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Involvement of clusterin in 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2-induced vascular smooth muscle cell differentiation☆

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Abstract

To establish an in vitro model of vascular smooth muscle cell (VSMC) differentiation, we examined the effect of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on the expression of VSMC differentiation markers. After the addition of 15d-PGJ_2 to confluent human umbilical artery smooth muscle cells synchronized in the G_0 phase, cells showed a "hill and valley" appearance and thereafter aggregated and formed macroscopic nodules. Cells forming nodules expressed high levels of SM2, the most specific VSMC differentiation marker, comparable to medial VSMCs in vivo. 15d-PGJ_2 significantly increased the mRNA and protein expression levels of clusterin, a secreted glycoprotein reported to induce nodule formation and differentiation of VSMCs. Moreover, addition of an anti-clusterin antibody completely inhibited the nodule formation induced by 15d-PGJ_2 and induced apoptosis. Our results suggested that clusterin is involved in 15d-PGJ_2-induced nodule formation and cell differentiation in VSMCs.

The dedifferentiation and subsequent abnormal proliferation of vascular smooth muscle cells (VSMCs) have been thought to play important roles in vascular remodeling. In normal arteries, VSMCs reside in the media in non-proliferating and fully differentiated states. However, once endothelial cell function is disturbed, VSMCs dedifferentiate, change phenotype to synthetic one, migrate into the intima, and proliferate [1]. These processes initiated by dedifferentiation are known to be promoted by a number of growth factors, cytokines, and vasoactive substances. In contrast, the molecular mechanism of VSMC redifferentiation remains largely unknown, at least in part, because of the lack of an established in vitro model for VSMC differentiation.

15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a potent endogenous ligand for peroxisome proliferator-activated receptor-γ (PPAR-γ), is known to promote the differentiation of macrophages by stimulating the expression of the scavenger receptor CD36 through PPAR-γ [2,3]. This PG also exerts several anti-inflammatory effects on macrophages such as the inhibition of inducible nitric oxide synthase expression [4], inhibition of inflammatory cytokine production [5], and inhibition of matrix metalloproteinase activity [6]. We reported that the lipocalin-type PGD synthase (L-PGDS), which converts PGH_2 to PGD_2, the precursor of 15d-PGJ_2, is expressed in vascular endothelial cells and intimal VSMCs of the human aorta and that PGD_2 and 15d-PGJ_2 are actually released by a laminar shear stress of physiological strength in endothelial cells [7]. These results suggest that 15d-PGJ_2 produced in the vascular wall plays an important role in the regulation of vascular cell functions.

We also reported that 15d-PGJ_2 induces the expression of VSMC-specific differentiation markers [8]. Differentiated VSMCs express large amounts of contractile proteins such as smooth muscle α-actin (SM α-actin), calponin-h1, h-caldesmon, and the smooth muscle myosin heavy chain (SM-MHC) isoforms SM1 and SM2. Expression levels of these proteins decrease in cells with the dedifferentiated synthetic phenotype [9]. 15d-PGJ_2 simply added to growing VSMCs significantly increased the expression of SM2, a most

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reliable VSMC differentiation marker expressed at the last stage of differentiation, however, its expression level was much lower than that in fully differentiated cells localized in the vascular media, suggesting that the stage of differentiation was not very late.

In the present study, we further investigated the effect of 15d-PGJ_2 on VSMC differentiation in different culture conditions by monitoring the expression of VSMC-specific differentiation markers, especially SM2. In this process, we found that clusterin, a secreted glycoprotein reported to induce the aggregation and differentiation of VSMCs, is involved in 15d-PGJ_2-induced VSMC differentiation.

Materials and methods

Chemicals. 15d-PGJ_2 was purchased from Cayman Chemical (Ann Arbor, MI).

Cell culture. VSMCs were isolated from the media of human umbilical arteries by explant. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 20% (vol/vol) fetal bovine serum (FBS) (Life Technologies, Rockville, MD), 5 ng/mL 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.

Reverse transcription-polymerase chain reaction. Reverse transcription-polymerase chain reaction (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 for SM1 and SM2, SMemb, calponin-h1, h-caldesmon, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were described previously [8]. PCR primers for clusterin synthesized based on a sequence in the GenBank database were 5′-AGAGTGTAAGCCCTGCCTGAA-3′ and 5′-TGGGAAA GAAGAAGTGAGGCC-3′. PCR products were electrophoresed on 2% agarose gel and visualized by staining with ethidium bromide. Amplified DNAs were identified by sequencing. The levels of ethidium bromide fluorescence of DNAs 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 DNAs were quantified at the number of cycles thereby determined.

Western blotting. Western blot analysis was performed as described [10] using a goat polyclonal antibody to human clusterin-β chain (sc-6419, Santa Cruz Biotechnology).

Nuclear staining. To detect nuclear fragmentation, both detached and adherent cells were harvested. Cells were fixed with 100 μL of 1% (vol/vol) glutaraldehyde for 30 min and stained with 4 μL of 1 mmol/L Hoechst 33258. The number of apoptotic cells was determined by observation of 1000 cells with a fluorescent microscope.

Statistics. Results are expressed as means ± SD of the number of observations. Statistical significance was assessed with Student's *t* test for paired or unpaired values.

Results

15d-PGJ_2-induced morphological changes in VSMCs

We previously reported that 15d-PGJ_2 induces the expression of differentiation markers for VSMCs, however, the expression level of SM2, a most reliable marker of VSMC differentiation, was much lower than that in freshly isolated (fully differentiated) VSMC [8]. To better induce the differentiation, we cultured VSMCs until they were in a confluent state and synchronized them in G_0 phase by 48 h serum starvation, since the expression of smooth muscle-specific differentiation markers such as SM-MHC and SM α-actin has been reported to increase in cells confluence and quiescence [11]. Thereafter we incubated the cells with 15d-PGJ_2. After incubation with 15d-PGJ_2 for 2 days, the cells exhibited a "hill and valley" appearance and aggregated toward the center of the dishes (Fig. 1A). They formed macroscopic nodules after incubation with 15d-PGJ_2 for 4 days (Figs. 1B and C).

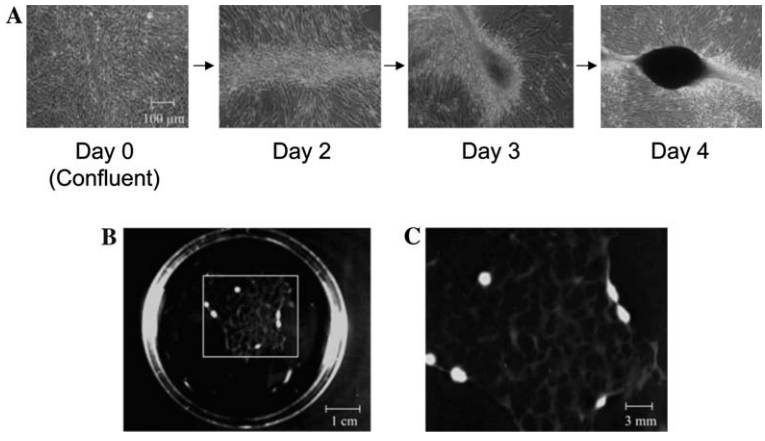

Fig. 1. 15d-PGJ_2-induced morphological change of VSMCs. (A) VSMCs were seeded in 60 mm plates and incubated until confluent. Then they were synchronized in G_0 phase by serum starvation for 48 h and stimulated with growth medium in the presence of 15d-PGJ_2 (12 μmol/L). Cells gradually showed a "hill and valley" appearance, aggregated, and formed nodules 4 days after stimulation. (B) Macro view. (C) Magnified view of the area surrounded by a square in (B).

Expression of differentiation markers

To clarify the meaning of the morphological changes in VSMCs cultured with 15d-PGJ_2, we examined the expression levels of VSMC-specific differentiation markers in adherent cells (A), cells contained in the nodules (N), and freshly isolated medial cells (F). Fig. 2 shows that SM-MHC (SM1 and SM2), calponin-h1, and h-caldesmon are expressed in all three types of VSMCs. In adherent cells cultured with 15d-PGJ_2, the mRNA levels of SM1 and SM2 slowly increased, whereas cells in nodules expressed a large amount of SM2, as much as freshly isolated VSMCs. The expression of SMemb, a non-muscle myosin heavy chain which increases in dedifferentiated VSMCs, decreased immediately after the treatment with 15d-PGJ_2 in both adherent cells and cells in nodules. The expression of h-caldesmon was significantly enhanced in cells in nodules, however, there was no difference in calponin-h1 levels between adherent cells and cells in nodules.

Effect of 15d-PGJ_2 on clusterin expression

Previous reports suggested that clusterin, a ubiquitous cell membrane protein, induces VSMC aggregation and differentiation [12–14]. Therefore, we examined whether clusterin is involved in the 15d-PGJ_2-induced formation of nodules and differentiation in VSMCs. The addition of 15d-PGJ_2 to exponentially growing cells dose-dependently increased the mRNA level of clusterin (Fig. 3A). 15d-PGJ_2 (12 μmol/L) induced clusterin mRNA 6 h after stimulation (Fig. 3B, upper panel). As shown in the lower panel, confluent and G_0-synchronized cells also expressed a large amount of clusterin. The expression rapidly decreased after mitogenic stimulation. However, 15d-PGJ_2 (12 μmol/L) maintained the mRNA expression of clusterin. The addition of 15d-PGJ_2 to exponentially growing cells dose- and time-dependently induced the protein expression of clusterin consistent with the mRNA expression (Fig. 3C).

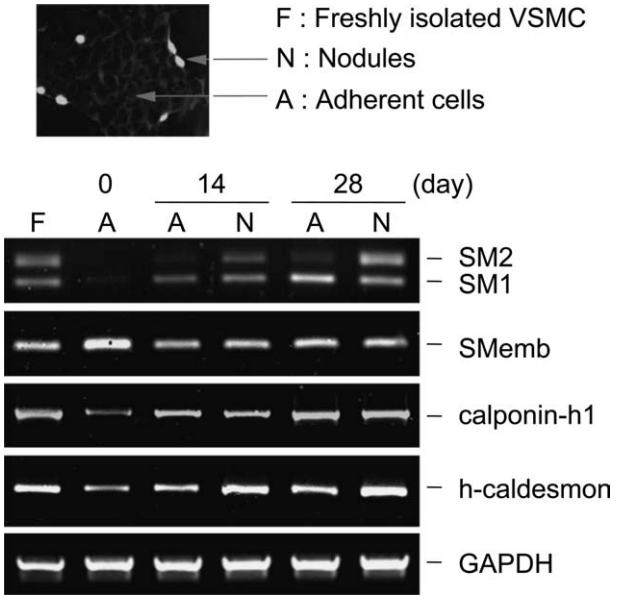

Fig. 2. The expression of VSMC-specific differentiation markers. G_0-synchronized cells in a confluent condition were incubated in growth medium in the presence of 15d-PGJ_2 (12 μmol/L) for the periods indicated. The medium was replaced every 2 days. Total cellular RNA extracted from the adherent cells (A), and cells in nodules (N) at the times indicated was analyzed for SM1/2, SMemb, calponin h1, h-caldesmon, and GAPDH by RT-PCR (32 cycles). The expression in freshly isolated VSMCs in vascular media (F) was also analyzed.

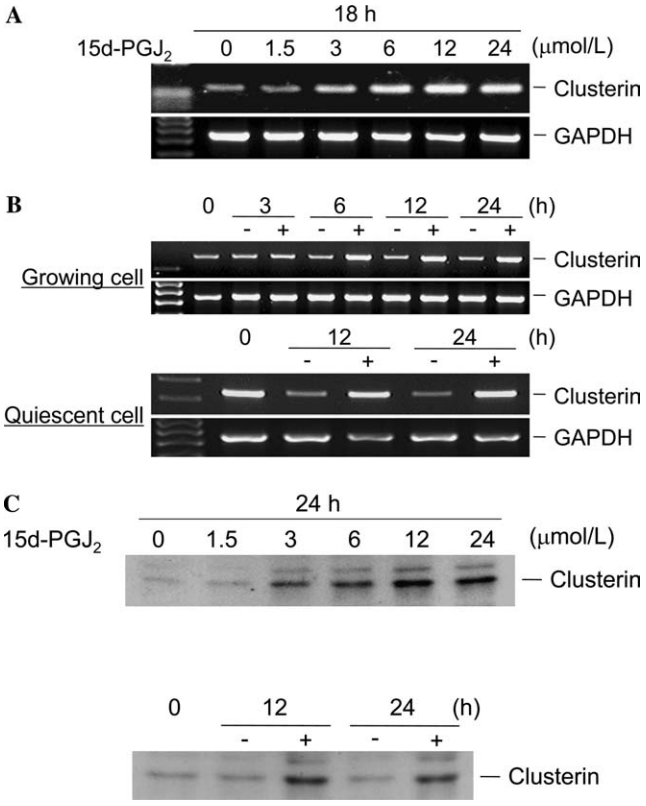

Fig. 3. The effects of 15d-PGJ_2 on the expression of clusterin. (A) Exponentially growing cells were stimulated with growth medium in the presence of the indicated concentration of 15d-PGJ_2. Total cellular RNA extracted 18 h after stimulation was analyzed for clusterin and GAPDH by RT-PCR. (B) Upper panel, exponentially growing cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μmol/L). Lower panel, G_0-synchronized cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μmol/L). Total cellular RNA extracted at the indicated times after stimulation was analyzed for clusterin and GAPDH by RT-PCR. (C) Proteins (10 μg/lane) were fractionated by SDS–PAGE and blotted with a goat polyclonal antibody to human clusterin (sc-6419, Santa Cruz Biotechnology). Upper panel, exponentially growing cells were stimulated with growth medium in the presence of the indicated concentration of 15d-PGJ_2 for 24 h. Lower panel, exponentially growing cells were stimulated with growth medium in the absence (−) or presence (+) of 15d-PGJ_2 (12 μmol/L).

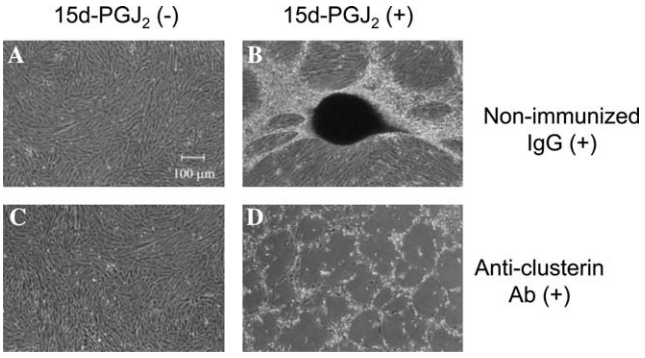

Fig. 4. The effect of clusterin antibody on VSMC aggregation. G_0-synchronized cells were stimulated with growth medium in the absence (−) (A,C) or presence (+) (B,D) of 15d-PGJ_2 (12 μmol/L) for 4 days. The medium was changed every 2 days. A non-immunized IgG (40 μg/mL) (A,B) or a monoclonal anti-clusterin antibody (40 μg/mL) (05-354, Upstate Biotechnology) (C,D) was added simultaneously with growth stimulation.

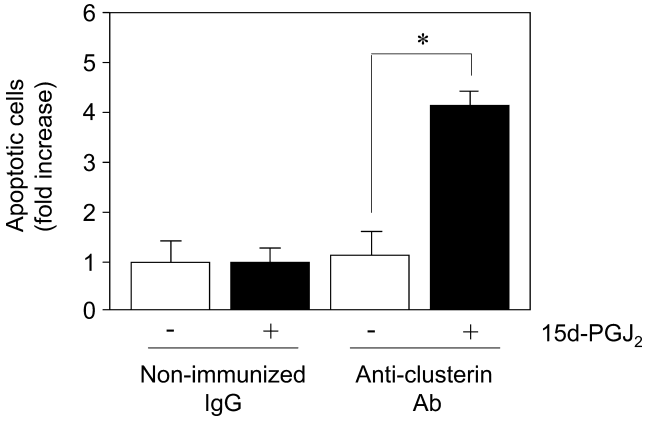

Fig. 5. The effect of clusterin antibody on VSMC apoptosis. G_0-synchronized cells were stimulated for 4 days as described in Fig. 1. After treatment, cells were stained with Hoechst 33258. The numbers of apoptotic cells are shown as a fold-increase against the value obtained in cells treated with non-immunized IgG in the absence of 15d-PGJ2 (12 μmol/L). $*P < 0.01$ ($n = 3$). Open columns, in the absence of 15d-PGJ_2; closed columns, in the presence of 15d-PGJ_2.

Anti-clusterin antibody completely inhibited VSMC aggregation

To examine whether the expression of clusterin induced by 15d-PGJ_2 is necessary for VSMC aggregation, we investigated the effect of a monoclonal anti-clusterin antibody (05-354, Upstate Biotechnology), which has been reported to inhibit clusterin function [15]. In the absence of 15d-PGJ_2, the addition of the anti-clusterin antibody (40 μg/mL) resulted in no visible changes (Fig. 4C) compared to cells treated with non-immunized IgG (Fig. 4A), whereas, in the presence of 15d-PGJ_2, the anti-clusterin antibody completely inhibited VSMC aggregation and nodule formation, and most of the cells were detached from the plates (Fig. 4D). We also examined the effect of the anti-clusterin antibody on apoptosis by nuclear staining of VSMCs with Hoechst 33258, since clusterin plays an important role in the regulation of apoptosis. The ratio of cells undergoing apoptosis was significantly increased in 15d-PGJ_2-treated cells in the presence of anti-clusterin antibody as compared with non-immunized IgG (Fig. 5).

Discussion

In the present study, we showed that 15d-PGJ_2 induces aggregation and nodule formation in VSMCs. The VSMCs in nodules expressed comparable levels of SM2 mRNA to those existing in vascular media, suggesting that the VSMCs in nodules were highly redifferentiated.

Several genes for contraction-related proteins including smooth muscle α-actin, SM22α, calponin, h-caldesmon, and the SM-MHC isoforms SM1 and SM2 are sequentially expressed in developing VSMCs [9]. Among them, SM2 emerges at the last stage of VSMC differentiation and its expression patterns in VSMCs with different phenotypes have been well examined [16–18]. Once they have dedifferentiated after vascular injury, VSMCs again express SM2 in the process of redifferentiation in vivo [19]. We used VSMCs explanted from umbilical artery in the present study, however, recent evidence showed that VSMCs of umbilical arteries display a mature phenotype resembling adult systemic VSMCs [20].

Some investigators have reported in vitro models of VSMC differentiation. Haller et al. [21] reported that PKC-α overexpression induces prosuction of α-SM actin. Cyclic mechanical strain [22] and gene transfer of C-type natriuretic peptide [23] have also been reported to upregulate SM2 expression in cultured rat VSMCs. However, none of these studies compared the expression levels of differentiation markers between cultured VSMCs and fully differentiated VSMCs in the media. We previously reported that 15d-PGJ_2 and the differentiation-inducing factor-1, a morphogen of *Dictyostelium*, induce SM2 [8,24] but the expression levels were quite low in comparison to fully differentiated VSMCs in the media. In the present study, VSMCs in nodules formed after 15d-PGJ_2 treatment expressed a comparable level of SM2 to those existing in vascular media. This may be the first report to demonstrate apparently complete redifferentiation in once dedifferentiated VSMCs in culture.

The nodule formation of VSMCs has been reported in porcine VSMCs [25,26]. Millis et al. focused on these phenomena and revealed that clusterin (also called complement cytolysis inhibitor, sulfated glycoprotein-2, testosterone-repressed prostate message-2, SP-40,40, and apolipoprotein J), an 80-kDa heterodimeric glycoprotein,

is secreted when cultured VSMCs change from a monolayer to nodular morphology [12]. They also recently reported that the lack of this molecule decreased the ability of cells to form nodules and furthermore, exogenous clusterin recovered this ability, suggesting that clusterin regulates VSMC differentiation [13,14]. In the present study, 15d-PGJ_2 increased the clusterin expression and furthermore, 15d-PGJ_2-induced aggregation and nodule formation of VSMCs were completely inhibited by the addition of a neutralizing antibody to clusterin, suggesting that these morphological changes of VSMCs induced by 15d-PGJ_2 were mediated by clusterin.

Recent reports suggested that clusterin also plays an important role in the regulation of apoptosis. Clusterin overexpression inhibits apoptosis in human renal cell carcinoma [27]. An antisense oligonucleotide of the clusterin gene enhances chemotherapy-induced apoptosis in cancer cells of prostate [28,29], bladder [30], and kidney [31]. Apoptotic cell debris has been reported to induce clusterin expression in fibroblasts [32]. Our result that an anti-clusterin antibody increased the number of apoptotic cells supports the previous reports. The aggregation and subsequent redifferentiation of VSMCs induced by clusterin may be one of the cytoprotective mechanisms against apoptosis.

Interestingly, the expression of clusterin is induced by physiological levels of laminar shear stress in vascular endothelial cells as well as L-PGDS, a rate-limiting enzyme upstream of 15d-PGJ_2 production [33]. This suggests that hemodynamic changes modulate the secretion of clusterin from vascular walls, at least in part, through 15d-PGJ_2. A recent clinical study reported that L-PGDS concentrations in both serum and urine increase in patients with essential hypertension [34]. From these results, the L-PGDS/15d-PGJ_2/clusterin pathway initiated by blood flow may play an important role in vascular remodeling.

In conclusion, we showed for the first time that 15d-PGJ_2 induces almost complete redifferentiation in cultured human VSMCs by constructing macroscopic nodules. This process may be promoted by aggregation through 15d-PGJ_2-induced clusterin expression.

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