

Inhibition of rac1 Reduces PDGF-induced Reactive Oxygen Species and Proliferation in Vascular Smooth Muscle Cells

In vascular smooth muscle cells, reactive oxygen species (ROS) were known to mediate platelet-derived growth factor (PDGF)-induced cell proliferation and NADH/NADPH oxidase is the major source of ROS. NADH/NADPH oxidase is controlled by rac1 in non-phagocytic cells. In this study, we examined whether the inhibition of rac1 by adenoviral-mediated gene transfer of a dominant negative rac1 gene product (Ad.N17rac1) could reduce the proliferation of rat aortic vascular smooth muscle cells (RASMC) stimulated by PDGF via decreasing intracellular ROS. RASMC were stimulated by PDGF (80 ng/mL) with or without N-acetylcysteine 1 mM or infected with 100 multiplicity of infection of Ad.N17rac1. Intracellular ROS levels were measured at 12 hr using carboxyl-2',7'-dichlorodihydrofluorescein diacetate confocal microscopy. At 72 hr, cellular proliferation was evaluated by cell number counting and XTT assay. Compared with control, ROS levels were increased by 2-folds by PDGF. NAC and Ad.N17rac1 inhibited PDGF-induced increase of ROS by 77% and 65%, respectively. Cell number was increased by PDGF by 1.6-folds compared with control. NAC and Ad.N17rac1 inhibited PDGF-induced cellular growth by 45% and 87%, respectively. XTT assay also showed similar results. We concluded that inhibition of rac1 in RASMCs could reduce intracellular ROS levels and cellular proliferation induced by PDGF.

Key Words : *Reactive Oxygen Species; Rac1 GTP-Binding Proteins; Gene Transfer Techniques*

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Received : 3 May 2001

Accepted : 11 July 2001

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*This work was supported by Korea Research
Foundation Grant (KRF-99-015-FP0064).

INTRODUCTION

Proliferation of vascular smooth muscle cell plays important roles in atherosclerosis and restenosis after coronary angioplasty (1, 2). However, the exact cellular mechanisms responsible for smooth muscle cell proliferation are unknown.

For many years, reactive oxygen species (ROS), such as superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2), have been thought of as toxic byproducts of aerobic living. But recently, a variety of evidence suggests that ROS are involved in the signal transduction in mammalian cells (3). In vascular smooth muscle cells, ROS were shown to mediate platelet-derived growth factor (PDGF)- or angiotensin II- induced cell proliferation (4-6). The production of ROS in blood vessel is enhanced in experimental models of hypercholesterolemia, hypertension, diabetes, and balloon injury to the coronary arteries (7-10). These evidences suggest that ROS could mediate the common mechanism of diseases characterized by vascular smooth muscle cell proliferation.

The NADH/NADPH oxidase is the major source of ROS induced by various growth factors in vascular smooth muscle cell (11). In non-phagocytic cells, small GTP-binding protein rac1 controls the level of ROS by regulating the NADH/NADPH oxidase complex (12-16). Recently, treatment with

antioxidants such as pyrrolidinedithiocarbamate (PDTC) or N-acetylcysteine (NAC) and overexpression of catalase have been shown to inhibit proliferation of vascular smooth muscle cell by decreasing the level of intracellular ROS (17, 18). These strategies decrease the preformed ROS.

In this study, we examined whether the inhibition of rac1 could inhibit the proliferation of rat aortic vascular smooth muscle cell (RASMC) stimulated by PDGF via decreasing the production of intracellular ROS. To inhibit NADH/NADPH oxidase effectively, we used adenoviral-mediated gene transfer of a dominant negative rac1 gene product (N17rac1) as shown in the previous study (12).

MATERIALS AND METHODS

Cell Cultures

Primary culture of RASMC was obtained as explants from the thoracic aorta of a 3-month-old Sprague-Dawley rat as previously described (19). Cells were maintained in DMEM (Gibco BRL, Grand Island, NY, U.S.A.) with 10% fetal calf serum (Gibco BRL, Grand Island, NY, U.S.A.) in a humidified atmosphere containing 5% CO_2 at 37°C. For experi-

ment, RASMCs were pre-cultured on the same constitution of media except 3% fetal bovine serum for 24 hr. All experiments were performed on cells between passage 5 and 12 grown to about 80% confluence in 6-well or 96-well plates.

To stimulate proliferation of RASMC, PDGF (BB isoform) (Upstate Biotechnology, Waltham, MA, U.S.A.) 80 ng/mL was added to the media. The antioxidant, *N*-acetylcysteine (Sigma, St. Louis, MO, U.S.A.) 1 mM was added along with PDGF addition to see the antioxidant effect.

Adenoviruses

One hundred multiplicity of infection (MOI) of Ad.N17rac1 was infected in 3% fetal calf serum media 24 hr before PDGF stimulation to allow sufficient gene expression in RASMC. Adenovirus Ad.N17rac1 contains the epitope-tagged dominant negative rac1 cDNA, which has a mutation changing amino acid 17 of rac1 from serine to asparagine. Construction of Ad.N17rac1 was done by homologous recombinant in 293 cells (13, 20). To assess the effects of adenoviral infection alone, a control virus Ad.dl312, which is deleted in the E1 region but lacks a recombinant transgene, was used (21). Viruses were amplified, purified, and titrated as previously described (22). All viruses were kindly presented by Dr. Finkel T. at the Laboratory of Molecular Biology, NHLBI.

Gene transfer efficiencies were assessed after infection with Ad.GAL, an adenovirus, which encodes the *Escherichia coli* *LacZ* gene product (22). Histochemical X-gal staining of Ad. GAL-infected cells was performed as previously described (22). Western blot analysis of rac1 expression used antibodies directed at the myc-epitope tag (9E10; Santa Cruz Biotech., Heidelberg, Germany), which identified the N17rac1 gene product (13). Twenty μ g of protein was loaded in each lane and bound immunoglobulins were detected by enhanced chemiluminescence (Tropix, Bedford, MA, U.S.A.). Confirmation of equal protein loading was assessed using an antibody directed at α -tubulin (Ab-1; Calbiochem-Novabiochem Corp., La Jolla, CA, U.S.A.).

Measuring Intracellular ROS

The level of intracellular ROS was measured at 12 hr after PDGF stimulation with or without NAC and Ad.N17rac1 treatment. Intracellular generation of ROS was detected using 5-(and 6)-carboxyl-2',7'-dichlorodihydrofluorescein diacetate (carboxyl-DCFH-DA) (Molecular Probes Inc., Eugene, OR, U.S.A.) (23-26). Carboxyl-DCFH-DA fluoresces green when oxidized by $O_2^{\cdot -}$ or H_2O_2 . The fluorescence was detected by confocal laser scanning microscope using excitation and emission wavelengths of 488 and 525 nm, respectively, by incubating cells for 5 min with 10 μ g/mL of carboxyl-DCFH-DA as previously described (6). Levels of Carboxyl-DCFH-DA fluorescence represent the values from at least 60 ran-

dom cells (mean \pm SD), based on an arbitrary scale of fluorescence intensity (6).

Assessment of RASMC proliferation

The degrees of RASMC proliferation were evaluated at 72 hr after PDGF stimulation with or without NAC and Ad.N17rac1 treatment. The RASMC proliferation was determined by two methods, cell number count using trypan blue exclusion and XTT assay. In cell number counts, only viable cells by trypan dye are counted in hemocytometer as cells/mL. 'Cell Proliferation Kit II (XTT)' (Boehringer Mannheim Corp., Mannheim, Germany) was used for XTT assay. Fifty μ L of XTT labeling mixture was added to each well of 96-well plate and readings were done 24 hr later at 492 nm, corrected against 690 nm.

Statistical analyses

Except when stated otherwise, all results are from triplicate cultures (mean \pm SD) and represent one of at least three similar experiments. Statistical significance was determined by first performing an ANOVA between the experimental groups ($p < 0.05$), and then a two-tailed unpaired t-test to compare N17rac1 with the other experimental groups.

RESULTS

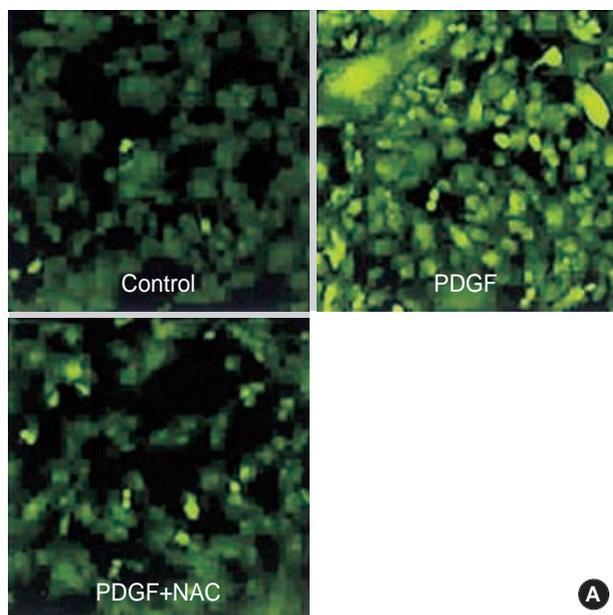
We used primary cultures of RASMC as an in vitro model for growth factor-induced ROS production and cell growth. Fetal calf serum concentration of 3% was used because it was the minimal concentration that showed linear growth pattern. To select the growth factor for RASMC stimulation, we tried angiotensin II, basic FGF and thrombin as well as PDGF. PDGF was chosen because it showed the most prominent ROS induction and cell growth. At 80 ng/mL, PDGF showed maximal ROS induction and cell growth, and we used this concentration for this experiment. NAC was used as an antioxidant, because we observed that PDTC and catalase induced significant cellular toxicity by trypan blue exclusion in the preliminary experiments.

As demonstrated in Fig. 1, compared to the cells without PDGF, the levels of intracellular ROS increased about 2-fold after stimulation with PDGF 80 ng/mL for 12 hr. To see the proliferative effect of PDGF, the cells were cultured with PDGF 80 ng/mL for 72 hr and then cell number count and XTT assay were performed. With PDGF, cell number increased about 1.6-fold compared with control (Fig. 2A). XTT assay showed a similar result with 1.8-fold increase in absorbance (Fig. 2B).

Next, we assessed the effects of treating with the cell permeant chemical NAC. Consistent with previous studies that demonstrated antiproliferative effects of ROS scavengers by

decreasing intracellular ROS levels (10, 17), treatment with NAC 1 mM inhibited the increase of intracellular ROS levels (Fig. 1) and the cell proliferation (Fig. 2) induced by PDGF. We have used 1 mM of NAC, because at that concentration, NAC showed the maximal inhibitory effects to induce ROS and promote cell growth.

Consistent with previous results in vascular smooth mus-



cle cells (27, 28), adenoviral infection at concentration of 100 MOI led to a successful gene transfer in >95% of infected cells (Fig. 3A). Similarly, Western blot analysis of Ad.N17rac1 infected cells demonstrated that the epitope-tagged form of dominant negative rac1 gene product was expressed at a high level (Fig. 3B).

After confirmation of the high efficiency of adenoviral-mediated gene transfer, we examined the effect of inhibition

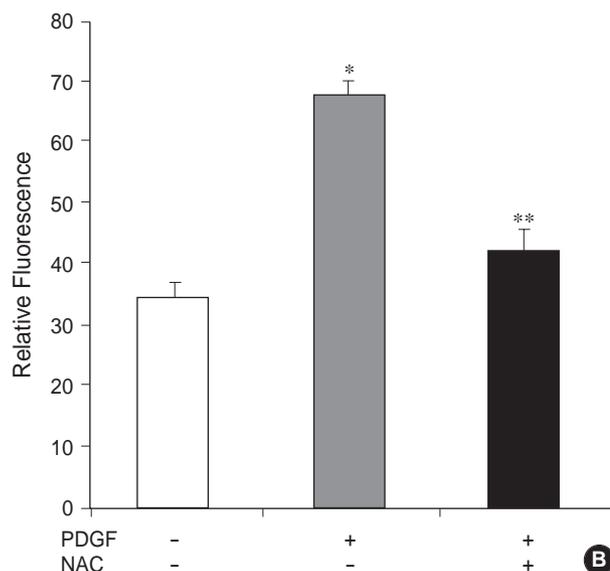


Fig. 1. Effects of PDGF and NAC on the levels of reactive oxygen species in rat aortic smooth muscle cells. (A) Representative images obtained by carboxyl-DCFH-DA confocal laser microscopy. Left upper panel: control cells. PDGF: cells incubated with PDGF 80 ng/mL for 12 hr. PDGF+NAC: cells co-incubated with PDGF 80 ng/mL and NAC 1 mM for 12 hr. (B) Levels of carboxyl-DCFH-DA fluorescence measured by confocal laser microscopy. Cells were incubated with PDGF 80 ng/mL (PDGF) or NAC 1 mM (NAC) for 12 hr. $p < 0.05$, *: PDGF (-) NAC (-) vs. PDGF (+) NAC (-), **: PDGF (+) NAC (-) vs. PDGF (+) NAC (+).

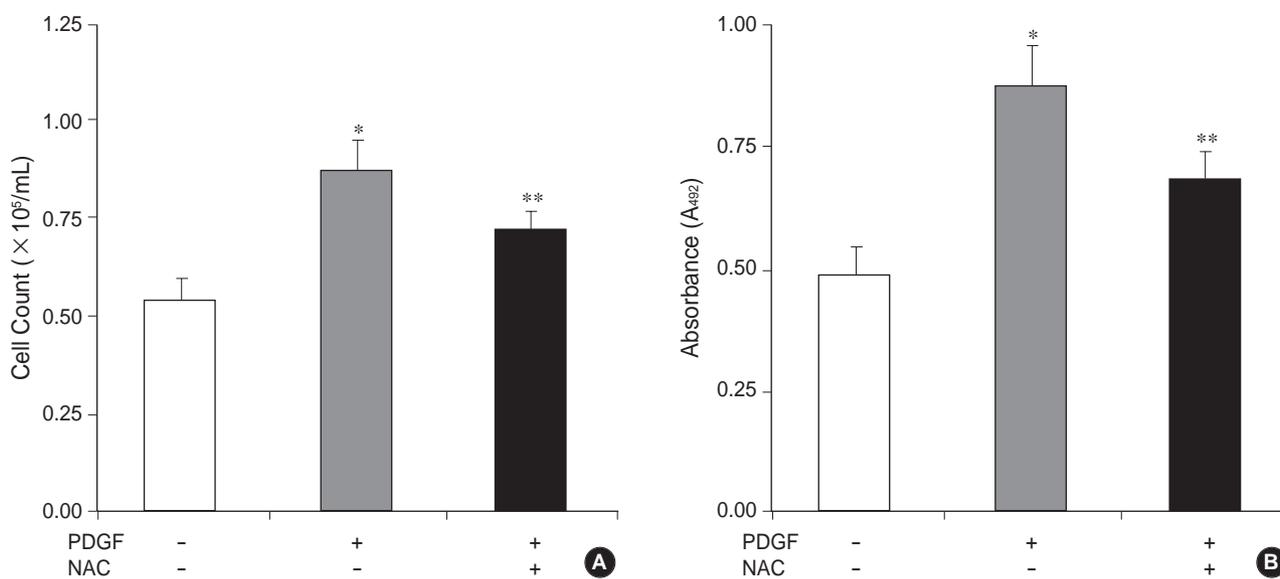


Fig. 2. Effects of PDGF and NAC on the proliferations of rat aortic smooth muscle cells. (A) Effects of PDGF and NAC on cell numbers. (B) Effects of PDGF and NAC on relative absorbance at 492 nm on XTT assay. Cells were incubated with PDGF 80 ng/mL (PDGF) or NAC 1 mM (NAC) for 72 hr. $p < 0.05$, *: PDGF (-) NAC (-) vs. PDGF (+) NAC (-), **: PDGF (+) NAC (-) vs. PDGF (+) NAC (+).

of *rac1*. To determine whether the inhibition of *rac1* could suppress the increase of intracellular ROS levels and cellular proliferation by PDGF stimulation, we used adenoviral-mediated gene transfer of a dominant negative *rac1* (Ad.N17*rac1*). Previous studies have implicated that *rac1* plays an impor-

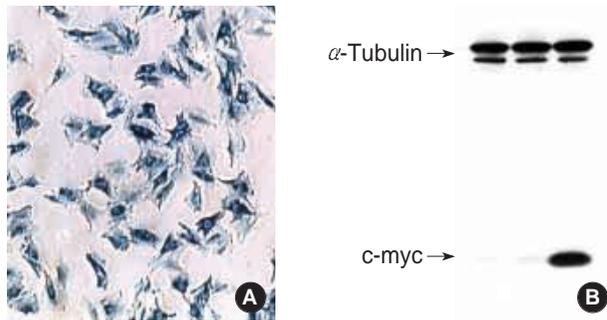
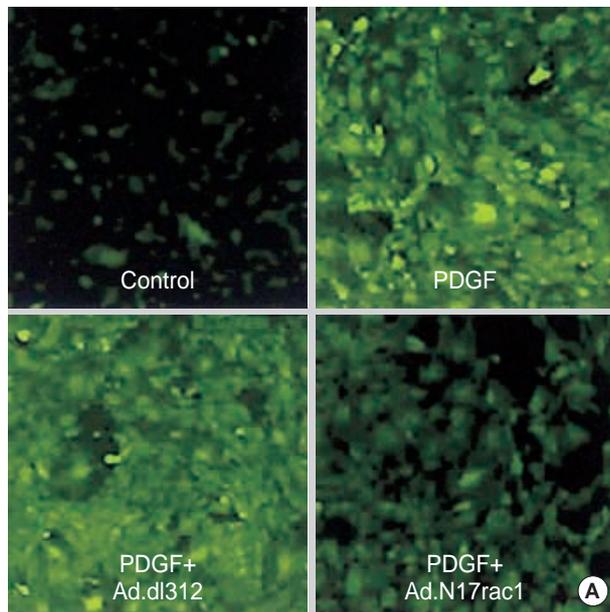


Fig. 3. Assessment of adenoviral-mediated gene transfer. (A) Histochemical X-gal staining 24 hr after infection with 100 multiplicity of infection of Ad.GAL, which encodes the *Escherichia coli* LacZ gene product, showed over 95% gene transfer efficiency. (B) Western blots of protein lysates (20 μ g) obtained from uninfected RASMC (left lane), RASMC infected with Ad.dl312 (middle lane), and Ad.N17*rac1* (right lane). Western blotting analysis was probed with α -tubulin to confirm equal loading and an antibody (21 kDa) recognizing the myc epitope tag of N17*rac1* construct.



tant role in ligand-stimulated ROS generation in non-phagocytic cells (13-15, 29, 30). Examination of intracellular ROS levels revealed that in uninfected cells, the stimulation with PDGF produced a significant increase in carboxyl-DCFH-DA fluorescence (Fig. 4). Similar increase in the fluorescence was seen in the cells infected with an E1-deleted adenovirus, which lacked a recombinant transgene (Ad.dl312; Fig. 4). By contrast, the cells infected with Ad.N17*rac1* showed a significant attenuation in the level of ROS, as assessed by carboxyl-DCFH-DA fluorescence, after stimulation with PDGF (Fig. 4).

Next, we assessed the effects of N17*rac1* expression on cell proliferation after stimulation with PDGF. In the cells treated with PDGF only or PDGF plus Ad.dl312, the number of cells was increased significantly, about 2-folds, compared with control (Fig. 5A). By contrast, in the cells infected with Ad.N17*rac1*, PDGF induced a significant attenuation in cell growth by approximately a half of the cell number (Fig. 5A). Similarly, according to the results by the XTT assay, the cells infected with Ad.N17*rac1* showed significant decreases of absorbance compared with the cells treated with PDGF only or PDGF plus Ad.dl312 (Fig. 5B).

DISCUSSION

We found that in RASMCs the inhibition of *rac1* by infec-

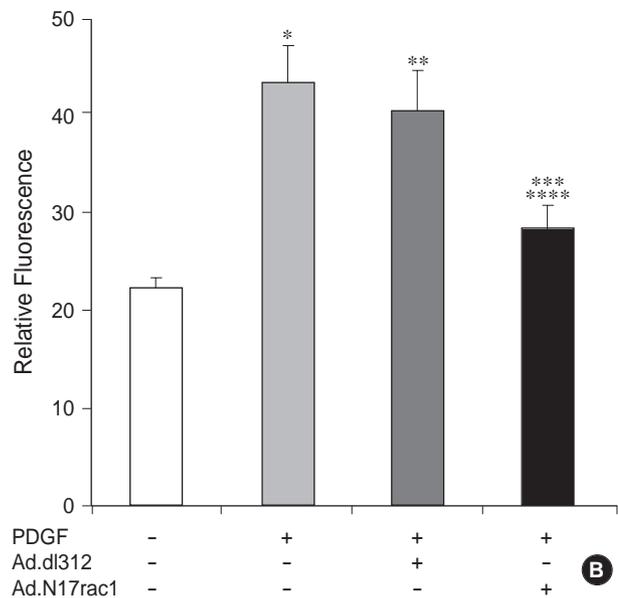


Fig. 4. Effects of Ad.N17*rac1* infection on the levels of reactive oxygen species in rat aortic smooth muscle cells. (A) Representative images obtained by carboxyl-DCFH-DA confocal laser microscopy. Control: uninfected control cells. PDGF cells incubated with PDGF 80 ng/mL. PDGF+Ad.dl312: cells incubated with PDGF 80 ng/mL and infected with 100 multiplicity of infection (MOI) of Ad.dl312. PDGF+ Ad.N17*rac1*: cells incubated with PDGF 80 ng/mL and infected with 100 MOI of Ad.N17*rac1*. Confocal images were obtained 12 hr after incubation with PDGF and viral infections were performed 24 hr before the start of incubation with PDGF. (B) Levels of carboxyl-DCFH-DA fluorescence measured by confocal laser microscopy. Cells were incubated with PDGF 80 ng/mL (PDGF) or infected with 100 MOI of Ad.dl312 or Ad.N17*rac1* as described in (A).

$p < 0.05$, from left to right, *: 1st vs. 2nd, **: 1st vs. 3rd, ***: 2nd vs. 4th, ****: 3rd vs. 4th bar.

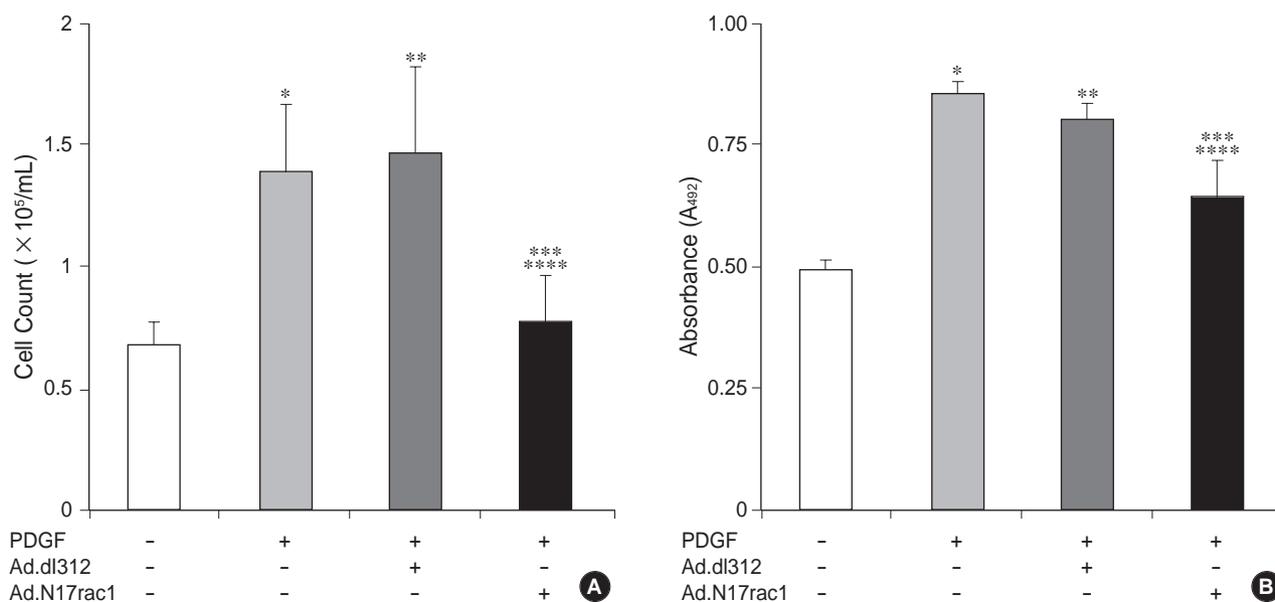


Fig. 5. Effects of Ad.N17rac1 infection on the proliferations of rat aortic smooth muscle cells. (A) Effects of Ad.N17rac1 infection on cell numbers. (B) Effects of Ad.N17rac1 infection on relative absorbance at 492 nm on XTT assay. Cells were incubated with PDGF 80 ng/mL (PDGF) or infected with 100 multiplicity of infection of Ad.dl312 or Ad.N17rac1. Cell number counting and XTT assay were performed 72 hr after incubation with PDGF and viral infections were performed 24 hr before the start of incubation with PDGF. $p < 0.05$, from left to right, *: 1st vs. 2nd, **: 1st vs. 3rd, ***: 2nd vs. 4th, ****: 3rd vs. 4th bar.

tion with AdN17rac1, an adenovirus containing gene encoding a dominant negative rac1 gene product, resulted in a high expression of N17rac1 in the cells, reduction in the levels of intracellular ROS, and suppression of DNA synthesis and cellular proliferation induced by PDGF stimulation.

Ligand-stimulated ROS production plays an important role in signal transduction in various cells (3, 31-33) and especially, growth factors, such as PDGF, epidermal growth factor, and angiotensin II trigger the rapid production of ROS. Also, ROS can stimulate proliferation of vascular smooth muscle cell (5, 34). Furthermore, in RASMC, PDGF stimulated H_2O_2 production and DNA synthesis, and both of these responses are blocked by antioxidant treatment (6). These reports and our study suggest that endogenously produced ROS have an important role in regulating vascular smooth muscle cell growth.

In phagocytic cells, a variety of stimuli cause burst of superoxide anion through NADPH oxidase (35). The small GTPase rac2 is one of the essential components of NADPH oxidase complex and controls the activity of the enzyme complex (29, 30). Recent evidences suggest that in non-phagocytic cells, similar enzyme complex, NADPH/NADH oxidase is controlled by rac1 (13, 14, 16). Furthermore, NADPH/NADH oxidase is the major source of ROS induced by various growth factors in vascular smooth muscle cell (11). In this experiment, we inhibited NADPH/NADH oxidase in RASMC using rac1 inhibition by adenoviral-mediated gene transfer of a dominant negative rac1 (N17rac1). And we demonstrated

that this resulted in a reduction of intracellular ROS levels and suppression of DNA synthesis and cellular proliferation. This is the first report to show that the over-expression of N17rac1 could inhibit cellular proliferation induced by PDGF.

Some reports have shown that ROS could induce apoptosis rather than proliferation in vascular smooth muscle cells (34, 36, 37). These findings are most likely due to the differences in the amount of ROS applied, but the exact mechanisms remain to be determined.

Usually to see the effects of growth factors, experiments are performed by serum deprivation or at the minimal serum concentration. In this experiment, we have used fetal calf serum of 3%, which was to observe cell growth on a linear growth phase. We thought this was more physiological than a serum-deprived condition. Due to relatively high serum concentration in controls, PDGF-induced increases in intracellular ROS levels, XTT absorbances and cellular proliferations were not so prominent.

Carboxyl-DCFH-DA confocal microscopy to measure the intracellular ROS levels can not discriminate between individual ROS, that is, superoxide anion ($\text{O}_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot\text{OH}$), and nitric oxide (NO). So, we could not observe the effects of individual ROS.

Nonetheless, our data suggest that under certain conditions, inhibition of rac1 in RASMC can reduce intracellular ROS levels and cellular proliferation induced by PDGF. The inhibition of rac1 protein function may be useful in variety of clinical settings, such as restenosis after coronary inter-

vention, in which the proliferation of vascular smooth muscle cells plays an important role in pathogenesis.

ACKNOWLEDGMENTS

We thank Dr. Toren Finkel and Ilsa I. Rovira (Lab. of Molecular Biology, NHLBI, NIH, U.S.A.) for providing adenoviral products and advices for this experiment.

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