Cell density-dependent expression of EDG family receptors and mesangial cell proliferation: role in lysophosphatidic acid-mediated cell growth

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LYSOPHOSPHATIDIC ACID (LPA; 1-acyl-2-hydroxy-sn-glycerol 3-phosphate), a ubiquitous simple glycerophospholipid, has been shown to exhibit a wide range of physiological and pathological actions. In addition to serving as a natural precursor for the formation of more complex membrane phospholipids, LPA has recently emerged as an important extracellular signaling molecule with a variety of growth factor-like properties (29). A growing body of evidence indicated that LPA is a potent signaling molecule involved in diverse physiological and pathological processes, such as cell proliferation, differentiation, cell-cell communication, cytoskeletal rearrangement, and tumor cell invasion (28, 38). LPA has also been shown to stimulate mesangial cell proliferation and contraction (20, 21) and implicated to play a role in the pathogenesis of renal injury (19). Circulating LPA has shown to be mainly generated by activated platelets, and the normal concentration of LPA range is between 2 and 20 μM (6). Additionally, LPA has also shown to be produced by activated noncirculating cell types, including glomerular mesangial cells, through the actions of group II phospholipase A2 (PLA2) and phospholipase D-like phosphodiesterases (40, 41, 43). The circulating LPA binds with high affinity to serum albumin while retaining its biological activity (37, 39).

The seminal studies from Moolenaar group (30, 42) demonstrated the participation of G proteins in LPA-induced fibroblast cell proliferation and in various other LPA responses. The recent cloning and identification of endothelial differentiation gene (EDG) family proteins as receptors for bioactive phospholipids further extended a great deal of interest in LPA signaling and diverse biological responses (10, 11). EDG family receptor was originally described as an orphan G protein-coupled receptor (GPCR) encoded by an immediate early response gene product cloned from human umbilical vein endothelial cells (16). Currently, eight related receptors within the EDG family cluster have been cloned and identified to serve as mammalian cell receptors for bioactive lipids LPA and sphingosine-1-phosphate (S1P) (11). EDG-2 (also referred in current nomenclature as LPA1) (26), EDG-4 (LPA2), and EDG-7 (LPA3) have been shown to be receptors for LPA (1, 2, 14). The other five members of EDG family genes, EDG-1, EDG-3, EDG-5, EDG-6, and EDG-8, are identified as receptors in mammalian cells, it was shown that all three LPA receptors (EDG-2, -4, -7) share 35% homology.

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tion of the small GTPase, Rho. Furthermore, pharmacological studies suggested that both EDG-2 and EDG-4 couple to at least three types of G proteins, $G_{i/o}$, $G_{12/13}$, and $G_q$, whereas EDG-7 couples with $G_{i/o}$ and $G_q$ (reviewed in Ref. 23). However, there are some studies showing differential response with respect to specific G protein coupling in different cell types, suggesting that the LPA receptor coupling may be dependent on the system of expression (11, 18).

Although the cellular overexpression studies are helpful in understanding generalized LPA receptor signaling, the relative contribution of specific endogenous LPA receptors in the multiple biological activities of LPA remains poorly defined. Using stable transfection of antisense oligonucleotides, recently it was shown that EDG-2 regulates LPA-induced preadipocyte proliferation and spreading (31). These studies also indicated that the differentiation of growing preadipocytes into quiescent adipocytes led to a strong reduction in the level of EDG-2 transcripts, suggesting the differential association of EDG-2 in growing vs. quiescent adipocytes. Using stereoselective agonists of the EDG receptors, Hooks et al. (17) showed that the mitogenic activity in rat hepatoma cell line RH7777 and HEK 293 cells and platelet aggregation responses to LPA are independent of EDG-2, -4, and -7. In this study, the ectophosphatase lipid phosphate phosphatase 1 (LPP1) has been shown to downregulate LPA-mediated mitogenesis. Additional work in this area showed that LPP1 can regulate LPA association with cells without significantly depleting bulk of LPA concentration in the extracellular medium, suggesting a novel mechanism of LPP1 for controlling EDG-2 receptor activation (46). These limited numbers of available studies suggest variable role of EDG receptors in different cell types. It is not clearly understood whether such variabilities in the involvement of specific EDG receptors for certain LPA responses are due to cell type-specific or the abundance of specific EDG receptors for LPA responses in various cell types. Particularly, with reference to cell growth, the abundance of specific EDG receptors in growing and quiescent state may differ considerably for LPA-mediated cell growth responses. Addressing the above issues related to EDG receptor(s) specificity for diverse LPA responses in various cell types would be of considerable importance in understanding the role of EDG receptors in the physiological and pathophysiological responses elicited by LPA.

To understand the role of EDG family LPA receptors in mesangial cell proliferation, we investigated the expression profile and abundance of EDG-2, EDG-4, and EDG-7 receptor genes in primary cultures of human glomerular mesangial cells and assessed the contribution of specific endogenous LPA receptors in mesangial cell proliferation stimulated by LPA. Specifically, we examined the cell density-dependent expression profile of these EDG receptor genes to assess differential expression profile of these LPA receptors and compared with cellular proliferative activity.

**MATERIALS AND METHODS**

**Materials.** LPA was obtained from Sigma (St. Louis, MO). [3H]Thymidine (6.7 Ci/mmol; 1 Ci = 37 GBq) was from Amersham Life Science (Arlington Heights, IL). Diacetylcerolpyrophosphate (DGPP; 8.0) was procured from Avanti Polar Lipids (Alabaster, AL). Oligonucleotide primers of EDG-2, EDG-4, and EDG-7 for RT-PCR analyses were obtained from Sigma-Genosys (Houston, TX). RNasey Mini Kit used for total RNA purification was from Qiagen (Valencia, CA). Glyceraldehyde 3-phosphate dehydrogenase primers were purchased from Clontech Laboratories (Palo Alto, CA). All other chemicals used were of analytic grade.

**Cell culture.** Cryopreserved primary cultures of normal human mesangial cells (*passage* 3) and the growth media were purchased from Cambrex Bio Science (Walkersville, MD). These cells were characterized by immunofluorescent staining for specific markers including: positive staining for fibronectin, α-actin, vimentin, desmin, myosin, and negative staining for von Willebrand (factor VIII) antigen. Cells were grown in MsGM mesangial cell growth media containing supplements and growth factors (FBS and gentamicin/amphotericin B; Cambrex Bio Science) for 5–7 days to attain $\sim 75\%$ confluency according to the growth instructions from the supplier (Cambrex Bio Science). Cells were subcultured and used between *passages* 4 and 6 for all the experiments.

The different number of cells (4,000; 2,000; and 1,000 cells/cm²) were initially plated into 12-well plates and grown in MsGM growth media for 72 h to attain $\sim 75$, 50, and 25% confluency. These different cell confluencies are referred in the text as sparse (25% confluency), subdense (50% confluency), and dense (75% confluency). The medium was replaced with basal medium containing 0.5% FBS and incubated for 24 h. After being washed with PBS, cells were collected for RNA isolation and PCR analyses.

**Cell proliferation assay.** For DNA synthesis studies, cells (at different density after synchronization) were stimulated with LPA (0–10 μM), added $^3$H-thymidine (0.5 μCi/ml), and incubated for 24 h. Cells were washed with ice-cold PBS and then with 10% trichloroacetic acid and digested with 0.25 N sodium hydroxide solution. An aliquot of cell digest was used to measure DNA synthesis and served as an index of cell proliferation.

**RT-PCR and real-time RT-PCR analysis.** Total RNA isolation and purification were carried out by RNeasy Mini Kit according to the procedures described in Qiagen’s manual instruction. RNase-free DNase I (Promega) was used to remove trace amounts of DNA from the total RNA samples. In brief, the RNA sample was treated with DNase I during split RW1 washings for 15 min at room temperature. For RT-PCR analysis, cDNA was synthesized from 0.4 μg of total RNA in 20 μl of reaction volume with 0.25 μl of reverse transcriptase (Promega). Parallel incubations in the absence of reverse transcriptase were used to ensure that no genomic DNA contaminated. Previously published primer sequences of human Edg-2 (31) were used to synthesize sense and antisense primers: sense, 5′-ATCTTTGGCTATGTTCGCCA-3′; antisense, 5′-TTGCTGTGAACTCCAGCCA-3′. EDG-4 and EDG-7 primers were based on the cDNA sequences from the GenBank, and sense and antisense primers were synthesized and obtained from Sigma-Genosys. Sense primers for EDG-4 and EDG-7 are 5′-CCGCA-CAGCCCGACTTTTCATTT-3′ and TCTGGACAGTGGTTGAGT-3′, respectively. Antisense primers for EDG-4 and EDG-7 are 5′-TTGCCGATGAGCAGCGCAAGCA-3′ and 5′-GGCATGAGGTTG-GCACATGTC-3′, respectively. For the real-time RT-PCR analysis, the method of reverse transcription for the first-strand cDNA was the same as used in RT-PCR. Similarly, we used the same primers and concentrations as used in RT-PCR to amplify EDG family genes using the Bio-Rad iCycler and 96-well PCR plate. Instead of three thermal cycles, two steps of amplification were used (i.e., 95°C for 15 s and 72°C for 40 s).

**Statistical analysis.** Results are presented as representative studies or by displaying means ± SE for three to four separate experiments, each assayed in triplicate. The data were compared by Student’s *t*-test between two groups and by one-way ANOVA followed by Bonferroni test when more than two groups were involved. A value of $P \leq 0.05$ was considered to be statistically significant.
RESULTS

Cell density-dependent mesangial cell proliferation. Initially, studies were performed to examine the mesangial cell-proliferative responses (DNA synthesis) with respect to different cell densities ranging from sparse (25% confluency) to dense (75% confluency) cell populations. As shown in Fig. 1A, cell proliferation rate (measured as the incorporation of \(^{3}H\)-thymidine into DNA as total cpm/1,000 cells seeded at time 0) was greatly reduced as a function of increasing cell density. The cell DNA synthetic rate in sparse cell density was ~4.5-fold greater compared with denser cell density (Fig. 1A). Additionally, we also calculated thymidine incorporation data (DNA synthesis, Fig. 1A) per microgram of cell protein at the time of harvest. The data for thymidine incorporation (cpm/µg cell protein) in sparse, subdense, and dense cell population were 47,911 ± 2,250, 14,710 ± 588, and 7,650 ± 268, respectively (data are statistically significant at \(P \leq 0.05\) compared between the groups by ANOVA). These DNA synthesis data calculated per unit of cell protein also followed a similar pattern to the Fig. 1A data presented per 1,000 cells seeded.

**LPA response on cell density-dependent mesangial cell proliferation.** Because LPA is one of the major mitogenic serum factors, we assessed the effect of LPA on mesangial cell proliferation in different cell density populations. Dose-response studies indicated that LPA between 5 and 10 µM concentration maximally stimulated cell proliferation by three- to fourfold compared with controls (data not shown). In all cell densities, LPA (10 µM) significantly increased mesangial cell proliferation (Fig. 1B). Parallel to control cells (Fig. 1A), cells stimulated with LPA also followed a qualitatively similar pattern of decreasing cell-proliferative activity with respect to increasing cell density (Fig. 1B).

Interestingly, the data in Fig. 2 indicated that the cell-proliferative rate by LPA was much higher in dense cell population (50–75% confluency) than the sparse cell density (25% confluency). The cell-proliferative rate by LPA (10 µM) was 1.5-fold greater than controls in sparse cell density, whereas the cell-proliferative rate was 3.5-fold greater than respective controls in dense (50–75% confluency) cell population (Fig. 2). These data indicate differential cell-proliferative response by LPA in sparse vs. dense cell population, suggesting potential variations in LPA receptors and/or differential mitogenic signaling as a function of cell density.

**EDG receptor expression profile in mesangial cells and association to cell proliferation.** Because LPA (a major serum mitogenic factor) may use EDG receptors for biological responses, the next series of experiments were performed to determine the expression profile of EDG receptors in mesangial cells and their association with cell-proliferative activity. The RT-PCR data indicated that all three LPA receptors EDG-2, EDG-4, and EDG-7 receptors are expressed in human primary mesangial cells (Fig. 3). Based on semiquantitative RT-PCR with comparable conditions, we noted that EDG-7...
was the most abundantly expressed LPA receptor, and EDG-2 was moderately abundant in mesangial cells. EDG-4 was weakly expressed in mesangial cells (Fig. 3A).

We then examined the EDG family receptor expression profile with respect to varied cell density. Interestingly, expression of EDG family receptors for LPA followed cell density-dependent pattern. As shown in Fig. 3A, EDG-7 was maximally expressed at sparse cell density (25% confluency) and minimally expressed in dense cell population (75% confluency). On the other hand, the EDG-2 expression was minimal at sparse cell density and maximal at dense cell population (Fig. 3A). No major changes in EDG-4 expression with respect to varied cell densities were noted (Fig. 3A). Confirmatory studies by real-time PCR also showed similar expression profile of EDG family LPA receptors as a function of cell density (Fig. 3B).

Comparative analysis of the association of EDG receptors with cell proliferation revealed that EDG-7 LPA receptor expression profile was positively associated with cell proliferative rate in varied cell density (Fig. 4). EDG-2 expression was negatively associated with cell proliferation rate with respect to cell density (Fig. 4). These data suggest differential association of EDG family LPA receptors with cell-proliferative rate.

**Participation of EDG-7 in LPA-induced mesangial cell proliferation.** Because EDG-7 is positively associated with cell-proliferative rate, we examined the participation of EDG-7 in LPA-induced mesangial cell proliferation. A short-chain phosphatidic acid analog, di-octyl glyceryl pyrophosphate (8:0 DGPP), has recently been shown to act as a selective antagonist for EDG-7 receptor (8). During the 24-h incubation period, DGPP did not alter the viability and cell number as assessed by Trypan blue exclusion criterion and by measuring cell number, respectively. The cell numbers in control and DGPP-treated cells were as follows (1 × 10⁵/dish, average of 2 determinations): 1.98 and 2.01, respectively. The viability of cells in control and DGPP-treated cells was similar (95–98% viable, as

![Graph showing DNA synthetic rate](image)

**Fig. 2.** Effect of LPA on mesangial cell proliferation rate as a function of cell density. Mesangial cell growth at different density population and other experimental conditions were exactly the same as noted in Fig. 1 and in MATERIALS AND METHODS. LPA response to induce DNA synthesis was assessed by calculating the fold increases in DNA synthesis over respective controls in sparse, subdense, and dense cell population. Data are means ± SE of 3 experiments. Statistical comparisons of data were done between sparse and subdense and between sparse and dense cell population groups.

![Graph showing EDG family receptors](image)

**Fig. 3.** Effect of cell density on endothelial differentiation gene (EDG)-2, EDG-4, and EDG-7 mRNA transcription. Mesangial cells were grown in MsGM growth media for 72 h to obtain sparse, subdense, and dense cell population as described in Fig. 1 and in MATERIALS AND METHODS. The medium was replaced with basal media containing 0.5% FBS and incubated for 24 h. After being washed with PBS, cells were collected for RNA isolation and RT-PCR or real-time RT-PCR analyses as described in MATERIALS AND METHODS. A: representative RT-PCR blot to show the expression of EDG-2, EDG-4, and EDG-7 in mesangial cells. B: representative graph showing the real-time RT-PCR mRNA transcription of EDG family receptors. We repeated these experiments 3 times and obtained similar pattern of data as shown in representative blot or graph.
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Fig. 4. Association between the expression of EDG family receptors and DNA synthetic rate as a function of cell density. Experimental conditions for mesangial cell growth at different density and RT-PCR analysis are exactly the same as described in Figs. 1 and 3, respectively. Quantitative analysis of EDG-2 and EDG-7 mRNA transcription was done by densitometric scanning of the transcriptional message and normalization with housekeeping gene GAPDH. DNA synthetic rate (proliferative rate) was calculated from the \(^{3}H\)-thymidine incorporation data (Fig. 1A) as cpm/1,000 cells seeded at time 0. The data are from the representative experiment. Three separate experiments were performed and obtained a similar data pattern as shown in the representative data.

assessed by Trypan blue exclusion method). In our studies, DGPP almost completely blocked LPA-induced mesangial cell proliferation (Fig. 5).

DISCUSSION

We showed that the transcription of EDG family LPA receptors is regulated by cell density-dependent processes in mesangial cells. Specifically, EDG-7 receptors are maximally expressed in sparse cell density (~25% confluency) and minimally expressed or downregulated in dense cell population (~75% confluency). Unlike the expression of EDG-7, the EDG-2 receptor expression was minimal in sparse and maximal in dense cell population. Furthermore, the expression profile of EDG-7 was positively associated with cell-proliferative rate with respect to cell density. On the other hand, EDG-2 expression pattern was negatively associated with cell-proliferative rate as a function cell density. To the best of our knowledge, this is the first report showing differential regulation of EDG family LPA receptors as a function of cell density and their specific association with cell-proliferative activity. Although our data suggested differential association of EDG-7 and EDG-2 with cell proliferation, further studies using overexpression and/or RNAi approaches are required to define the important roles of these EDG receptors in mesangial cell proliferation. Furthermore, additional studies are warranted to define the regulatory mechanisms of EDG-2 and EDG-7 transcription and the signaling network involved in mesangial cell proliferation as a function of cell density. It would also be important to further understand whether EDG-2 is an antiproliferative effector in mesangial cells.

The cloning and characterization of EDG family LPA receptors during the recent years have generated a great interest and rapid growth in LPA-related research. Additionally, the recent molecular genetic approaches in the area of EDG family LPA receptors (e.g., overexpressing or generation of EDG receptor-null mice) provided clear opportunities to understand the functional role of these receptors in LPA signaling (10, 47).

However, the cellular and molecular mechanisms that regulate the transcription of EDG family LPA receptors are not well defined. In our studies, it is intriguing to note that EDG-7 and EDG-2 transcription are oppositely regulated as a function of increasing cell density. These data suggest that different transcriptional signaling may be operated in regulating the expression of EDG-7 and EDG-2 receptors.

Although the molecular events involved in cell density-dependent transcription and proliferative activity are largely unknown, recent studies are emerging to suggest the participation of redox signaling and alterations in protein tyrosine phosphorylation milieu in cell density-mediated responses. Pani and associates (32) showed that the decreased proliferative rate in dense confluent cells was, at least in part, due to a decrease in the steady-state level of intracellular reactive oxygen species (ROS) and the consequent impairment of mitogenic redox signaling. These studies also showed that the decrease in the concentration of ROS in dense confluent cells was associated with diminished activity of the small GTPase Rac-1 and increased tyrosine phosphatase activity. Parallel to these studies, Fiaschi et al. (7) showed that the low molecular mass protein-tyrosine phosphatase is upregulated in dense cell cultures compared with sparse cell density. These authors also demonstrated that the platelet-derived growth factor (PDGF) receptor phosphorylation was markedly reduced in dense cell population than the sparse cell density. Further studies indicated that the tyrosine phosphorylation of Src, paxillin, and focal adhesion kinase was increased with increasing cell density (3). These observations point to the alterations of ROS and tyrosine phosphorylation/dephosphorylation signaling with respect to cell density. Additionally, density-dependent cellular and biochemical changes have been previously reported in various cell types including mesangial cells with respect to growth, changes in intracellular calcium levels to hormonal stimulation, extracellular matrix synthesis and composition, cytoskeletal protein expression, smooth muscle actin expres-

Fig. 5. Involvement of EDG-7 in LPA-induced mesangial cell proliferation. Mesangial cells were grown in MsGM growth media to obtain subdense cell population as described in Fig. 1 and in MATERIALS AND METHODS. The medium was replaced with basal medium containing 0.5% FBS and incubated for 24 h. Cells were preincubated with diacylglycerolpyrophosphate (DGPP; 20 µM; an antagonist for EDG-7) for 1 h, and cells were pulsed with \(^{3}H\)-thymidine (0.5 µCi/ml) in the absence or presence of LPA (10 µM) for 24 h. Cells were washed and digested in 0.25 N NaOH. An aliquot of cell digest was used to measure DNA synthesis and served as an index of cell proliferation. Data are means ± SE of 3 experiments. Statistical analyses were done between control vs. control + DGPP, LPA vs. LPA + DGPP, and LPA vs. control subgroups.
sion, ganglioside GM3 expression, and expression of distinct transforming growth factor-β receptor phenotypes (5, 13, 24, 25, 27, 34). Our data regarding decreased cellular proliferative rate with increasing cell density are parallel with previous observations in rat mesangial cell proliferation stimulated with 10% FBS (45) and fibroblasts (32). It is interesting to note that the secretion of fibronectin, collagen, and total protein synthesis in mesangial cells also followed similar pattern as of cell proliferation rate as a function of cell density (45). However, in these studies cell-associated matrix proteins did not follow this cell density-dependent pattern. As mesangial hypercellularity and matrix deposition are associated with glomerular injury, density-dependent modulation of cell growth and matrix protein synthetic activity may have a pathobiological significance in renal disease. Further studies are needed to understand whether altered ROS formation and other related tyrosine phosphorylation signaling regulate EDG family LPA receptor transcription in mesangial cells with respect to changes in cell density.

Although LPA has long been known as an important bioactive endogenous molecule to induce diverse cellular responses, only recent studies have investigated the role of LPA in mesangial cell physiology and renal disease. Plasma concentrations of LPA are shown to be about threefold higher in patients with renal failure on hemodialysis compared with healthy control subjects (35). In this study, a higher molar ratio of the plasma lysophosphatidylethanolamine/phosphatidylethanolamine was also noted in renal failure patients on hemodialysis compared with controls. Platelet aggregation has been commonly observed in the glomerular capillaries in many renal diseases (4), and the LPA released by activated platelets can enter the glomerulus to impact various responses. Extracellular LPA can also be generated through secretory phospholipase A2 through microvesicular shedding from blood cells challenged with inflammatory stimuli (9), suggesting the generation of LPA at the site of injury and inflammation. Indeed, such extracellular generation of LPA was observed in mesangial cells through group II PLA2 under inflammatory conditions (33, 36, 44). These observations suggest that the local concentrations of LPA within the mesangial area may be even higher in pathological conditions associated with increased glomerular phospholipase activity.

Inoue and associates (21) showed that LPA can stimulate rat mesangial cell proliferation, and LPA synergistically increased PDGF-mediated mesangial cell proliferation. In this study, LPA (10–30 μM) alone exhibited weaker proliferative activity (~1.4-fold) in rat mesangial cells. These studies also demonstrated that LPA activated MAP kinase, and preincubation with pertussis toxin inhibited both cell proliferation and MAP kinase activation by LPA in mesangial cells, suggesting the participation of G protein-mediated events in mesangial cell LPA signaling. Further studies indicated that LPA serves as a survival factor for mesangial cells suppressing PDGF-induced mesangial cell death (22). In another independent study, LPA (20 μM concentration) was also shown to stimulate rat mesangial cell proliferation by ~1.9-fold compared with controls (12). However, LPA at higher concentration (100 μM) significantly inhibited serum-induced mesangial cell proliferation, suggesting dual actions of LPA on mesangial cell growth (12). In our studies with human primary mesangial cells, LPA increased mesangial cell proliferation by 3–3.5-fold in dense cell population (50–75% confluency) and ~1.5-fold in sparse cell density compared with the respective cell density controls. As noted in our study, the differences in the degree of mesangial cell proliferation by LPA between our studies in human mesangial cells and other studies in rat mesangial cells may be due to variations in cell density-related processes and/or cellular species origin. Nevertheless, increased circulating LPA infiltrated into the mesangium or LPA generated by intrinsic glomerular cells in response to activation or inflammation may serve as endogenous pathobiological mediator for mesangial cell proliferation. Because EDG family receptors mediate LPA signaling, we further examined the involvement of EDG family receptor in LPA-induced mesangial cell proliferation. Recently, two independent groups have reported the characterization of selective antagonists for EDG family LPA receptors. Fisher et al. (8) found that a short-chain phosphatidic acid analog DGPP is an effective antagonist at the EDG-7 receptor with a reported IC50 of 106 nm at inhibiting calcium mobilization in RH7777 expressing EDG-7 receptor. Heise et al. (15) reported that an N-acyl ethanolamide phosphate (NAEPA) with benzyl-4-oxybenzyl group at the 2-position of the ethanol backbone selectively inhibited LPA-stimulated GTPγS binding in stably transfected EDG-2- and EDG-7-expressing RH7777 cells, with IC50 values of 137 and 428 nM, respectively. Either DGPP or NAEPA antagonists had any specificity for EDG-4 receptor, and currently there is no antagonist reported for EDG-4 receptor. Because EDG-7 was shown to be associated with mesangial cell-proliferative rate, using DGPP as a specific antagonist, we assessed whether EDG-7 is involved in LPA-mediated mesangial cell proliferation. Our data indicated that the preincubation of cells with DGPP almost completely blocked mesangial cell proliferation in both sparse and dense cell population. These data clearly suggest that LPA uses EDG-7 to mediate mesangial cell proliferation.

In our studies, DGPP also inhibited cell proliferation in control cells. In these studies, cells were incubated in media containing 0.5% FBS in the absence or presence of DGPP for 24 h. Because LPA is a component of serum, it may be possible that small amounts of LPA in the 0.5% FBS-containing media used during cell proliferation studies may have, at least in part, contributed for the ability of DGPP to inhibit DNA synthesis in control cells. Additionally, we showed that EDG-7 was positively associated with DNA synthetic rate. The antagonist activity of DGPP for EDG-7 receptor function may also have contributed to certain extent the ability of DGPP to inhibit DNA synthesis in control cells. In parallel with positive association of EDG-7 to cell proliferation, EDG-7 may be necessary for basal level proliferative activity. Blockage of EDG-7 function by DGPP may have inhibited DNA synthesis in control cells.

It was interesting to note that the response of LPA to induce mesangial cell proliferation was much higher in dense cell population (3.5-fold over control) compared with sparse cell density (1.5-fold over control). Such differential LPA response to induce cell proliferation has also been observed in fibroblasts (Xing Y and Kamanna VS, personal observation). We are not able to explain fully this differential cell proliferation response by LPA in sparse vs. dense cell density. As specific EDG family LPA receptors are shown to be positively or negatively associated with mesangial cell-proliferative rate as a function of cell density, it may be possible that the differential...
response of LPA to stimulate cell proliferation in varied cell density may be through differential regulation of the expression of EDG family LPA receptors in a cell density-dependent manner. Additionally, the potential mechanisms for this differential LPA response may be multifactorial and may include the differential response of LPA to induce specific growth factors in sparse vs. dense cells, variations in ROS generation in these different cell density, interactive effects with LPA, modulation in specific mitogenic signaling, etc. Further studies are required to understand the mechanisms of differential cell proliferation response by LPA as a function of cell density.

In summary, we demonstrated that cell density regulates the expression of EDG family LPA receptors in mesangial cells. Specifically, the expression of EDG-7 decreased with increasing cell density, whereas the expression of EDG-2 increased with increasing cell density. Comparative studies with mesangial cell proliferation indicated that EDG-7 expression pattern was positively associated with mesangial cell-proliferative rate as a function of cell density. However, EDG-2 expression was negatively associated with mesangial cell proliferation rate with respect to cell density. Further studies showed that the mesangial cell-proliferative response by LPA was greater in dense cell population compared with sparse cell density. Blockage of EDG-7 by specific antagonist completely inhibited LPA-induced mesangial cell proliferation. These studies suggest that EDG family LPA receptors play an important role in mesangial cell growth. Understanding the regulatory transcriptional mechanisms of these LPA receptors may provide novel tools to control mesangial cell proliferation seen in mesangial proliferative glomerular diseases. Further in vitro and in vivo studies are warranted to extend these studies and to establish the role of EDG family receptors in renal disease.

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