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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 Induces G_1 Arrest and Differentiation Marker Expression in Vascular Smooth Muscle Cells

YOSHIKAZU MIWA, TOSHIYUKI SASAGURI, HIROYASU INOUE, YOJI TABA, AKIO ISHIDA, and TAKEO ABUMIYA

Departments of Bioscience (Y.M., T.S., Y.T., A.I.), Pharmacology (H.I.), and Epidemiology (T.A.), National Cardiovascular Center Research Institute, Osaka, Japan; Second Department of Internal Medicine, Faculty of Medicine, Kyushu University, Fukuoka (Y.M.); and Third Department of Internal Medicine, University of the Ryukyus School of Medicine, Okinawa, Japan (Y.T., A.I.)

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ABSTRACT

In search of substances useful for the treatment of atherosclerotic vascular diseases, we studied the effects of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a natural ligand for peroxisome proliferator-activated receptor γ, on the proliferation and differentiation of vascular smooth muscle cells (VSMCs). 15d-PGJ_2 but not WY14643, an agonist for peroxisome proliferator-activated receptor α, dose-dependently inhibited VSMC proliferation; the effect was maximal at 12 μM. This compound strongly suppressed the activities of cyclin-dependent kinases (Cdk) 4, 6, and 2, thereby preventing the phosphorylation of the retinoblastoma protein. These Cdks seemed to be inhibited through two mechanisms: the down-regulation of cyclin D1 and the up-regulation of Cdk inhibitor $p21^{Cip1/Waf1/Sdi1}$. 15d-PGJ_2 was found to inhibit the phosphatidylinositol 3-kinase/protein kinase B signaling pathway, which mediates cyclin D1 expression. Mitogenic stimulation of quiescent cells decreased the level of mRNA for the smooth muscle-specific myosin heavy-chain SM1, whereas this reduction was prevented by 15d-PGJ_2. A long-term treatment of exponentially growing VSMCs with 15d-PGJ_2 markedly elevated the mRNA level of SM1 and, moreover, induced SM2, another isoform expressed exclusively in mature VSMCs. 15d-PGJ_2 also increased the expression levels of calponin-h1 and smooth muscle α-actin. These results suggest that 15d-PGJ_2 induces G_1 arrest by two distinct mechanisms and promotes VSMC differentiation.

Vascular smooth muscle cells (VSMCs) in the arterial media are fully differentiated to play their physiological roles as regulators of vascular wall tension. However, in atherosclerotic and restenotic lesions, their phenotypes have been converted to dedifferentiated (immature) ones (Owens, 1995). Dedifferentiated VSMCs migrate into the intima, proliferate, and synthesize extracellular matrices, thereby contributing to the formation of neointima. Therefore, to prevent VSMC hyperplasia in vivo, substances capable of promoting the differentiation of VSMCs may be more effective than those that simply inhibit their proliferation. A number of substances have been reported to inhibit VSMC proliferation, but few are also able to prevent phenotype conversion and induce differentiation.

Prostaglandins (PGs) of the J_2 family, including PGJ_2, Δ^{12}-PGJ_2, and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), are naturally occurring metabolites of PGD_2 (Kikawa et al., 1984). We previously reported that PGJ_2 and Δ^{12}-PGJ_2 strongly inhibit VSMC proliferation, although the underlying mechanisms remain undetermined (Sasaguri et al., 1992). Recently, PGs of the J_2 family were found to be natural ligands for peroxisome proliferator-activated receptor (PPAR) γ, a member of the ligand-activated transcription factor nuclear receptor superfamily that includes receptors for steroid, retinoid, and thyroid hormones (Forman et al., 1995; Kliewer et al., 1995). PPARγ has been implicated in the induction of differentiation in adipocytes (Forman et al., 1995; Kliewer et al., 1995) and monocyte/macrophages (Tontonoz et al., 1998) by the suggestion that it increases the expression of genes specific to these cell species. Among

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; PG, prostaglandin; 15d-PGJ_2, 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor; MHC, myosin heavy chain; SM-MHC, smooth muscle-specific myosin heavy chain; Cdk, cyclin-dependent kinase; GST, glutathione *S*-transferase; pRb, retinoblastoma protein; PAGE, polyacrylamide gel electrophoresis; PI3K, phosphatidylinositol 3-kinase; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Akt, protein kinase B; MAPK, mitogen-activated protein kinase; GM, growth medium.

members of the J_2 family, 15d-PGJ_2, a further metabolite of Δ^{12}-PGJ_2, is the most effective activator for PPARγ; indeed, 15d-PGJ_2 promotes differentiation in these cells (Forman et al., 1995; Kliewer et al., 1995; Tontonoz et al., 1998).

From this background, we explored whether 15d-PGJ_2 is able to induce VSMC differentiation. To begin with, we examined the effect of 15d-PGJ_2 on VSMC proliferation with regard to cell cycle events that occur between G_0 and S phases, because an exit from cell cycle may be prerequisite for cell differentiation. VSMC differentiation was assessed by the expression of smooth muscle-specific myosin heavy chains (SM-MHCs), calponin-h1, SM22α, h-caldesmon, and smooth muscle (SM) α-actin. We report here for the first time that 15d-PGJ_2 induces G_1 arrest by two distinct mechanisms (i.e., the inhibition of cyclin D1 expression and the stimulation of $p21^{Cip1/Waf1/Sdi1}$ expression); moreover, we show that this compound up-regulates the expression of VSMC differentiation markers.

Materials and Methods

Chemicals. 15d-PGJ_2 was purchased from Cayman Chemical Co. (Ann Arbor, MI). WY14643 [4-chloro-6-(2,3-xylidino)-2-pyrimidylthioacetic acid] was from Biomol Research Laboratories (Plymouth Meeting, PA). Wortmannin was from Wako Pure Chemicals Industries (Osaka, Japan). LY294002 [2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one] was from Calbiochem (La Jolla, CA).

Cell Culture. VSMCs were isolated from the media of human umbilical arteries by explant. Cells were maintained in Dulbecco's modified Eagle's medium containing 20% (v/v) fetal bovine serum (Life Technologies, Rockville, MD), 10 ng/ml recombinant human basic fibroblast growth factor (Amersham Pharmacia Biotech, Uppsala, Sweden), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B (growth medium). Synchronization in G_0 phase was achieved by serum starvation for 48 h.

Transfection of Oligonucleotides. Oligonucleotides were introduced into cells using LipofectAMINE PLUS reagent according to the manufacturer's protocol (Life Technologies).

DNA Synthesis Assay. DNA synthesis was assessed by the level of [^{3}H]thymidine incorporation as described in Ishida et al. (1997).

DNA Flow Cytometry. Cells dispersed by trypsinization and suspended in PBS were stained with propidium iodide using Cycle TEST PLUS DNA Reagent Kit (Becton Dickinson, Franklin Lakes, NJ), and the fluorescence of DNA was measured using a flow cytometer (FACSCalibur; Becton Dickinson). Cell cycle distribution was analyzed using computer software (ModFit LT; Becton Dickinson).

Immunoprecipitation and Western Blotting. Cell lysates were immunoprecipitated and analyzed by Western blotting as described in Ishida et al. (1997).

Cyclin-Dependent Kinase (Cdk) Assay. The immunoprecipitates with anti-Cdk antibodies were suspended in 40 μL of 20 mM Tris·HCl, pH 7.4, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM [γ-^{32}P]ATP (3.7 MBq/ml; Amersham Pharmacia Biotech), and 20 μg/ml glutathione *S*-transferase (GST)-fused retinoblastoma protein (pRb) carboxyl terminal (Santa Cruz Biotechnology, Santa Cruz, CA), and then incubated for 30 min at 30°C with occasional mixing. The reaction was terminated with an equal volume of 2× loading buffer as described in Ishida et al. (1997). The sample was boiled for 3 min, and then the beads were precipitated by centrifugation. The supernatant was fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to a polyvinylidene difluoride membrane. Phosphorylated proteins were visualized and quantified using a Bioimage analyzer (BAS-2500; Fuji Photo Film Co., Tokyo, Japan).

Phosphatidylinositol 3-Kinase (PI3K) Assay. Cell lysates were immunoprecipitated with an anti-phosphotyrosine antibody (PY20; Transduction Laboratories, San Diego, CA). L-α-Phosphatidylinositol (Santa Cruz Biotechnology) dissolved in chloroform was dried under nitrogen and suspended in water by sonication. After three washes with the kinase buffer (50 mM Tris·HCl, pH 7.8, 50 mM NaCl, 2 mM $MgCl_2$, and 0.5 mM EDTA), the precipitates were suspended in the same buffer containing 0.5 mg/ml L-α-phosphatidylinositol micelles and 37 kBq [γ-^{32}P]ATP and incubated at 30°C for 10 min. The reaction was terminated by the addition of 0.5 ml of 1 M HCl and 2 ml of chloroform/methanol (2:1, v/v). The lower organic phase was dried under nitrogen, dissolved in a small volume of chloroform, spotted on a silica-gel thin-layer plate (silica gel 60; Merck, Darmstadt, Germany), and developed with chloroform/methanol/28% NH_3/water (70:100:15:25, v/v). Radioactive spots on the plate were visualized using the BAS-2500.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and Northern Blotting. Total cellular RNAs were extracted with Isogen (Nippon Gene, Tokyo, Japan) from cultured cells or medial smooth muscle strips homogenized with Physcotron (Microtec Co., Funabashi, Japan).

RT-PCR was performed using Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech). Total cellular RNAs (1 μg) were used for the RT reaction and the products were amplified using DNA Thermal Cycler 480 (Perkin-Elmer Cetus Instruments, Norwalk, CT). PCR primers were synthesized as follows, based on the GenBank database: SM1 and SM2, 5′-ATGAGGCCACGGAGAGCAACGA-3′ and 5′-CCATTGAAGTCTGCGTCTCGA-3′; SMemb, 5′-GAGGAAGCAGAAGAAGAAGCGA-3′ and 5′-TTTCTGTGTCATCGTCGGAGAG-3′; calponin-h1, 5′-CTTCATGGACGGCCTCAAAGA-3′ and 5′-GTAGTTGTGTGCGTGGTGGTT-3′; SM22α, 5′-GGATCATAGTGCAGTGTGGCC-3′ and 5′-GGAGGAGACAGTAGAGGTGATG-3′; h-caldesmon, 5′-AGACAAGGAAAGAGCTGAGGCA-3′ and 5′-GCTGCTTGTTACGTTTCTGCTC-3′; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′. PCR products were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. Amplified DNA was identified by sequencing. The levels of ethidium bromide fluorescence of DNA obtained in every PCR cycle were plotted on a semilogarithmic graph, to determine an appropriate PCR cycle number at which all the samples were plotted within a linear range of the graph. The amounts of DNA were quantified at the cycles thereby determined. Northern blotting was performed as described in Ishida et al. (1997).

Statistics. Results are expressed as the mean ± S.D. of the number of observations. Statistical significance was assessed by Student's *t* test for paired or unpaired values.

Results

15d-PGJ_2 Inhibited VSMC Proliferation. First we examined the effect of 15d-PGJ_2 on VSMC proliferation. When G_0-synchronized cells were stimulated with growth medium, DNA synthesis began about 15 h after the stimulation and the S phase lasted for about 15 h (Sasaguri et al., 1996). Therefore, to examine the effect of 15d-PGJ_2 on DNA synthesis, we measured [^{3}H]thymidine incorporation in the presence of various concentrations of 15d-PGJ_2 for 30 h after mitogenic stimulation (Fig. 1A). 15d-PGJ_2 inhibited DNA synthesis in a dose-dependent manner; the maximal effect was obtained at 12 μM, where the incorporation was reduced to a level below the basal. In contrast, WY14643 (100 μM), an agonist for PPARα, did not inhibit DNA synthesis. The increase in cell number was also strongly suppressed by 15d-PGJ_2 (Fig. 1B). However, proliferation was slowly recovered after 15d-PGJ_2 was removed from the medium.

To determine where the action point of 15d-PGJ_2 is located, the cell cycle distribution was analyzed by flow cytometry (Fig. 1C). When G_0 cells were stimulated with growth

medium for 24 h, the cell populations in S and G_2/M phases increased. However, 15d-PGJ_2 nearly completely inhibited these increases, suggesting that the PG inhibits a process that precedes the entry into S phase.

The antiproliferative effect of 15d-PGJ_2 was unlikely to be caused by cytotoxicity or apoptosis, because 15d-PGJ_2 did not increase the number of detached cells, trypan blue-stained cells, hypodiploid cells in flow cytometry, or ladder-like DNA fragments in agarose gel electrophoresis (data not shown).

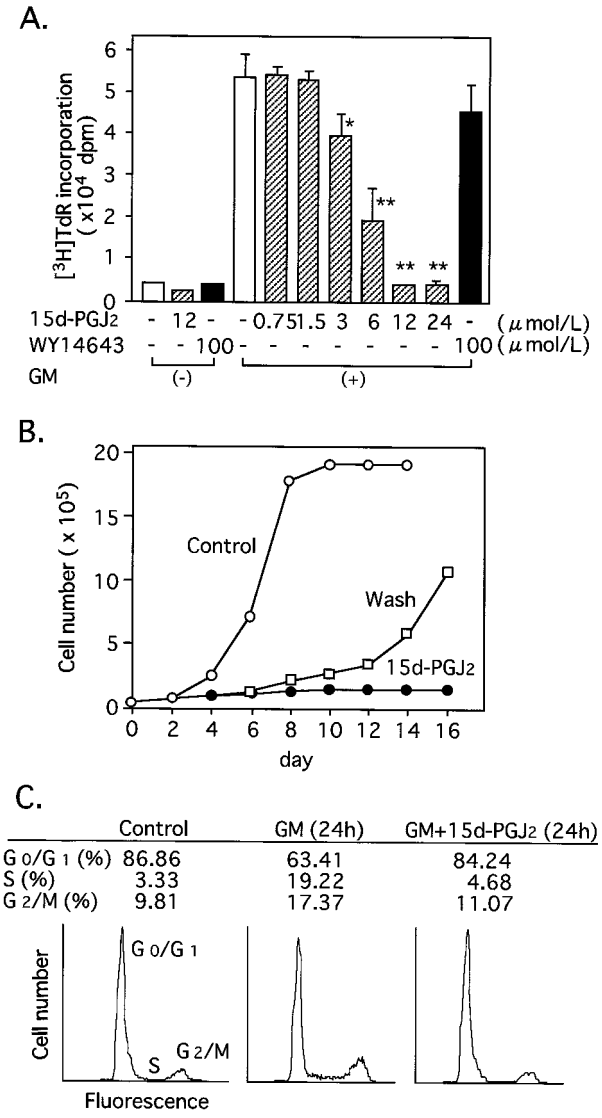

Fig. 1. Effect of 15d-PGJ_2 on VSMC proliferation. A, VSMCs seeded in 24-multiwell plates and synchronized in G_0 phase were stimulated with growth medium (GM) in the presence of [^{3}H]thymidine (37 kBq/ml) and the indicated concentrations of 15d-PGJ_2 or WY14643. Incorporated radioactivities were determined at 30 h after stimulation. Data represent the means ± S.D. of three independent experiments performed in triplicate. *$P < .05$, **$P < .01$ against control. B, VSMCs seeded in 6-well plates (4×10^4/well) were cultured in growth medium. 15d-PGJ_2 (12 μM) was added after 2 days and removed from some of the wells after 4 days. The medium was changed and cells enumerated every 2 days. Demonstrated are representative data of three independent experiments performed in triplicate. ○, growth medium; ●, growth medium + 15d-PGJ_2; □, wash. C, G_0 cells were cultured in GM in the absence or presence of 15d-PGJ_2 (12 μM) for 24 h. Thereafter, they were trypsinized and stained with propidium iodide as described under *Materials and Methods*. The DNA content of 2×10^4 cells was analyzed by flow cytometry. Demonstrated are representative data of three independent experiments. Percentages of the cell number in each phase are also shown.

15d-PGJ_2 Inhibited Cyclin D1 Expression. To determine the mechanism by which 15d-PGJ_2 prevents the entry into S phase, we examined the effect of the PG on cell cycle events that occur between G_0 and S phases. As shown in Fig. 2A, 15d-PGJ_2 nearly completely inhibited the phosphorylation of pRb, which is essential to enter S phase. Mitogenic stimulation for 18 h slowed the mobility of pRb, indicating it was hyperphosphorylated, whereas 15d-PGJ_2 strongly inhibited this shift. We confirmed this interpretation by blotting the precipitates with an antiboby that recognizes only the underphosphorylated form of pRb. No band was detected in cells stimulated with growth medium in the absence of 15d-PGJ_2, whereas a 110-kDa band was clearly detected in cells treated with 15d-PGJ_2. Therefore 15d-PGJ_2 seemed to block an event that preceded pRb phosphorylation.

Then we measured the activities of Cdk4, Cdk6, and Cdk2, which are responsible for pRb phosphorylation. As shown in Fig. 2B, 15d-PGJ_2 strongly suppressed the activities of all these kinases without inhibiting their expressions, suggesting that this PG prevented pRb phosphorylation by inhibiting Cdk activities.

Because Cdk4 and Cdk6 are activated by associating with cyclin D1, and Cdk2 is subsequently activated by cyclin E, we examined the effects of 15d-PGJ_2 on the expression of these G_1 cyclins. As demonstrated in Fig. 3A, 15d-PGJ_2 inhibited the mRNA expression of cyclin D1, which normally increased

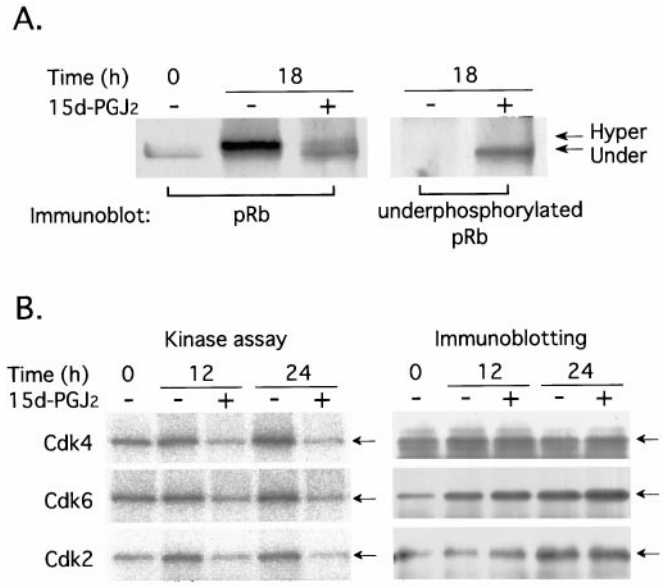

Fig. 2. Effect of 15d-PGJ_2 on pRb phosphorylation and Cdk activities. G_0-synchronized cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM). A, cell lysates prepared at the times indicated were immunoprecipitated by an anti-pRb monoclonal antibody (PharMingen, San Diego, CA) and blotted with another monoclonal antibody to pRb (PharMingen) or an antibody that recognizes only underphosphorylated pRb (PharMingen). The arrows indicate hyperphosphorylated (Hyper) and underphosphorylated (Under) pRb. B, cell lysates prepared at the times indicated were immunoprecipitated by anti-Cdk4, Cdk6 (Santa Cruz Biotechnology), and Cdk2 (Upstate Biotechnology, Lake Placid, NY) polyclonal antibodies. After the kinase reaction using GST-fused murine pRb carboxyl terminal as the substrate, the assay mixtures were fractionated by SDS-PAGE, electroblotted onto a membrane, and visualized by autoradiography. For immunoblotting, the immunoprecipitates were fractionated by SDS-PAGE and blotted with the same antibodies as used for immunoprecipitation.

soon after mitogenic stimulation. The mRNA level of cyclin E, which normally increased from the late G_1 phase, was also suppressed by 15d-PGJ_2. Consistent with the result of Northern blotting, 15d-PGJ_2 also suppressed the expression of cyclin D1 protein (Fig. 3B).

15d-PGJ_2 Inhibited the PI3K/Protein Kinase B (Akt) Signaling Pathway. Cyclin D1 induction is one of the earliest events in G_1 phase. To determine the upstream signals that 15d-PGJ_2 interrupts to prevent cyclin D1 expression, we examined the effect of 15d-PGJ_2 on mitogen-activated protein kinase (MAPK) and PI3K, because these mediators are activated after growth factor receptor stimulation and have been suggested to mediate cyclin D1 expression (Liu et al., 1995; Lavoie et al., 1996; Muise-Helmericks et al., 1998; Gille and Downward, 1999; Takuwa et al., 1999).

15d-PGJ_2 enhanced rather than inhibited mitogen-induced phosphorylation of p44/42^{MAPK} without influencing the protein expression (Fig. 4A). Mitogenic stimulation elevated PI3K activity and the phosphorylation level of Akt, which mediates PI3K signal transduction (Burgering and Coffer, 1995) (Fig. 4, B and C). However, 15d-PGJ_2 strongly suppressed PI3K as did PI3K inhibitors wortmannin and LY294002 (Fig. 4B). This PG also inhibited the phosphorylation of Akt (Fig. 4C). Because wortmannin and LY294002 inhibited cyclin D1 mRNA expression in our cells (Miwa et al., 2000), 15d-PGJ_2 seemed to inhibit cyclin D1 expression at least in part by inhibiting a PI3K/Akt-dependent pathway.

15d-PGJ_2 Induced Cdk Inhibitor p21 Expression. To examine whether there is additional mechanisms for the G_0/G_1 arrest induced by 15d-PGJ_2, we examined the effect of 15d-PGJ_2 on the expression of Cdk inhibitor proteins. The

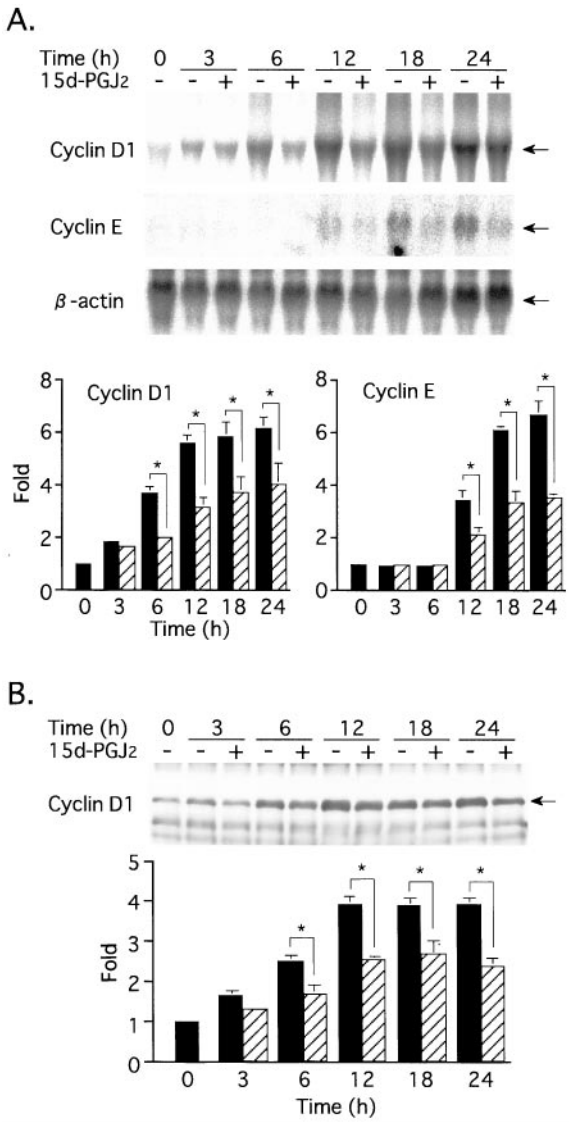

Fig. 3. Effect of 15d-PGJ_2 on the expression of G_1 cyclins. G_0 cells were stimulated with GM in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM). A, total cellular RNAs were extracted at the indicated times. Equal amounts of RNA (10 μg/lane) were fractionated by electrophoresis and hybridized with ^{32}P-labeled cDNA probes for cyclin D1, cyclin E, and β-actin. The cyclin mRNA levels were normalized to those of β-actin and are shown as fold increase against the values obtained at time 0. Data represent the mean ± S.D. ($n = 3$). *$P < .05$. Filled columns, GM; hatched columns, GM + 15d-PGJ_2. B, cell lysates prepared at the times indicated were immunoprecipitated with a polyclonal antibody to cyclin D (Upstate Biotechnology). Proteins were fractionated by SDS-PAGE and blotted with a monoclonal antibody to cyclin D1 (Santa Cruz Biotechnology). The expression levels of cyclin D1 were quantified and are shown as fold increase against the value obtained at time 0. Data represent the mean ± S.D. ($n = 3$). *$P < .05$. Filled columns, GM; hatched columns, GM + 15d-PGJ_2.

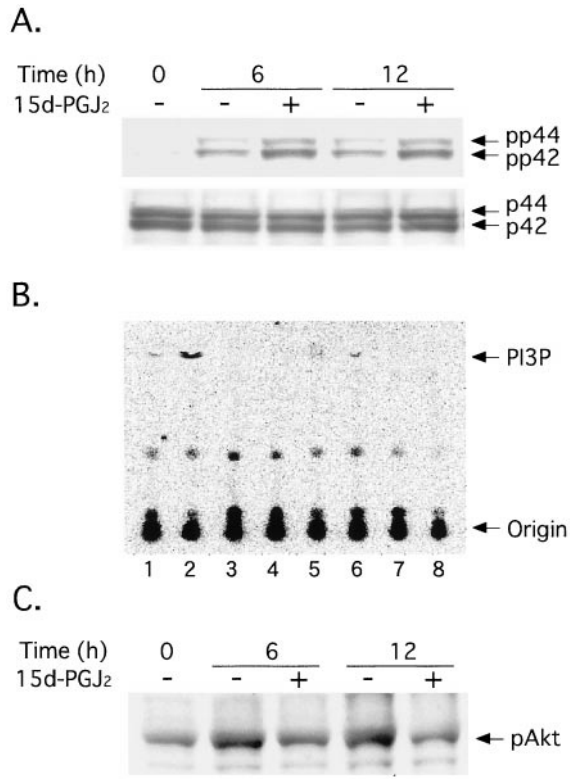

Fig. 4. Effect of 15d-PGJ_2 on the activities of MAPK and PI3K. A, G_0 cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM) for the periods indicated. Cell lysates were fractionated by SDS-PAGE and blotted with polyclonal antibodies to p44/42^{MAPK} (Santa Cruz Biotechnology) and phosphorylated p44/42^{MAPK} (pp44 and pp42) (New England Biolabs, Beverly, MA). B, Equal amounts of proteins obtained from G_0 cells (lane 1) and cells stimulated with growth medium for 6 h (lanes 2–8) were immunoprecipitated with an antiphosphotyrosine antibody (PY20). PI3K activity in the precipitates was determined as described under *Materials and Methods*. 15d-PGJ_2 (12 μM, lanes 3 and 6), wortmannin (300 nM, lanes 4 and 7), or LY294002 (25 μM, lanes 5 and 8) was added to the kinase assay mixture (lanes 3–5) or to living cells simultaneously with mitogenic stimulation (lanes 6–8). C, G_0 cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM) for the periods indicated. Cell lysates were were fractionated by SDS-PAGE and blotted with a polyclonal antibody to phosphorylated Akt (pAkt) (New England Biolabs).

mRNA expression of p21 was down-regulated after the middle of G_1 phase, whereas it was elevated and thereafter sustained until 24 h when 15d-PGJ_2 was added (Fig. 5A). The level of p21 protein changed in parallel to that of the mRNA (Fig. 5B). The expressions of other inhibitors $p27^{Kip1}$, $p57^{Kip2}$, $p16^{Ink4A}$, and $p15^{Ink4B}$ were not increased by 15d-PGJ_2 (Fig. 5C). It was likely, therefore, that p21 is involved in the Cdk inhibition induced by 15d-PGJ_2.

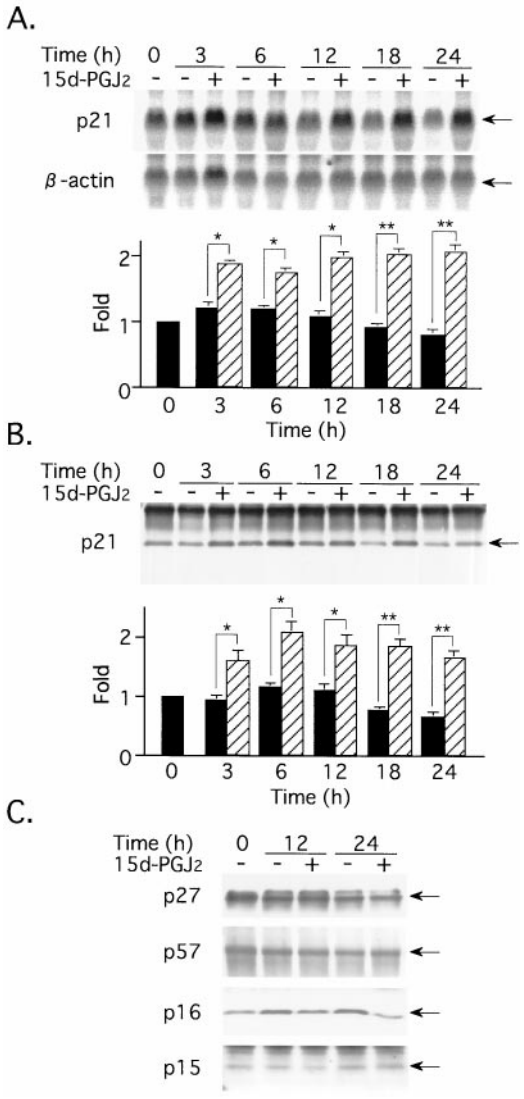

Fig. 5. Effect of 15d-PGJ_2 on the expression of Cdk inhibitors. G_0 cells were stimulated with GM in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM). A, total cellular RNAs were extracted at the times indicated. Equal amounts of RNA (10 μg/lane) were fractionated by electrophoresis and hybridized with ^{32}P-labeled cDNA probes for p21 and β-actin. The mRNA levels of p21 were normalized to those of β-actin and are shown as fold increase against the value obtained at time 0. Data represent the mean ± S.D. ($n = 3$). *$P < .05$, **$P < .01$. Filled columns, GM; hatched columns, GM + 15d-PGJ_2. B, cell lysates prepared at the times indicated were immunoprecipitated with a polyclonal antibody to p21 (Santa Cruz Biotechnology). Proteins were fractionated by SDS-PAGE and blotted with a monoclonal antibody to p21 (PharMingen). The protein levels of p21 were quantified and are shown as fold increase against the value obtained at time 0. Data represent the mean ± S.D. ($n = 3$). *$P < .05$, **$P < .01$. Filled columns, GM; hatched columns, GM + 15d-PGJ_2. C, immunoprecipitates obtained with antibodies (Santa Cruz Biotechnology) to p27 (polyclonal), p57 (goat polyclonal), p16 (monoclonal), and p15 (polyclonal) were fractionated by SDS-PAGE and blotted with antibodies to p27 (monoclonal, PharMingen), p57 (rabbit polyclonal; Santa Cruz Biotechnology), p16 (polyclonal; Santa Cruz Biotechnology), and p15 (same antibody as used for immunoprecipitation), respectively.

Antiproliferative Effect of 15d-PGJ_2 Unlikely To Be Mediated by PPARγ. 15d-PGJ_2 is the most effective activator for PPARγ. To investigate whether PPARγ is involved in the antiproliferative effect of 15d-PGJ_2, we inhibited PPARγ with an antisense oligonucleotide for PPARγ. As shown in Fig. 6A, transfection with the antisense oligonucleotide reduced the protein level of PPARγ, but the antiproliferative effect of 15d-PGJ_2 was not attenuated by the transfection (Fig. 6B), suggesting that the effect of the PG may not be mediated by PPARγ.

15d-PGJ_2 Induced VSMC Differentiation Marker Expression. To explore whether 15d-PGJ_2 regulates VSMC differentiation, we examined the effect of 15d-PGJ_2 on the expression of SM-MHC isoforms SM1 and SM2, well-established markers for VSMC differentiation (Aikawa et al., 1993). The gene expression levels of SM1 and SM2 were determined by RT-PCR using a single pair of PCR primers that cover the sequence specific to SM2, because these two isoforms are produced from a single gene by alternative splicing. SM1 was abundantly expressed in cells synchronized in G_0 phase, but the expression of SM2 was undetectable (Fig. 7A). Mitogenic stimulation decreased the expression level of SM1, whereas 15d-PGJ_2 delayed this reduction. The expression of SM2 was not clearly detected during these 24 h even when the PCR products were amplified by 40 thermal cycles (not shown). 15d-PGJ_2 did not influence the expression of SMemb (also designated MHC-B), a nonmuscle-type MHC.

Next we examined whether a long-term incubation with 15d-PGJ_2 is able to induce SM-MHC isoforms. As shown in Fig. 7B, freshly isolated medial SM cells expressed a large

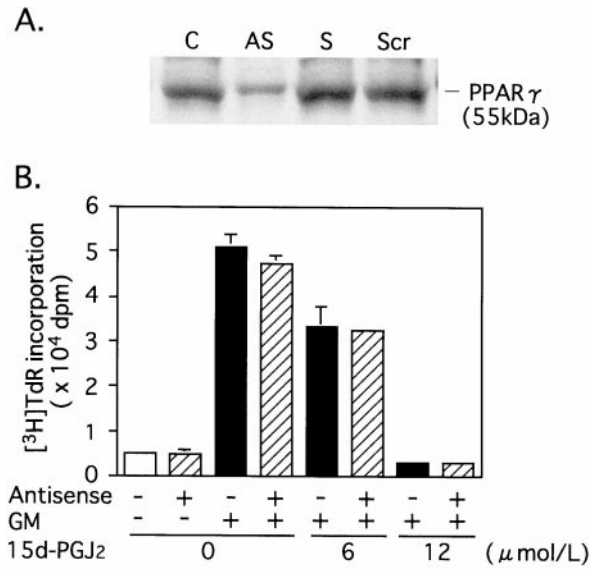

Fig. 6. Effect of PPARγ inhibition on the antiproliferative effect of 15d-PGJ_2. A, G_0 cells were transfected with indicated phosphorothioate oligonucleotides as described under *Materials and Methods*. C, control (vehicle); AS, antisense (5′-ATGGTCATTTATGAGGCCAC-3′); S, sense (5′-GTGGCCGCAGAAATGACCAT-3′); Scr, scramble (5′-ACGTTGCGTCTCGATTCAGC-3′). Whole-cell lysates were fractionated by SDS-PAGE and blotted with a polyclonal antibody to PPARγ (Santa Cruz Biotechnology). B, cells transfected with vehicle and the antisense oligonucleotide were stimulated with growth medium (GM) in the presence of indicated concentrations of 15d-PGJ_2. The levels of [^{3}H]thymidine incorporation for 24 h were determined as described under *Materials and Methods*.

amount of SM2 mRNA predominantly over the amount of SM1 mRNA. In cells in primary culture, SM2 was still expressed, although its level was much less than that in freshly isolated cells. However, subcultured cells seemed to be completely dedifferentiated to the synthetic phenotype, because they expressed a small amount of SM1 and no SM2. When cells were cultured in the presence of 15d-PGJ_2 for 24 days, the level of SM1 was markedly elevated during the first 14 days and thereafter sustained; moreover, the expression level of SM2 also gradually elevated from day 14 to 24. In contrast, the level of SMemb was reduced soon after addition of 15d-PGJ_2 and thereafter sustained.

We also examined the effect of 15d-PGJ_2 on the expression of calponin-h1, a myofibrillar thin filament actin-binding protein expressed exclusively in smooth muscle cells (Samaha et al., 1996) (Fig. 7B). In parallel with the expression of SM1 and SM2, the mRNA level of calponin-h1 was markedly elevated by 15d-PGJ_2 during the first 14 days and thereafter sustained. And moreover, 15d-PGJ_2 also up-regulated the expression of SM α-actin (Fig. 7C). However, SM22α and h-caldesmon, other smooth muscle markers, were already expressed in subcultured cells and they were no longer elevated after addition of the PG (Fig. 7B).

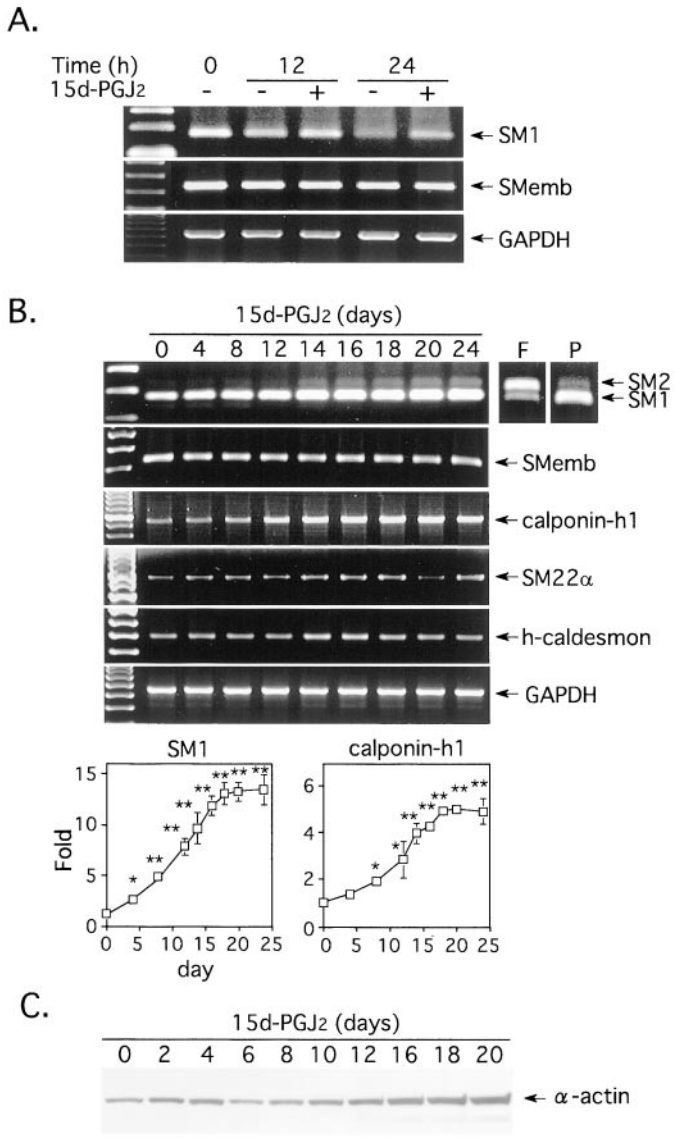

Fig. 7. Effect of 15d-PGJ_2 on VSMC differentiation marker expression. A, cells synchronized in G_0 were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μM). Total cellular RNAs extracted at the indicated times were analyzed for SM1/2, SMemb, and GAPDH by RT-PCR. PCR cycle numbers were 32, 32, and 24, respectively. The arrows indicate the PCR products for SM1 [178 base pairs (bp)], SMemb (226 bp), and GAPDH (452 bp). B, exponentially growing cells were incubated in growth medium in the presence of 15d-PGJ_2 (12 μM) for the periods indicated. The medium was replaced with fresh one containing the PG every 2 days. RT-PCR was performed for SM1/2, SMemb, calponin-h1, SM22α, h-caldesmon, and GAPDH. PCR cycle numbers were 39, 32, 32, 32, 32, and 24, respectively. The levels of SM1 and calponin-h1 were normalized to those of GAPDH and are plotted as fold increase ($n = 3$). $^*P < .05$, $^{**}P < .01$. The arrows indicate the PCR products for SM1, SM2 (217 bp), SMemb, calponin-h1 (713 bp), SM22α (764 bp), h-caldesmon (395 bp), and GAPDH. For SM-MHCs, RNAs extracted from freshly isolated medial SM cells (F) and those from cells in primary culture (P) also were analyzed. C, cells were treated as described in B. Cell lysates (0.5 μg of protein) were fractionated by SDS-PAGE and blotted with an anti-SM α-actin polyclonal antibody (Boehringer Mannheim, Mannheim, Germany).

Discussion

15d-PGJ_2 strongly inhibited VSMC proliferation like the other PGs of the J_2 family (Sasaguri et al., 1992). This effect seemed to be mediated by at least two negative growth signals: the inhibition of cyclin D1 expression and the induction of p21. Cdk4, Cdk6, and Cdk2, which are responsible for the phosphorylation of pRb, are regulated positively by G_1 cyclins such as cyclin D1 and cyclin E and negatively by Cdk inhibitors such as p21. When hyperphosphorylated, pRb releases transcription factors of the E2F family, which initiate DNA synthesis. Consistent with this scenario, 15d-PGJ_2 inhibited the activities of Cdk4, Cdk6, and Cdk2, the phosphorylation of pRb, and DNA synthesis.

The action of 15d-PGJ_2 is unique among antiproliferative substances studied so far. p21 is induced by several physiological stimuli that inhibit cell proliferation (Gartel et al., 1996), such as nerve growth factor, transforming growth factor-β, interferons, 1,25-dihydroxyvitamin D_3, retinoids, PGA_2, nitric oxide (Ishida et al., 1997, 1999), and fluid shear stress loaded on vascular endothelial cells (Akimoto et al., 2000). However, substances that inhibit cyclin D1 expression are rare (Miwa et al., 2000), let alone those that also induce p21.

The mitogen-activated protein kinase (MAPK) cascade (Liu et al., 1995; Lavoie et al., 1996) and the PI3K pathway (Gille and Downward, 1999; Takuwa et al., 1999) have been suggested to mediate the gene expression of cyclin D1. In fact, the MAPK kinase inhibitor PD98059 and PI3K inhibitors wortmannin and LY294002 all inhibited cyclin D1 expression and DNA synthesis in our cells (data not shown). Therefore, we examined the effect of 15d-PGJ_2 on these two signaling pathways. However, MAPK was unlikely to be involved, because 15d-PGJ_2 stimulated rather than inhibited $p44/42^{MAPK}$ phosphorylation induced by mitogenic stimulation, although the possibility remains that the PG interrupts the MAPK-dependent pathway downstream of its phosphorylation by MAPK kinase. On the other hand, recent evidence suggests that $p44/42^{MAPK}$ can mediate antiproliferative effect (Bornfeldt et al., 1997; Herrera et al., 1998). However, the enhancement of $p44/42^{MAPK}$ phosphorylation induced by 15d-PGJ_2 was unlikely to be involved in the antiproliferative effect, because PD98059 did not influence the PG-induced

inhibition of DNA synthesis (not shown). In contrast, however, 15d-PGJ_2 strongly suppressed the activity of PI3K and the phosphorylation of Akt kinase. Therefore, 15d-PGJ_2 seemed to prevent cyclin D1 expression at least in part by inhibiting a PI3K/Akt-dependent pathway. A post-transcriptional mechanism could also contribute to the suppression of cyclin D1 expression by 15d-PGJ_2, because not only the transcription but also the translation of cyclin D mRNA has been reported to be enhanced by a PI3K/Akt-dependent pathway (Muise-Helmericks et al., 1998).

PPARγ agonists, such as the thiazolidinedione class of antidiabetic drugs and 15d-PGJ_2, modulate several atherogenic processes. They inhibit macrophage activation by inhibiting the induction of nitric oxide synthase gene (Ricote et al., 1998), the production of inflammatory cytokines (Jiang et al., 1998), and the activation of matrix metalloproteinases (Marx et al., 1998b; Ricote et al., 1998). PPARγ stimulated by the lipid components of oxidized low-density lipoprotein promotes macrophage differentiation by up-regulating scavenger receptor CD36 (Nagy et al., 1998; Tontonoz et al., 1998). Troglitazone inhibits VSMC proliferation and intimal hyperplasia (Law et al., 1996). PPARγ agonists inhibit matrix metalloproteinase-9 expression and platelet-derived growth factor-BB-induced migration in VSMCs (Marx et al., 1998a). Moreover, PPARγ ligands inhibit vascular cell adhesion molecule-1 expression in vascular endothelial cells (Jackson et al., 1999).

Our VSMCs expressed PPARγ consistent with the results of others (Marx et al., 1998a). However, it was unlikely that the antiproliferative effect of 15d-PGJ_2 was mediated by PPARγ, because the antisense oligonucleotide for PPARγ had no influence on the effect of 15d-PGJ_2. This interpretation is in agreement with the fact that PGA_2, which is also a cyclopentenone PG but is much less potent in the ability to stimulate PPARγ, inhibited VSMC proliferation as effectively as 15d-PGJ_2 (Sasaguri et al., 1992). Moreover, the antiproliferative effect of troglitazone was small compared with that of 15d-PGJ_2 (data not shown). Recently, it has been suggested that some of the biological effects of 15d-PGJ_2 are mediated by PPARγ-independent mechanisms. 15d-PGJ_2 inhibits H_2O_2 production in neutrophils (Vaidya et al., 1999) and inducible nitric oxide synthase expression in microglia (Petrova et al., 1999), both independently of PPARγ. More recently, the inhibition of nuclear factor-κB by cyclopentenone PGs such as PGA_1 and 15d-PGJ_2 has been shown to be based on the direct inhibition of IκB kinase (Rossi et al., 2000).

15d-PGJ_2 seemed to interrupt a very early stage of the cell cycle, because the induction of cyclin D1 expression is one of the earliest events after mitogenic stimulation. Considering that cell differentiation may be induced after an exit from the cell cycle, this nature of 15d-PGJ_2 led us to speculate that this substance could induce VSMC differentiation. In skeletal myoblasts, a forced expression of cyclin D1 inhibits the ability of MyoD to transactivate muscle-specific genes (Skapek et al., 1995). This effect may be caused by the cyclin D1-induced nuclear translocation of Cdk4 and the subsequent direct interaction of MyoD with Cdk4 (Zhang et al., 1999). These studies suggest that the expression level of cyclin D1 regulates cell differentiation. Furthermore, p21 also has been suggested to be involved in the process of differentiation in some cell species (Gartel et al., 1996). Therefore, we explored whether 15d-PGJ_2 promotes VSMC differentiation.

Several genes for contraction-related proteins are sequentially expressed in developing VSMCs, including SM α-actin, SM22α, h-caldesmon, calponin-h1, and SM-MHC isoforms SM1 and SM2 (Owens, 1995). SM-MHC isoforms may be the most established markers for VSMC differentiation, because the pattern of their expression in different phenotypes has been well examined (Aikawa et al., 1993). SM1 is abundant and SM2 is exclusively expressed in mature VSMCs, whereas SM1 is diminished and SM2 is undetectable in immature cells. Fetal systemic VSMCs are biochemically and functionally immature, however VSMCs of umbilical arteries display a mature phenotype resembling adult systemic VSMCs (Arens et al., 1998). Indeed, medial smooth muscle cells freshly isolated from umbilical arteries abundantly expressed SM2.

In our study, 15d-PGJ_2 reduced the rate of the reduction of SM1 when added to G_0 cells simultaneously with mitogens, and it markedly elevated the level of SM1 and induced SM2 expression when added to exponentially growing cells. VSMCs once dedifferentiated after vascular injury again express SM2 in the process of redifferentiation in vivo (Aikawa et al., 1997). In general, however, it is difficult to induce the expression of SM2 in cultured VSMCs. The level of SM2 expression achieved by the treatment with 15d-PGJ_2 was comparable with that in cells in primary culture; however, it was much lower than the level in freshly isolated medial cells. It takes more than 5 months to recover SM2 expression in once dedifferentiated cells in vivo (Aikawa et al., 1997). Therefore, 15d-PGJ_2 may spend much more time to achieve full recovery of SM2 expression, or some additional factors that exist in vascular wall may be required for full differentiation. In rat VSMCs, cyclic mechanical strain has been shown to induce SM1 and SM2 expression (Reusch et al., 1996), overexpression of cGMP-dependent protein kinase up-regulates SM2 and calponin (Boerth et al., 1997), and gene transfer of C-type natriuretic peptide induces G_1 arrest and SM2 expression (Doi et al., 1997). Recently, we showed that differentiation-inducing factor-1, a morphogen of *Dictyostelium discoideum*, induces SM1 and SM2 in human VSMCs (Miwa et al., 2000). However, the present study is one of the earliest reports to demonstrate that a physiological substance is able to induce SM2 in cultured VSMCs. The expression levels of calponin-h1 and SM α-actin also increased in parallel with SM-MHC expression. Therefore, 15d-PGJ_2 may be able to induce redifferentiation of once dedifferentiated cells.

SM22α and h-caldesmon, which are first detectable in developing VSMCs during embryogenesis (Owens, 1995), were already expressed in exponentially growing cells and the expressions were no longer elevated by incubation with 15d-PGJ_2. SM1 is expressed in fetal arteries of the early gestational stage, whereas the expression of SM2 is up-regulated during late fetal and postnatal development (Aikawa et al., 1993). Therefore, our subcultured cells may be dedifferentiated to a fetal stage, but they may not be so immature as they lose the expressions of SM22α, h-caldesmon, and SM1.

In parallel studies, we obtained evidence that endothelial cells produce 15d-PGJ_2 in response to blood flow (Taba et al., 2000) and that 15d-PGJ_2 inhibits endothelial cell apoptosis (Y. Taba and T. Sasaguri, unpublished observations). There-

fore, we hypothesize that 15d-PGJ_2 produced by endothelial cells maintains vascular homeostasis by functioning as an antiatherogenic factor (i.e., by inhibiting activation and promoting differentiation in macrophages, inhibiting adhesion molecule expression and preventing apoptosis in endothelial cells, and inhibiting proliferation and migration and inducing differentiation in VSMCs). Moreover, supposing that 15d-PGJ_2 acts in the same way in vivo as it does in vitro, this PG or other substances of a similar nature may provide novel preventive and therapeutic strategies for the treatment of vascular diseases.

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Send reprint requests to: Toshiyuki Sasaguri, M.D., Ph.D., Department of Bioscience, National Cardiovascular Center Research Institute, 5-7-1 Fujishiro-dai, Suita, Osaka 565-8565, Japan. E-mail: sasaguri@ri.ncvc.go.jp