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Single intraluminal delivery of antisense cdc2 kinase and proliferating-cell nuclear antigen oligonucleotides results in chronic inhibition of neointimal hyperplasia

(restenosis/angioplasty/smooth muscle cells/gene therapy/hemagglutinating virus of Japan)

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ABSTRACT **To develop an effective strategy to prevent neointima formation after angioplasty injury, we have identified cell-cycle regulatory proteins as targets for inhibition by using antisense oligonucleotides (ODNs). We utilized an intraluminal molecular delivery method that employs the protein coat of a Sendai virus complexed with liposomes that enhances markedly the efficiency of ODNs uptake. First, we examined the effect of antisense cdc2 kinase and proliferating-cell nuclear antigen (PCNA) ODNs *in vitro*. Cotransfection of antisense cdc2 kinase and PCNA ODNs inhibited serum-stimulated vascular smooth muscle cell growth, whereas antisense cdc2 kinase ODNs alone or PCNA ODNs alone failed to show any inhibitory effect. Transfection of the combination of antisense cdc2 kinase and PCNA ODNs into balloon-injured arteries *in vivo* provided a marked decrease in cdc2 and PCNA mRNA expression as determined by reverse transcription–PCR, compared to sense controls. Antisense ODN treatment significantly inhibited the increase in DNA synthesis induced by balloon injury. Moreover, antisense ODN administration inhibited completely neointima formation at 2 weeks after angioplasty in an apparent dose-dependent manner. Moreover, the inhibitory effect of antisense ODN on neointima formation persisted up to 8 weeks after a single transfection. The present study documents that a single intraluminal molecular delivery of combined cdc2 kinase and PCNA antisense ODNs results in a sustained inhibition of neointima formation in the rat carotid balloon-injury model.**

The introduction of percutaneous transluminal angioplasty has been one of the major advancements in the treatment of stenotic vascular lesions. However, its long-term efficacy is limited by the development of restenosis due to neointima formation in 30–40% of patients (1, 2). To date, no effective pharmacological therapy for preventing restenosis in humans has been reported (3–5). This failure may reflect the difficulty in identifying appropriate drug targets due to the complexity of the pathophysiological process of neointima formation and/or the inability to deliver sufficient quantities of drugs to the site of injury.

Neointima formation after angioplasty involves a complex interaction between multiple growth factors that promote vascular smooth muscle cell (VSMC) proliferation and migration including: thrombin, platelet-derived growth factor, and basic fibroblast growth factor, to name a few (6). Given the multiplicity of growth factors involved, it appears unlikely that selective inhibition of a particular growth factor will completely prevent lesion formation (2–5, 7, 8). Growth-factor-induced cell proliferation involves the sequential activation of intracellular proteins that promote cell-cycle progression (9, 10). Accordingly, we hypothesized that restenosis could be prevented by the blockade of genes regulating cell-cycle progression—the final common pathway. Previous studies have established that proliferating-cell nuclear antigen (PCNA; a nuclear protein required for DNA synthesis by DNA polymerase Δ) and $p34^{cdc2}$ (a serine/threonine protein kinase) are principle components of the final common pathway regulating cell proliferation (9–12). PCNA and cdc2 kinase appear to play an essential role in the transition through both the G_1/S and G_2/M phases of the cell cycle. We therefore employed antisense oligonucleotides (ODNs) directed at the translation initiation sites of PCNA and cdc2 mRNAs to inhibit neointimal hyperplasia. The effectiveness of the antisense approach *in vivo* has been reported by Simon *et al.* (13). Our results demonstrated the efficacy of combined administration of these ODNs on VSMC proliferation *in vitro* and *in vivo*.

To enhance the efficiency of cellular uptake and the stability of antisense ODNs while minimizing nonspecific toxicity, we developed a viral protein-mediated ODN transfer technique. Phosphorothioate ODNs were complexed with liposomes and the protein coat of the inactivated hemagglutinating virus of Japan (HVJ) (14–17). This method resulted in a more rapid cellular uptake and a 10-fold higher transfection efficiency of ODN or plasmid DNA than lipofection or passive uptake methods (17). Using the HVJ method, we demonstrated that a single intraluminal administration of the combination of antisense cdc2 kinase and PCNA ODNs effectively inhibited neointima formation after angioplasty in the rat carotid model.

MATERIALS AND METHODS

Synthesis of Oligomers and Selection of Sequence Targets. The sequences of ODNs against mouse cdc2 kinase and rat PCNA used in this study were as follows: antisense cdc2 kinase [5′-GTCTTCCATAGTTACTCA-3′, positions −9 to +9 of the mouse sequence (18); there is 98% amino acid sequence homology across species], sense cdc2 kinase (5′-TGAGTAACTATGGAAGAC-3′), antisense PCNA (5′-GATCAGGCGTGCCTCAAA-3′, positions +4 to +22 of rat sequence) (19), and sense PCNA (5′-TTTGAGGCACGCCTGATC-3′). We have confirmed previous observations that these antisense sequences block the synthesis of the targeted gene products in VSMCs and nonvascular cells *in vitro* (refs. 11 and 12 and unpublished observations). We also examined 4-bp mismatched ODNs (mismatched cdc2 kinase, 5′-GTCTGCCGTCGTTAGTCA-3′; mismatched PCNA, 5′-

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Abbreviations: ODN, oligonucleotide; PCNA, proliferating-cell nuclear antigen; HVJ, hemagglutinating virus of Japan; RT, reverse transcription; BrdUrd, bromodeoxyuridine.

GATTAGTCGTACCTAAAA-3'). Synthetic ODNs were washed by 70% ethanol, dried, and dissolved in sterile Tris/EDTA buffer (10 mM Tris/1 mM EDTA). The supernatant was purified over a NAP-10 column (Pharmacia) and quantitated by spectrophotometry (20).

Preparation of HVJ–Liposomes. Phosphatidylserine, phosphatidylcholine, and cholesterol were mixed in a weight ratio of 1:4:8:2 (14–17). The lipid mixture (10 mg) was deposited on the sides of a flask by removal of tetrahydrofuran in a rotary evaporator. Dried lipid was hydrated in 200 μl of balanced salt solution (BSS = 137 mM NaCl/5.4 mM KCl/10 mM Tris·HCl, pH 7.6) containing sense or antisense ODNs (120 nmol). The control group liposomes did not contain ODN (BSS, 200 μl). Liposomes were prepared by shaking and sonication. Purified HVJ (Z strain) was inactivated by UV irradiation (110 ergs per mm^2 per sec) for 3 min just before use. The liposome suspension (0.5 ml, containing 10 mg of lipids) was mixed with HVJ (10,000 hemagglutinating units) in a total volume of 4 ml of BSS. The mixture was incubated at 4°C for 5 min and then for 30 min with gentle shaking at 37°C. Free HVJ was removed from the HVJ–liposomes by sucrose density gradient centrifugation. The top layer of the sucrose gradient was collected for use. The final concentration of antisense ODN is equivalent to 3 μM, as calculated (14–17). We also prepared HVJ–liposomes that contained 15 μM ODN by using this protocol. This preparation method has been optimized to achieve maximal transfection efficiency as reported (14–17). In this study, we also confirmed the optimization in VSMCs.

***In Vitro* Transfection.** Rat aortic VSMCs (passage 4–8) were isolated and cultured (21). They were maintained in Waymouth's medium (GIBCO) with 5% (vol/vol) calf serum, penicillin (100 units/ml), and streptomycin (1000 μg/ml). After confluence, cells were made quiescent by placing them for 48 h prior to the transfection in a defined serum-free (DSF) medium, as reported (21). Cells were washed with BSS, and 500 μl of HVJ–liposomes (3 μM encapsulated ODNs) was then added to the wells. The cells were incubated at 4°C for 5 min and then at 37°C for 30 min. Cells were maintained in fresh DSF or medium with 5% serum, and cell numbers were determined by Coulter counter 3 days after transfection.

***In Vivo* Transfer of ODN.** A no. 2 French Fogarty catheter was used to induce vascular injury in male Sprague–Dawley rats (400–500 g; Charles River Breeding Laboratories) as described (22). These rats were anesthetized, and a cannula was introduced into the left common carotid via the external carotid artery. After vascular injury of the common carotid, the distal injured segment was transiently isolated by temporary ligatures. The HVJ–liposome complex was infused into the segment and incubated for 15 min at room temperature as described above. After a 15-min incubation, the infusion cannula was removed. After the transfection, blood flow to the common carotid was restored by release of the ligatures. No adverse neurological or vascular effects were observed in any animal undergoing this procedure.

Reverse Transcription–PCR (RT–PCR). RNA was extracted from antisense or sense ODN (combination of 15 μM PCNA and 15 μM cdc2 kinase wrapped in liposomes)-treated injured vessels by RNAzol (Tel-Test, Friendswood, TX) at 1 day after transfection. Levels of PCNA, cdc2, and β-actin mRNAs were measured by RT–PCR as described (23). The PCNA 5' primer (nt 150–177 of rat PCNA cDNA) was 5'-ACTCTGCGCTCCGAAGG-3'; the 3' primer (nt 451–468) was 5'-TCTCCAATTAGGCTAAG-3'. The cdc2 5' primer (nt 54–75) was 5'-GGAGAAGGTACCTATGGAGTTG-3'; the 3' primer (nt 307–328) was 5'-GAATCCATGTACTGACCAGGAG-3' (24). The 5' primer complementary to the rat β-actin gene was 5'-TTGTAACCAACTGGGACGATATGG-3'; the 3' primer was 5'-GATCTTGATCTTCATGGTGCTAGG-3' (Clontech). Extreme care was taken to avoid contamination of tissue samples with trace amounts of experimental RNA. Aliquots of RNA derived from intact and injured vessels were amplified simultaneously by PCR (30 cycles) and compared with a negative control (primers without RNA). Amplification products were electrophoresed through 2% agarose gels and stained with ethidium bromide.

Measurement of DNA Synthesis. For bromodeoxyuridine (BrdUrd) staining, BrdUrd was injected into rats after vascular injury (100 mg/kg subcutaneously and 30 mg/kg intraperitoneally at 18 h prior to sacrifice and then 30 mg/kg intraperitoneally at 12 h prior to sacrifice) (25). Rats were sacrificed on day 4 after transfection. The carotid artery was removed after perfusion–fixation (110 mmHg; 1 mmHg = 133 Pa) with 4% (wt/vol) paraformaldehyde and processed for immunohistochemistry by using anti-BrdUrd antibodies (Amersham). The proportion of BrdUrd-positive cells was determined by cell counts under a light microscopy in a blinded fashion. Measurement of DNA was performed at 4 days after transfection using bisbenzimide trihydrochloride (Pierce).

Morphometric Analysis. At 2, 4, and 8 weeks after transfection, rats were sacrificed and vessels were perfusion-fixed with 4% paraformaldehyde. Three individual sections from the middle of transfected segments were analyzed. In addition, three sections from the middle section of the injured untransfected region were also analyzed. Animals were coded so that operation and analysis were performed without knowledge of which treatment individual animals received.

Statistical Analysis. All values are expressed as mean ± SEM. All experiments were repeated at least three times. Analysis of variance with subsequent Duncan's test was used to determine significant differences in multiple comparisons. $P < 0.05$ was considered significant.

RESULTS

Confluent quiescent VSMCs treated with 5% serum for 3 days exhibited a 40% greater cell density compared to vehicle-treated cells. This mitogenic response was unaffected by the administration of either PCNA ODN alone (3 μM) or cdc2 ODN alone (3 μM). However, cotransfection of both antisense PCNA at 3 μM and cdc2 at 3 μM inhibited significantly VSMC proliferation in response to serum stimulation (Table 1). There was no significant effect of antisense ODN on the cell number of confluent quiescent VSMCs maintained under serum-free conditions (data not shown). Moreover, the 4-bp missense control of PCNA and cdc2 antisense ODNs failed to inhibit serum-stimulated VSMC mitogenesis (Table 1). Based on these *in vitro* results, we examined the effect of cotransfection of the combination of

Table 1. Effect of cdc2 kinase and PCNA antisense phosphorothioate ODNs on serum-stimulated increase inVSMC growth

ODN	Treatment	Cells, no. per well
PCNA/cdc2 kinase	Untreated	133,800 ± 10,320
	Sense	168,840 ± 16,410
	Mismatched	149,700 ± 20,070
	Antisense	79,890 ± 7,020*
PCNA	Untreated	200,500 ± 10,770
	Sense	190,740 ± 19,620
	Antisense	206,400 ± 4,890
cdc2 kinase	Untreated	165,300 ± 9,750
	Sense	131,640 ± 6,450
	Antisense	200,700 ± 4,890

ODNs were added to 3 μM. Treatments: untreated, untreated VSMCs; sense, sense-ODN-treated VSMCs; mismatch, 4-bp mismatched-ODN-treated VSMCs; AS, antisense-ODN-treated VSMCs. *, $P < 0.01$ vs. other serum-stimulated treatments.

PCNA and cdc2 antisense ODNs on neointima formation *in vivo*.

PCNA and cdc2 kinase mRNA levels *in vivo* were determined by RT–PCR as shown in Fig. 1. Analysis of RNA extracted from sense-ODN-treated injured vessels at 1 day after transfection showed detectable levels of cdc2 kinase and PCNA mRNAs. Treatment with a combination of antisense cdc2 kinase and PCNA ODNs resulted in the simultaneous inhibition of PCNA and cdc2 mRNA expression to undetectable levels at 1 day after transfection. As a control for the integrity of the extracted RNA and for the PCR protocol, an aliquot of each RNA sample was also amplified with oligonucleotide primers complementary to the rat β-actin gene. There was no significant change in β-actin mRNA levels between sense- and antisense-ODN-treated injured vessels.

We first studied the combined effects of antisense ODNs directed against PCNA and cdc2 (each at 3 μM) on vascular DNA synthesis and content (4 days) and on neointimal size (2 weeks after vascular injury). The combination of PCNA and cdc2 antisense ODNs significantly inhibited BrdUrd incorporation (a marker of DNA synthesis) by 50% in the vessel wall 4 days after injury as compared to the sense-ODN-treated controls (antisense, 10% labeled cells; sense, 22%; $P < 0.01$; Fig. 2*A*). There was no significant difference in the labeling index between untreated injured and sense-ODN-transfected injured vessels. In intact untreated vessels, <1% of the cells in the tunica media stained positively for BrdUrd by immunohistochemistry (data not shown). Moreover, the combination of PCNA and cdc2 antisense ODNs also inhibited the increase in vascular DNA content induced by balloon injury (antisense, 1.0 mg/g of tissue; sense, 1.6 mg/g of tissue; $P < 0.05$; Fig. 2*B*). Neointima formation was assessed by morphometry of perfusion-fixed specimens 14 days after balloon injury in a blinded fashion. The combination of PCNA and cdc2 antisense ODNs at the same concentration that inhibited DNA synthesis by 50% also inhibited neointima formation by 50–60% ($n = 7$) compared to untreated injured vessels ($n = 5$) or sense-ODN-treated vessels ($n = 8$) (Fig. 2*C*). The antisense ODN had no significant effect on medial area.

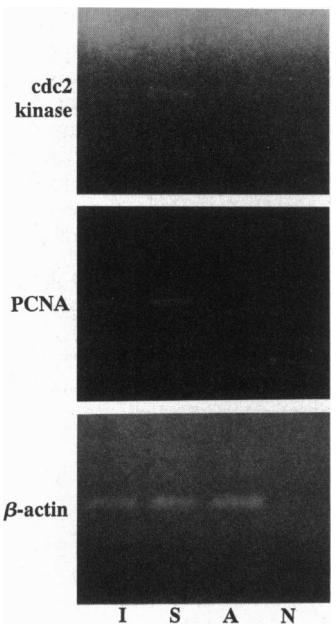

FIG. 1. RT–PCR of RNA from vessels treated with sense and antisense ODNs 1 day after transfection. Lanes: I, intact (uninjured) vessels; S, vessels treated with a PCNA and cdc2 sense ODN combination; A, vessels treated with a PCNA and cdc2 antisense ODN combination; N, negative control.

We postulated that the degree of neointima inhibition may be dose-dependent. Accordingly, we examined the effect of a higher dose (15 μM) of ODN. Indeed, a single administration of the 15 μM PCNA and 15 μM cdc2 antisense ODN combination completely abolished neointima formation ($n = 8$), whereas the sense control ODNs (each at 15 μM) had no effect ($n = 8$) (Fig. 3 *A* and *B*). In accordance with our *in vitro* findings, intraluminal transfection of PCNA antisense ODNs alone at 15 μM failed to inhibit neointima formation (neointimal area, 0.237 ± 0.016 mm²; sense control area, 0.214 ± 0.022 mm²; $P > 0.05$). The selectivity of the antisense ODN effect was further confirmed by the observation that the inhibition of the neointima formation was limited to the area of intraluminal transfection along the injured carotid artery. In contrast, the adjacent injured carotid segments outside the area of antisense transfection exhibited neointimal lesions similar to the sense-ODN-treated control injured carotid artery [neointimal area in untransfected region, 0.287 ± 0.059 mm² ($n = 8$); antisense-ODN-transfected area, 0.019 ± 0.014 mm² ($n = 8$); $P < 0.01$].

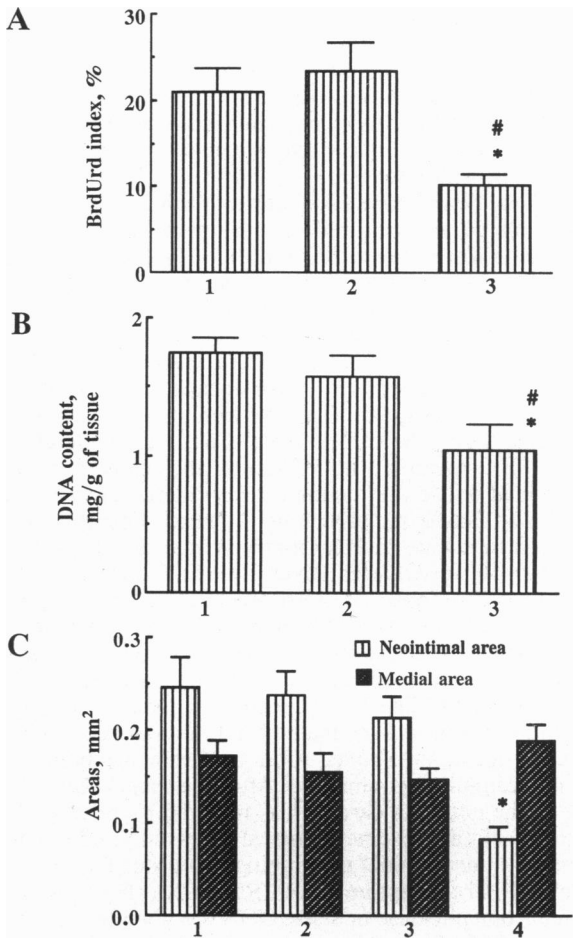

FIG. 2. Effect of combined 3 μM PCNA and 3 μM cdc2 kinase antisense ODNs on BrdUrd index and DNA content (4 days) and neointima area (14 days). (*A*) Effect of cdc2 kinase and PCNA antisense ODNs on BrdUrd index. (*B*) Effect of cdc2 kinase and PCNA antisense ODNs on DNA content. (*A* and *B*) Bars: 1, without HVJ–ODN treatment; 2, sense HVJ–ODN treatment (3 μM each); 3, antisense HVJ–ODN treatment (3 μM each). *, $P < 0.01$ vs. sense treatments; #, $P < 0.01$ vs. control treatment. (*C*) Effect of cdc2 kinase and PCNA antisense and sense ODNs on intimal and medial areas. Bars: 1, injured vessels without any treatment; 2, injured vessels treated with HVJ complex without ODN; 3, injured vessels treated with sense HVJ–ODN; 4, injured vessels treated with antisense HVJ-ODN. *, $P < 0.01$ vs. sense treatment.

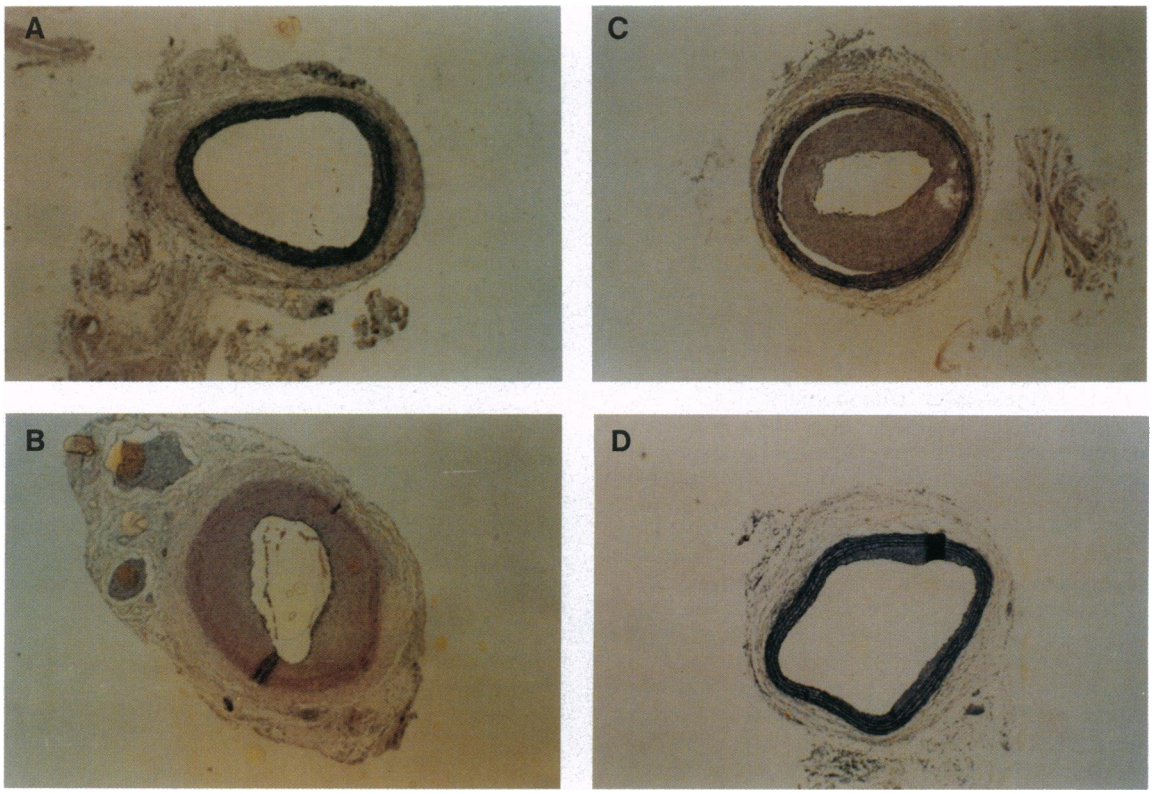

FIG. 3. Effect of the combination of 15 μM cdc2 kinase and 15 μM PCNA antisense ODNs on neointima formation. Representative cross-sections are shown. (*A*) Uninjured rat carotid artery. (*B*) Injured carotid without HVJ–ODN treatment. (*C*) Injured carotid treated with sense HVJ–ODN (15 μM). (*D*) Injured carotid treated with antisense HVJ–ODN (15 μM). (×25.)

Given that neointima formation results from an initial acute phase of medial smooth muscle cell replication, we hypothesize that blockade of PCNA and cdc2 kinase during this critical early phase would have a sustained inhibitory effect on the development of the neointima lesion. Indeed, the single administration of the 15 μM PCNA and 15 μM cdc2 antisense ODN combination significantly inhibited the extent of neointima formation for a period of 8 weeks after transfection (Fig. 4). Although there was evidence for a neointimal lesion at 4 weeks after an antisense treatment, the lesion was significantly inhibited compared to a sense treatment ($P < 0.01$). Furthermore, no further progression of the lesion was observed at 8 weeks after a single antisense administration (Fig. 5).

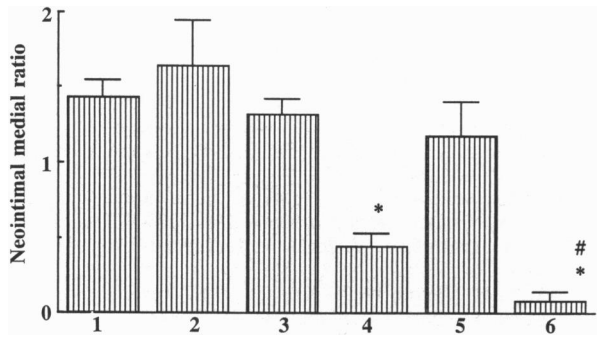

FIG. 4. Dose effect of cdc2 kinase and PCNA antisense and sense ODNs on intimal/medial area ratio. Medial, lumen, and intimal areas were measured on a digitizing tablet (model 2200, Southern Micro Instruments, Atlanta). Bars: 1, injured vessels without any treatment; 2, injured vessels treated with HVJ–liposomes without ODN; 3, injured vessels treated with 3 μM sense HVJ–ODN; 4, injured vessels treated with 3 μM antisense HVJ–ODN; 5, injured vessels treated with 15 μM sense HVJ–ODN; 6, injured vessels treated with 15 μM antisense HVJ–ODN. *, $P < 0.01$ vs. sense treatment; #, $P < 0.01$ vs. 3 μM antisense ODN treatment.

DISCUSSION

The problem of restenosis after angioplasty is an important challenge facing cardiovascular medicine (1, 2). Given that neointima formation after angioplasty involves a complex interaction between multiple growth factors that promote VSMC proliferation and migration (6), it appears unlikely that selective inhibition of a particular growth factor will completely prevent lesion formation. Given that growth-factor-induced cell proliferation involves the sequential activation of intracellular proteins that promote cell-cycle progression (9, 10), we hypothesized that restenosis could be prevented by the blockade of genes regulating the final common pathway of cell-cycle progression. This study provides evidence that complete prevention of neointima formation can be

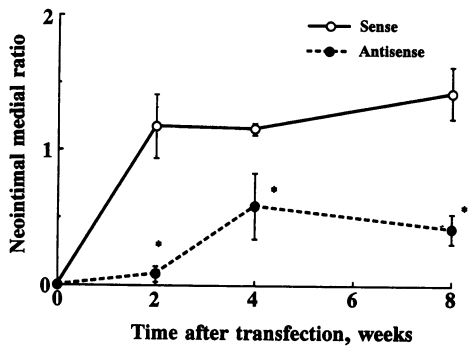

FIG. 5. Long-term efficiency of cdc2 kinase and PCNA antisense and sense ODNs on intimal/medial area ratio. **, $P < 0.01$ vs. sense treatment.

achieved by a single administration of antisense ODN directed against cell-cycle regulatory genes via an intraluminal delivery system.

Current approaches that seek to utilize antisense ODN as therapeutic agents *in vivo* are limited by the inefficiency of cellular uptake, the short half-life, and nonspecific toxicity at high doses (26, 27). In the present study, we introduce a molecular delivery system that employs the viral coat of HVJ in a liposome–ODN complex (14–17). This HVJ–ODN method markedly enhances ODN therapeutic efficiency by increased uptake, nuclear localization, and intracellular stability. Utilizing this method, we were able to document that a single intraluminal administration of the combination of PCNA and cdc2 kinase antisense ODNs inhibited neointima formation up to 8 weeks after balloon injury.

The specificity of the inhibitory effect of the antisense ODN on neointima formation was suggested by several lines of evidence: (*i*) This antisense ODN combination inhibited VSMC proliferation *in vitro*, whereas the sense and missense control ODNs did not. (*ii*) Antisense ODN directed against either PCNA or cdc2 failed to inhibit VSMC proliferation *in vitro* when administered alone at similar concentrations. (*iii*) The effects of the antisense PCNA and cdc2 ODNs on VSMC proliferation *in vitro* were synergistic not additive. (*iv*) We have confirmed previous reports that these antisense ODNs selectively inhibit the expression of the targeted cell-cycle regulatory proteins by Western blot *in vitro*. (*v*) The *in vivo* experiments using RT–PCR documented marked inhibition of mRNA expression of both PCNA and cdc2 kinase genes but not β-actin gene expression in antisense-ODN-treated injured vessels. (*vi*) This antisense ODN combination specifically reduced two quantitative markers of cell-cycle progression *in vivo* (BrdUrd labeling and vascular DNA content). (*vii*) The inhibitory effect of the antisense ODN combination on neointima formation appeared to be dose dependent. (*viii*) The prevention of neointima formation was limited to the area transfected with the antisense ODN. (*ix*) The administration of PCNA antisense ODN alone served as a negative control that failed to inhibit neointima formation.

Overall, this study provides evidence that neointima lesion formation after balloon angioplasty can be prevented by a single intraluminal administration of antisense oligonucleotides directed against cell-cycle regulatory genes. This therapeutic strategy fulfills the criteria needed for success—i.e., a proper drug target, an efficient drug delivery method, and an intraluminal approach. In defining the strategy to prevent restenosis in humans, the method of drug delivery is critically important. Intraluminal delivery is desirable since it can be carried out concomitantly with the transluminal angioplasty—a practical issue of clinical relevance. The blockade of cell-cycle progression is particularly attractive since it maintains the cells in a quiescent differentiated phenotype without inducing cell injury. During the course of this investigation, it was reported that c-*myb* antisense ODNs inhibited neointima formation when administered in concentrations >150 μM via pluronic gels applied to the adventitia of the rat carotid artery (13). Unfortunately, this periadventitial polymer delivery system is not a feasible methodology for the prevention of restenosis after percutaneous transluminal angioplasty in humans. The HVJ–ODN intraluminal method substantially increases the efficiency of uptake and the stability of ODN *in vivo* (unpublished observations) thereby avoiding the potential toxicity of high concentrations of ODN (i.e., >150 μM). We anticipate that the modification of antisense ODN pharmacokinetics by use of the HVJ–liposome complex will facilitate the potential clinical utility of these agents by (*i*) allowing for an abbreviated intraluminal incubation time to preserve organ perfusion, (*ii*) prolonging the duration of biological action, (*iii*) enhancing specific activity, and (*iv*) reducing the cost of ODN therapy. Given that the virus is inactivated by ultraviolet irradiation, there is little potential for biological hazard compared to retroviral *in vivo* gene transfer approaches (28). Nevertheless, it is anticipated that isolation of the specific protein(s) that facilitates the cellular uptake of ODN will further enhance the clinical applicability of this method. The use of this method in humans will also require the development of a catheter that will permit instillation/incubation of the HVJ complex and simultaneously maintain tissue perfusion (e.g., a double-balloon perfusion catheter) (29). We speculate that continued development of these methodologies will facilitate the use of antisense ODN technology to further characterize the biological role of gene products activated in response to vascular injury and to provide additional therapeutic agents for use in humans.

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1. McBride, W., Lange, R. A. & Hillis, D. A. (1988) *N. Engl. J. Med.* **318,** 1734–1737.
2. Califf, R. M., Fortin, D. F., Frid, D. J., Harlan, W. R., Ohman, E. M., Bengtson, J. R., Nelson, C. L., Tcheng, J. E., Mark, D. B. & Stack, R. S. (1991) *J. Am. Coll. Cardiol.* **17,** 2B–13B.
3. Pukac, L. A. (1991) *Am. J. Pathol.* **139,** 1501–1509.
4. Lindner, V. & Reidy, M. A. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 3739–3743.
5. Powell, J. S., Clozel, J. P., Muller, R. K. M., Kuhn, H., Hefti, F., Husang, M. & Baumgartner, H. R. (1989) *Science* **245,** 186–188.
6. Libby, P., Schwartz, D., Brogi, E., Tanaka, H. & Clinton, S. K. (1992) *Circulation* **86,** III-47–III-52.
7. Ferns, G. A. A., Raines, E. W., Sprugel, K. H., Motani, A. S., Reidy, M. A. & Ross, R. (1991) *Science* **253,** 1129–1132.
8. Majesky, M. W., Lindner, V., Twardzik, D. R., Schwartz, S. M. & Reidy, M. A. (1991) *J. Clin. Invest.* **88,** 904–910.
9. Wagner, S. & Green, M. R. (1991) *Nature (London)* **352,** 189–190.
10. Hunter, T. & Karin, M. (1992) *Cell* **70,** 375–387.
11. Furukawa, Y., Piwnica-Worms, H., Ernst, T. J., Kanakura, Y. & Griffin, J. D. (1990) *Science* **250,** 805–808.
12. Jaskulski, D., DeRiel, J. K., Mercer, W. E., Calabretta, B. & Baserga, R. (1988) *Science* **240,** 1544–1546.
13. Simons, M., Edelman, E. R., DeKeyser, J. L., Langer, R. & Rosenberg, R. D. (1992) *Nature (London)* **359,** 67–70.
14. Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T. & Okada, Y. (1991) *J. Biol. Chem.* **266,** 3361–3364.
15. Kaneda, Y., Iwai, K. & Uchida, T. (1989) *Science* **243,** 375–378.
16. Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, H., Kaneda, Y. & Ogihara, T. (1992) *Biochem. Biophys. Res. Commun.* **186,** 129–134.
17. Morishita, R., Gibbons, G. H., Kaneda, Y., Ogihara, T. & Dzau, V. J. (1993) *J. Clin. Invest.* **91,** 2580–2585.
18. Lee, M. G. & Nurse, P. (1987) *Nature (London)* **327,** 31–35.
19. Matsumoto, K., Moriuchi, T., Koji, T. & Nakane, P. K. (1987) *EMBO J.* **6,** 637–642.
20. Itoh, H., Mukoyama, M., Pratt, R. E. & Dzau, V. J. (1992) *Biochem. Biophys. Res. Commun.* **188,** 1205–1213.
21. Itoh, H., Pratt, R. E. & Dzau, V. J. (1990) *J. Clin. Invest.* **86,** 1690–1697.
22. Rakugi, H., Jacob, H. J., Krieger, J. E., Ingelfinger, J. R. & Pratt, R. E. (1993) *Circulation* **87,** 283–290.
23. Venturelli, D., Travali, S. & Calabretta, B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 5963–5967.
24. Sala, A. & Calabretta, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 10415–10419.
25. Hanke, H., Strohschneider, T., Oberhoff, M., Betz, E. & Karsch, K. R. (1990) *Circul. Res.* **67,** 651–659.
26. Marcus-Sekura, C. J. (1988) *Anal. Biochem.* **172,** 289–295.
27. Stein, C. A. & Cohen, J. S. (1988) *Cancer Res.* **48,** 2659–2668.
28. Thompson, L. (1992) *Science* **257,** 1854.
29. Nabel, E. G., Plautz, G., Boyce, F. M., Stanley, J. C. & Nabel, G. J. (1989) *Science* **244,** 1342–1344.