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Overexpression of Protein Kinase C β Isoform by Adenoviral Gene Transfer Accelerated Intimal Hyperplasia in Rat Carotid Artery

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Abstract: Vigorous restenosis is responsible for high failure rates of angioplasty in diabetic patients. Since protein kinase C (PKC) β isoform activation is associated with vascular complications of diabetes, we have determined whether PKC β isoform overexpression can stimulate growth and migration of vascular smooth muscle cells (SMCs) and cause an acceleration of arterial intimal hyperplasia. Overexpression of PKC β 1 (adv-PKC β 1) or β -galactosidase (adv- β gal) were achieved using replication-deficient adenovirus vectors in cultured rat aortic SMCs and rat carotid arteries after balloon injury. Rat aortic SMCs infected with adv-PKC β 1 at 10^9 PFU/ml increased PKC activity and PKC β 1 isoform protein level by more than 10-fold compared to adv- β gal infected SMCs at 4 days after infection. The growth and migration rates in PKC β 1 overexpressed SMCs were increased by 1.5- to 1.9-fold compared to adv- β gal infected SMCs. Protein levels of PKC β 1 isoform in adv-PKC β 1 infected arteries were increased by 4.2- and 2.0-fold compared to non-infected and adv- β gal infected arteries, respectively, at 7 days after adenovirus infection. Ratio of intima/media area, a marker of restenosis, were 0.26 ± 0.08 and 0.54 ± 0.14 ($p < 0.05$) at 7 days after infection in adv- β gal and adv-PKC β 1 infected arteries, respectively. Thus, overexpression of PKC β isoform can enhance vascular SMCs migration and growth, causing an accelerated rate of intimal hyperplasia, and may involve the restenotic process after angioplasty in diabetic patients.

Key words: protein kinase C, restenosis, gene transfer, diabetes.

Introduction

High failure rates in balloon angioplasty and vascular grafts due to accelerated restenosis are associated with cardiovascular morbidity and mortality in diabetic patients^{3, 15, 51}). The development of arterial restenosis could be due to arterial smooth muscle cells (SMC) accumulation induced by migration of SMC into the intima and its proliferation. Although a great deal of studies have fo-

cused on the mechanism regarding the arterial restenosis process, there have been relatively few directed specificity at factors involved in the diabetic state^{40, 41}).

One possible mechanism by which the restenosis process can be accelerated in the diabetic state is the activation of diacylglycerol (DAG)-protein kinase C (PKC) pathway which has been demonstrated to affect many vascular functions including vascular SMC migration and growth and platelet aggregation²⁹). Recent studies have documented

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that hyperglycemia and diabetic condition will increase DAG levels and PKC activities in the vascular tissues of diabetic animals and patients. The increases in PKC activity in diabetes are associated with retinal and renal hemodynamic changes, cardiac dysfunctions and hypertrophy, cytokine expression and angiogenesis²⁹). PKC is a family of serine-threonine kinases of at least twelve isoforms (α , β 1, β 2, γ , δ , ϵ , ζ , ν , η , θ , λ , μ) of varying substrate preference, intracellular localization and mode of activation^{6, 33, 35, 36, 44}). Among the different PKC isoforms, hyperglycemia and diabetes are reported to activate, preferentially, β -isoform in the retina, heart, aorta and renal glomeruli^{23, 25, 29}). Functionally, using a PKC β isoform selective inhibitor LY333531, the activation of PKC β isoform appears to be partly responsible for abnormalities of retinal and renal blood flow, growth factors expression and extracellular matrix production²⁴). In addition, transgenic mice overexpressing PKC β isoform targeted specificity to the myocardium developed cardiac hypertrophy and dysfunction⁴⁶). However, a role of PKC β isoform on the pathogenesis of restenosis is still unclear.

In the present study, we have characterized the effects of overexpressing PKC β 1 isoform both in cultured cell and in vivo using adenoviral vectors containing the full length cDNA of PKC β 1 isoform. For in vivo studies, the effect of PKC β 1 overexpression on intimal hyperplasia after balloon injury was studied, whereas the effect of PKC β 1 isoform on migration and growth of arterial SMCs were also characterized.

Methods

Materials

Male Sprague-Dawley rats (body weight 350g) were used for animal studies. Dulbecco's modified Eagle medium (DMEM) and calf serum (CS) were purchased from Gibco BRL (Grand Island, NY). Anti-PKC β 1 antibody was obtained from Santa-

Cruz Biotech (Santa Cruz, CA). [γ -³²P]-ATP was purchased from New England Nuclear (Boston, MA). 293 cells were obtained from Microbix (Toronto, Canada). Beta-gal staining kit was purchased from Boehringer Mannheim (Indianapolis, IL).

Construction of replication deficient adenovirus containing PKC cDNA

Recombinant adenovirus containing full length cDNA encoding PKC β 1 was generated as described previously^{4, 5}). Briefly, the mouse PKC β 1 cDNA obtained from Dr.P.Ashendal (Purdue University, IA) was ligated into the adenoviral shuttle vector, pACCMVpLpA, downstream of the CMV-promoter and upstream of a SV40 polyA tail signal. The resulting plasmid and pJM17, the E1A transcription factor deficient adenoviral genomic vector, were purified and co-transfected into E1A-transformed 293 cells by calcium phosphate precipitation method. Subsequent generation of recombinant adenovirus expressing PKC β 1 (adv-CMV-PKC β 1) in 293 cell lysates was confirmed by Southern blot analysis. For the control, recombinant adenovirus containing beta-galactosidase (adv- β gal) was generated. The viruses were purified by cesium chloride gradient ultracentrifugation, desalted and then assayed for infectivity in a 293 cell plaque assay. Purified adenovirus stocks were stored in 20% glycerol at -80 °C.

Cell culture and in vitro infection with adenovirus

Primary rat aortic SMCs were obtained from male Sprague-Dawley rats (100-150 g body weight) by aortic media explant technique²²). The cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% calf serum. The cells were used within 10 passages for the various experiments. After washing the subconfluent SMCs with serum-free DMEM, adenovirus infection was performed by incubation with either adv- β gal or adv-PKC β 1 at 1×10^9 PFU/ml for one hour at 37 °C as described^{4, 5}). To determine the overex-

pression of β -gal protein, cells were stained with β -gal staining kit (Boehringer Mannheim, Indianapolis, IL). Cell viability was assessed by trypan blue exclusion.

Intra-arterial administration of adenovirus in balloon-injured rat carotid arteries

After rats were anesthetized with pentobarbital sodium solution (Abbott Laboratories, North Chicago, IL) at 1 mg/kg body weight, the left common carotid artery was isolated and injured with 2 Fr size Fogarty catheter (Baxter Healthcare Co. Irvine, CA) as described previously^{10, 27, 30, 45}. After balloon injury, another catheter was introduced into the lumen of the balloon injured arterial segments. The lumen of the artery was washed with DMEM, 1×10^9 PFU/ml of adenovirus solution was instilled into the isolated arterial segments and incubated for 15 minutes with the artery clamp distal to the injury site. After incubation, the carotid artery was declamped and blood flow was restored. At seven days after balloon injury with or without adenoviral infection, rats were sacrificed and the infected segments of common carotid artery were isolated for the various analysis.

Partial purification of PKC

Rat aortic SMCs and carotid arterial tissues were washed with ice-cold phosphate buffered saline (PBS). The carotid arterial tissues were frozen in liquid nitrogen and crashed into frozen powder, immediately. All samples were homogenized with lysis buffer A, consisting of 20 mmol/l Tris-HCl, pH 7.5, 2 mmol/l EDTA, 0.5 mmol/l sucrose and leupeptin (1 μ g/ml) with polytron for 20 seconds and with 60 strokes of a Dounce homogenizer. The samples were centrifuged at 2,500 rpm for 10 minutes at 4 °C. The cytosolic and membrane fractions were fractionated with ultracentrifugation method as described previously²². The resulting supernatants were passed through 0.5 ml DEAE column (Pharmacia, Uppsala, Sweden), washed twice with 3 ml of buffer B (buffer A without su-

crose), and then finally eluted with 0.4 ml of buffer B containing 200 mmol/l NaCl. Protein concentrations of the samples were measured by Bradford's method⁹.

Assay of PKC activity

PKC activities were measured by their ability to transfer ³²P from [γ -³²P] ATP into specific substrate octapeptide (RKRTLRL) in the presence of Ca²⁺, phosphatidylserine, and diacylglycerol (Avanti, Pelham, AL) as described previously^{20, 38, 50}. PKC activities were calculated by subtracting the non-specific kinase activities obtained in the absence of Ca²⁺, phosphatidylserine, or diacylglycerol.

Immunoblotting of PKC β 1

Partially purified PKC fractions from rat carotid arteries or total cell lysate were separated on 8% SDS-polyacrylamide gels under reducing conditions and transferred to PDVF membranes (Schleicher&Schuell, Keene, NH). Immunoblots were incubated with anti-PKC β 1 isoform antibody for 1 hour at room temperature. The blots were washed and incubated with peroxidase-conjugated anti-rabbit IgG. Specific bands were visualized by ECL (Amersham, Arlington Heights, IL) and quantified by a computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Morphometric analysis

The rat common carotid arteries were harvested at the described days after balloon injury and adenoviral infection. The arteries were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned to 5 μ m, and stained with hematoxylin and eosin to quantitate the neointimal hyperplasia. The areas of intima, between the internal elastic lamina to the luminal surface, and the medial layer in each section were measured by using a microscopical image analyzing system, AX-70 (Olympus, Tokyo, Japan). Measurements of intima and medial areas were made on three cross sections per rat

taken from the middle of each carotid segment. This analysis was performed by investigators blinded to this experimental group.

Growth assay

Cultured rat aortic SMCs which were infected with either adv- β gal or adv-PKC β 1 as described above. After 2 days, cells were seeded in 24-well plates at a density of 5,000 cells/well and cultured with DMEM containing 1% calf serum for 24 hours. The cells were then stimulated with DMEM containing 2%, 5% calf serum or 10 ng/ml of PDGF-B chain, and cultured for three more days. In another experiment, the infected rat aortic SMCs were stimulated with DMEM containing 10% calf serum for 6 hours after cells were seeded, and then cultured with DMEM containing 2% calf serum for 18 or 64 hours. Total cellular DNA contents were measured using Hoechst-33258 dye and a fluorometer (model TKO-100; Hoefer Scientific Instruments, San Francisco, CA) as described⁴⁹.

Migration assay

Transwells which have polycarbonate membranes with 8 μ m pores (Corning Coster Co., Cambridge, MA) were used for migration assay. These membranes were coated with type I collagen (Vitrogen Collagen Co., Pleasanton, CA). Subconfluent rat aortic SMCs infected with either adv- β gal or adv-PKC β 1 were incubated with 0.1% bovine serum albumin (BSA) in DMEM for 14 hours at 37°C. Cells were washed three times in PBS and were removed from the culture dish with trypsin for the minimum period of time required to obtain a monocellular suspension. Cells were washed twice in DMEM with 0.1% BSA, and resuspended at a density of 1×10^5 cells/ml. Conditioned media was added into the lower wells and cell suspensions were loaded to the top of the filter, and incubated for 4 hours at 37°C in 95% air/5% CO₂. At the end of the incubation time, the upper surface of transwell was wiped completely to remove the cells,

and the cells on the bottom surface of transwell were fixed with 70% ethanol and stained with SYTOX green nucleic acid stain (Molecular Probes, Eugene, OR). Cells on the bottom side of the filter were counted by using a video image processing system.

Statistics

All results are expressed as mean \pm standard deviations. The unpaired Student's *t* test was used for comparison of two groups. For experiments containing more than two groups, comparisons were made by ANOVA with Dunnett *t* test. Differences are considered to be statistically significant at the $p < 0.05$.

Results

Time course of protein expression after adenovirus infection

First, we examined the infectivity of adenovirus to vascular SMCs and the time course of protein overexpression in the cells after adenovirus infection. As shown in Fig. 1, percentage of the cells positively stained with X-gal was reached 100% at

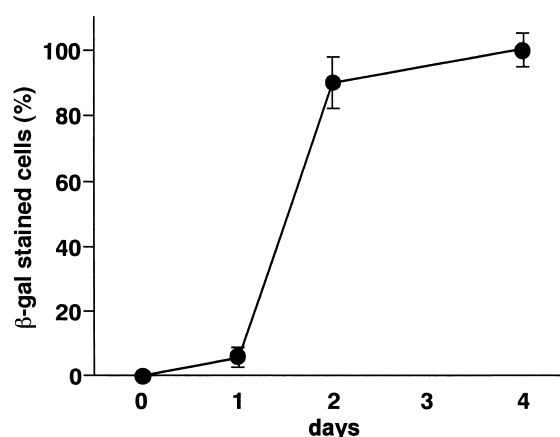


Fig. 1. Time course of β gal protein expression in rat aortic SMCs infected with adv- β gal. The percentage of positively stained cell number in one plate was calculated. Results are expressed as mean \pm SD derived from different four experiments.

4 days after infection with adenovirus (adv- β gal) solution at 1×10^9 PFU/ml for one hour. The PKC β 1 isoform protein levels were increased by 2.5-, 18.0- and 8.0-fold at 1, 4 and 7 days after adenovirus (adv-PKC β 1) infection at 1×10^9 PFU/ml, respectively (Fig. 2). All of these cells were still viable (>98%) as assessed by trypan blue exclusion.

PKC β 1 isoform level and PKC activity after adenovirus infection in rat aortic SMCs

PKC β 1 isoform level in adv-PKC β 1 infected SMCs was increased by more than ten fold compared to non-infected SMCs and adv- β gal infected SMCs at four days after adenovirus infection (Fig. 3A). PKC activity in adv - PKC β 1 infected SMCs was increased by 17.0- and 12.2-fold in cytosolic fraction, and by 4.4- and 5.8-fold in membrane fraction compared to non-infected SMCs and adv- β gal infected SMCs, respectively (Fig. 3B).

The effect of PKC β 1 isoform overexpression on SMC growth

The effect of PKC β 1 isoform overexpression on the proliferation and migration of rat aortic SMC

in vitro were investigated since these biological actions may have major roles in the restenotic process. The growth rate of PKC β 1 overexpressed rat aortic SMCs as measured by total DNA levels was not changed compared to adv- β gal infected SMCs at 6 hours after cells were seeded. But, at 24 and 72 hours after seeded, total DNA content was significantly higher in PKC β 1 overexpressed SMCs compared to adv- β gal infected SMCs (Fig. 4A). There was no significant difference in growth rate between non-infected SMCs and adv- β gal infected SMCs.

Four days after seeding cells, the growth of PKC β 1 overexpressed rat aortic SMCs as measured by total DNA levels was significantly increased by 1.5- to 1.7-fold compared to adv- β gal infected SMCs cultured with DMEM containing 2%, 5% calf serum or 10 ng/ml of PDGF-BB (Fig. 4B).

The effect of PKC β 1 isoform overexpression on SMC migration

The migration of PKC β 1 overexpressed rat aortic SMCs was significantly enhanced by 1.7-fold compared to adv- β gal infected SMCs in the basal

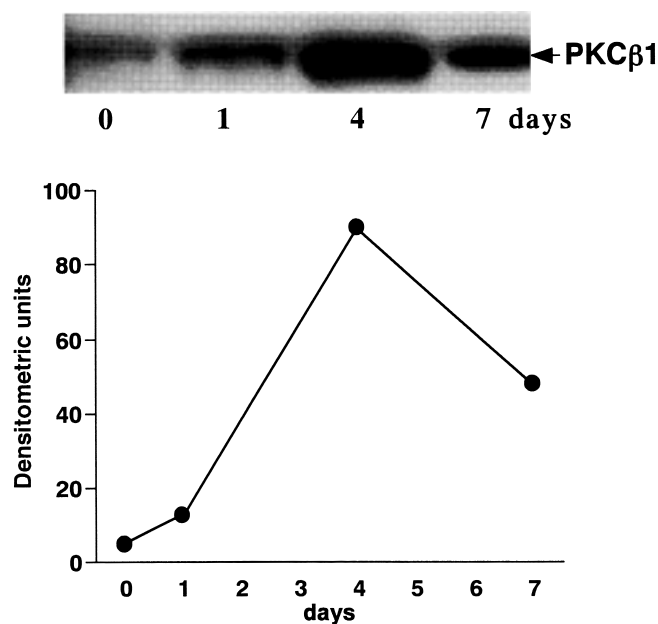


Fig. 2 . Time course of PKC β 1 isoform protein levels in rat aortic SMCs infected with adv-PKC β 1. Upper panel shows immunoblots with anti-PKC β 1 antibody in rat aortic SMCs at 0, 1, 4 and 7 days after adv-PKC β 1 infection. Lower panel shows densitometric quantification of the immunoblot.

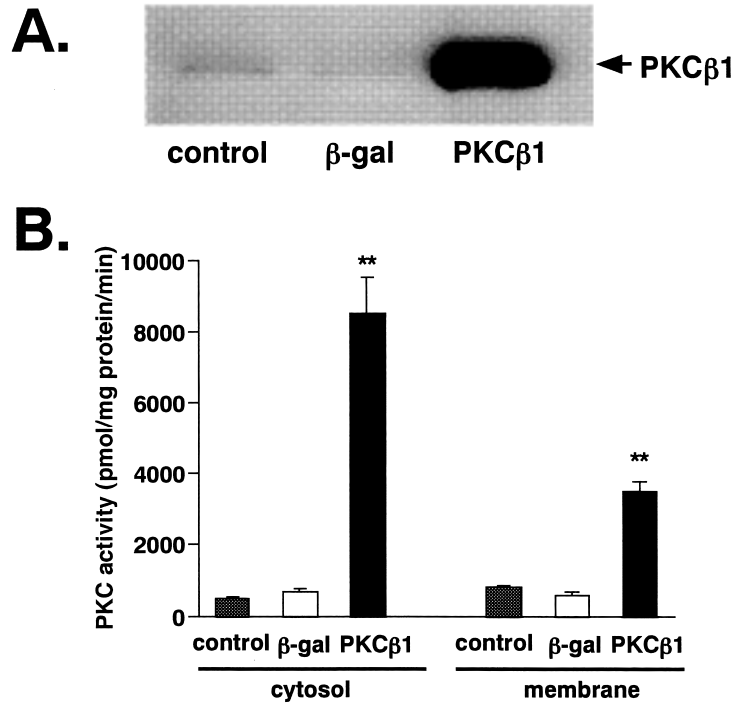


Fig. 3 . PKC β 1 isoform protein level and PKC activity in rat aortic SMCs after adenovirus infection. (A). Immunoblots for PKC β 1 isoform in control, adv- β gal and adv-PKC β 1 infected rat aortic SMCs 4 days after infection. (B). PKC activities in cytosolic and membrane fraction of control, adv- β gal and adv-PKC β 1 infected rat aortic SMCs 4 days after infection. Results are presented as mean \pm SD derived from three different experiments. ** p <0.01 vs control, adv- β gal infected SMCs.

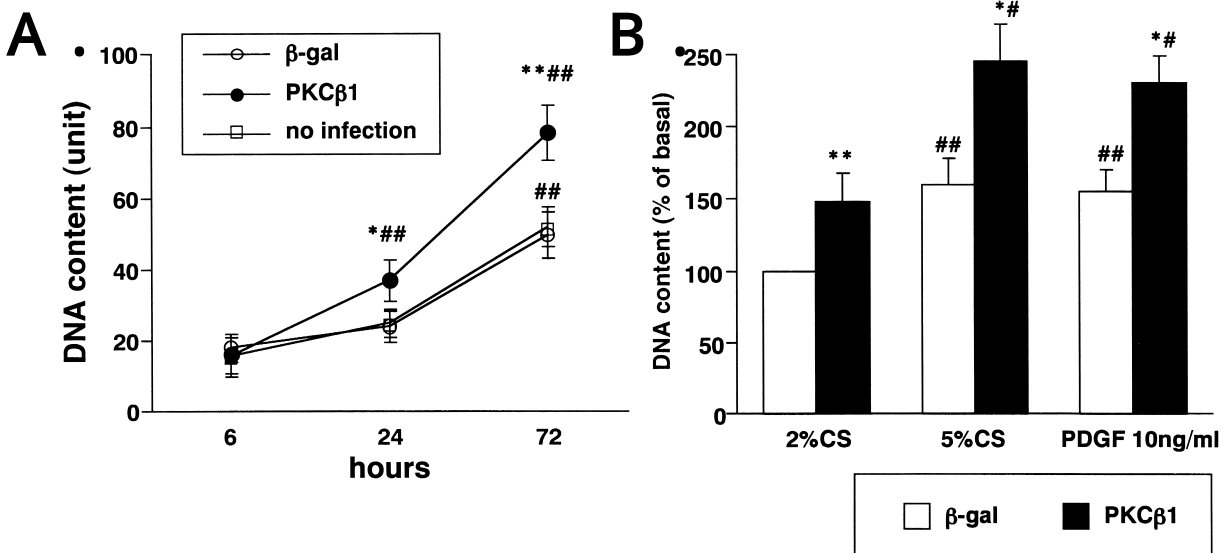


Fig. 4 . Growth of PKC overexpressed rat aortic SMCs. (A). Rat aortic SMCs which were infected with either adv- β gal, adv-PKC β 1 or non-infected SMCs were seeded and cultured in 10% calf serum for 6 hours, then cultured in 2% calf serum for appropriate time. DNA content was measured at 6, 24 and 72 hours after seeded. Results are expressed as mean \pm SD derived from four different experiments. * p <0.05, ** p <0.01 vs adv- β gal infected SMCs and non-infected SMCs. ## p <0.01 vs 6 hours. (B). Rat aortic SMCs were infected with either adv- β gal or adv-PKC β 1 were cultured in DMEM containing 1% calf serum for one day and then cultured for 3 days in DMEM containing 2%, 5% calf serum or 10 ng/ml of PDGF-BB. DNA content was measured at 4 days after seeding. Values are expressed as % of basal DNA contents, and presented as mean \pm SD derived from four different experiments. * p <0.05, ** p <0.01 vs adv- β gal infected SMCs. # p <0.05, ## p <0.01 vs 2% calf serum.

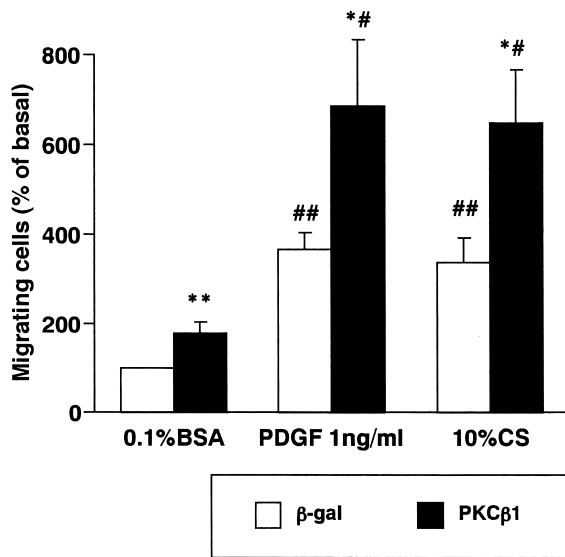


Fig. 5. Migration assay of PKC β 1 overexpressed rat aortic SMC and effects of 1 ng/ml of PDGF-BB or 10% calf serum. Values are expressed as % of basal migrating cell number and presented as mean \pm SD derived from four different experiments. * p <0.05, ** p <0.01 vs adv- β gal infected SMCs. # p <0.05, ## p <0.01 vs 0.1% BSA.

condition maintained with 0.1% BSA. Stimulation with 1ng/ml PDGF-BB or 10% calf serum enhanced migration of β gal and PKC β 1 overexpressed aortic SMCs by 3.4-to 3.7-fold compared to the basal. Moreover, migration of PKC β 1 overexpressed SMCs was significantly accelerated by 1.8-fold with 1 ng/ml PDGF-BB, and by 1.9-fold with 10% calf serum compared to β gal infected SMCs (Fig. 5).

PKC β 1 isoform overexpression in rat carotid artery

The potential role of PKC β 1 isoform on arterial intimal hyperplasia was confirmed further by overexpressing PKC β 1 isoform protein using adv-PKC β 1 in balloon injured rat carotid arteries. Sixteen rats were used for this experiment. As shown in Fig. 6, PKC β 1 isoform protein levels were significantly increased by 2.1-, 2.0- and 4.2-fold in balloon injured (no adenoviral infection), adv- β gal infected and adv-PKC β 1 infected arteries compared to non-injured control arteries at 7 days

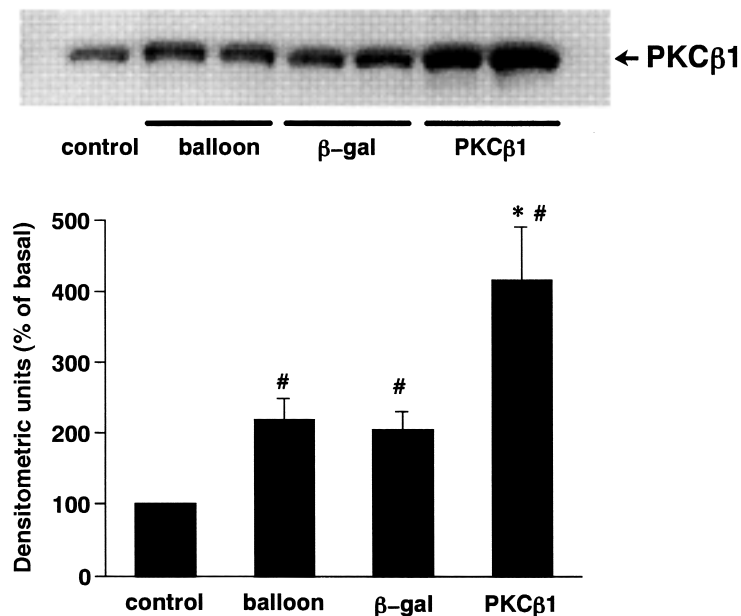


Fig. 6. PKC β 1 isoform protein levels in balloon injured rat carotid arteries infected with adv-PKC β 1. Upper panel shows immunoblots for PKC β 1 isoform in control non-injured carotid arteries, carotid arteries at 7 days after balloon injury without adenovirus infection or carotid arteries at 7 days after balloon injury and infection with either adv- β gal or adv-PKC β 1. Lower panel shows densitometric quantification of PKC β 1 isoform protein levels of carotid arteries. Results were shown as mean percentage of control \pm SD (n=4). * p <0.05 vs balloon injured (no adenovirus infection) and adv- β gal infected arteries. # p <0.01 vs control arteries.

after infection, respectively. And, significant increases in total PKC activities of adv-PKC β 1 infected arteries by 1.6-fold compared to adv- β gal infected arteries were observed at 7 days after balloon injury and adenovirus infection.

The effect of PKC β 1 isoform on intimal hyperplasia

The biological effect of PKC β 1 isoform overexpression on neointimal formation was studied by measuring the ratio of intimal area/medial area after balloon injury and infection with either adv- β gal or adv-PKC β 1. Fourteen rats were used for this experiment. Cross sections of carotid arteries at 7 days after balloon injury and adenovirus infection were shown in Fig. 7A. Histological examination demonstrated that neointimal thickening in adv-PKC β 1 infected arteries were significantly greater than adv- β gal infected arteries. The ratio

of intima/media of adv-PKC β 1 infected arteries were quantitated as described in the method section and found to be significantly increased by 2.1-fold compared to adv- β gal infected arteries. No significant differences were detected in medial area between two groups (Fig. 7B).

Discussion

Present study demonstrated that overexpression of PKC β 1 isoform could enhance growth and migration of vascular SMCs and that PKC β 1 overexpression in rat carotid arteries after balloon injury accelerated the rate of intimal hyperplasia. To obtain the sufficient amount of PKC β 1 protein, we used recombinant adenovirus vector for both in vitro and in vivo studies. As shown in other studies^{13,19}, using our adenovirus system, up to

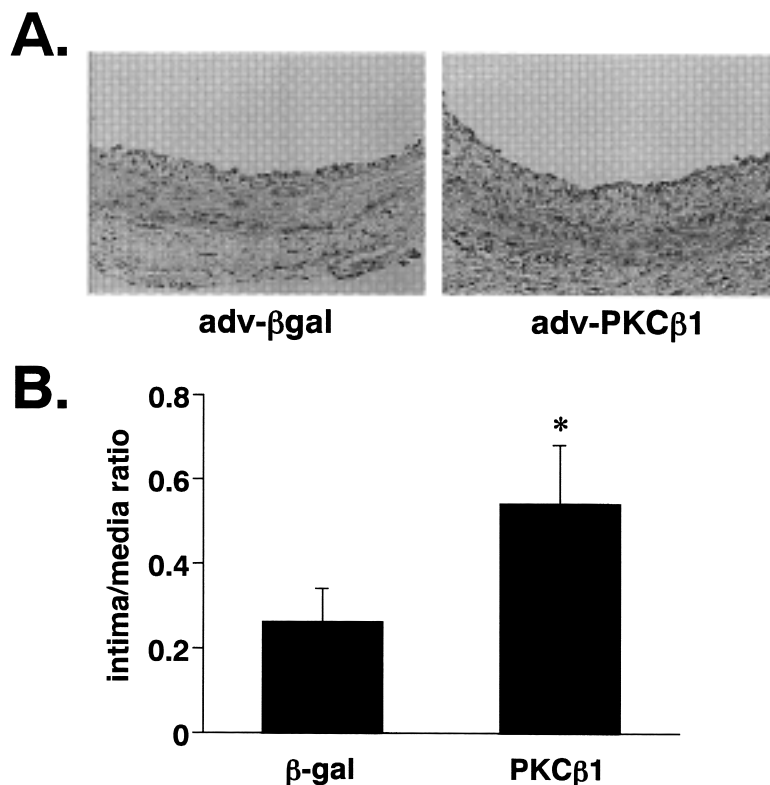


Fig. 7 . (A). Cross sections of carotid arteries at 7 days after balloon injury and infection with either adv- β gal or adv-PKC β 1 were stained with hematoxylin and eosin. Magnification of all sections; x100. (B). The ratio of intima/media of carotid arteries at 7 days after balloon injury and infection with either adv- β gal or adv-PKC β 1. Measurements of intima and medial areas were made on three cross sections per rat taken from the middle of each carotid segment. Values are mean \pm SD (n=7). *p<0.05.

98% of SMCs could be induced to express β -gal protein 2 days after adenovirus infection as demonstrated by β -gal staining, and protein levels reached a maximum level 4 days after transfection. Previous paper has suggested that adenovirus vectors, especially at higher titer, cause cell damage and/or vascular inflammation, because of the translation of viral open reading frame³⁴). Our data suggested that adenovirus vectors (adv- β gal and adv-PKC β 1) at 10^9 PFU/ml had no cytotoxic effect as determined by trypan blue exclusion and that adv- β gal overexpression had no effect on PKC β 1 protein levels and PKC activities compared to non-infected SMCs. Furthermore, attachment of SMCs on culture dishes, which was determined by the DNA content of the attached cells 6 hours after harvest by trypsinization, was not different between non-infected, adv- β gal and adv-PKC β 1 infected SMCs. Nevertheless, growth rate of PKC β 1 overexpressed SMCs was significantly higher compared to adv- β gal infected SMCs after 24 or 72 hours stimulation with serum or PDGF. It has been shown that PKC β 1 isoform could stimulate cell growth of R6 rat embryo fibroblast cell line^{7 21}), bovine aortic endothelial cells^{39 49}) and bovine arterial fibroblasts¹²). These are supportive data for our present study which showed PKC β 1 isoform overexpression enhanced cell growth of vascular SMCs. However, other reports demonstrated that PKC β isoform inhibited the growth of colon cancer cell line¹⁸) and keratinocyte cell line³⁷). Moreover, it has been reported that PKC α ⁴⁷), and PKC δ ¹⁷) decreased growth rate of vascular SMCs. These data indicates that the effect of PKC β isoform on SMC growth could be cell type-specific and isoform-specific. We showed here that the ratio of intima/media area after balloon injury was significantly increased in adv-PKC β 1 infected arteries with increased PKC β 1 isoform protein level compared to adv- β gal infected arteries in vivo. These data suggests that activation of PKC β isoform which is seen in diabetic condition might involve in an acceleration of intimal hyper-

plasia after angioplasty.

In restenosis process, platelet deposition and release of growth factors, such as PDGF and bFGF, have been postulated to be important for initiating the cellular growth response after vascular injury^{1 11 32 42 43 48}). PDGF-B chain is known to activate mitogen-activated protein (MAP) kinases and to be the most potent known chemoattractant for vascular SMCs⁵²). The signal transduction pathways which mediate the chemotactic effects of PDGF-BB on SMCs are still unknown, but several classes of cellular components are implicated, including focal adhesion kinase (p125FAK), small GTP-binding protein of the rho family and certain down-stream signaling of the PDGF beta receptor, such as MAPK/ERK or PI3 kinase pathway¹). PKC has been shown to regulate localization of cytoskeletal proteins and phosphorylation of FAK induced by alpha v beta 5³¹). It has been established that PKC plays a pivotal role in signal transduction pathways that influence numerous cellular functions including cell proliferation and migration²⁶). Several lines of evidence demonstrated that classical type of PKC (α , β , γ), but not novel type of PKC (δ , ζ , ϵ), are able to activate c-Raf, MEK/MAPK pathway^{2 14 16 28}). These findings suggest that the activation of PKC could influence cellular responses to PDGF or other growth factors in restenosis process.

Among the various isoforms of PKC, PKC β isoform appeared to be preferentially activated in vascular tissues such as aorta and heart in diabetic rats^{8 46}). Furthermore, it has been reported that treatment of diabetic rats with PKC β isoform selective inhibitor could ameliorate several abnormalities in vascular functions²⁴). These data suggest that PKC β isoform plays a pivotal role in the pathogenesis of diabetic vascular complications. However, specific substrate which is activated by PKC β , and leads to the activation of downstream signal transduction pathways is still unknown. Further studies will be needed.

In summary, the successful overexpression of

PKC β 1 isoform in cultured vascular SMCs and in rat carotid arteries has clearly demonstrated that PKC β 1 isoform overexpression can cause the abnormal migration and proliferation of vascular SMC and lead to accelerated intimal hyperplasia after balloon injury in rat carotid arteries. These results suggest that PKC β isoform may be proposed as a new potential therapeutic target for restenosis after angioplasty in diabetes.

References

- 1) Abedi H, Zachary I. Signalling mechanisms in the regulation of vascular cell migration. *Cardiovasc. Res.* 30: 544 556, 1995.
- 2) Alessandrini A, Crews CM, Erikson RL. Phorbol ester stimulates a protein-tyrosin / threonine kinase that phosphorylates and activates the *Erk-1* gene product. *Proc. Natl. Acad. Sci.* 89: 8200 8204, 1992.
- 3) Barsness GW, Peterson ED, Ohman EM, Nelson CL, DeLong ER, Reves JG, Smith PK, Anderson RD, Jones RH, Mark DB, Califf RM. Relationship between diabetes mellitus and long-term survival after coronary bypass and angioplasty. *Circulation* 96: 2551 2556, 1997.
- 4) Becker TC, BeltrandelRio H, Noel RJ, Johnson JH, Newgard CB. Overexpression of hexokinase I in isolated islet of Langerhans via recombinant adenovirus. Enhancement of glucose metabolism and insulin secretion at basal but not stimulatory glucose levels. *J. Biol. Chem.* 269: 21234 21238, 1994.
- 5) Becker TC, Noel RJ, Coats WS, Gomez-Foix AM, Gerard RD, Newgard CB. Use of recombinant adenovirus for metabolic engineering of mammalian cells. *Methods Cell Biol.* 43PtA: 161 189, 1994.
- 6) Blobe GC, Khan WA, Halpern AE, Obeid LM, Hannun YA. Selective regulation of expression of protein kinase C β isoenzymes occurs via alternative splicing. *J.Biol.Chem.* 268: 10627 10635, 1993.
- 7) Borner C, Ueffing M, Jaken S, Parker PJ, Weinstein IB. Two closely related isoforms of protein kinase C produce reciprocal effects on the growth of rat fibroblasts. *J. Biol. Chem.* 270: 78 86, 1995.
- 8) Bowling N, Walsh RA, Song G, Estridge T, Sandusky GE, Fouts RL, Mintze K, Pickard T, Roden R, Bristow MR, Sabbah HN, Mizrahi JL, Gromo G, King GL, Vlahos CJ. Increased protein kinase C activity and expression of Ca²⁺-sensitive isoforms in the failing human heart. *Circulation* 99: 384 391, 1999.
- 9) Bradford MM. A rapid and sensitive method of the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248 254, 1976.
- 10) Chen L, Daum G, Forough R, Clowes M, Walter U, Clowes AW. Overexpression of human endothelial nitric oxide synthase in rat vascular smooth muscle cells and in balloon-injured carotid artery. *Circ.Res.* 82: 862 870, 1998.
- 11) Clowes A, Reidy M, Clowes M. Mechanism of stenosis after arterial injury. *Lab. Invest.* 49: 208 215, 1993.
- 12) Das M, Stenmark KR, Ruff LJ, Dempsey EC. Selected isoenzymes of PKC contribute to augmented growth of fetal and neonatal bovine PA adventitial fibroblasts. *Am. J. Physiol.* 273: L1276 1284, 1997.
- 13) de Martin R, Raidl M, Hofer E, Binder BR. Adenovirus-mediated expression of green fluorescent protein. *Gene Ther.* 4: 493 495, 1997.
- 14) Downward J, Graves JD, Warne PH, Rayter S, Cantrell DA. Stimulation of p21ras upon T-cell activation. *Nature* 346: 719 726, 1990.
- 15) Erbel R, Haude M, Hopp HW, Franzen D, Rupprecht HJ, Heublein B, Fischer K, Jaegere P, Serruys P, Rutsch W, Probst P. Coronary-artery stenting compared with balloon angioplasty for restenosis after initial balloon angioplasty. *N. Engl. J. Med.* 339: 1672 1678, 1998.

- 16) Force T, Bonventre JV. Growth factors and mitogen-activated protein kinases. *Hypertension* 31: 152 161, 1998.
- 17) Fukumoto S, Nishizawa Y, Hosoi M, Koyama H, Yamakawa K, Ohno S, Morii H. Protein kinase C δ inhibits the proliferation of vascular smooth muscle cells by suppressing G1 cyclin expression. *J.Biol.Chem.* 272: 13816 13822, 1997.
- 18) Goldstein DR, Cacace AM, Weinstein IB. Overexpression of protein kinase C beta 1 in the SW480 colon cancer cell line causes growth suppression. *Carcinogenesis* 16: 1121 1126, 1995.
- 19) Guzman RJ, Lemarchand P, Crystal RG, Epstein SE, Finkel T. Efficient and selective adenovirus - mediated gene transfer into vascular neointima. *Circulation* 88: 2838 2848, 1993.
- 20) Hofmann J. The potential for isoenzyme-selective modulation of protein kinase C. *FASEB J.* 11: 649 669, 1997.
- 21) Housey GM, Johnson MD, Hsiao WLW, O'Brian CA, Murphy JP, Kirschmeier P, Weinstein IB. Overproduction of protein kinase C causes disordered growth control in rat fibroblasts. *Cell* 52: 343 354, 1988.
- 22) Igarashi M, Wakasaki H, Takahara N, Ishii H, Jiang ZY, Yamauchi T, Kuboki K, Meier M, Rhodes CJ, King GL. Glucose or diabetes activates p38 mitogen-activated protein kinase via different pathways. *J.Clin.Invest.* 103: 185 195, 1999.
- 23) Inoguchi T, Battan R, Handler E, Sportsman JR, Heath WF, King GL. Preferential elevation of protein kinase C isoform β 2 and diacylglycerol levels in the aorta and heart of diabetic rats: Differential reversibility to glycemic control by islet cell transplantation. *Proc.Natl. Acad.Sci.* 89: 11029 11063, 1992.
- 24) Ishii H, Jirousek MR, Koya D, Takagi C, Xia P, Clermont A, Bursell SE, Kern TS, Ballas LM, Heath WF, Stramm LE, Feener EP, King GL. Amelioration of vascular dysfunctions in diabetic rats by an oral PKC β inhibitor. *Science* 272: 728 731, 1996.
- 25) Ishii H, Koya D, King GL. Protein kinase C activation and its role in the development of vascular complications in diabetes mellitus. *J. Mol.Med.* 76: 21 31, 1998.
- 26) Kariya K, Kawahara Y, Tsuda T, Fukuzaki H, Takai Y. Possible involvement of protein kinase C in platelet-derived growth factor-stimulated DNA synthesis in vascular smooth muscle cells. *Atherosclerosis* 63: 251 255, 1987.
- 27) Kim S, Lin H, Barr E, Chu L, Leiden JM, Parmacek MS. Transcriptional targeting of replication-defective adenovirus transgene expression to smooth muscle cells in vivo. *J.Clin. Invest.* 100: 1006 1014, 1997.
- 28) Kolch W, Heldecker G, Kochs G, Hummel R, Vahldl H, Mischak H, Flnkenzeller G, Marme D, Rapp UR. Protein kinase C α activates RAF-1 by direct phosphorylation. *Nature* 364: 249 252, 1993.
- 29) Koya D, King GL. Protein kinase C activation and the development of diabetic complications. *Diabetes* 47: 859 866, 1998.
- 30) 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.* 73: 797 807, 1993.
- 31) Lewis JM, Cheresh DA, Schwartz MA. Protein kinase C regulates alpha v beta 5 - dependent cytoskeletal associations and focal adhesion kinase phosphorylation. *J. Cell Biol.* 134: 1323 1332, 1996.
- 32) Libby P, Schwartz D, Brogi E, Tanaka H, Clinton SK. A cascade model for restenosis: a special case of atherosclerosis progression. *Circulation* 86: III 47 III 52, 1992.
- 33) Mellor H, Parker PJ. The extended protein kinase C superfamily. *Biochem. J.* 332: 281 292, 1998.
- 34) Newman KD, Dunn PF, Owens JW, Schulick AH, Virmani R, Sukhova G, Libby P, Dichek DA. Adenovirus-mediated gene transfer into

- normal rabbit arteries results in prolonged vascular cell activation, inflammation, and neointimal hyperplasia. *J. Clin. Invest.* 96: 2955 2965, 1995.
- 35) Nishikawa K, Toker A, Johannes FJ, Songyang Z, Cantley LC. Determination of the specific substrate motifs of protein kinase C isozymes. *J.Biol.Chem.* 272: 952 960,1997.
- 36) Nishizuka Y. Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. *Science* 258: 607 614, 1992.
- 37) O'Driscoll KR, Madden PV, Christiansen KM, Viage A, Slaga TJ, Fabbro D, Powell CT, Weinstein IB. Overexpression of protein kinase C beta 1 in a murine keratinocyte cell line produces effects on cellular growth, morphology and differentiation. *Cancer Lett.* 83: 249 259, 1994.
- 38) Olivier FJ, de la Rubia G, Feener EP, Lee ME, Loeken MR, Shiba T, Quertermous T, King GL. Stimulation of endothelin-1 gene expression by insulin in endothelial cells. *J. Biol. Chem.* 266: 23251 23256, 1991.
- 39) Rosales OR, Isaacs CM, Bhargava J. Overexpression of protein kinase C alpha and beta1 has distinct effects on bovine aortic endothelial cell growth. *Cell Signal* 10: 589 597, 1998.
- 40) Ross R, Aigius L. The process of atherogenesis-cellular and molecular interaction: from experimental animal models to humans. *Diabetologia* 35 (Suppl2): S34 S40, 1992.
- 41) Ross R. The pathogenesis of atherosclerosis: a perspective for 1990s. *Nature* 362: 801 809, 1993.
- 42) Rutherford C, Martin W, Salame M, Carrier M, Anggard E, Ferns G. Substantial inhibition of neo-intimal response to balloon injury in rat carotid artery using a combination of antibodies to platelet-derived growth factor-BB and basic fibroblast growth factor. *Atherosclerosis* 130: 45 51, 1997.
- 43) Schwartz SM. Perspectives series: Cell adhesion in vascular biology: Smooth muscle migration in atherosclerosis and restenosis. *J. Clin. Invest.* 99: 2814 2817, 1997.
- 44) Simpson PC. B-protein kinase C and hypertrophic signaling in human heart failure. *Circulation* 99: 334 337, 1999.
- 45) Ueno H, Yamamoto H, Ito S, Li JJ, Takeshita A. Adenovirus-mediated transfer of a dominant - negative H-ras suppresses neointimal formation in balloon-injured arteries in vivo. *Arterioscler. Thromb. Vasc. Biol.* 17: 898 904, 1997.
- 46) Wakasaki H, Koya D, Schoen FJ, Jirousek MR, Ways DK, Hoit BD, Walsh RA, King GL. Targeted overexpression of protein kinase C β 2 isoform in myocardium causes cardiomyopathy. *Proc.Natl.Acad.Sci.* 94: 9320 9325, 1997.
- 47) Wang S, Desai D, Wright G, Niles RM, Wright GL. Effect of protein kinase C alpha overexpression on A7r5 smooth muscle cell proliferation and differentiation. *Exp. Cell Res.* 236: 117 126, 1997.
- 48) Wilcox JN. Molecular biology: insight into the causes and prevention of restenosis after arterial intervention. *Am. J. Cardiol.* 72: 88E 95E, 1993.
- 49) Xia P, Aiello LP, Ishii H, Jiang ZY, Park DJ, Robinson GS, Takagi H, Newsome WP, Jirousek MR, King GL. Characterization of vascular endothelial growth factor's effect on the activation of protein kinase C, its isoforms, and endothelial cell growth. *J.Clin.Invest.* 98: 2018 2026, 1996.
- 50) Yasuda I, Kishimoto A, Tanaka S, Tominaga M, Sakurai A, Nishizuka Y. A synthetic peptide substrate for selectively assay of protein kinase C. *Biochem. Biophys. Res.* 166: 1220 1227, 1990.
- 51) Yasuhara H, Shigematsu H, Muto T. Risk factors for restenosis after balloon angioplasty in focal iliac stenosis. *Surgery* 123: 658 665, 1998.
- 52) Zou Y, Hu Y, Metzler B, Xu Q. Signal transduction in arteriosclerosis: mechanical stress-activated MAP kinases in vascular smooth muscle cells. *Int. J. Mol. Med.* 1: 827 834, 1998.

Protein Kinase C β の過剰発現は，バルーン傷害後のラット頸動脈の内膜肥厚を促進する

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目 的

糖尿病では閉塞性動脈硬化性疾患に対する血行再建後の再狭窄が高頻度に発生する。糖尿病の心血管組織では protein kinase C β (PKC β) が特異的に活性化されており，心血管合併症の重要な要因と考えられている。今回我々は，PKC β の過剰発現が血管平滑筋細胞の増殖，遊走能および，バルーンによる血管傷害後の内膜肥厚に及ぼす影響について検討した。

方 法

アデノウイルスベクターを用いて血管平滑筋細胞に PKC β 1 を過剰発現し，その増殖，遊走能を検討した。また，ラット頸動脈を 2Fr Fogarty バルーンカテーテルを用いて内膜傷害した後，アデノウイルスベクターにて血管壁に PKC β 1 蛋白を過剰発現し，7日後の内膜肥厚の変化を検討した。

結 果

PKC β 1 を過剰発現した血管平滑筋細胞の PKC β 1 蛋白量及び PKC 活性はコントロールウイルス (β -galactosidase) を過剰発現したそれより 10 倍以上増加した。PKC β 1 を過剰発現した血管平滑筋細胞の増殖，遊走能は対照に比し，それぞれ 1.5 倍，1.9 倍の増加を認めた。傷害 7 日後のラット頸動脈の P K

C β 1 蛋白量は，PKC β 1 過剰発現群でバルーン未施行群， β -gal 過剰発現群に対し，それぞれ 4.2 倍，2.0 倍に上昇した。7 日後の intima/media 比は β -gal，PKC β 1 過剰発現群でそれぞれ 0.26 ± 0.08 ， 0.54 ± 0.14 であった。

考 察

PKC β の過剰発現は，血管平滑筋の増殖，遊走能を亢進し，内膜傷害後の血管再狭窄を増悪する可能性が示唆された。