Synergistic effects of PDGF-BB and cAMP-elevating agents on expression of connexin43 in mesangial cells

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Submitted 1 April 2005; accepted in final form 26 October 2005


The gap junction plays an important role in the regulation of cell growth, migration, and differentiation. Platelet-derived growth factor (PDGF) is reported to be a potent inhibitor of gap junctional intercellular communication (GJIC). Short-term exposure of cells to PDGF causes rapid and transient disruption of GJIC without altering connexin43 (Cx43) protein level. In this study, we investigated long-term effects of PDGF-BB on Cx43 expression in mesangial cells (MCs). Exposure of MCs to PDGF-BB affected neither the Cx43 protein level nor GJIC. However, in the presence of cAMP-elevating agents, PDGF-BB dramatically increased the expression of Cx43, which was accompanied by obviously augmented membrane distribution of Cx43 and functional GJIC. The increased expression of Cx43 was closely correlated with reduction in α-actin, a dedifferentiation marker of MCs. The effect of PDGF on Cx43 was largely prevented by inhibitors of phosphatidylinositol 3'-kinase or mitogen-activated protein kinase, but not by inhibition of protein kinase C. Exposure of MCs to PDGF-BB caused elevation in intracellular cAMP, and it was abolished by indomethacin, a cyclooxygenase inhibitor. However, indomethacin did not affect the synergistic effect. In addition, PDGF-BB also did not affect the degradation of Cx43. With the use of MCs transfected with a Cx43 promoter-luciferase vector, cooperative activation of Cx43 promoter by PDGF and cAMP was found. Together, our data reveal, for the first time, unexpected synergy between PDGF-BB and cAMP-elevating agents in the induction of Cx43 and MC differentiation. Regulation of GJIC could be an important mechanism by which PDGF modulates MC phenotypes.

Among them, connexin43 (Cx43) is predominantly expressed in a variety of cell types (19, 32).

Glomerular mesangial cells (MCs) are the specialized smooth muscle cells (SMCs) that play a pivotal role in the regulation of glomerular hemodynamics. One of the striking features of MCs is that these cells possess an extremely high density of GJs (3, 30). Like SMCs, Cx43 is considered to be the predominant GJ protein in MCs (3). GJs in MCs are critically involved in the transmission of intracellular signals and in the coordination of MC contraction (12, 39). GJs also may participate in the control of other MC behavior. For example, the critical roles of GJs in cell growth, migration, and differentiation have been extensively documented (9, 16, 19, 25, 32). In addition, the dysfunction of GJs has been considered as an important factor in the initiation and progression of vascular pathologies (8, 28, 32, 33).

Among cytokines and growth factors implicated in the regulation of MC functions, platelet-derived growth factor (PDGF) is one of important molecules. In vivo studies using mutant mice deficient in PDGF-BB or its receptor have demonstrated a crucial and indispensable role of PDGF-BB in the development of the mesangium (20, 35). Systemic administration of PDGF-BB or local transfection with a PDGF-BB gene results in mesangial proliferation without other renal abnormalities (1, 11). In vitro studies have identified PDGF as the most potent mitogen for MCs. In addition, PDGF also promotes migration, survival, and secretion of multiple cytokines and growth factors in MCs (1, 11). Because GJIC is known to be important for most of these cellular functions, GJs could be involved in the PDGF-induced alteration of MC behavior. Indeed, it is known that PDGF induces rapid and transient disruption of GJIC in several cell types, including MCs (14, 15, 40). In 3T3 cells, mutation of Cx43 abrogates the growth-promoting action of PDGF (25). However, information is still incomplete concerning the regulation of GJ by PDGF. In particular, it is unclear how long-term exposure to PDGF affects GJ function and whether PDGF interacts with other molecules in the regulation of GJs. The purpose of this study was to address these questions.

cAMP is the most important second messenger in the up-regulation of GJs in a variety of cell types (10, 29, 31, 37, 38). The molecular targets of the effect of cAMP on connexins are multiple, including modulation of mRNA and protein levels, phosphorylation states, trafficking, and GJ conductance. Inter-

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Estingly, the cAMP signaling pathway also can be activated by PDGF in several cell types (4, 13, 22). In those cells, PDGF induces activation of mitogen-activated protein kinase (MAPK) and subsequently induces cyclooxygenase activation and prostaglandin E2 (PGE2) release, leading to activation of the cAMP pathway. This has been considered as a negative feedback pathway against its mitogenic effect. These findings promoted us to speculate that PDGF may regulate GJ via this negative signaling pathway. This hypothesis was also tested in this study.

Using cultured MCs, we investigated chronic effects of PDGF-BB on the expression of Cx43 and cell differentiation under basal and cAMP-stimulated conditions. Our data revealed, for the first time, unexpected cooperation between PDGF-BB and cAMP-elevating agents in induction of Cx43 expression and MC differentiation.
MATERIALS AND METHODS

Reagents. The cAMP Biotrak enzyme immunoassay system was purchased from Amersham Biosciences. The reporter plasmid pCRE-SEAP and Great EscAPE SEAP detection kit were obtained from BD Biosciences (Palo Alto, CA). Cx43 promoter vector (pCx43 1686-Luc) was the kind gift of Dr. Stephen J. Lye (Samuel Lunenfeld Research Institute, University of Toronto, Ontario, Canada). Anti-vasodilator-stimulated phosphoprotein (anti-VASP) at serine157 was obtained from Chemicon International (Temecula, CA). Phospho-p44/42 antibody was obtained from New England Biolabs (Beverly, MA). Tetramethylrhodamine isothiocyanate-conjugated swine anti-rabbit immunoglobulin was purchased from DAKO (Glostrup, Denmark), and cisteamine was obtained from Wako (Osaka, Japan). All other reagents were obtained from Sigma (St. Louis, MO).

Mesangial cells. Establishment and characterization of rat MCs were established and characterized as described previously (17, 39, 40). Cells were maintained in Dulbecco’s modified Eagle’s medium ( GibCO BRL, Gaithersburg, MD) supplemented with 5% fetal bovine serum (FBS). Medium containing 1% FBS was generally used for experiments. Stable transfection. With the use of a calcium phosphate coprecipitation method, MCs were transfected with pCRE-SEAP (BD Bioscience) TOgether with pcDNA3.1 (Invitrogen, Carlsbad, CA), which encodes neomycin phosphotransferase (17). pCRE-SEAP introduces a secreted alkaline phosphatase (SEAP) gene under the control of the cAMP response element (CRE). Stable transfectants were selected with 0.7 mg/ml G418, and a clone with the lowest background level and the highest SEAP inducibility was selected and used for studies. Immunocytochemistry. Immunocytochemical staining for Cx43 was performed as previously reported (39–41). Evaluation of GJC. GJC was assessed by transfer of the membrane-impermeant fluorescent dye Lucifer yellow following single-cell microinjection with the use of an automated microinjection system (Zeiss, Jena, Germany), as described previously (39–41). Western blot analysis. Equal amounts of cell lysates were separated with 10% SDS-polyacrylamide gels and electrotransferred onto 0.4 µM polyvinylidene difluoride (PVDF) membranes. After being blocked with 3% bovine serum albumin (BSA) in PBS, the membranes were incubated with the first antibodies (anti-Cx43, anti-β-actin, anti-α-actin, anti-phosphorylated MAPK, and anti-VASP phosphorylated at serine157). After being washed with PBS containing 0.1% Tween 20, the membranes were probed with horseradish peroxidase-conjugated sheep anti-rabbit IgG or rabbit anti-mouse IgG. Immunoreactivity was detected using the enhanced chemiluminescence system (ECL; Amersham Biosciences). Densitometric analysis of individual bands was performed using Scion Image software (Scion, Frederick, MD). Data shown are representative of at least three independent experiments with similar results.

Northern blot analysis. MCs were treated with various agents for 12 h. Equal amounts of RNA (5 µg) extracted from cells were separated with electrophoresis and transferred onto nylon membranes (Hybond N+; Amersham Biosciences). The level of Cx43 was examined as described previously (38). The staining of 28S and 18S ribosomal RNA by ethidium bromide was used for loading control.

Biotinylation of cell-surface proteins. MCs in confluent culture were treated with either 250 µM 3-isobutyl-1-methylxanthine (IBMX) for 15 min and exposed to PDGF-BB for the indicated times and concentrations. After stimulation, the cells were lysed and assayed for cAMP according to the instructions for the cAMP Biotrak enzyme immunoassay system. Cellular lysates were also used for protein assay with the Micro BCA protein assay kit (Pierce). SEAP assay. Activity of SEAP was evaluated using the Great EscAPE detection kit following the protocol provided by the manufacturer. In brief, MCs in 96-well plates were exposed to various stimuli for 24 h. Aliquots of supernatants were sampled from the cultures and centrifuged at 12,000 g for 2 min. Dilution buffer (15 µl) was mixed with 5 µl of sample, and the mixture was incubated at 65°C for 30 min to eliminate the endogenous alkaline phosphatase activity. Assay buffer (20 µl) was subsequently added to the mixture and incubated for an additional 10 min at room temperature. The chemiluminescent CSPD substrate at a concentration of 1.25 mM was prepared by dilution with 20× chemiluminescence enhancer, and 20 µl of the diluted substrate were added to each sample, followed by 10-min incubation at room temperature. The intensity of the chemiluminescent signal was determined using a luminometer (Gene Light 55; Microtech Nition, Chiba, Japan).

Transfection experiment. MCs in subconfluent culture were transfected with pCx43 luciferase vector (1686-Luc) (23, 24) by using Lipofectamine 2000 according to the manufacturer’s instructions.
REGULATION OF GAP JUNCTION BY PDGF AND cAMP

F1086

PDGF-BB potentiates cAMP-elicited Cx43 expression and GJIC. Our previous study showed that short-term (within 2 h) exposure of MCs to PDGF-BB resulted in rapid and transient disruption of GJIC without affecting the protein level of Cx43 (40). To determine the long-term effects of PDGF-BB on the level of Cx43, we exposed MCs to PDGF-BB for 48 h and performed Western blot analysis. As shown in Fig. 1A, PDGF-BB (30 ng/ml) did not influence the level of Cx43. However, in the presence of the cAMP-elevating agent IBMX (250 μM), a nonspecific phosphodiesterase inhibitor and a known stimulator of Cx43 synthesis in several cell types (10, 29, 31, 37), PDGF-BB dramatically increased the relative level of Cx43 as follows: control, 1.00 ± 0.08; IBMX, 2.21 ± 0.28; PDGF, 1.19 ± 0.18; PDGF + IBMX, 29.55 ± 3.11 (means ± SE; n = 3). This effect of PDGF-BB was concentration dependent, but in the absence of IBMX, PDGF induced Cx43 only modestly even at high concentrations (Fig. 1B). Similarly, treatment of MCs with IBMX alone resulted in only modest induction of Cx43, but in the presence of PDGF-BB, the effect of IBMX was markedly enhanced in a dose-dependent manner (Fig. 1C). Time course analysis revealed that the synergistic effect between PDGF-BB and IBMX was detected at 12 h after the stimulation and lasted for at least 60 h (Fig. 1D).

The potentiating effect of PDGF-BB on the Cx43 level also was observed in the presence of other cAMP-elevating agents, including adenylyl cyclase activator ( forskolin), cAMP analog (8-bromo-cAMP), and specific phosphodiesterase (PDE) 3 and 4 inhibitors (cilostamide and rolipram) (Fig. 1E). In the Western blot, Cx43 protein was detected as triplet bands (Fig. 1A, P0, P1, and P2). These bands correspond to the native (P0), phosphorylated (P1), and hyperphosphorylated (P2) isoforms of Cx43 (27). Our data show that the co-stimulation of MCs with PDGF-BB and cAMP-elevating agents induced both the native and phosphorylated forms of Cx43 protein.

To examine whether PDGF and IBMX affect Cx43 expression at the transcriptional level, we performed Northern blot analysis. Consistent with the increase in protein level, mRNA expression of Cx43 also was elevated by treatment with PDGF and IBMX in combination (Fig. 1F). PDGF or IBMX alone did not obviously affect Cx43 mRNA expression.

The synergistic effect of PDGF-BB and IBMX on Cx43 expression was confirmed by immunofluorescent staining (Fig. 2). Cx43 was mainly localized at cell-to-cell contact and perinuclear regions in control cells (Fig. 2A) (39, 40). Exposure of MCs to IBMX caused a modest but clear enhancement of Cx43, especially at the region of cell-to-cell contact (Fig. 2B).

PDGF-BB alone affected neither Cx43 expression nor distribution (Fig. 2C). However, the combination of PDGF with IBMX markedly enhanced the Cx43 staining. Under costimulation, the characteristic Cx43 particles were much larger and brighter than those in untreated cells or cells with a single stimulus (Fig. 2D). They were distributed not only at cell-to-cell contact and perinuclear regions but also in the cytoplasm.

The increased level of Cx43 at plasma membrane under the combined stimulation was confirmed by biotinylation of cell-surface proteins. The amount of membrane-associated Cx43 obviously increased in cells exposed to PDGF plus IBMX, whereas little alteration was observed in cells with a single stimulus (Fig. 2E).

The increased level of Cx43 at the plasma membrane was associated with enhanced function of GJIC when analyzed using the Lucifer yellow dye-transfer assay (39–41). As shown in Fig. 3, A and B, IBMX (250 μM) alone induced modest promotion of GJIC. This effect of IBMX on GJIC was significantly enhanced by PDGF, whereas PDGF (30 ng/ml) alone did not influence GJIC. The numbers of dye-coupled cells were as follows: control, 5.83 ± 1.64; IBMX, 13.70 ± 2.91; PDGF, 6.41 ± 1.76; and PDGF + IBMX, 18.92 ± 4.12 (means ± SE; n = 12–13).

RESULTS

PDGF-BB potentiates cAMP-elicited Cx43 expression and GJIC. Our previous study showed that short-term (within 2 h) exposure of MCs to PDGF-BB resulted in rapid and transient disruption of GJIC without affecting the protein level of Cx43 (40). To determine the long-term effects of PDGF-BB on the level of Cx43, we exposed MCs to PDGF-BB for 48 h and performed Western blot analysis. As shown in Fig. 1A, PDGF-BB (30 ng/ml) did not influence the level of Cx43. However, in the presence of the cAMP-elevating agent IBMX (250 μM), a nonspecific phosphodiesterase inhibitor and a known stimulator of Cx43 synthesis in several cell types (10, 29, 31, 37), PDGF-BB dramatically increased the relative level of Cx43 as follows: control, 1.00 ± 0.08; IBMX, 2.21 ± 0.28; PDGF, 1.19 ± 0.18; PDGF + IBMX, 29.55 ± 3.11 (means ± SE; n = 3). This effect of PDGF-BB was concentration dependent, but in the absence of IBMX, PDGF induced Cx43 only modestly even at high concentrations (Fig. 1B). Similarly, treatment of MCs with IBMX alone resulted in only modest induction of Cx43, but in the presence of PDGF-BB, the effect of IBMX was markedly enhanced in a dose-dependent manner (Fig. 1C). Time course analysis revealed that the synergistic effect between PDGF-BB and IBMX was detected at 12 h after the stimulation and lasted for at least 60 h (Fig. 1D).

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Potentiating effect of PDGF-BB on Cx43 expression is mediated by phosphatidylinositol 3'-kinase- and MAPK-dependent pathways but not by PKC activation. Previous studies demonstrated that acute disruption of GJIC by PDGF-BB in MCs was mediated by phosphatidylinositol 3'-kinase (PI3K)- and MAPK-dependent signaling pathways (12, 13, 36). To determine whether similar signaling mechanisms contribute to the synergistic effect of PDGF-BB with cAMP, we examined the level of Cx43 in the presence of the PKC inhibitor calphostin C or under conditions of depletion of PKC by pretreatment with 12-O-tetradecanoylphorbol 13-acetate (10^{-6} M) for 24 h (14). As shown in Fig. 5, B and C, neither of these manipulations interfered with the synergistic effect of PDGF-BB and IBMX on Cx43.

Effect of PDGF-BB on Cx43 expression is not mediated by amplification of cAMP signaling pathway. Because cAMP is a well-characterized second messenger involved in the induction

Fig. 3. Promotion of gap junctional intercellular communication (GJIC) by PDGF-BB and IBMX. A: microphotographic analysis of dye-coupled cells. Confluent cultures of MCs were left untreated (a) or treated with 250 μM IBMX (b), 30 ng/ml PDGF-BB (c), or IBMX + PDGF-BB (d) for 24 h, and Lucifer yellow dye was injected into a single cell. Dye diffusion into adjacent cells was recorded by a video camera. Magnification, ×320. B: quantitative analysis of dye-coupled cells. The number of cells into which Lucifer yellow was transferred from the injected cells within 3 min is shown. Data are means ± SE (n = 12–13). *P < 0.01 vs. untreated control. #P < 0.01 vs. untreated control and single stimulations.

Fig. 4. Effect of phosphatidylinositol 3'-kinase (PI3K) and mitogen-activated kinase kinase (MAPKK) inhibitors on PDGF-BB-induced activation of extracellular signal-regulated kinase (ERK) and Cx43 phosphorylation. A: induction of ERK activation and Cx43 phosphorylation by PDGF. MCs were treated with 30 ng/ml PDGF for indicated lengths of time. Cellular proteins were analyzed by Western blot with anti-phosphorylated ERK (pERK), anti-Cx43, or anti-β-actin antibody. B: inhibition of PDGF-induced activation of ERK and phosphorylation of Cx43 by PI3K and MAPK inhibitors. MCs were treated with or without 100 μM LY-294002 or PD-98059 for 15 min and exposed to 30 ng/ml PDGF-BB for an additional 15 min. Cellular proteins were analyzed by Western blot with anti-phosphorylated ERK (pERK), anti-Cx43, or anti-β-actin antibody.
of Cx43 and promotion of GJIC (10, 29, 31, 37), one possible mechanism underlying the synergistic effect of PDGF-BB on IBMX could be amplification of the cAMP signaling pathway. To examine this possibility, we tested the effect of PDGF-BB on the level of intracellular cAMP. Exposure of MCs to PDGF-BB (25 ng/ml) caused a time-dependent increase in cAMP that peaked at 15 min and gradually returned to near basal level (Fig. 6A). This effect of PDGF-BB was concentration dependent, and significant elevation was detected even at 1 ng/ml (Fig. 6B). The effect of PDGF-BB on intracellular cAMP was mediated by the products of cyclooxygenase, as described in other cell types (4, 5, 22), because the specific cyclooxygenase inhibitor indomethacin completely abrogated the elevation of cAMP (Fig. 6C). A positive control, PGE2 (10^{-5} M), induced a significant increase in cAMP in MCs.

PDGF-BB also induced activation of PKA that was not preventable by indomethacin (Fig. 6D), as revealed by the phosphorylation of VASP at serine157, a validated substrate of cAMP-dependent protein kinase (6). Activation of PKA subsequently triggers CRE, leading to expression of genes that have CREs in their regulatory regions. Because the promoter region of the rat Cx43 gene contains CRE (34) and cAMP-induced elevation in CRE activity is correlated with upregulation of Cx43 in MCs (38), we evaluated the activity of CRE. For this purpose, MCs were stably transfected with pCRE-SEAP, and reporter cells were established. The cells were then exposed to PDGF (30 ng/ml) with or without cAMP-elevating agents (250 μM IBMX, 30 μM forskolin, 500 μM 8-bromo-cAMP), and activity of SEAP in conditioned medium was evaluated. As shown in Fig. 6E, cAMP-elevating agents induced significant activation of CRE. This effect was not significantly altered by PDGF-BB. PDGF-BB alone also did not elicit activation of CRE.

To assess the role of the elevated intracellular cAMP in the synergistic action of PDGF-BB, we examined Cx43 expression in the presence or absence of indomethacin. As shown in Fig. 7, the Cx43 level was not significantly affected by indomethacin. Furthermore, PGE2 was found to induce Cx43 only weakly even in the presence of IBMX. It is therefore unlikely that the action of PDGF-BB was mediated by the elevated cAMP.

**Effect of PDGF-BB on Cx43 expression is not attributable to decreased degradation of Cx43.** The accumulation of Cx43 can result from increased synthesis and/or decreased degradation of Cx43. To determine the role of PDGF on Cx43 degradation, we incubated MCs with IBMX in the presence or absence of PDGF for 16 h and exposed them to cycloheximide to block protein synthesis. The Cx43 protein level at different time points was evaluated using Western blot analysis. As shown in Fig. 8, incubation of MCs with PDGF plus IBMX for 16 h markedly increased Cx43 protein level compared with cells treated with IBMX alone. Exposure of cells to cycloheximide led to gradual reduction in the amount of Cx43. No obvious effect on the degradation of Cx43 was observed in cells treated with PDGF. It is therefore unlikely that decreased degradation of Cx43 contributes to PDGF-induced accumulation of Cx43 in MCs.

**PDGF activates Cx43 promoter in the presence of cAMP.** Previous studies have demonstrated the presence of activator protein-1 (AP-1) and CRE in the promoter region of Cx43 (2, 7, 24). Because PDGF and IBMX are well-known activators of AP-1 and CRE, respectively, the agents may upregulate Cx43 via activation of these regulatory elements. To test this hypothesis, we examined the ability of PDGF and/or IBMX to activate the Cx43 promoter. For this purpose, MCs were transiently transfected with the luciferase gene linked to the Cx43 promoter (1686-Luc) (23, 24). As shown in Fig. 9, exposure of MCs to the combined stimulation of PDGF and IBMX caused a significant increase in luciferase activities, whereas PDGF or IBMX alone induced only a modest increase (relative induction: control, 1 ± 0.07; IBMX, 1.46 ± 0.15; PDGF, 1.52 ± 0.08; 4.34 ± 0.43; means ± SE; n = 4).

**PDGF-BB induces MC differentiation in the presence of cAMP-elevating agents.** Gap junction is an important factor in the control of cell differentiation (9, 10, 31). To test whether the increased Cx43 expression and GJIC were accompanied by alteration of cellular phenotype, we examined a dedifferentiation marker of MCs, α-actin, using Western blotting and immunofluorescent staining. As shown in Fig. 10A, in the
presence of IBMX, PDGF-BB reduced the level of α-actin in a dose-dependent manner, whereas PDGF or IBMX alone showed little impact on the level of α-actin. Similar results were also observed in the presence of other cAMP-elevating agents, including forskolin, 8-bromo-cAMP, and PDE3/PDE4 inhibitors (Fig. 10B).

The reduction of α-actin in PDGF- and IBMX-treated cells was also confirmed by immunofluorescent staining. As shown in Fig. 6, PDGF-BB treatment led to a decrease in the expression of α-actin, which was further supported by immunoblot analysis using a specific antibody for VASP phosphorylated at serine157 (pVASP).

**Fig. 6. Effects of PDGF-BB on cAMP signaling pathway.**

A: time course effects of PDGF-BB on intracellular cAMP. MCs were treated with 100 μM IBMX for 15 min and exposed to 25 ng/ml PDGF-BB for the indicated intervals. Cellular proteins were extracted and assayed for intracellular cAMP. Results shown are means ± SE; n = 4. *P < 0.01 vs. untreated control.

B: dose-dependent induction of cAMP by PDGF-BB. MCs were exposed to various concentrations of PDGF-BB in the presence of 100 μM IBMX for 15 min. Results shown are means ± SE; n = 4. *P < 0.01 vs. untreated control.

C: abrogation of the PDGF-BB effect on intracellular cAMP by indomethacin. MCs were treated with or without 100 μM indomethacin for 15 min and exposed to PDGF-BB for an additional 15 min. Cellular proteins were extracted and assayed for cAMP. Results shown are means ± SE; n = 4. As a positive control, an effect of 10^{-5} M PGE2 is shown. *P < 0.01 vs. untreated control.

D: effect of PDGF-BB on phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) at serine157 (pVASP). MCs were treated with or without 100 μM indomethacin for 15 min and exposed to PDGF-BB for the indicated durations. Cellular protein was extracted and subjected to Western blot analysis using a specific antibody for VASP phosphorylated at serine157 (pVASP).

E: influence of PDGF-BB on the activity of cAMP response element (CRE). MCs engineered to produce secreted alkaline phosphatase (SEAP) under the control of CRE were exposed to cAMP-elevating agents (250 μM IBMX, 30 μM forskolin, 500 μM 8-Br-cAMP) in the presence or absence of 30 ng/ml PDGF-BB for 24 h. Conditioned media were harvested and assayed for SEAP activity. Results shown are means ± SE; n = 4. *P < 0.01 vs. untreated control.
in Fig. 10C, untreated MCs exhibited positive staining of α-actin along with well-organized stress fibers. α-Actin was not obviously affected by IBMX or PDGF-BB (30 ng/ml) alone. However, in the presence of IBMX (250 μM), reduction and disorganization of α-actin was observed after treatment with PDGF. In addition, the cell shape was changed from well-spreading to spindle-like morphology with branched processes.

**DISCUSSION**

In this study, we examined the chronic effect of PDGF-BB on GJIC in MCs. Our data showed that PDGF-BB potentiated cAMP-elicited expression of Cx43 and GJIC. This effect of PDGF-BB was mediated by PI3K- and MAPK-dependent signaling pathways. We also demonstrated an inverse relationship between Cx43 expression and α-actin level, suggesting a potential role of PDGF in the control of GJIC and MC differentiation. PDGF together with the cAMP signaling may play an important part in the regulation of the integrated cell-to-cell communication in the mesangium.

The synergistic action of PDGF-BB and cAMP-elevating agents on Cx43 expression and GJIC was unexpected, because PDGF-BB has been described as a potent inhibitor of GJIC (14, 15, 40). Counteracting effects between PDGF-BB and cAMP-elevating agents in the control of cell growth, migration, and other cell behaviors have been extensively documented (18, 21, 26), whereas little is known about the synergism between PDGF-BB and cAMP.

Because cAMP is a well-characterized second messenger in promoting GJIC and Cx43 expression (10, 29, 31, 37), PDGF-mediated modification of the cAMP signaling pathway could be a possible mechanism. Indeed, PDGF-BB has the ability to...
induce intracellular cAMP and to activate PKA in several cell types (4, 5, 22). In the present study, a significant elevation in intracellular cAMP was detected after exposure of MCs to PDGF. In agreement with the previous reports (4, 5, 22), this effect of PDGF-BB was also mediated by mechanisms involving cyclooxygenase activation, because it was completely prevented by the specific cyclooxygenase inhibitor indomethacin. We also found that PDGF-BB induced activation of PKA in MCs that was not preventable by indomethacin. However, these effects of PDGF-BB on the cAMP pathway did not explain its effect on Cx43, because 1) treatment of cells with indomethacin had no obvious effect on Cx43 expression; 2) the main cyclooxygenase product, PGE2, did not completely mimic the superadditive action; and 3) the activation of PKA by PDGF-BB did not lead to subsequent activation of CRE.

Of note, although PGE2 caused an increase in intracellular cAMP, it had little effect on Cx43 expression in MCs under both basal and costimulated conditions. The reasons for this

Fig. 10. Effects of PDGF-BB and IBMX on α-actin expression. A: dose-dependent inhibition of α-actin expression by PDGF-BB in the presence of IBMX. MCs were treated for 48 h with different concentrations of PDGF-BB in the presence or absence of 250 μM IBMX, and expression of Cx43 and α-actin was evaluated. Den- sitometric analysis of the data is shown. Values are expressed as percentages of basal α-actin control (means ± SE, n = 4). *P < 0.01 vs. single stimulations. #P < 0.01 vs. both untreated control and single stimulations. B: inverse relationship between Cx43 and α-actin levels. MCs were treated with various cAMP-elevating agents (250 μM IBMX, 30 μM forskolin, 500 μM 8-Br-cAMP, 20 μM cilostamide + 20 μM rolipram, and 10−7 M PGE2) together with or without 30 ng/ml PDGF-BB for 48 h. Cellular proteins were extracted and analyzed for expression of Cx43 and α-actin. C: immunofluorescent staining of α-actin. MCs were left untreated (a) or treated with 250 μM IBMX (b), 30 ng/ml PDGF-BB (c), or IBMX + PDGF-BB (d) for 48 h and subjected to immunofluores- cent staining of α-actin. Magnification, ×600.
unresponsiveness are presently unclear. It is possible that PGE₂ is a mild stimulus in intracellular cAMP, which is not strong enough to induce Cx43 expression. The unresponsiveness to PGE₂ excluded the mediating role of PGE₂ in the synergistic action.

The accumulation of Cx43 could be a result of increased synthesis and/or decreased degradation of Cx43. After protein synthesis was blocked with cycloheximide, the rate of reduction of Cx43 protein in cells treated with PDGF was not different from that in untreated cells. This result indicates that altered degradation of Cx43 is not causative of the synergistic effect of PDGF. In addition, treatment of cells with inhibitors of proteasome or lysosome, two major pathways involved in the degradation of Cx43 (32), in combination with IBMX did not mimic the synergistic effect of PDGF (unpublished data).

Thus the mechanism of synergistic induction of Cx43 could be due to the increased Cx43 synthesis. Indeed, PDGF and IBMX induced a marked increase in the level of Cx43 mRNA. In addition, PDGF and IBMX together induced pronounced activation of the Cx43 promoter. These results indicate that transcriptional regulation of Cx43 by PDGF and cAMP signaling pathway is responsible for the increased expression of Cx43.

Further study is needed to determine the regulatory elements involved in the synergistic activation of the Cx43 promoter.

The data showing the inverse relationship between Cx43 expression and α-actin level in MCs indicate a possibility that PDGF controls MC differentiation via modification of GJ function. Previous reports have demonstrated that PDGF plays a crucial role in the generation of the mesangium during development (20, 35). Recently, a critical role of PDGF-BB in the conversion of bone marrow cells to mesangial-like cells was also reported (36). Our current data together with previous reports suggest that PDGF-BB may function not only as a promigratory and mitogenic factor but also as a differentiation factor for MCs. Generation of the mesangium should include establishment of integrated cell-cell and cell-matrix interactions. From this point of view, the important role of PDGF in regulating GJs is not surprising. At present, solid evidence supporting a direct link between the upregulated Cx43 and MC differentiation is lacking. Our preliminary data show that blockade of GJIC by heptanol (39) does not affect α-actin expression (data not shown). Further investigation is needed to clarify this issue.

Our study provided additional evidence supporting the existence of MAPK-mediated positive and negative signaling pathways in the PDGF-mediated control of cell behavior. In several cell types, activation of the MAPK cascade by PDGF-BB can lead to either proliferation or growth inhibition, depending on the extent of level of growth inhibitory prosta-glandins generated (4, 13, 22). Similarly, our previous (40) and current studies have demonstrated that PI3K and MAPK signaling can lead to acute disruption and chronic promotion of GJIC in MCs. Interestingly, the upregulation of GJs by PDGF is dependent on the availability of the cAMP signaling. The opposite, binary roles of the MAPK pathway in the PDGF-mediated regulation of cell growth and GJs indicate that both events may be closely interrelated. It remains to be determined how the complex molecular and cellular events triggered by PDGF are coordinated.

Our data have revealed synergistic effects of PDGF-BB and cAMP-elevating agents on Cx43 expression and MC differentiation. Together with our previous data (40), PDGF-BB has been shown to exert both positive and negative control of GJs. Regulation of GJ may be an important mechanism by which PDGF modulates MC behavior. Our findings may open a new window toward further understanding of the biological effects of PDGF and GJ in various pathophysiological situations.

GRANTS

This study was supported in part by Grants-in-Aid for Scientific Research Nos. 15590845 (to J. Yano) and 14657257 (to M. Kitamura) from the Ministry of Education, Science, Sports and Culture, Japan.

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16. Huang GY, Cooper ES, Waldo K, Kirby ML, Gilula NB, and Lo CW. Junction mediated regulation of cell growth and GJs indicate that both pathways in the PDGF-mediated control of cell behavior. In several cell types, activation of the MAPK cascade by PDGF-BB can lead to either proliferation or growth inhibition, depending on the extent of level of growth inhibitory prostaglandins generated (4, 13, 22). Similarly, our previous (40) and current studies have demonstrated that PI3K and MAPK signaling can lead to acute disruption and chronic promotion of GJIC in MCs. Interestingly, the upregulation of GJs by PDGF is dependent on the availability of the cAMP signaling. The opposite, binary roles of the MAPK pathway in the PDGF-mediated regulation of cell growth and GJs indicate that both events may be closely interrelated. It remains to be determined how the complex molecular and cellular events triggered by PDGF are coordinated.

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