Jikeikai Med J 2002 ; 49 : 149-55

Prevention of Arterial Stenosis after Balloon Injury Using an Adenoviral Vector Encoding Deoxycytidine Kinase cDNA with Cytosine Arabinoside

Keiji IWANO[1], Yoshinobu MANOME[2], and Tsuneya OHNO[2]

[1]*Division of Cardiology, Department of Internal Medicine, The Jikei University School of Medicine*
[2]*Department of Microbiology, The Jikei University School of Medicine*

ABSTRACT

Restenosis occurs in 25% to 50% of patients after percutaneous transluminal angioplasty. Therefore, many patients must undergo angiography or angioplasty because restenosis is unresponsive to most pharmacologic therapies. Recent *in vivo* gene-therapy trials targeting arterial smooth muscle have successfully prevented restenosis. Therefore, we have developed a novel gene-therapy approach to a balloon arterial injury which uses a replication-incompetent adenoviral vector encoding the human deoxycytidine kinase (dCK) gene and cytosine arabinoside (ara-C), an inactive prodrug converted by dCK to a cytotoxic metabolite. The activated prodrug is incorporated into DNA in a cell-cycle-dependent fashion and thus is cytotoxic to the proliferating cells. We found that that adenoviral vector-mediated gene transfer of human dCK cDNA into A7r5 rat arterial smooth-muscle cells significantly increased their sensitivity to ara-C *in vitro* and prevented arterial stenosis in animals after balloon injury. These results suggest that the *in situ* transduction of arterial smooth muscle cells with the dCK gene by means of transluminal catheter and adenoviral vectors increases their sensitivity to ara-C and may thus play a role in the treatment of arterial restenosis after angioplasty. (Jikeikai Med J 2002 ; 49 : 149-55)

Key words : angioplasty, restenosis, deoxycytidine kinase, cytosine arabinoside, gene therapy

INTRODUCTION

Restenosis after arterial balloon injury remains an important problem affecting the long-term success of coronary angioplasty. Restenosis develops within 6 months in 25% to 50% of patients who undergo angioplasty. These patients frequently must undergo angiography again and occasionally require further angioplasty. Restenosis is also costly (estimated at $2 billion annually in the United States) and refractory to many pharmacologic treatments[1].

The poor results of conventional therapy for restenosis have increased interest in alternative forms of treatment such as gene therapy. *In vivo* gene transfer is a promising therapeutic strategy for many diseases, and restenosis is considered a good target for this approach because effective methods for local delivery of vectors have been established and appropriate targets have been identified.

One particularly promising strategy of gene therapy involves the transfer of a "chemosensitization" or "suicide" gene into target cells, thus increasing their sensitivity to otherwise innocuous prodrugs or conventional chemotherapeutic agents. The prototypic

Received for publication, August 31, 2002
岩野　圭二，馬目　佳信，大野　典也
Mailing address : Keiji IWANO, Division of Cardiology, Department of Internal Medicine, The Jikei University School of Medicine, 3-25-8, Nishi-Shimbashi, Minato-ku, Tokyo 105-8461, Japan.
E-mail address : iwano@jikei.ac.jp

chemosensitization gene is the herpes simplex virus thymidine kinase (HSV-tk) gene. The transduction of the HSV-tk gene into arterial smooth-muscle cells using replication-incompetent retroviral and adenoviral vectors renders these cells susceptible to the antiviral nucleoside analog ganciclovir. Proliferating smooth-muscle cells incorporate activated prodrug into their DNA, resulting in chain termination; however, innocuous, nondividing host cells are unaffected by ganciclovir. This approach has successfully been used to inhibit arterial stenosis in animal models[2–5].

Recently, many suicide genes other than HSV-tk have been reported and investigated for efficacy. We have also previously proposed a novel suicide-gene system in which the deoxycytidine kinase (dCK) suicide gene is transduced to cancer cells, which are then treated with the prodrug cytosine arabinoside (ara-C); this system has cytocidal effects both *in vitro* and *in vivo* against dividing brain-tumor cells[6].

In this report, we demonstrate that the adenoviral transduction of the dCK gene to arterial smooth-muscle cells confers sensitivity to ara-C. The effect on arterial smooth muscle cells was confirmed to be stronger than that previously reported on 9L tumor cells *in vitro*. Because arterial smooth-muscle cells appear to be a good target for this system, we further evaluated the effectiveness of this system in a rat model of arterial balloon injury.

Materials and Methods

1. *Cell line and animals*

The A7r5 embryonal thoracic-aorta smooth-muscle cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Adult male Sprague-Dawley rats (400 to 450 g) were purchased from Clea Japan (Tokyo).

2. *Recombinant adenovirus*

The construction of the recombinant adenoviruses Ad.CMV-βgal and Ad.CMV-dCK have been described previously[6]. After expansion, viruses were purified with two cesium chloride ultracentrifugations, dialyzed against 10% glycerol, 10 mM Tris (pH 8.0) and 1 mM $MgCl_2$, and stored at $-80^{\circ}C$. The virus titers were determined as plaque-forming units (pfu) assayed in semisolid cultures consisting of 293 cells.

3. *In vitro cytotoxic assay*

An *in vitro* cytotoxic assay was performed by calculating the survival cell ratio after treatment with the adenovirus and ara-C[7]. Briefly, A7r5 rat smooth-muscle cells were infected with different multiplicities of infection (MOIs) of Ad.CMV-dCK. Forty-eight hours after infection, the cells were exposed to different concentrations of ara-C and the cell number was determined after a further 48 hours of incubation.

Animal Studies

4. *Left carotid arterial balloon injury*

All animal procedures were approved by the Research Committee of the Jikei University School of Medicine. After pentobarbital (50 mg/kg, i.p.) was administered, the right femoral artery of Sprague-Dawley rats was surgically exposed. A 2-French Fogarty balloon catheter was inserted from the right femoral artery and passed to the left common carotid artery. The tip of the catheter was controlled by direct observation through the exposed left carotid artery wall. A segment of the left common carotid artery was injured by passing the inflated balloon through it three times.

5. *Experimental gene therapy for arterial stenosis of the balloon-injured artery*

After the left common carotid artery was injured with the balloon, a small surgical clip was used to occlude its distal end. The adenoviral vector (5×10^9 pfu) was injected through the catheter and directly transferred to the injured segment of the artery. After 40 minutes of incubation, the vector was withdrawn and the vessel wall was washed with phosphate-buffered saline (PBS). Next, the surgical

clip and catheter were removed, and the wound was sutured. The rats were then allowed to recover from anesthesia. To examine the efficacy of gene transfer, the rats in which Ad.CMV-βgal had been transduced were killed and the injured arteries were fixed with 3% paraformaldehyde in PBS and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) and PBS containing 10 mM $K_3Fe(CN)_6$, 10 mM $KFe(CN)_6$, and 2 mM $MgSO_4$. All reactions were carried out at 37°C for 2 to 16 hours.

In other experiments, 72 hours after infection, the infected animals were treated with a subcutaneous injection of ara-C (200 mg/kg) every 24 hours for 4 days. Fourteen days after injury, the rats were subjected to histologic examinations (Fuji Biomedics Co., Saitama). The thickness of the carotid arterial wall was assessed, and the intima/media (I/M) ratio was determined with a color image-analyzer (SP500F, Olympus, Tokyo). Statistical analysis was performed with the two-sample t-test. A P value less than 0.05 indicated significance.

Results

1. Vascular restenosis model after arterial balloon injury

Repeated passage of a 2-French Fogarty balloon catheter into the left carotid artery did not produce vascular responses, which sometimes occur under direct carotid manipulation, such as after arterectomy, but did produce arterial stenosis with intimal hypertrophy and marked smooth muscle proliferation in all animals. The I/M ratio was as high as 100% to 130% after 14 days.

2. Transgene expression after adenoviral vector infection at the site of carotid-artery injury

The enzymatic activity of the β-galactosidase was expressed mainly in the injured segment (Fig. 1). In contrast, the uninjured walls did not demonstrate a high degree of β-galactosidase activity.

3. Sensitivity of aortic smooth muscle cells to ara-C after dCK gene transduction

Although naive A7r5 cells were relatively resistant to ara-C, they became sensitive to ara-C in an MOI-dependent manner (Table 1). The calculated inhibitory concentrations of 50% (IC_{50}) were as follows: MOI=0, 595 nM; MOI=10, 47.5 nM; and MOI=50, 32.0 nM.

Table 1. Sensitivity of A7r5 smooth-muscle cells to ara-C after adenovirus-mediated transduction of the dCK gene.

	Survival cells (% control cells)		
ara-C (nM)	MOI=0	MOI=10	MOI=50
0	100.0±16.1	100.0±10.1	100.0±10.9
50	102.5±16.6	48.3±3.0	21.6±8.2
100	95.8±11.2	10.9±2.8	7.2±4.7
200	78.4±6.9	6.4±2.3	0.0±0.0
400	63.5±7.2	3.4±1.1	0.0±0.0
800	36.0±5.2	1.9±0.3	0.0±0.0

Fig. 1. X-gal staining of the carotid artery after Ad.CMV-βgal infection. Seventy-two hours after infection, the artery was fixed and stained with X-Gal. The β-galactosidase activity (blue) was limited to the injured portion. The activity also reached the media of the vessel wall.

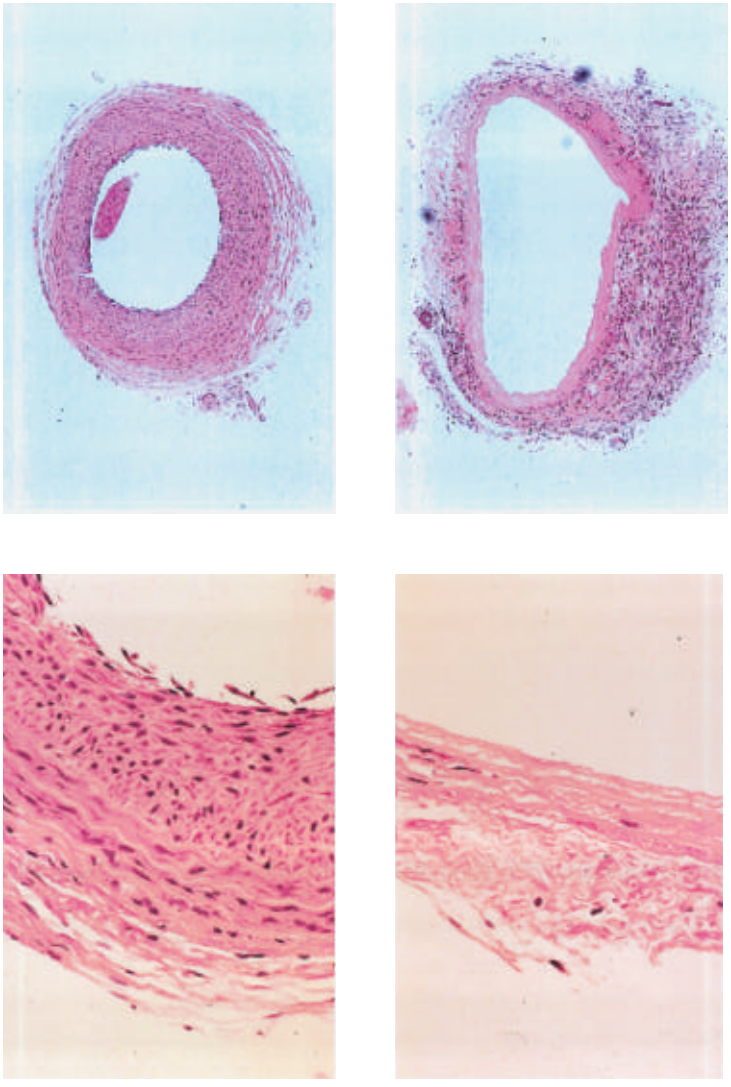

Fig. 2. Cross sections of the carotid artery after treatment. Whereas animals treated with dCK/ara-C showed less intimal proliferation, control animals showed marked hypertrophy of the neointima and stenotic lesions of the carotid artery. (hematoxylin and eosin staining; upper, low-power magnification; lower, high-power magnification).

4. Inhibition of neointima formation after arterial injury by Ad.CMV-dCK infection and ara-C treatment

Encouraged by these findings, we measured the intimal thickness by arterial balloon injury and the consequences of Ad.CMV-dCK gene transfer and ara-C treatment *in vivo*. After arterial injuries were produced, the injury sites were infected with either Ad.CMV-dCK or a control viral vehicle for 40 minutes. After 72 hours, the rats received injections of either ara-C at a daily dose of 200 mg/kg, s.c., for 4 days or the same volume of saline as a control. The animals were then killed and subjected to histologic examination.

Neointima formation in the carotid artery was markedly less in treated rats than in control rats (Fig. 2). This result was then confirmed in additional animals. The I/M ratio was signficantly lower in rats treated with Ad.CMV-dCK-infected/ara-C ($n=4$, $18.1\pm36.2\%$) than in control rats treated with Ad.CMV-dCK/saline ($140.6\pm59.1\%$, $n=5$, $P<0.01$), vehi-

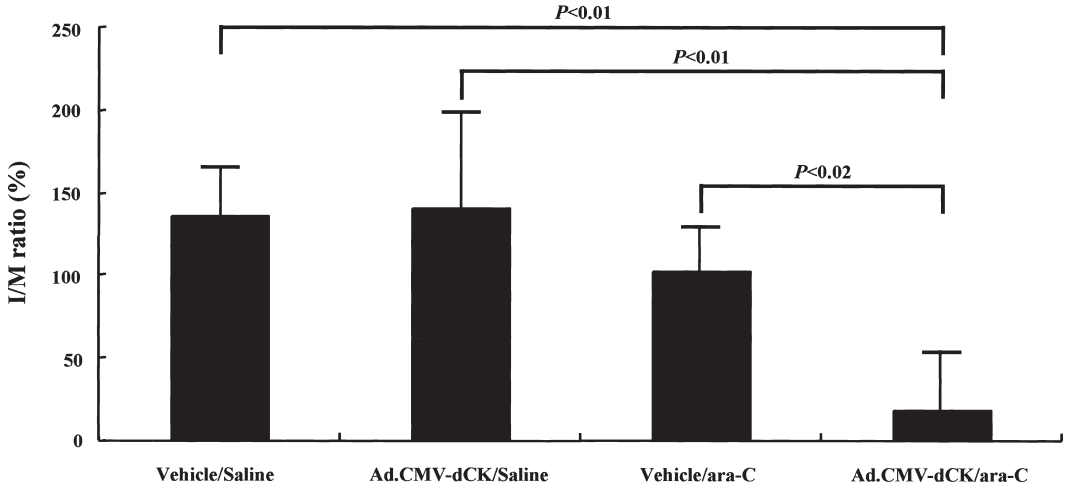

Fig. 3. The I/M ratio of the carotid artery after balloon injury and adenoviral vector-mediated dCK gene transfer. Three days after infection, the rats were treated with either ara-C (800 mg/kg) or the same amount of saline. The I/M ratio was determined after scanning each histologic section (vehicle injection, treated with saline, $n=3$; vehicle injection, treated with ara-C, $n=3$; Ad.CMV-dCK infection, treated with saline, $n=5$; and Ad.CMV-dCK infection, treated with ara-C, $n=4$).

cle/saline (136.4±29.8%, $n=3$, $P<0.01$), or vehicle/ara-C (102.6±27.2%, $n=3$, $P<0.02$; Fig. 3).

Discussion

We have demonstrated that the adenoviral transduction of dCK cDNA followed by ara-C administration inhibits the proliferation of arterial smooth muscle cells *in vitro* and also prevents the development of stenosis after arterial balloon injury *in vivo*.

The pathogenesis of restenosis involves numerous processes, including the local release of growth factors, the activation of intracellular signals, the induction of early response genes or cell cycle regulating genes, the proliferation of smooth muscle cells, the adherence of leukocytes or platelets, the infiltration of inflammatory cells, thrombus formation, and vascular remodeling. Because all these processes play a role in the formation of stenotic lesions, they are potential targets for treatment. The proliferation of smooth muscle cells, an extensively studied process, is considered a particularly promising target.

Recently, antiproliferative approaches shown to inhibit smooth muscle proliferation and neointima formation have included antisense nucleotides to proto-oncogenes, cell cycle regulating genes, and transcriptional factors, such as c-myb[8], cdc2 kinase, proliferating cell nuclear antigen, cyclin B1, cdc2. cdk2[9-12], c-myc[13,14], cyclin G1[15], and NF-kB p65 subunit[16]. These antisense oligonucleotides were designed to be antiproliferative or cytostatic molecules, which block the translation of messenger RNA to target proteins and ultimately abolish the growth of rapidly dividing cells.

Despite these preliminary results, we decided to use a cytocidal approach rather than a cytostatic approach to prevent restenosis. The reason for this decision was that various methods, such as the local administration of antisense oligomers to proliferating cell nuclear antigen or cdc2 kinase[17], c-myb[18], or thrombin receptor[19] do not effectively inhibit arterial stenosis after balloon injury. The pathogenesis of restenosis is more complicated than previously thought and is thus considered to be a clinically intractable cycle. In fact, anticancer drugs[20] and intraluminal irradiation[21,22], which are intended to eradicate proliferating cells, have been recommended for the treatment of restenosis. Therefore, we chose a cytotoxic gene-therapy approach to treat proliferating smooth muscle cells and then examined its efficacy using an adenoviral vector.

In vivo gene transfer to the vascular wall was originally accomplished with a retroviral vector and liposome[23]. However, transfection with a retroviral

vector is less efficient in nonmalignant tissue in which only a small number of cells are actively proliferating at a given time. In contrast, adenoviral vectors have several advantages for gene transfer to the vascular wall. One major advantage is adenoviral vectors is high gene-transfer efficiency[24,25]. Unlike a retrovirus, an adenovirus can transduce a gene into nondividing cells as well as dividing cells. An adenovirus can also be concentrated and easily stored as a high-titer stock. Adenoviruses can transfer genes to the vascular wall[26] despite blood flow. Moreover, an adenoviral vector transduces a gene especially to the vessel wall, which lacks an endothelium. This may be due to the relatively small number of receptors to adenoviruses on normal endothelial cells. Because angioplasty usually damages endothelial cells, gene delivery could thereby be facilitated, particularly in the treated region.

A great advantage of suicide-gene therapy is that sensitivity is selectively conferred to proliferating cells. Even if an adenoviral vector delivers a gene to both nondividing cells and dividing cells, only dividing cells will be affected by the prodrug. In this context, adenoviral gene delivery of HSV-tk together with ganciclovir has successfully been used to treat arterial stenosis *in vivo*[2-5].

Recently, several suicide-gene systems have been used to treat cancer[6,27,28]. We have demonstrated that the dCK gene transduction and ara-C inhibit rat 9L brain tumor cells and prolong survival[6]. This dCK/ara-C system has several advantages over the previously reported HSV-tk/ganciclovir or cytosine deaminase/5-fluorocytosine suicide system[29]. First, ara-C is a widely used drug whose pharmacokinetics are well understood. The standard protocol for treatment of such patients has been established and most of the adverse effects have already been clarified. In fact, in the present animal study we observed few pathologic changes except for extramedullary hematopoiesis in the liver and spleen, which was anticipated. Second, because the dCK gene is of human origin, the potential hazards of immunologic reaction are reduced. Our comparison of the cytocidal effect and the IC_{50} shows that after dCK gene transfer arterial smooth-muscle cells are more sensitive to ara-C than are 9L brain tumor cells[6]. This increased sensitivity is a distinct advantage for the treatment of restenosis.

In summary, we have demonstrated that the transduction of A7r5 arterial smooth-muscle cells with the dCK gene by means of replication-incompetent adenoviral vectors significantly increases the sensitivity of these cells to ara-C *in vitro* and inhibits arterial stenosis after injury *in vivo*. Although our results cannot be directly compared with those of the HSV-tk system, the dCK/ara-C system seems to have some advantages for use with arterial smooth-muscle cells and, therefore, may be an effective treatment for restenosis after angioplasty.

Acknowledgements: This work was supported in part by the Japan Heart Foundation and an IBM Japan Research Grant for 1996. The authors also sincerely thank Yuko Aikawa and Yoko Manome for their expert technical assistance.

REFERENCES

1. Feldman LJ, Tahlil O, Steg G. Perspectives of arterial gene therapy for the prevention of restenosis. Cardiovasc Res 1996; 32: 194-207.
2. Chang MW, Ohno T, Gordon D, Lu MM, Nabel GJ, Nabel EG, et al. Adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene inhibits vascular smooth muscle cell proliferation and neointima formation following balloon angioplasty of the rat carotid artery. Mol Med 1995; 1: 172-81.
3. Guzman RJ, Hirschowitz EA, Brody SL, Crystal RG, Epstein SE, Finkel T. *In vivo* suppression of injury-induced vascular smooth muscle cell accumulation using adenovirus-mediated transfer of the herpes simplex virus thymidine kinase gene. Proc Natl Acad Sci USA 1994; 91: 10732-6.
4. Ohno T, Gordon D, San H, Pompili VJ, Imperiale MJ, Nabel GJ, et al. Gene therapy for vascular smooth muscle cell proliferation after arterial injury. Science 1994; 265: 781-4.
5. Steg PG, Tahlil O, Aubailly N, Caillaud JM, Dedieu JF, Berthelot K, et al. Reduction of restenosis after angioplasty in an atheromatous rabbit model by suicide gene therapy. Circulation 1997; 96: 408-11.
6. Manome Y, Wen PY, Dong Y, Tanaka T, Mitchell BS, Kufe DW, et al. Viral vector transduction of the human deoxycytidine kinase cDNA sensitizes glioma cells to the cytotoxic effects of cytosine arabinoside *in vitro* and *in*

vivo. Nat Med 1996 ; 2 : 567-73.
7. Manome Y, Abe M, Hagen MF, Fine HA, Kufe DW. Enhancer sequences of the DF3 gene regulate expression of the Herpes Simplex virus thymidine kinase gene and confer sensitivity of human breast cancer cells to ganciclovir. Cancer Res 1994 ; 54 : 5408-13.
8. Simons M, Edelman ER, DeKeyser JL, Langer R, Rosenberg RD. Antisense c-myb oligonucleotides inhibit initial arterial smooth muscle cell accumulation *in vivo*. Nature 1992 ; 359 : 67-70.
9. Morishita R, Gibbons GH, Kaneda Y, Ogihara T, Dzau VJ. Pharmacokinetics of antisense oligodeoxyribonucleotides (cyclin B1 and CDC 2 kinase) in the vessel wall *in vivo* : enhanced therapeutic utility for restenosis by HVJ-liposome delivery. Gene 1994 ; 149 : 13-9.
10. Morishita R, Gibbons GH, Ellison KE, Nakajima M, Zhang L, Kaneda Y, et al. Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia. Proc Natl Acad Sci USA 1993 ; 90 : 8474-8.
11. Abe J, Zhou W, Taguchi J, Takuwa N, Miki K, Okazaki H, et al. Suppression of neointimal smooth muscle cell accumulation *in vivo* by antisense cdc2 and cdk2 oligonucleotides in rat carotid artery. Biochem Biophys Res Commun 1994 ; 198 : 16-24.
12. Morishita R, Gibbons GH, Ellison KE, Nakajima M, von der Leyen H, Zhang L, et al. Intimal hyperplasia after vascular injury is inhibited by antisense cdk 2 kinase oligonucleotides. J Clin Invest 1994 ; 93 : 1458-64.
13. Shi Y, Hutchinson HG, Hall DJ, Zalewski A. Downregulation of c-myc expression by antisense oligonucleotides inhibits proliferation of human smooth muscle cells. Circulation 1993 ; 88 : 1190-5.
14. Shi Y, Fard A, Galeo A, Hutchinson HG, Vermani P, Dodge GR, et al. Transcatheter delivery of c-myc antisense oligomers reduces neointimal formation in a porcine model of coronary artery balloon injury. Circulation 1994 ; 90 : 944-51.
15. Zhu NL, Wu L, Liu PX, Gordon EM, Anderson WF, Starnes VA, et al. Downregulation of cyclin G1 expression by retrovirus-mediated antisense gene transfer inhibits vascular smooth muscle cell proliferation and neointima formation. Circulation 1997 ; 96 : 628-35.
16. Autieri MV, Yue TL, Ferstein GZ, Ohlstein E. Antisense oligonucleotides to the p65 subunit of NF-kB inhibit human vascular smooth muscle cell adherence and proliferation and prevent neointima formation in rat carotid arteries. Biochem Biophys Res Commun 1995 ; 213 : 827-36.
17. Robinson KA, Chronos NA, Schieffer E, Palmer SJ, Cipolla GD, Milner PG, et al. Endoluminal local delivery of PCNA/cdc2 antisense oligonucleotides by porous balloon catheter does not affect neointima formation or vessel size in the pig coronary artery model of postangioplasty restenosis. Cathet Cardiovasc Diagn 1997 ; 41 : 348-53.
18. Villa AE, Guzman LA, Poptic EJ, Labhasetwar V, D'Souza S, Farrell CL, et al. Effects of antisense c-myb oligonucleotides on vascular smooth muscle cell proliferation and response to vessel wall injury. Circ Res 1995 ; 76 : 505-13.
19. Herbert JM, Guy AF, Lamarche I, Mares AM, Savi P, Dol F. Intimal hyperplasia following vascular injury is not inhibited by an antisense thrombin receptor oligodeoxynucleotide. J Cell Physiol 1997 ; 170 : 106-14.
20. Axel DI, Kunert W, Goggelmann C, Oberhoff M, Herdeg C, Kuttner A, et al. Paclitaxel inhibits arterial smooth muscle cell proliferation and migration *in vitro* and *in vivo* using local drug delivery. Circulation 1997 ; 96 : 636-45.
21. Carter AJ, Laird JR. Experimental results with endovascular irradiation via a radioactive stent. Int J Radiat Oncol Biol Phys 1996 ; 36 : 797-803.
22. Waksman R, Robinson KA, Crocker IR, Gravanis MB, Cipolla GD, Seung KB, et al. Intracoronary radiation decreases the second phase of intimal hyperplasia in a repeat balloon angioplasty model of restenosis. Int J Radiat Oncol Biol Phys 1997 ; 39 : 475-80.
23. Nabel EG, Plautz G, Nabel GJ. Site-specific gene expression *in vivo* by direct gene transfer into the arterial wall. Science 1990 ; 249 : 1285-8.
24. Lee SW, Trapnell BC, Rade JJ, Virmani R, Dichek DA. *In vivo* adenoviral vector-mediated gene transfer into balloon-injured rat carotid arteries. Circ Res 1993 ; 73 : 797-807.
25. Mazur W, Ali NM, Grinstead WC, Schulz DG, Raizner AE, French BA. Lipofectin-mediated versus adenovirus-mediated gene transfer *in vitro* and *in vivo* : comparison of canine and porcine model systems. Coronary Artery Dis 1994 ; 5 : 779-86.
26. Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient and selective adenovirus-mediated gene transfer into vascular neointima. Circulation 1993 ; 88 : 2838-48.
27. Dong Y, Wen P, Manome Y, Parr M, Hirshowitz A, Chen L, et al. *In vivo* replication-deficient adenovirus vector- mediated transduction of the cytosine deaminase gene sensitizes glioma cells to 5-fluorocytosine. Human Gene Ther 1996 ; 7 : 713-20.
28. Manome Y, Wen PY, Chen L, Tanaka T, Dong Y, Yamazoe M, et al. Gene therapy for malignant gliomas using replication incompetent retroviral and adenoviral vectors encoding the cytochrome P450 2B1 gene together with cyclophosphamide. Gene Ther 1996 ; 3 : 513-20.
29. Kreuzer J, Denger S, Reifers F, Beisel C, Haack K, Gebert J, et al. Adenovirus-assisted lipofection : efficient *in vitro* gene transfer of luciferase and cytosine deaminase to human smooth muscle cells. Atherosclerosis 1996 ; 124 : 49-60.