after vein grafting, the inhibition of hyperplasia in transplanted hearts has also been reported by Suzuki et al. Transfection of antisense cdk2 kinase ODN resulted in significant inhibition of VSMC growth in the transplanted heart *(59)*. The first

antisense drug appeared on the market in the United States at the end of 1999 as a novel drug to treat cytomegaloviral-mediated retinopathy.

Another strategy for combating disease processes by targeting to the transcriptional process is the use of ribozymes. We demonstrated the utility of ribozyme oligonucleotides against TGF-β by targeting the common sequence of *TGF*-β genes among humans, rats, and mice to treat restenosis in a balloon injury carotid artery model *(60)*. In addition, we also reported inhibition of production of lipoprotein (a) (Lp [a]), which is a risk factor for atherosclerosis, restenosis after angioplasty, cardiac disease, and stroke, without affecting plasminogen level using a ribozyme strategy *(61)*. Nevertheless, similar to antisense strategy, application of ribozyme technology to human gene therapy may require enhancement of the efficiency of cellular uptake and the stability of ribozyme oligonucleotides, because ribozyme oligonucleotides are easily degraded by nucleases because of their RNA backbone.

Decoy-Based Gene Therapy

Transfection of *cis*-element ds ODN (decoy) has been reported as a powerful tool in a new class of antigene strategies for gene therapy *(49)*. Transfection of ds ODN corresponding to the *cis* sequence will result in the attenuation of authentic *cis*-trans interaction, leading to the removal of transfactors from the endogenous *cis*-element, with subsequent modulation of gene expression. Therefore, the decoy approach may also enable us to treat diseases by modulation of endogenous transcriptional regulation. Recently, several studies have demonstrated application of the "decoy" ODN strategy as in vivo gene therapy *(62–64)*, and provide evidence of in vivo application of this novel molecular approach as a therapeutic strategy against cardiovascular disease. Many researchers have employed antisense technology as a "loss-of-function" approach at the transcriptional and translational levels, whereas the *cis*-element decoy strategy is also applicable as a "loss-of-function" approach at the pretranscriptional and transcriptional levels to study transcription factors.

As discussed above, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes, which results in mitosis. A critical element of cell-cycle progression regulation involves the complex formed by E2F, cyclin A, and cdk 2. The dissociation of the transcription factor E2F from the Rb gene product is proposed to play a pivotal role in the regulation of cell proliferation by inducing coordinated transactivation of genes involved in cell-cycle regulation including c-*myc*, c-*myb*, cdc 2, PCNA, and thymidine kinase. Accordingly, we hypothesized that transfection of VSMC with a sufficient quantity of decoy ODN containing the E2F *cis* element (consensus sequence "TTTTCGGCGC") would effectively bind E2F, prevent it from transactivating the gene expression of essential cell cycle regulatory proteins and thereby inhibit VSMC proliferation and neointimal formation. Transfection of E2F decoy ODN into rat balloon-injured carotid arteries resulted in almost complete inhibition of neointimal formation at 2 wk after balloon injury, accompanied by a reduction in mRNA of PCNA and cdc 2 kinase, but not β-actin *(62)*. Of importance, sustained inhibition of neointimal formation by a single administration of E2F decoy ODN was observed for at least 8 wk after treatment. Inhibition of neointimal formation by E2F decoy with hydrogel catheter delivery was also demonstrated using a porcine coronary artery model *(65)*. Modification of the delivery of ODN by a hydrogel catheter may overcome issues, such as the low transfection efficiency observed with coronary infusion. Based on these results, we started a clinical trial using hydrogel catheter delivery of E2F decoy to treat

restenosis after angioplasty in April 2000. As of March 2002, we have treated five patients with E2F decoy ODN. We did not observe any side effects up to 6 mo, although the clinical outcome has not yet been evaluated.

In addition, in 1996, clinical application of "decoy" against E2F by Dr. Dzau at Harvard University was also approved by the FDA to treat neointimal hyperplasia in vein bypass grafts, which results in failure in up to 50% of grafts within a period of 10 yr. A proof-of-concept study, the Project in Ex-Vivo Vein Graft Engineering Via Transfection (PREVENT I) study, was the first clinical trial using genetic engineering techniques to inhibit cell-cycle activation in vein grafts *(66)*. This prospective, randomized, controlled trial demonstrated the safety and biological efficacy of intraoperative transfection of human bypass vein grafts with E2F decoy oligonucleotides in a high-risk human patient population with peripheral arterial occlusion. They demonstrated successful inhibition of graft occlusion, accompanied by selective inhibition of PCNA and c-myc expression *(66)*. More recently, similar results were obtained in PREVENT II, a randomized, double-blind, placebo-controlled trial investigating the safety and feasibility of E2F decoy oligonucleotides in preventing autologous vein graft failure after coronary artery bypass surgery *(67)*. The interim results confirmed the safety and feasibility of using this product. Analysis of the secondary end points using quantitative coronary angiography and three-dimensional intravascular ultrasound demonstrated increased patency and positive vascular remodeling (inhibition of neointimal size and volume) in the treated group at 12 mo. Patients examined at follow-up were found to have, on average, a 40% reduction in critical stenosis. Further assessment of this encouraging therapeutic approach should be completed by 2005 in adequately powered phase III studies in coronary and peripheral vessel disease, to determine definitively the extent and duration of clinical benefit. Because E2F has been postulated to play an important role in the pathogenesis of numerous diseases, e.g., vasculopathy after transplantation *(68)*, the development of the E2F decoy strategy may provide a useful therapeutic tool for treating these proliferative diseases.

On the other hand, the transcription factor NFκB also plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes whose activation has been postulated to be involved in numerous diseases, such as myocardial infarction. These diseases are, importantly, potentially amenable to ODN-based gene therapy, because treatment of these diseases is extremely difficult because of the lack of effective pharmacological agents. The pathophysiology of myocardial infarction is quite complicated. Numerous cytokines including interleukin-1, -2, -6, -8, and TNF-α, to name a few, regulate this process. However, gene regulation of many cytokines is relatively simple, because the transcription factor NFκB has been reported to upregulate these cytokines. Interestingly, adhesion molecules such as VCAM and ICAM are also known to be upregulated by NFκB. Accordingly, we hypothesized that myocardial infarction and glomerulonephritis could be prevented by the blockade of genes regulating cell inflammation—the final common pathway that is induced by NFκB binding (*see* Fig. 5). The necessity to block cytokine and adhesion molecule genes at more than one point to achieve maximum inhibitory effects may be a result of the redundancy and complexity of the interactions of these genes.

Importantly, increased NFκB binding activity has been confirmed in balloon-injured blood vessels *(69)*. Our recent study provided the first evidence of the feasibility of a decoy strategy against NFκB in treating restenosis *(69)*. Transfection of NFκB decoy ODN into balloon-injured carotid artery or porcine coronary artery markedly reduced

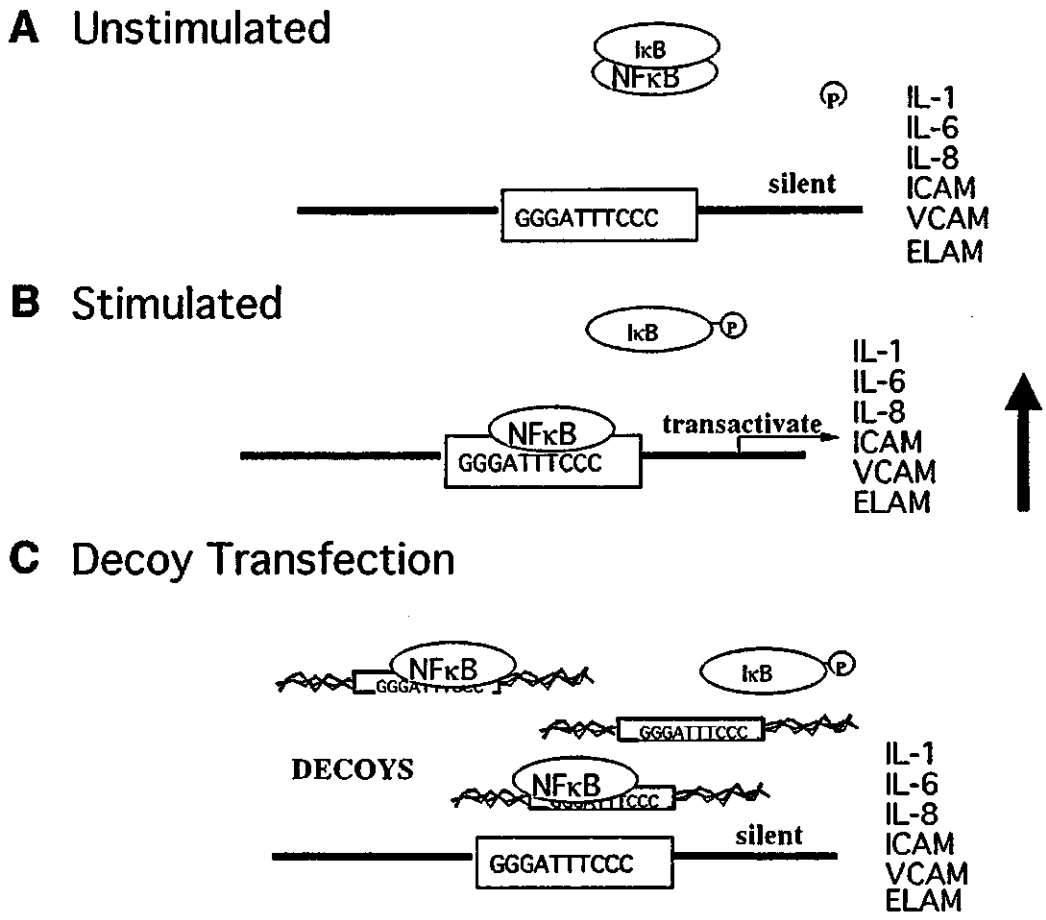

Fig. 5. Mechanisms of NFκB decoy ODN.

neointimal formation, whereas no difference was observed between scrambled decoy ODN-treated and untransfected blood vessels *(69,70)*. Based on the therapeutic efficacy of this strategy, we obtained permission for a second clinical trial using the decoy strategy to treat restenosis from 2001. In addition, the inhibition of VSMC replication was confirmed by the observation that transfection of NFκB decoy ODN inhibited the progression of vasculopathy in cardiac transplantation models *(71,72)*. Blockade of NFκB is also effective in treating reperfusion myocardial injury *(63,64)*. Transfection of NFκB decoy ODN into rat coronary artery prior to left-ascending artery (LAD) occlusion markedly reduced the area of damaged myocytes 24 h after reperfusion. The therapeutic efficacy of this strategy via intracoronary administration immediately after reperfusion, similar to the clinical situation, was also examined. NFκB decoy ODN reduced the damage of myocytes resulting from reperfusion, in contrast with rats treated with scrambled control decoy or vehicle.

Because NFκB has been postulated to play an important role in the pathogenesis of numerous diseases, e.g., cancer and arthritis, the development of a NFκB decoy strategy may provide a useful therapeutic tool for treating these diseases. Furthermore, modifications of ODN composition to prolong decoy stability in vivo and/or development of a delivery system into the cardiovascular organs/tissues will be critical to enhance potential therapeutic efficacy *(73,74)*. Despite these limitations, development of this technology offers great promise as a new tool for defining biological processes and treating

pathological conditions. Overall, this approach is particularly attractive for several reasons: (1) the potential drug targets (transcription factors) are plentiful and readily identifiable; (2) the synthesis of sequence-specific decoys is relatively simple and can be targeted to specific tissues; (3) knowledge of the exact molecular structure of the targeted transcription factor is unnecessary; and (4) decoy ODN may be more effective than antisense ODN in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same *cis* element. Thus, the decoy strategy may be useful for treating a broad range of human diseases. In contrast, because an important concern regarding the decoy strategy revolves around the potential inhibition of normal physiological responses, the application of decoy strategy as gene therapy may be limited to treatment of acute conditions, namely transcription factor-driven diseases.

Unresolved Issues in ODN-Based Gene Therapy

ODN-based gene therapy still has many unsolved problems, such as the short half-life, low efficiency of uptake, and degradation by endocytosis and nucleases. Therefore, many groups currently are focusing on modifications of the gel approach using a catheter delivery system. Further modification of ODN pharmacokinetics will facilitate the potential clinical utility of the agents by: (1) allowing a shorter intraluminal incubation time to preserve organ perfusion; (2) prolonging the duration of biological action; and (3) enhancing efficacy, such that the nonspecific effects of high doses of ODN can be avoided. Regarding the ODN-based strategy as gene therapy, one of the major concerns is nonspecific effects, particularly those of phosphorothioate-substituted ODN *(75–78)*. To overcome these issues, carefully controlled experiments must be performed to eliminate the potential nonspecific effects of ODN-mediated therapy. For gene therapy using an ODN-based strategy, the toxicity of phosphorothioate ODN may also be important. Although low-dosage administration does not seem to cause any toxicity, bolus infusions may be dangerous. Higher doses over prolonged periods of time may cause kidney damage, as evidenced by proteinuria and leukocytes in the urine in animals *(77)*. Liver enzymes may also be increased in animals treated with moderate to high doses. Several phosphorothioate ODN have been shown to cause acute hypotensive events in monkeys *(79,80)*, probably as a result of complement activation *(81)*. These effects are transient, if managed appropriately, and relatively uncommon. This toxicity can be avoided by giving intravenous infusions rather than bolus injections. More recently, prolongation of prothrombin, partial thromboplastin, and bleeding times has been reported in monkeys *(82)*. Alternatively, we recently developed new modification of decoy ODN in order to increase their stability against nucleases. Although the chemical modifications of ODN, such as phosphorothioation and methylphosphonation, were employed, problems with these modified ODNs became apparent including insensitivity to RNaseH, lack of sequence specificity, and immune activation, as described previously. To overcome these limitations, covalently modified ODN were developed by enzymatically ligating two identical molecules, thereby preventing their degradation by exonucleases (Fig. 6) *(83)*. In fact, the transfection of novel AP-1 decoy ODN with circular ribbon structure, prior to the balloon injury procedure, prevented neointimal formation in the rat balloon-injured artery more effectively than nonmodified decoy ODN *(83)*.

PERSPECTIVES IN GENE THERAPY

Gene therapy in the field of cardiovascular disease would be useful for the treatment of many diseases, including PAD, myocardial infarction, restenosis after angioplasty,

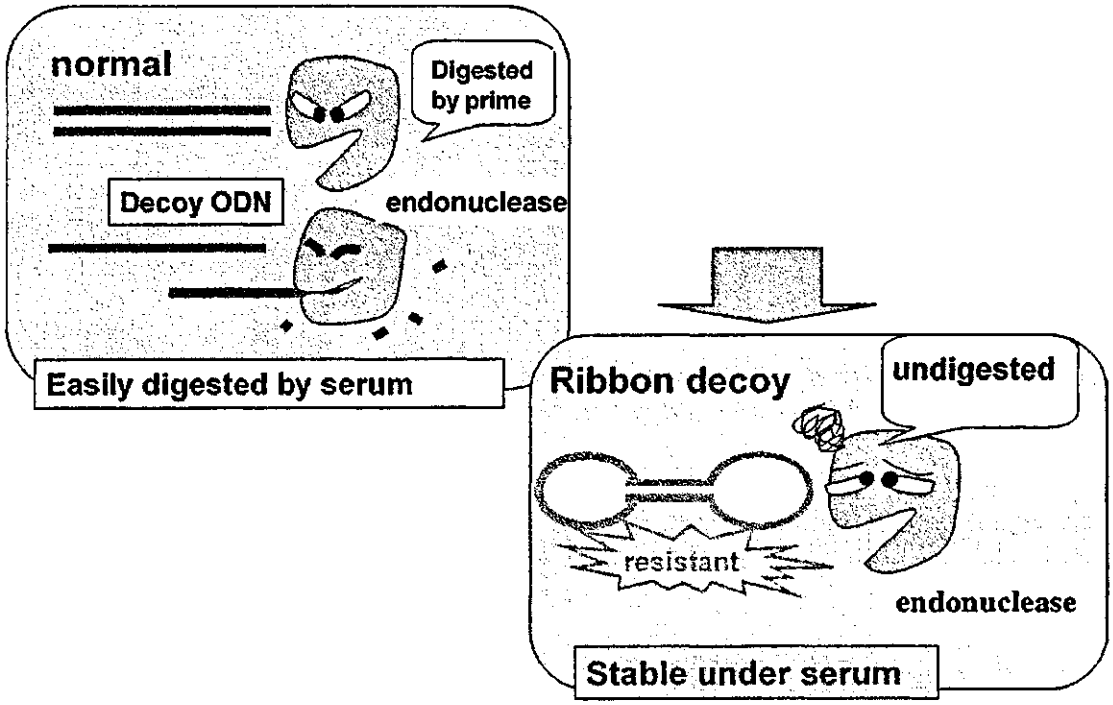

Fig. 6. Development of ribbon-type decoy ODN that has the potent resistance to endonuclease.

and rejection in heart transplantation. The first federally approved human gene therapy protocol started on September 14, 1990 for adenosine deaminase deficiency patients. Ten years since the commencement of the first trial, over 4000 patients have been treated by gene therapy. The objectives are generally to evaluate: (1) the in vivo efficacy of the gene transfer method; (2) the safety of the gene transfer method, and (3) the possible therapeutic efficacy. Although there are still many unresolved issues in the clinical application of gene therapy, gene therapy for cardiovascular disease now appears to be not far from reality and it is time to take a hard look at practical issues that will determine the real clinical potential. These include: (1) further innovations in gene transfer methods, (2) well-defined disease targets, (3) cell-specific targeting strategies, and (4) effective and safe delivery systems.

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