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Pathophysiological Roles of Gap Junction in Glomerular Mesangial Cells

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Abstract Glomerular mesangial cells (MCs) are specialized vascular smooth muscle cells that play a critical role in the control of glomerular hemodynamics. One of the intriguing features of MCs is their extraordinary abundance in gap junctions (GJs). It has long been speculated that GJs may bridge MCs together and provide the mesangium with the characteristics of a functional syncytium. Accumulating scientific evidence supports this idea. GJs are reported to be critically involved in important physiological processes like tubuloglomerular feedback and glomerular filtration. In addition, GJs are implicated in the control of many cellular processes of MCs, including growth, differentiation and survival. This article summarizes the current knowledge on the roles of GJs in glomerular pathophysiology.

Key words Mesangial cell · Gap junction · Connexin · Signal transduction · Glomerular pathophysiology

Introduction

Gap junctions (GJs) are intercellular channels that allow the direct exchange of ions, nutrients and small signaling molecules from one cell to its nearest neighbors (Saez et al., 2003). Studies on GJs have focused on the cardiovascular and central nervous systems because of the critical roles of GJs in these organs. Recently, however, GJs have been recognized to be important in the majority of organs and to take part in the control of a variety of cell behavior. Deregulation of GJs has been considered a common mediator for various pathologies. The importance of GJs is also reflected by the exponentially growing number of publications in this field, as well as rapid expansion of information concerning their function in a variety of cell types. In this article, we summarize the latest data on the pathophysiological roles of GJs in glomerular mesangial cells (MCs) and provide a brief review of the function of GJs in relevant fields.

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Gap Junctions in MCs

Interconnection of MCs by Functional GJ Channels

MCs represent a glomerular projection of the medial layer of the afferent arteriole invading both the glomerulus and juxtaglomerular apparatus. These cells are embedded in the matrix of glomerular tufts and occupy an intermediary position between the macular densa and the afferent arteriole (Fig. 1a). MCs share many properties of vascular smooth muscle cells (SMCs) and are considered to play an essential role in the regulation of glomerular hemodynamics (Stockand & Sansom, 1998).

Pricam et al. (1974) first reported the presence of abundant GJs in both extra- and intraglomerular MCs using a freeze-fracture technique. They speculated that all MCs might be bridged together by GJs. The idea was later confirmed by Inkyo-Hayasaka et al. (1996), who examined the distribution of MCs by histochemical analysis using successive glomerular section. They clearly demonstrated that, within glomeruli, MCs connect with each other and form a branching network from the vascular pole to the

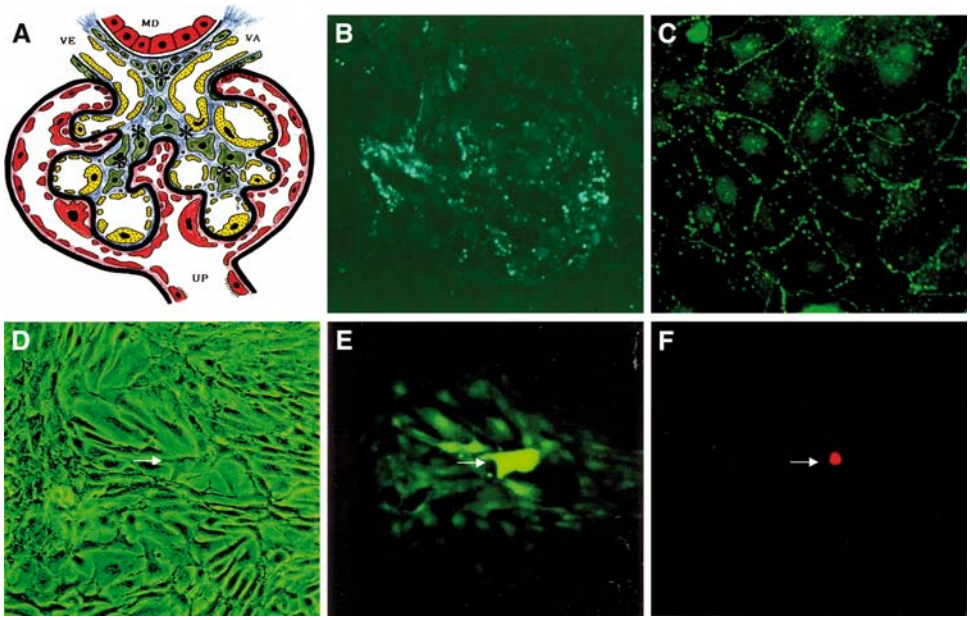

Fig. 1 Presence of Cx43 and functional GJIC in MCs. (**a**) Cartoon depicting the distribution of MCs (*area of mesangium) in the glomerulus. (**b, c**) Immunofluorescent staining of Cx43 in renal sections and cultured MCs. (**d–f**) Diffusion of Lucifer yellow dye from microinjected MC. (**d**) Phase-contrast image of MCs. (**e**) Lucifer yellow diffusion after single-cell injection. (**f**) Ethidium bromide staining of microinjected cell. VE: vessel of efferent arteriole, VA: vessel of afferent arteriole, MD: macular densa, UP: urinary pole. Arrow indicate the injected cell

peripheral capillaries. Subsequently, several investigators reported that renal cortex and glomerular cells express several types of connexins (Cxs) using histochemical analysis and reverse transcription-polymerase chain reaction (Barajas, Liu & Tucker, 1994; Guo, Liu & Barajas, 1998; Hillis et al., 1997; Yao et al., 2002; Yao, Morioka & Oite, 2000). Although information is still limited, MCs are known to express Cx43 (Barajas et al., 1994; Yao et al., 2000) and possibly Cx40 and Cx45, which are present in vascular SMCs (Severs et al., 2001).

We have examined Cx43 expression in both kidney sections and cultured rat MCs (Oite, Yao & Morioka, 2003; Yao et al., 2000, 2002; Yaoita et al., 2002). As shown in Fig. 1b, there was an extraordinarily high density of Cx43 in the areas of afferent arteriolar and extraglomerular MCs. There was also positive punctate Cx43 staining along the distribution of intraglomerular MCs. In cultured MCs, Cx43 was found in the cell membranes at the regions of cell-to-cell contact and around the nucleus (Fig. 1c). GJ proteins in MCs were proved to be functional using a dye transfer assay. Single-cell microinjection of a low-molecular weight fluorescent dye, Lucifer yellow, led to diffusion of this dye to more than 10 surrounding cells (Fig. 1e). These results support the idea that MCs are interconnected with each other via functional GJ channels.

Roles of GJ Channels in MCs

Transmission of intercellular signal

GJs provide a pathway for the intercellular transmission of signaling molecules. Different from the extracellularly regulating pathways via growth factors, hormones or neurotransmitters, the intercellular pathway bypasses the transduction across the cell membrane. Signaling molecules less than 1.2 kDa pass through GJs, and most second messengers such as cyclic adenosine monophosphate (cAMP), adenosine triphosphate (ATP), inositol 1,4,5-trisphosphate (IP_3) and calcium (Ca^{2+}) can been transferred. Of these, transmission of the Ca^{2+} signal via GJs has been extensively investigated (Jorgensen et al., 1997; Rottingen & Iversen, 2000).

MCs possess GJs abundantly and may form a sophisticated communication system via GJ channels (Goligorsky et al., 1997; Pricam et al., 1974; Taugner et al., 1978). To examine this possibility, we examined the GJ-mediated intercellular Ca^{2+} signal in MCs. We focused on Ca^{2+} because it is a basic signaling molecule with versatile functions. Most MC behavior is known to be controlled by intracellular Ca^{2+} (Bonventre, 1996; Stockand & Sansom, 1998). The mechanisms underlying intercellular Ca^{2+} signaling have been well documented. Two different pathways have been proposed to contribute to the propagation of the intercellular Ca^{2+} wave, i.e., the paracrine pathway mediated by the secreted diffusible messenger ATP and the direct intercellular pathway mediated by GJs (Jorgensen et al., 1997; Rottingen & Iversen, 2000; Yao et al., 2002, 2003). Mechanical stimulation of a single MC initiated a rise in Ca^{2+} in the stimulated cells, which was followed by transmission of the signal to surrounding cells (Fig. 2). The spreading of the Ca^{2+} signal in MCs was mediated by GJ channels because it was completely prevented by GJ inhibitors (Yao et al., 2002).

The GJ-mediated intercellular pathway in MCs may have significant pathophysiological implications. (1) MCs are exposed to various mechanical stresses (fluid shear

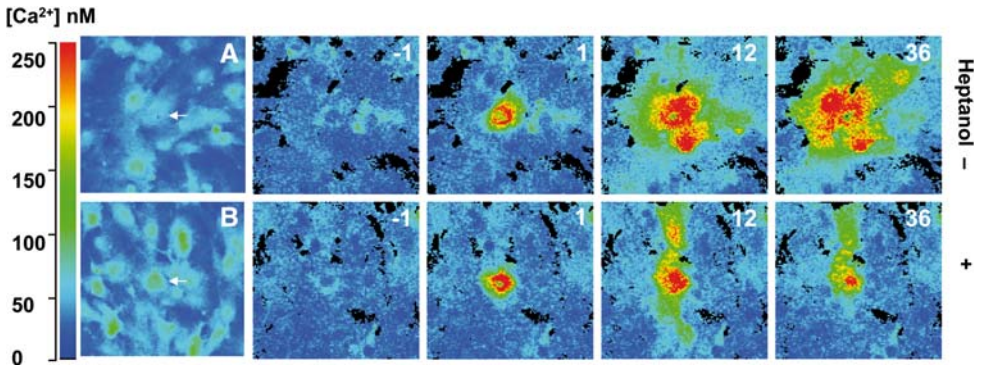

Fig. 2 Intercellular calcium wave transmission in cultured MCs. A single MC (*arrow*) was mechanically stimulated during fluorescence ratio-metric imaging. Time (seconds) before or after stimulation is indicated on each panel. The pseudocolor map represents estimated calcium concentrations. (*Top row*) MC without treatment. (*Bottom row*) MCs pretreated with heptanol for 30 min before stimulation

stress, hydrostatic pressure, triaxial stretch) caused by vascular flow. This is because MCs extend their cytoplasmic processes toward the peripheral basement membrane. During pressure-induced glomerular expansion, the outward displacement of these anchoring points caused by distension of the capillaries and the mesangium results in intense stretching of MCs. This situation is similar to the mechanical stress used for the *in vitro* Ca^{2+} wave study. The displacement of the anchoring points of MCs may, therefore, evoke an intercellular Ca^{2+} transmission *in vivo*. GJs might provide an important pathway to transmit intraglomerular signals to extraglomerular MCs or other effector cells and thereby produce synchronizing responses to the mechanical strain. (2) MCs also reside in the juxtaglomerular interstitium, surrounded by afferent and efferent arterioles and the macular densa. Histochemical analyses revealed that intra- and extraglomerular MCs and afferent arteriolar cells are tightly coupled by GJs (Goligorsky et al., 1997; Pricam et al., 1974; Taugner et al., 1978; Yao et al., 2002). Intercellular communication via GJs may be involved in the signal transduction from the macula densa to the afferent arteriole. Several reports support this hypothesis. For example, selective abrasion of MCs by administration of an anti-Thy-1 antibody led to a diminished or even abrogated response of tubuloglomerular feedback (TGF) (Aizawa et al., 1991; Ren, Carretero & Garvin, 2002). A similar result was obtained with disruption of GJ intercellular communication (GJIC) via the GJ inhibitor heptanol in isolated juxtaglomerular apparatus (Ren et al., 2002). A recent study by Peti-Peterdi (2006) demonstrates the occurrence of a calcium wave in the isolated rabbit juxtaglomerular apparatus (JGA)-glomerulus complex after activation of TGF by increasing tubular flow rate. The calcium wave spread from the macula densa toward distant cells of the JGA and glomerulus (including extraglomerular MCs), which could be largely prevented by GJ uncoupling (Peti-Peterdi, 2006). These data indicate that GJs in MCs play a key role in transmitting the TGF signal initiated by the macula densa to the afferent arteriole.

Coordination of MC function

The transmission of intercellular signals via GJs leads to synchronized cell behavior. This is well evidenced by the roles of GJs in the cardiovascular system, where they maintain the coordinated vascular responses in concert with hormones, neruotransmitters and other factors. It has been reported that GJIC affects both agonist-induced vasodilation and vasoconstriction (Christ et al., 1996).

MCs are specialized vascular SMCs in the glomerulus. The contractile response in MCs is also regulated by GJIC. This is supported by the following observations. (1) Single-cell injection of IP_3, a mediator responsible for the propagation of the Ca^{2+} wave in MCs, led to contraction of not only the injected cell but also neighboring cells (Fig. 3) (Yao et al., 2002). (2) Addition of a GJ inhibitor to MC-embedded collagen gels significantly attenuated serum-induced gel contraction (Yao et al., 2002). (3) Pharmacological blockade of the calcium wave triggered by TGF led to significant reduction in the diameter of the glomerular tuft (Peti-Peterdi, 2006). These observations indicate that exchange of the intercellular signal via GJ might permit amplification and integration of signals, causing coordinated cell responses. The mechanism involved in the coordination of cellular contractile responses by GJs is illustrated in Figure 4. The participation of GJs in MC contraction suggests that they may have a role to play in the regulation of glomerular hemodynamics.

Participation in MC growth and differentiation

The GJ is known to be an important regulator in cell growth and differentiation. Accumulating evidence

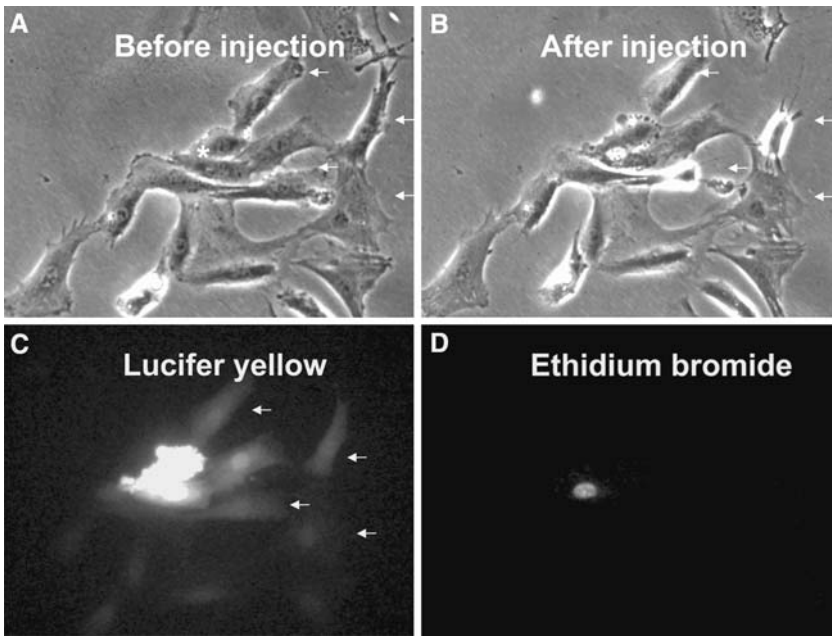

Fig. 3 MC contraction induced by single-cell injection of IP_3. A single MC was injected with IP_3 together with Lucifer yellow and ethidium bromide (*impaled cell). (**a**) Micrography of MC before injection. (**b**) Phase-contrast image of MCs after IP_3 injection. (**c**) Lucifer yellow diffusion after single-cell injection. (**d**) Ethidium bromide staining of the microinjected cell. *White arrows* indicate diminution of cell planar area and shortening of cytoplasmic extensions

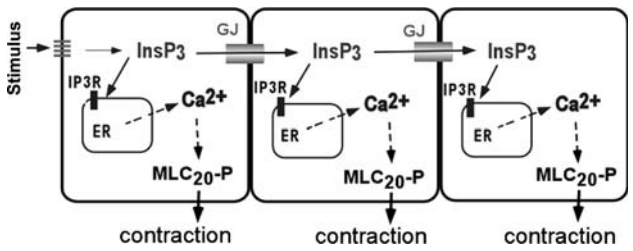

Fig. 4 Mechanisms involved in GJ-mediated propagation of intercellular calcium signal and coordination of cell contraction

supports a close link between growth/differentiation and GJIC. In most cases, an inverse relationship exists between cell growth/differentiation and Cx/GJIC. For example, the number of GJs and the activity of GJIC are reduced or absent in many neoplastic cells (Klaunig & Ruch, 1990), and transfection of Cx genes into GJIC-deficient tumor cells leads to establishment of GJIC and partially reverses the tumorigenic phenotype (Klaunig & Ruch, 1990). Also, GJIC can be inhibited by mitogens including peptide growth factors, chemical carcinogens and oncogenes (Saez et al., 2003). Viral proteins that transform cells (e.g., polyomavirus middle T antigen, simian virus 40 and adenovirus E1A) generally disrupt GJIC. In contrast, GJIC in fibroblasts is enhanced by growth inhibitors, transforming growth factor-β and retinoids, all of which are considered to be antimitogenic/differentiation factors. Additionally, communication-independent regulation of the tumor cell phenotype by GJ has been reported (Zhang, Kaneda & Morita, 2003). Collectively, these data support the notion that GJs play a role in regulating cell proliferation, either via direct intercellular exchange of positive or negative growth signals or via other unknown mechanisms.

The critical role of GJs in the control of cell growth led us to hypothesize that they may be implicated in some pathological processes such as mesangial proliferative glomerulonephritis, where abnormal MC proliferation is one of the major characteristic pathological features. As the first step toward understanding of the role of GJs in MC proliferation, we examined GJIC in MCs after exposure to platelet-derived growth factor (PDGF), a well-established pathogenic factor responsible for mitogenesis of MCs (Abboud, 1995). Addition of PDGF into MC culture caused a rapid and reversible inhibition of GJIC (Fig. 5), which was completely blocked by a phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002 (Yao et al., 2000). Because the PDGF-triggered, PI3K-mediated signaling pathway mediates MC proliferation (Choudhury et al., 1997), the closure of GJIC may be causative of MC growth induced by PDGF. Indeed, a study using 3T3 fibroblasts supports this hypothesis. Transfection of 3T3 fibroblasts with a mutant Cx43 gene resulted in a lack of closure of GJIC and cellular proliferation in response to PDGF (Moorby & Gherardi, 1999). These results indicate that disruption of GJIC in MCs may be involved in abnormal MC proliferation.

GJs are also an important factor in the control of cell differentiation. In several cell types, the induction of Cx43 expression is closely correlated with the expression of differentiation markers (Dowling-Warriner & Trosko, 2000; Romanello et al., 2001). However, currently, the role of GJs in MC differentiation is unclear. PDGF, as an indispensable factor for the generation of the mesangium, is required for converting bone marrow cells into MCs

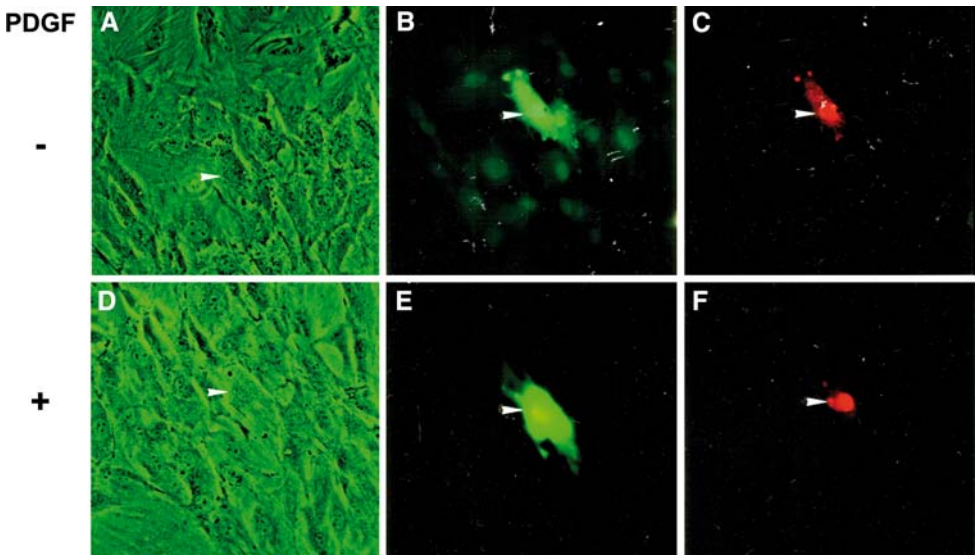

Fig. 5 Disruption of GJIC by PDGF. (**a–c**) MCs without PDGF pretreatment. (**d, e**) MCs pretreated with PDGF for 15 min before injection. (**a, d**) Phase-contrast micrographs. (**b, e**) Lucifer yellow fluorescence. (**c, f**) Ethidium bromide staining of the microinjected cell. *Arrowheads* indicate the microinjected cell

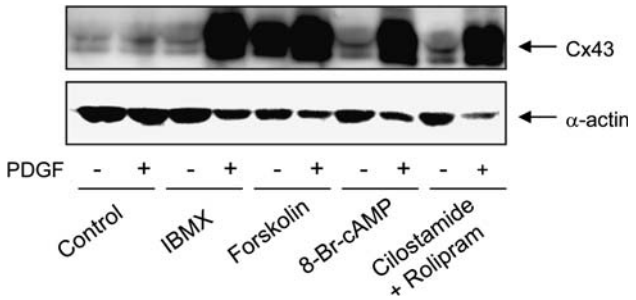

Fig. 6 Inverse relationship between Cx43 and α-actin levels in MCs. MCs were treated with various cAMP-elevating agents (3-isobutyl-1-methylxanthine [*IBMX*], forskolin, 8-bromo-cAMP, cilostamide plus rolipram) together with (+) or without (–) PDGF-BB. Cellular proteins were extracted and Western blot analysis was performed for Cx43 (*top row*) and α-actin (*bottom row*)

(Suzuki et al., 2004). This suggests a potential role for PDGF in MC differentiation. We recently demonstrated that long-term exposure of MCs to PDGF in the presence of cAMP-elevating agents induced a significant increase in Cx43 levels and GJIC. This was accompanied by an obvious reduction in the level of α-actin, a dedifferentiation marker of MCs (Yao et al., 2004, 2006) (Fig. 6). Although a direct link between increased Cx43/GJIC and reduced α-actin remains to be established, GJ may also be involved in the control of MC differentiation.

GJs were recently implicated in high glucose-induced senescence in MCs (Zhang et al., 2006). Exposure of MCs to a high glucose condition led to cell senescence, as evaluated by the increased number of cells expressing senescence-associated β-galactosidase (SA-β-gal), which was accompanied by a concomitant reduction of Cx43. Overexpression of Cx43 significantly decreased, while knocking down Cx43 significantly increased the percentage of SA-β-gal-stained cells. These results further support the critical role of GJs in the control of MC phenotype.

The control of MC phenotype by GJs may have pathophysiological relevance. Because MC proliferation and dedifferentiation are major pathological features in several types of glomerulonephritis, deregulation of GJs in MCs may be implicated in pathological processes. If so, enhancement of GJIC or Cx expression via genetic or pharmacological approaches could be potentially useful for attenuation of MC proliferation and induction of MC differentiation in glomerulonephritis.

Providing a life-supporting pathway for MCs

In physiological situations, intercellular exchange of ions, nutrients and small signaling molecules via GJs provides an important pathway for the maintenance of homeostasis. In contrast, under pathological conditions, GJ may allow transmission of not only ‘‘good signals’’ but also ‘‘harmful signals’’ to surrounding cells, resulting in either suppression or promotion of cell injuries. Indeed, contradictory roles of GJs have been well documented in several cell types. For example, exposure of cells to irradiation induces injury or death in not only the irradiated cells but also nonirradiated bystander cells; this bystander effect is mediated by GJIC (Azzam, de Toledo & Little, 2001). In contrast, it has also been reported that GJIC-mediated signals from bystander cells protect cells expressing herpes simplex virus thymidine kinase from ganciclovir-induced cytotoxicity (Wygoda et al., 1997). Conflicting roles of GJs have also been reported in *in vivo* situations, e.g., ischemic brain damage (Nakase et al., 2004; Rawanduzy et al., 1997).

GJs may also regulate the survival of MCs. In particular, because there are no capillaries in the extraglomerular

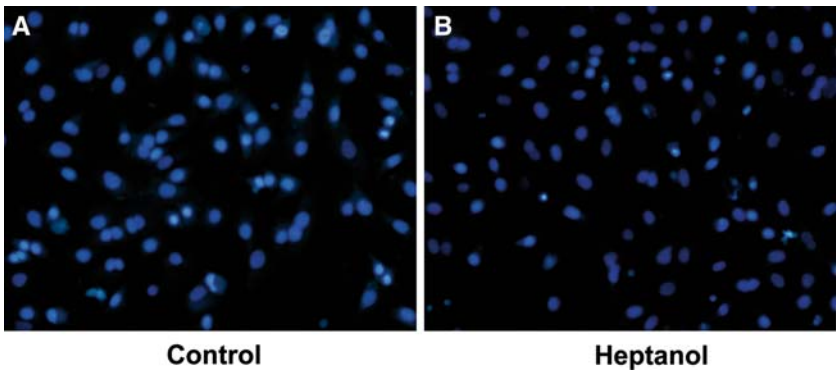

Fig. 7 Induction of apoptosis by GJ inhibitors in MCs cultured under serum- and matrix-free conditions. Apoptotic cells were assessed by staining the nuclei with Hoechst 33258 to detect nucleus condensation. Note the obvious increase in the number of apoptotic cells in the presence of the GJ inhibitor heptanol (*right*) compared to the control (*left*)

mesangium, their abundant GJs may contribute to exchange of nutrients and other metabolites and thereby maintain MC survival. We designed a study to examine this possibility. MCs were cultured in serum-free medium in culture plates precoated with polylysine in the presence or absence of the GJ inhibitor heptanol. Morphological and immunocytochemical studies revealed that MCs under these culture conditions quickly attached, spread and formed GJs. In the presence of GJ inhibitor, significant induction of apoptosis was observed (Fig. 7). This result indicates that GJIC is necessary for the survival of MCs.

Regulation of GJs in MCs

Unlike most of the structural proteins in the adherent junctions, GJ proteins have a very short half-life (1–5 h). This characteristic is in good accord with its roles in signal transduction. Because intercellular signals via GJs must be rapidly and dynamically regulated by a number of factors, the short half-life of Cx is advantageous for the fine regulation of signal transduction pathways.

The function of GJs is regulated by phosphorylation, synthesis, assembly, trafficking and degradation of Cx. Phosphorylation of Cx proteins is a form of posttranslational modification that regulates gap junctional trafficking, assembly into plaques, channel conductance and turnover (Saez et al., 2003; Warn-Cramer & Lau, 2004). The phosphorylation of several Cx molecules such as Cx43 is closely related to acute disruption of GJIC. Growth-regulating kinases such as protein kinase C (PKC), mitogen-activated protein kinase (MAPK) and Src kinase are able to phosphorylate Cx43. Disruption of GJIC by growth factors, tumor promoters, oncogene proteins, hormones and inflammatory mediators is mediated by one or more of these kinases (Warn-Cramer & Lau, 2004).

We analyzed the signaling mechanisms underlying the abrupt disruption of GJIC by PDGF in MCs (Yao et al., 2000). The closure of GJIC by PDGF was not accompanied by alteration in Cx43 protein levels but was closely associated with Cx43 phosphorylation. PDGF induced activation of PI3 and MAP kinases, while blockade of these kinases prevented both Cx43 phosphorylation and closure of GJIC. Phosphorylation of Cx43 by these kinase-related mechanisms is, therefore, an important regulatory step in the PDGF-induced disruption of GJIC in MCs (Oite et al., 2003; Yao et al., 2000).

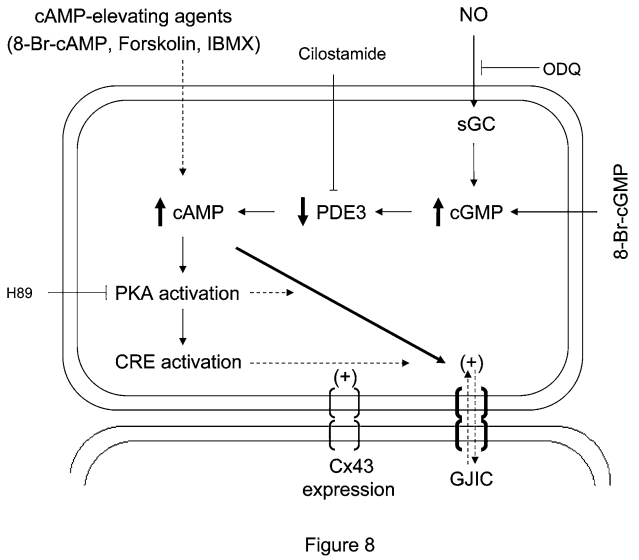

Fig. 8 Schematic diagram illustrating potential mechanisms involved in the NO-mediated regulation of Cx43 expression and GJIC. NO activates soluble guanylate cyclase (*sGC*), causing the generation and action of cyclic guanosine monophosphate (*cGMP*). The cGMP-dependent inhibition of phosphodiesterase 3 (*PDE3*) activity results in an increase in the cAMP level and subsequent activation of protein kinase A (*PKA*), leading to the enhancement of Cx43 expression and GJIC. *IBMX* 3-isobutyl-1-methylxanthine; *CRE* cAMP response element; *ODQ* 1H-[1,2,4] oxadiazolo [4,3-α-] quinoxalin-1-1

Regarding factors and mechanisms implicated in the long-term regulation of GJ protein expression and GJIC in MCs, information is still limited. At present, only cAMP is a known second messenger that is able to induce Cx43 expression and to promote GJIC in various cell types, including MCs (Iijima, Moore & Goligorsky, 1991; Yao et al., 2005, 2006; Zhu et al., 2006). Recently, we identified nitric oxide (NO) as a potent stimulator of Cx43 expression

and GJIC in MCs (Yao et al., 2005). This effect of NO is also mediated by the cAMP signaling pathway. The detailed, putative mechanisms are illustrated in Fig. 8.

Besides PDGF, cAMP-elevating agents and NO, other factors including the PKC activator phorbol myristate acetate, Ca^{2+} ionophore ionomycin, reactive oxygen intermediates (ROIs) and cellular acidification have also been reported to alter GJIC in MCs (Iijima et al., 1991). Because PKC, Ca^{2+}, ROI and acidification are involved in various pathophysiological conditions in the glomerulus, regulation of GJs could be important mechanisms by which these factors affect glomerular functions.

Conclusion

Several lines of evidence support the important roles of GJs in MC functions. (1) MCs are extensively connected by functional GJs. (2) GJs in MCs participate in the regulation of important physiological processes, including TGF and glomerular filtration. (3) GJs are implicated in the control of MC behavior including proliferation, differentiation and survival. (4) Factors critically involved in various processes of glomerular pathophysiology are potent regulators of Cx43 expression and GJIC in MCs. These data suggest that GJs may participate in glomerular pathophysiology. However, ubiquitous expression of Cxs in the body and a lack of their specific blockers are the current hurdles for the evaluation of GJ roles *in vivo*. Recently, with the availability of genetically modified animals, information about the pathophysiological roles of GJs *in vivo* has been growing. Deregulation of GJs is currently documented in various pathologies including ischemic injury, inflammation, vascular atherosclerosis and hypertension (De Maio, Vega & Contreras, 2002; Haefliger, Nicod & Meda, 2004; Kwak et al., 2003; Nakase et al., 2004). Because these pathological situations similarly occur in glomeruli, understanding the roles and regulation of GJs in MCs may not only provide new insights into the pathogenesis of glomerular diseases but also lead to the development of new therapeutic strategies.

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