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SUPPRESSION OF NEOINTIMAL SMOOTH MUSCLE CELL ACCUMULATION IN VIVO BY ANTISENSE CDC2 AND CDK2 OLIGONUCLEOTIDES IN RAT CAROTID ARTERY

Jun-ichi Abe[1], Wei Zhou[1], Jun-ichi Taguchi[2], Noriko Takuwa[3], Keisaburo Miki[4], Hiroshi Okazaki[1], Kiyoshi Kurokawa[2], Mamoru Kumada[3], and Yoh Takuwa[1†]

Departments of [1]Cardiovascular Biology, [2]Internal Medicine,and [3]Physiology,Faculty of Medicine, University of Tokyo, Tokyo 113, Japan

[4]Terumo Corporation, Kanagawa 259-01, Japan

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SUMMARY : Deendothelializing balloon injury of rat carotid artery results in progressive intimal smooth muscle cell accumulation and luminal stenosis over 14 days after injury. We have found transient rises (approximately 3-fold maximal increases over the uninjured control value) of the kinase activities of both cdc2 and cdk2, key molecules for cell cycle progression, in the injured carotid artery along with the development of intimal proliferation. The topical application of the antisense, but not the sense, cdc2 and cdk2 phosphorothioate oligodeoxynucleotides dissolved in F127 pluronic gel around the freshly injured artery resulted in reductions of the intimal smooth muscle cell accumulation by 47 % and 55 %, respectively, as estimated by an intimal to medial cross-sectional area ratio, with concomitant decreases in cdc2 and cdk2 kinase activities. These results indicate that both cdc2 and cdk2 kinases are involved in intimal smooth muscle cell accumulation after balloon angioplasty and suggest a potential usefulness of the antisense cdc2 and cdk2 oligonucleotide therapy for arterial stenosis.

Balloon angioplasty is a well established therapeutic intervention for ischemic coronary heart disease, in which a inflated balloon at the tip of a catheter mechanically extends the narrowed lumen. However, restenosis occurs in as high as 30-50 % of patients because of formation of a thick intimal layer (neointima) due to medial smooth muscle cell migration and proliferation (1, 2). A variety of bioactive substances have been implicated in the development

† All correspondence should be addressed to Yoh Takuwa, M. D.,at Department of Cardiovascular Biology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113, Japan. Fax: 81-3-5800-6845.

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of intimal smooth muscle cell proliferation after angioplasty (1-3). They include platelet degranulation products (platelet derived growth factor, epidermal growth factor and serotonin), locally produced growth factors (basic fibroblast growth factor, thrombin, insulin-like growth factor-I and angiotensin II) and circulating humoral factors (vasopressin and catecholamines). However, precise molecular mechanisms by which neointimal formation is brought about after balloon angioplasty are not yet fully understood. To date, these is no effective therapy established for preventing arterial restenosis after angioplasty (1-3).

Recent studies have revealed that growth stimulation of higher eukaryotic cells induces the activation of cyclin-dependent kinases cdc2 and cdk2, which play critical roles in cell cycle progression (4, 5). Thus, stimulation with growth factors causes gradual increases in mRNA and protein levels as well as changes in phosphorylation states of cdk2 and cdc2 starting from late G1 or G1/S boundary, which is immediately followed by increases in the activities of these cyclin-dependent kinases (6-9). Accumulating evidence has demonstrated that in mammalian cells cdc2 kinase is essential for entry into mitosis (4, 10, 11), whereas cdk2 is implicated in G1/S transition (9, 11-13).

In an effort to develop an effective means to inhibit restenosis after angioplasty, we examined the usefulness of locally applied oligodeoxynucleotides (ODNs) antisense to cdk2 and cdc2 for suppressing smooth muscle cell accumulation in rat balloon-injured carotid artery. The results demonstrate that the antisense cdc2 and cdk2 ODNs efficiently prevent intimal proliferation, indicating the involvement of both cdc2 and cdk2 in vascular smooth muscle cell proliferation *in vivo*.

MATERIALS AND METHODS

Preparation of Balloon Injury Model Male 14-week-old normotensive Wister rats (300-500 g) were anesthetized with Nembutal (4 mg per 100 g) and ethyl ether. The left common carotid artery was cleared of surrounding connective tissue and cannulated with a 2 French Fogarty catheter (Baxter Healthcare, Santa Ana, CA). The catheter was introduced into the left internal carotid artery. The balloon was air-inflated to distend the left common carotid artery and passed three times up and down to produce deendothelializing injury. The right common carotid artery was dissected in exactly the same way, but not subjected to angioplasty, and used as a normal control. Eighteen-base antisense phosphorothioate ODNs complementary to codons -3 to +3 of either cdc2 (5′-ATC TTC CAT AGT TAG TCA-3′), or cdk2 (5′-GTT CTC CAT GAA GCG CCA-3′) or corresponding sense ODNs synthesized by 380B DNA synthesizer (Applied Biosystems) were dissolved in 20 % (w/v) F127 pluronic gel solution

(BASF Wyandotte Corporation, Wyandotte, MI) at a concentration of 1 mg/ml. Immediately after balloon injury, 200 μl of the gel solution containing ODN was applied to surround the exposed region of the common carotid artery (13) and the wounds were then closed. During the whole procedure, care was taken to prevent infection. A part of animals received an i. v. bolus of Evans blue in lactated Ringer's solution (60 mg/kg) 1 hour prior to fixation to histologically confirm removal of the endothelium. At indicated time, rats were anesthetized as described above and sacrificed for morphometric analysis of neointimal formation and measurements of kinase activities. Morphometric analysis was performed as follows: the intimal and medial areas in 6 cross sections (3 μm thickness) every 2 mm in length from each common carotid artery were measured and the mean values of both intimal and medial areas were calculated. Then the intimal to medial (I/M) cross-sectional area ratio was determined (14).

Measurement of cdc2 and cdk2 Kinase Activity in the Carotid Artery At indicated time, both injured (left) and uninjured (right) common carotid arteries were exised, and dissected in 12 mm length in ice-cold Dulbecco's phosphate buffered saline. Each artery was then immediately homogenized at 4 °C in 300 μl of a buffer containing 50 mM 2-amino-2-hydroxymethyl-1, 3-propanediol (Tris)-HCl (pH 8.0), 120 mM NaCl, 0.5 % Nonidet-P 40, 100 mM NaF, 1 mM Na_3VO_4, 0.1 % sodium dodecylsulfate (SDS), 2 mM [ethylenebis (oxyethylenenitrilo)] tetraacetic acid (EGTA), 80 μg/ml each of leupeptin and aprotinin, and 0.6 mM phenylmethylsulfonyl fluoride. The supernatant after centrifugation at 10,000 x g for 5 min was subjected to immunoprecipitaion using 25 μl of a rabbit polyclonal antibody (IgG fraction) raised against the carboxyl-terminal sequence of either cdc2 or cdk2 as described in detail previously (15, 16). Histone H1 kinase activity of the anti-cdc2 or -cdk2 immunoprecipitate was measured at 25 °C for 10 min in the reaction mixture (40 μl) containing 0.4 mg/ml of histone H1 (Boehringer-Mannheim), 60 μM ATP, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM Tris-HCl (pH 7.4) and 2 μCi of [γ-^{32}P]ATP. The reaction was terminated by adding 4 x electrophoresis sample buffer and boiling for 5 min. Samples were analyzed on 12.5 % SDS-polyacrylamide gel electrophoresis (PAGE), followed by autoradiography. The radioactivity in the spot corresponding to histone H1 was quantitated by Fuji BAS 2000 Bio-Image Analyzer (15, 16).

Statistics All data are presented as the mean ± standard error (S. E.). The statistical significance of differences in results was determined using nonpaired *t* test for groups of unequal sample sizes. Multiple comparisons were then made using Duncan's multiple-range test.

RESULTS

Shown in Fig.1 (upper) is the time course of intimal smooth muscle cell accumulation in the rat left common carotid artery in response to balloon arterial injury. As reported previously (20), as the ratio of intimal to medial cross-sectional areas (I/M ratio) of the carotid artery increased sharply after a lag period of 3 days and reached a maximal level of approximately 1.0 at day 14. Be contrast, the uninjured right carotid artery did not show any thickening of the intima throughout the observation period of four weeks. We studied whether there is any

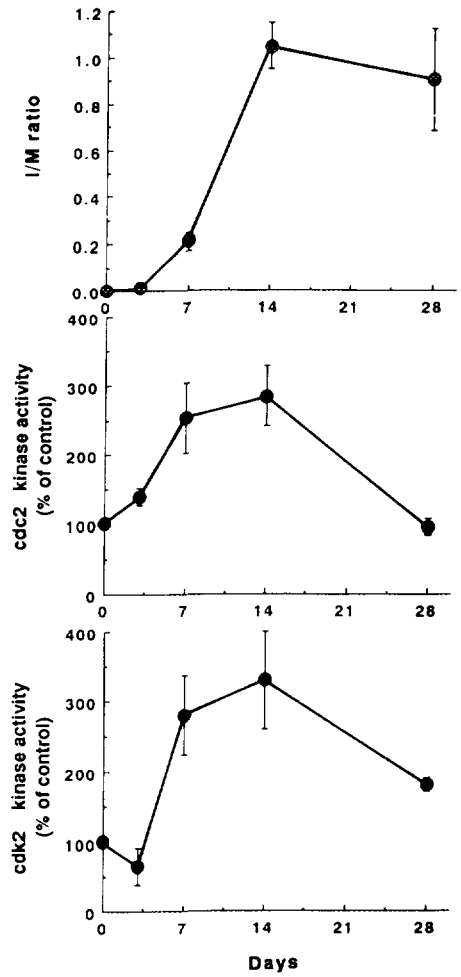


Figure 1. Time courses of intimal thickening, cdc2 kinase activity and cdk2 kinase activity in rat carotid artery following balloon angioplasty.

The injured and uninjured control arteries were removed at indicated time points after balloon angioplasty and processed for the measurement of intimal and medial cross-sectional areas or homogenized for the measurement of cdc2 and cdk2 kinase activity. Intimal thickening is expressed as intimal to medial (I/M) cross-sectional area ratio(upper). The kinase activity of cdc2 (middle) and cdk2 (lower) is expressed as % of the respective control activity of uninjured artery. Each value represents the mean ± S. E. of 6 to 11 determinations.

change in cdc2 and cdk2 kinase activities in injured carotid artery during the course of development of intimal thickening. When the kinase activity in the anti-cdc2 immunoprecipitates from homogenates of both injured and uninjured carotid arteries was measured with histone H1 as a substrate in vitro, the injured carotid artery from day 3 rats displayed a 1.4-fold higher kinase activity as compared with the uninjured artery (Fig.1 middle). The cdc2 kinase activity in the injured artery continued to increase to reach a maximal

value 2.5-fold higher than that in the uninjured artery at day 7, and stayed at this level till day 14. At day 28, when intimal smooth muscle accumulation had already ceased, the cdc2 kinase activity in the injured artery fell back to a level which was not different from the control value in the uninjured artery. The cdk2 kinase activity also showed a transient rise with a similar time course after angioplasty: it reached a maximal level of 3.3-fold over the control value at day 14 and then declined toward the baseline control value (Fig.1 lower). These results suggest that both cdc2 and cdk2 are involved in intimal smooth muscle cell accumulation.

In order to investigate more directly the roles of the cyclin-dependent kinases in the intimal smooth muscle proliferation and to test potential usefulness of inhibition of the cyclin-dependent kinases as a therapy for preventing arterial stenosis, we examined the effects of locally administered antisense cdc2 and cdk2 ODNs on intimal thickening in response to arterial injury. Two hundred μg of either antisense or corresponding sense phosphorothioate ODNs dissolved in F127 pluronic solutions was applied around the left common carotid artery immediately after angioplasty . The effects of the ODNs were examined in rats at day 14 that showed a maximal extent of neointimal formation (Fig. 1). As shown in Fig. 2 (left), the cdc2 kinase activity of the injured artery treated with the antisense cdc2 ODN was reduced by 50 % (the mean of 11 determinations) as compared with that of the untreated injured artery. By contrast, the injured artery treated with the sense cdc2 ODN or pluronic gel alone showed no

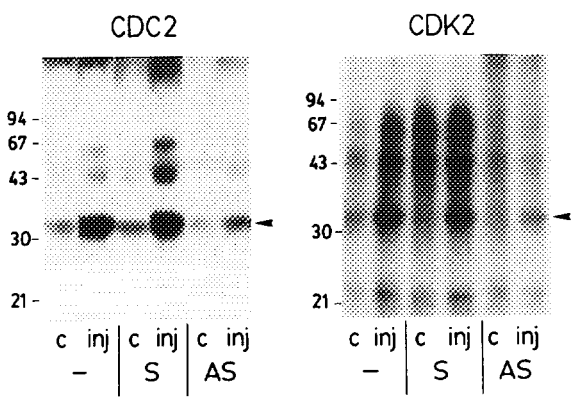


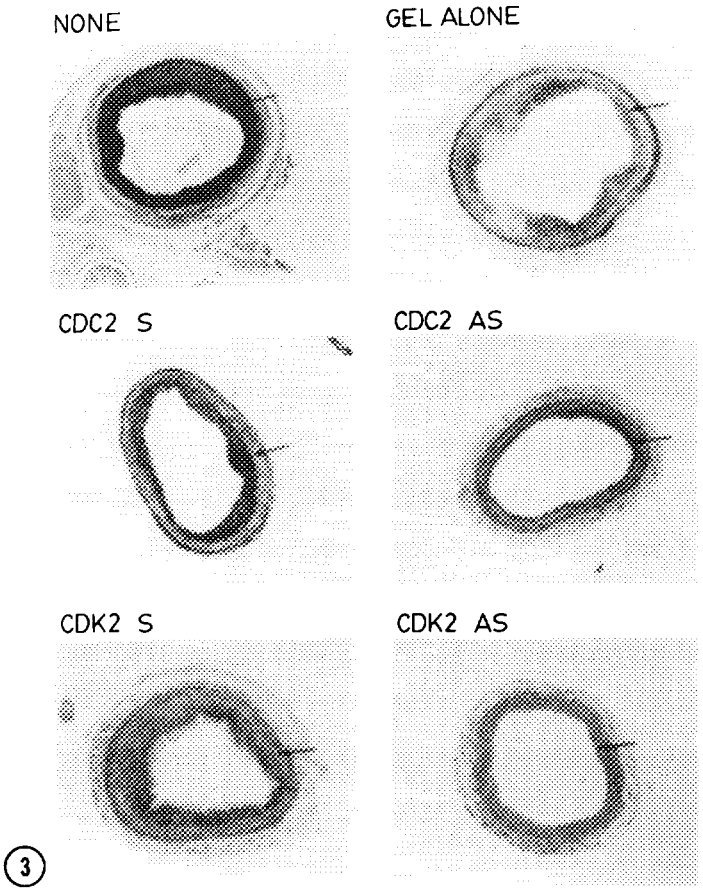
Figure 2. Effects of antisense and sense cdc2 and cdk2 oligodeoxynucleotides on the kinase activity of cdc2 and cdk2 in balloon-injured carotid arteries. The injured artery treated with either F127 pluronic gel alone or 200 μg of sense or antisense oligodeoxynucleotide dissolved in F127 pluronic gel, and uninjured control arteries were removed 14 days after balloon angioplasty and homogenized for the measurement of kinase activities of cdc2 and cdk2. Representative autoradiograms of the kinase assay samples separated on SDS-PAGE are shown. Arrowheads indicate the position of histone H1. Numbers on the left indicate molecular masses in kD.

significant reduction in the cdc2 kinase activity. Likewise, local administration of the antisense cdk2 ODN, but not the sense ODN or gel alone, induced a reduction by 45 % in the cdk2 kinase activity of the injured artery on day 14 (the mean of 13 determinations) (Fig.2 right). Thus, both of the antisense cdc2 and cdk2 ODNs given locally were effective in inhibiting the activation of the respective cyclin-dependent kinase in the injured carotid artery. Under the same experimental condition the administration of either antisense cdc2 or antisense cdk2 ODNs substantially reduced neointimal formation (Figs.3and 4). The intimal cross-sectional area as well as the I/M ratio were significantly reduced in injured arteries treated with either of the antisense ODNs, but not with sense ODNs or gel alone (Fig. 4). The extent of inhibition of the neointimal formation with the antisense cdc2 and cdk2 ODNs was 47 % and 55 % , respectively (Fig. 3 and 4) and was comparable to the extent of inhibition of the respective kinase activity with the antisense ODNs (Fig. 2). The antisense ODNs did not alter the medial surface area, suggesting that the antisense cdc2 or cdk2 ODNs did not affect the viability of medial smooth muscle cells. Morphological examinations revealed that either of the antisense ODNs did not seem to change histological appearance of neointimal cells or extracellular matrix.

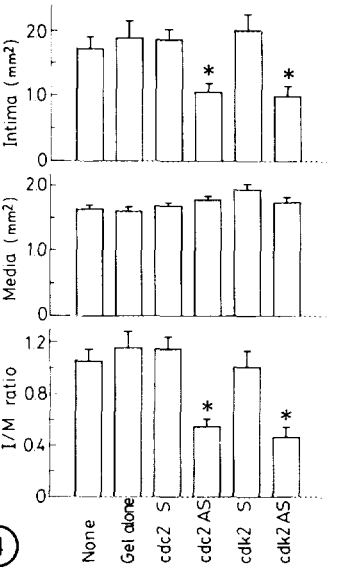
DISCUSSION

The present study demonstrates that topical application of the antisense cdc2 and cdk2 ODNs, but not the corresponding sense ODNs, reduces intimal smooth muscle proliferation after balloon angioplasty in the rat carotid artery model (Figs.3and4). The suppression of neointimal formation by the antisense ODNs is accompanied by concomitant inhibition of the activation of cdc2 and cdk2 kinases (Fig. 2). These results indicate that the antisense cdc2 and cdk2 ODNs produce sequence-specific inhibition of the activity of the respective kinases and that both cdc2 and cdk2 are involved in intimal smooth muscle cell accumulation *in vivo* after balloon angioplasty. In addition, the present results confirm effectiveness of direct local application of the antisense ODN dissolved in pluronic gel to the injured vessel in combination with the use of a phosphorothioate ODN which is more stable to nuclease-mediated degradation than a phosphodiester ODN, as first reported by Simons et al. (19).

Cdc2 and cdk2 have been shown to function as key regulatory kinases in the control of cell cycle progression in the mammalian cell growth model such as cultured fibroblasts (6, 8-



(3)



(4)

Figure 3. Representative cross sections of balloon-injured carotid arteries at day 14 untreated or treated with either F127 pluronic gel alone, or cdc2 or cdk2 oligodeoxynucleotides. The cross-sections of carotid arteries untreated or treated with F127 pluronic gel alone, or sense (S) or antisense (AS) ODN were subjected to Elastica Van Gieson staining. The magnification was x30. Arrows indicate internal elastica lamina (1EL).

Figure 4. Effects of antisense and sense cdc2 and cdk2 oligodeoxynucleotides on the neointimal formation in balloon-injured carotid artery. The injured artery untreated or treated with either F127 plurogenic gel alone, or 200 μg of sense (S) or antisense (AS) oligodeoxynucleotides dissolved in F127 pluronic gel were removed 14 days after balloon angioplasty, and the intimal and medial cross sectional areas and the ratio of intimal to medial cross-sectional areas (I/M ratio) were determined . The data are expressed as the means ± S. E. of 10-19 determinations. The symbol (*) denotes statistically significant difference ($p < 0.001$) as compared with the values of "none", "gel alone", and respective "sense ODN" groups.

10) and T lymphocytes (13). The present results demonstrate that both cdc2 and cdk2 indeed play critial roles in *in vivo* proliferation of vascular smooth muscle cells, suggesting the apparent universality of the intracellular machinery governing eukaryotic cell growth. Recent studies have revealed that the activities of both cdc2 and cdk2 kinases are regulated at multiple

steps by growth factors. Thus, the expression of cdc2 is induced at G/S boundary in growth factor-stimulated quiescent cells (6, 8), whereas the protein level of cdk2 is relatively more constant throughout the cell cycle (8, 9). Cdc2 and cdk2 are both subjected to cell-cycle dependent phosphorylation and dephosphorylation at threnoine and tyrosine residues which affect the kinase activities (17, 18). The expression of the regulatory subunits, cyclins (cyclins A and B for cdc2 and cyclins A, D and E for cdk2) is also stimulated by growth factors (19-22). In the present study the administration of the antisense cdc2 or cdk2 ODNs caused a partial inhibition of the kinase activities by approximately 50 % (Fig. 2). This may have been brought about by incomplete inhibition of cdc2 and cdk2 expression due to insufficient penetration of the antisense ODNs into the arterial wall and/or their limited life span within cells. The combined application of antisense cyclin ODNs together with the antisense cdc2 and cdk2 ODNs might produce more satisfactory suppression of both the kinase activities and the neointimal formation.

Multiple growth factors of different classes, including platelet-derived growth factor and fibroblast growth factor that activate the receptor tyrosine kinases, and thrombin and angiotensin II that activate G protein-coupled receptors, have been implicated in the development of intimal smooth muscle proliferation following balloon angioplasty (1-3). Recent progress in the understanding of the signal transduction mechanisms utilized by these growth factors indicates that the multiple signalling pathways activated by different classes of mitogens finally converge into the common net work pathways including the expression of protooncogenes and the activation of mitogen-activated protein (MAP) kinase and several cyclin-dependent kinases including cdc2 and cdk2 (16, 23, 24). Therefore, it may be advantageous to target a molecule located in the common downstream pathway rather than to inhibit the action of a single growth factor by administering a receptor antagonist or an antibody. In this context, it is of note that Simons et al. (13) obtained potent suppression of neointimal formation (80 % inhibition as estimated by intimal to medial cross-sectional area ratio) following balloon angioplasty with local delivery of ODN antisense to a protooncogene, c-myb.

In conclusion, the present study demonstrates that topically given antisense ODNs which target cdc2 and cdk2, key molecules in the mitogenic machinery, suppress intimal smooth muscle proliferation in response to balloon injury in the rat carotid artery model. The local

administration of these antisense ODNs may represent a potentially useful therapeutic strategy for preventing arterial restenosis after balloon angioplasty.

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