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# Modulation of Cell Proliferation and Hypertrophy by Gangliosides in Cultured Human Glomerular Mesangial Cells

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Glomerular mesangial cells (GMCs) in diverse renal diseases undergo cell proliferation and/or hypertrophy, and gangliosides have been reported to play an important role in modulating cell structure and function. This study compared the effects of transforming growth factor-β1 (TGF-β1) and the effects of the application of exogenous gangliosides on GMCs and investigated whether the application of exogenous gangliosides regulated cellular proliferation and hypertrophy. Human GMCs were cultured with exogenous gangliosides and TGF-β1 in a media containing 10% fetal bovine serum and in a media without the fetal bovine serum. Exogenous gangliosides biphasically changed the proliferation of human GMCs (0.1-1.0 mg/mL). A low concentration (0.1 mg/mL) of gangliosides mainly increased the number of human GMCs, whereas cellular proliferation was significantly reduced by raising the concentration of exogenous gangliosides. TGF-β1 greatly reduced the number of human GMCs in a concentration-dependent manner (1-10 ng/mL). Serum deprivation accelerated the gangliosides- and TGF-β1-induced inhibition of mesangial cell proliferation to a greater extent. Gangliosides (1.0 mg/mL) and TGF-β1 (10 ng/mL) both caused a significant increase in the incorporation of [$^3$H]leucine per cell in the serum-deprived condition, whereas it was completely reversed in serum-supplemented condition. Similar results to the [$^3$H]leucine incorporation were also observed in the changes in cell size measured by flow cytometric analysis. These results show that exogenous gangliosides modulate cell proliferation and hypertrophy in cultured human GMCs, and these cellular responses were regulated differently based on whether the media contained serum or not. Results from the present study raise new possibilities about the potential involvement of gangliosides in the development of mesangial cell proliferation and hypertrophy.

**Key words:** Gangliosides, TGF-β1, Proliferation, Hypertrophy, Human glomerular mesangial cells

## INTRODUCTION

Glomerular mesangial cells (GMCs) are the target of a variety of glomerular diseases such as secondary focal segmental glomerulosclerosis and diabetic nephropathy. When these glomerular diseases occur GMCs undergo significant morphological changes. Among the various cytokines involved in the progression of glomerular sclerosis, transforming growth factor-β (TGF-β) is a prominent factor. Stimulation of GMCs by TGF-β induces an increase in the expression of α-smooth muscle actin (α-SMA, Dubus *et al.*, 2002), a decrease in cell proliferation and stimulates cell hypertrophy (Border and Noble, 1997). In pathological conditions, there are sequential and/or simultaneous changes in GMC phenotypes including initial mesangial proliferation and hypertrophy, transformation into myofibroblast-like cells and mesangial cell-mediated pathological extracellular matrix remodeling (Kagami *et al.*, 2000; Floege *et al.*, 1992). Activated GMCs have been shown to proliferate as a result of an increase in the production of interstitial collagens and have been shown to hypertrophy as a result of an enhanced expression of α-SMA. Therefore, it is conceivable that proliferation and

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hypertrophy of GMCs are two specific responses that may result from mesangial cell activation, and these responses are important in the pathology and potential progression of glomerular sclerosis and end-stage renal diseases. Investigation of the cellular and molecular mechanisms responsible for morphological changes of GMCs may help us to understand the pathogenesis of progressive glomerular sclerosis.

On the other hand, gangliosides are sialic acid (NeuAc)-conjugated glycosphingolipids that are a component of the plasma membrane of all vertebrate cells. They play an important role in a large variety of biological processes such as cell differentiation, proliferation, and cell-cell interaction (Hakomori, 1990). Gangliosides are particularly abundant in kidney tissue (Shayman and Radin, 1991). Due to their abundance and electronegative charge, it is widely accepted that alteration of ganglioside expression may be an important factor in the pathogenesis of glomerular diseases. Recently, it has been suggested that glomerular hypertrophy is characterized by a decrease in ganglioside expression in streptozotocin-induced diabetic rats, and that gangliosides may negatively regulate the high-ambient glucose-induced proliferation of cultured rat GMCs (Kwak *et al.*, 2003; Rho *et al.*, 2004). Based on these findings, we considered that gangliosides may act as an endogenous molecule to regulate cellular responses of GMCs in a pathological state. However, despite the variety of cellular functions that have been suggested for gangliosides, little is known about their pathophysiological involvement in the development of mesangial cell proliferation and hypertrophy. In this report, using pharmacological manipulation for cellular ganglioside contents and comparing the effects of TGF-β1, we provide direct evidence that gangliosides are a potent modulator of cellular proliferation and hypertrophy in cultured human GMCs.

## MATERIALS AND METHODS

### Mesangial cell culture

Human GMCs, which were a generous gift from Dr. Hun Joo Ha (College of Pharmacy, Ewha Woman's University, Seoul, Korea), were cultured in a RMPI 1640 media containing 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 20 mM $NaHCO_3$, 20% heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in 95% air/5% $CO_2$ condition. In the experiments, confluent human GMCs were transferred to 24-well plates ($2\times10^4$ cells/well) or 10 cm dishes ($1\times10^6$ cells/dish) and cultured with the RPMI 1640 media containing 20% FBS for 24 h, followed by incubation with serum-free media for 48 h to arrest and synchronize cell growth. Then, the human GMCs were further incubated with exogenous gangliosides and TGF-β1 for 24 h in the serum-free or 10% serum-supplemented condition. The human GMCs used in this study were between passages 5 and 10.

### Extraction and determination of gangliosides

To examine the effects of gangliosides on cell proliferation and hypertrophy of cultured human GMCs, we used gangliosides from the cerebral cortex of male Sprague-Dawley rats (10-week-old). Gangliosides were extracted and purified according to the method outlined by Harth *et al.* (1978), and total ganglioside-NeuAc was determined using the resorcinol method (Svennerholm, 1957). This study used monodimensional ascending chromatography for ganglioside separation, which was carried out on Silica G high-performance thin-layer chromatography (HPTLC) plates (10×10 cm) according to the method outlined by Harth *et al.* (1978) using three different solvent systems: (1) chloroform to the top; (2) chloroform-methanol-water (70:30:4, v/v/v); (3) chloroform-methanol-0.25% $CaCl_2$ (50:42:11, v/v/v). Ganglioside-NeuAc in the fractions obtained by thin-layer chromatography was determined according to the method outlined by Horgan (1981). The percentage distribution of ganglioside GM3, GM2, GM1, GD1a, GD1b, GT1b, and GQ1b were 10.5, 9.6, 23.4, 24.7, 13.7, 15.2, and 2.9, respectively. These results were very similar to previously published results (Yamagishi *et al.*, 2003).

### Measurement of cell proliferation and viability

After the 24 h stimulation period, the human GMCs were washed 3 times with ice-cold phosphate-buffered saline (PBS, pH 7.4) and collected by trypsinization. At the same time, the culture media was collected and centrifuged at 500 g for 10 min to obtain the cells detached from the culture plates, and the cells were then combined with the GMCs which adhered to plates. All cells were suspended with PBS. Trypan blue solution (0.4%) was added to the aliquots of the cell suspension in a 1:1 ratio, and the mixture was allowed to stand for 10 min at room temperature. A hemocytometer was used to count live and dead cells, and percent viability was calculated.

### Measurement of [$^3$H]leucine incorporation

The incorporation of [$^3$H]leucine was measured to assess de novo protein synthesis using the method outlined by Wolf and Neilson (1990) with slight modifications. Briefly, after the human GMCs in the 24-well plates ($1\times10^5$ cells/well) were made quiescent for 48 h in RPMI 1640 without serum, the cells were then stimulated for 24 h with gangliosides and/or TGF-β1 in the presence or absence of 10% FBS. [$^3$H]leucine (0.1 μCi/mL) was incubated for the last 12 h. At the end of the stimulation period, the

GMCs were washed twice with ice-cold PBS and collected by trypsinization. Cellular proteins were subsequently precipitated with ice-cold 10% trichloroacetic acid. After redissolving the precipitates in 0.5 M NaOH containing 0.1% Triton X-100, 5 mL of scintillation cocktail (Roth, Karlsruhe, Germany) was added, and radioactivity was measured by liquid scintillation spectroscopy (Beckman, LS6500). The number of cells in each well was measured simultaneously, and the ratio of [$^3$H]leucine incorporation to the number of cells was determined.

### Measurement of cell size by flow cytometry

After stimulation with exogenous gangliosides and TGF-β1 for 24 h in the serum-free or 10% serum-supplemented condition, the GMCs were washed twice with ice-cold PBS and harvested with 5 mM EDTA and resuspended in the RPMI 1640 media containing 10% FBS, and then placed on ice. Aliquots of the cells were counted and centrifuged at 500 g for 10 min at 4°C. The cells were resuspended at a concentration of $10^6$ cells/mL. The cells were kept on ice and then analyzed with a Becton Dickinson Facscan fluorescence-activated cell sorter using the forward light scatter mode. Results were obtained as plots of cell population (granules versus cell size).

### Measurement of protein contents

The ratio of total protein contents to the numbers of cell was also investigated as another parameter of cellular hypertrophy. At the end of stimulation period, the GMCs were trypsinized, scraped off the plates with a rubber policeman, and washed twice with ice-cold PBS. A small cell aliquot was used to count the number of cells following the resuspension of the cells in PBS. The remaining cells were lysed in 0.5 mM NaOH, and total protein contents were measured by a modified Lowry method using bovine serum albumin as the standard.

### Materials and reagents

Penicillin G, streptomycin, and *N*-acetyl neuraminic acid were obtained from Sigma Chemical (St Louis, MO). RPMI 1640 media and FBS were purchased from Gibco BRL (Gaithersburg, MD). HPTLC plate, Sep-Pak $C_{18}$ column, and [$^3$H]leucine (5 μCi, 142 Ci/mmol) were obtained from Merck (Darmstadt, Germany), Waters (Milford, MA), and Amersham Pharmacia Biotech (Piscataway, NJ), respectively. All other chemicals were of the highest grade and obtained from commercial sources.

### Statistical analysis

Results are expressed as mean ± S.E. Analysis of variance (ANOVA) and Student's *t*-test were performed on the quantitative data. For all experiments $p$ values <0.05 were considered statistically significant.

## RESULTS

### Change in the number of cells

Since the serum contains a variety of biologically active substances that may potentially regulate the structure and function of GMCs through several different pathways (Stephenson *et al.*, 1998), we hypothesized that the serum would affect the ganglioside- and TGF-β1-induced proliferation and/or hypertrophy of GMCs. Consequently, this study examined this hypothesis using experimental conditions supplemented with or without sera. Fig. 1 shows concentration-dependent changes in the number of cells by gangliosides and TGF-β1 in cultured human GMCs. The application of exogenous gangliosides (0.1-1.0 mg/mL) to GMCs biphasically modulated cellular proliferation in both the serum-free and 10% serum-supplemented conditions (Fig. 1A). Low concentration of gangliosides significantly increased the number of cells,

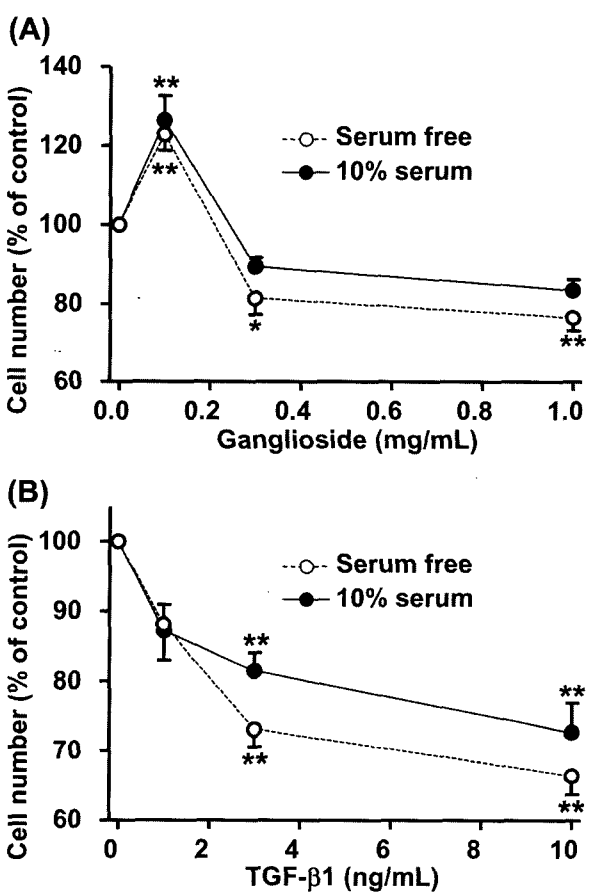


**Fig. 1.** Effects of gangliosides and TGF-β1 on the proliferation of human glomerular mesangial cells. After arresting the cell cycle and growth for 48 h, cells were treated with various concentrations of exogenous gangliosides (A) or TGF-β1 (B) for 24 h in the serum-free and 10% serum-supplemented condition. Results are expressed as mean ± S.E. of seven independent experiments. *$p$<0.05, **$p$<0.01 *vs.* control.

whereas high concentrations (≥0.3 mg/mL) greatly reduced the numbers of GMCs. In contrast to this, TGF-β1 (1-10 ng/mL) caused a significant reduction in the number of cells in a concentration-dependent manner (Fig. 1B). Although these cellular responses to exogenous gangliosides and TGF-β1 were not significantly different between the serum-deprived and 10% serum-supplemented conditions, serum deprivation accelerated gangliosides-, and TGF-β1-induced inhibition of mesangial cell proliferation to a greater extent. None of the conditions significantly change cell viability (data not shown).

### [$^3$H]Leucine incorporation

Since new protein synthesis is a useful method to measure cellular hypertrophy (Monkawa *et al.*, 2002), cellular hypertrophy was measured by assessing [$^3$H]leucine incorporation. Fig. 2 shows the ganglioside- and TGF-β1-induced changes of [$^3$H]leucine incorporation in the serum-free and 10% serum-supplemented conditions.

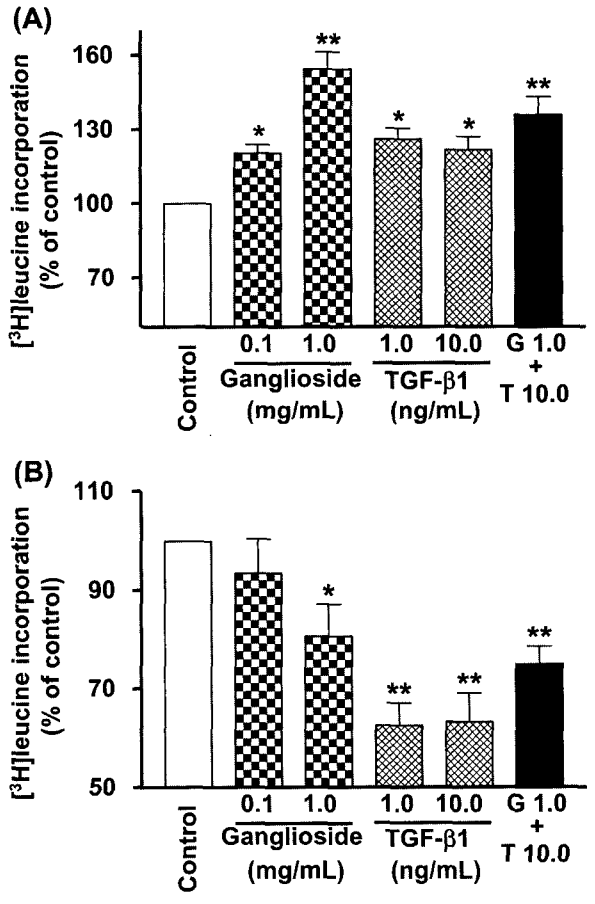


**Fig. 2.** Effects of gangliosides and TGF-β1 on [$^3$H]leucine incorporation in human glomerular mesangial cells. Cells were treated with exogenous gangliosides or TGF-β1 for 24 h in the serum-free (A) and 10% serum-supplemented (B) condition, and incorporation of [$^3$H]leucine per cell was measured. Results are expressed as mean ± S.E. of seven independent experiments. *$p$<0.05, **$p$<0.01 *vs.* control.

Exogenous gangliosides (0.1 and 1.0 mg/mL) and TGF-β1 (1.0 and 10 ng/mL) significantly increased incorporation of [$^3$H]leucine in the serum-free condition, and ganglioside (1.0 mg/mL)-induced increase of [$^3$H]leucine incorporation was partially inhibited by simultaneous treatment of GMCs with 10.0 ng/mL of TGF-β1 (Fig. 2A). In contrast to this, when GMCs were incubated with exogenous gangliosides and TGF-β1 in the presence of the serum, incorporation of [$^3$H]leucine was significantly lower compared to the control, and TGF-β1 (10.0 ng/mL) partially restored the ganglioside (1.0 mg/mL)-induced decrease of [$^3$H]leucine incorporation (Fig. 2B). Changes in total protein contents were closely correlated with the results observed in the incorporation of [$^3$H]leucine per cell (data not shown).

### Flow cytometry

Flow cytometric analysis was also used to assess cellular hypertrophy. Fig. 3 shows the distribution of cell size in the various different conditions. Regardless of the agents added, human GMCs cultured in the 10% serum-supplemented condition appeared to be larger than those cultured in the serum-free condition. The cells treated with gangliosides (panel A-2; 0.1 mg/mL, panel A-3; 1.0 mg/mL) and TGF-β1 (panel A-4; 1.0 ng/mL, panel A-5; 10 ng/mL) in the serum-free condition were larger than those in the control (panel A-1), and ganglioside (1.0 mg/mL)-induced increase in cell size was partially reversed by simultaneous treatment of GMCs with TGF-β1 (10 ng/mL, panel; A-6). In contrast to this, the population of human GMCs treated with gangliosides (panel B-2; 0.1 mg/mL, panel B-3; 1.0 mg/mL) and TGF-β1 (panel B-4; 1.0 ng/mL, panel B-5; 10 ng/mL) in the serum-free condition were smaller than those in the control (panel B-1), and gangliosides (1.0 mg/mL)-induced decrease in cell size was partially reversed by simultaneous treatment of cells with TGF-β1 (10 ng/mL, panel; B-6). These results correspond well with the results shown in Fig. 2.

## DISCUSSION

Our study shows that exogenous gangliosides and TGF-β1 modulate the proliferation and hypertrophy of cultured human GMCs, and that these cellular responses were regulated differently based on whether the culture media contained serum or not. Exogenous gangliosides biphasically modulated mesangial cell proliferation in both the serum-free and 10% serum-supplemented conditions. Specifically, they stimulated proliferation at low concentrations and inhibited proliferation at high concentrations. TGF-β1 inhibited the proliferation of human GMCs in both the serum-free and 10% serum-supplemented conditions. Interestingly, both exogenous gangliosides and TGF-β1 stimulated the hypertrophy of human GMCs in the serum-

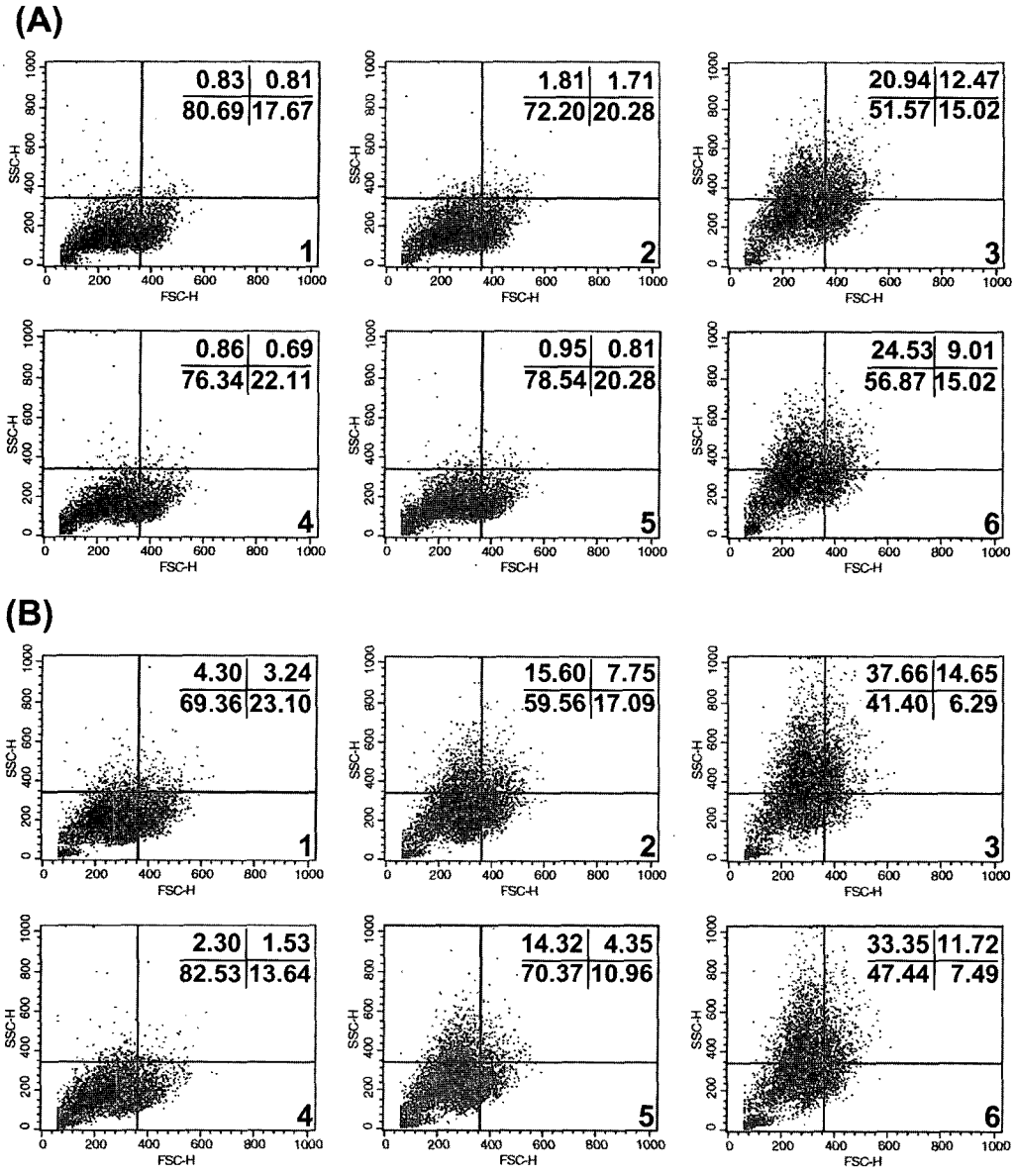


**Fig. 3.** Forward light scatter flow cytometry of human glomerular mesangial cells treated with gangliosides and TGF-β1. Cells were treated with exogenous gangliosides or TGF-β1 for 24 h in the serum-free (A) and 10% serum-supplemented (B) condition. Cell population is plotted on the x-axis (cell size) and y-axis (granule). 1; control, 2; 0.1 mg/mL gangliosides, 3; 1.0 mg/mL gangliosides, 4; 1.0 ng/mL TGF-β1, 5; 10.0 ng/mL TGF-β1, 6; 1.0 mg/mL gangliosides *plus* 10.0 ng/mL TGF-β1.

free condition, whereas cellular hypertrophy was antagonized by these agents in 10% serum-supplemented condition. These results provide a new rationale, suggesting that gangliosides and TGF-β1 appear to play a pathophysiological role in the proliferation and hypertrophy of human GMCs, which are prominent characteristics observed in the pathogenesis of progressive glomerular sclerosis.

Progressive sclerosis of glomeruli is the common pathway by which several forms of renal disease occurs, which all result in the activation of mesangial cells caused by inflammatory events. Several *in vivo* studies have demonstrated the significance of mesangial cell proliferation in the early stage of diabetic glomerulopathy, and GMCs may undergo hyperplasia and hypertrophy simultaneously (Mahadevan *et al.*, 1996; Young *et al.*, 1995). These reports suggest that the predicted evolution of diabetic glomerulopathy is comprised of an early, self-limited degree of mesangial cell proliferation and hypertrophy, and that these mesangial responses predict the slow progression into glomerular sclerosis. Therefore, the investigation of the cellular mechanisms responsible for proliferation and hypertrophy of GMCs may help us to understand the pathogenesis of progressive glomerular sclerosis. In fact, the utilization of human GMCs is made

more difficult by their limited life span and their dedifferentiation in culture. To avoid these problems, the human GMCs were used at a limited passage (5 to 10) in this study, and they had most of the properties of mesangial cells as described in a previous report (Dubus *et al.*, 2002). Moreover, it has been generally accepted that serum has a mitogenic property, and the factors are probably diverse. This means that serum contained in the culture media possibly modulates the proliferation and hypertrophy of human GMCs. It may be scientifically meaningful to understand the cellular mechanisms involved in the ganglioside-induced proliferation and hypertrophy of human GMCs in the presence or absence of serum.

Gangliosides, a sialic acid-conjugated glycosphingolipid, play an important role in a large variety of biological processes such as cell differentiation, proliferation, and cell-cell interaction (Hakomori, 1990). In early studies (Hakomori and Igarashi, 1995; Simons *et al.*, 1999), gangliosides had been exogenously applied to a wide range of cells *in vitro,* and this pharmacological strategy is generally considered to be a useful method to study the function of gangliosides in the plasma membrane. It became clear that after adding the exogenous gangliosides to the cells, they could not be removed by extensive washing and were inserted into the exoplasmic leaflet of the plasma membrane by operationally defined modes (Schwarzmann, 2001). Application of exogenous gangliosides to cells affects various biological systems such as cell adhesiveness, differentiation and growth. Despite the considerable effort that has gone into analyzing the method of ganglioside uptake by cell surface membrane, biochemical and molecular mechanisms involved in ganglioside uptake by human GMCs are not entirely clear. The present findings provide new evidence, suggesting that the application of exogenous gangliosides to human GMCs can be inserted into the plasma membrane and may modulate the growth of these cells by influencing the overall structure of the plasma membrane, in particular on the formation of cholesterol-sphingolipid rafts (Simons *et al.*, 1999). However, many questions with regard to the biochemical and biophysical mechanisms underlying ganglioside uptake by human GMCs remain to be investigated.

Previous studies have demonstrated that glomerular hypertrophy is characterized by a decrease in ganglioside expression in streptozotocin-induced diabetic rats (Kwak *et al.*, 2003), and ganglioside GM3 negatively regulates the high-ambient glucose-induced proliferation of rat GMCs in culture (Rho *et al.*, 2004). These findings indicate that gangliosides may act as an endogenous regulator for mesangial cell proliferation and hypertrophy. To confirm these findings, using a pharmacological strategy, this study examined whether exogenous gangliosides could regulate the proliferation and hypertrophy of human GMCs in culture. This study used gangliosides isolated from the cerebral cortex of rats, and were identified as a mixture of seven different gangliosides: GM3 (10.5%), GM2 (9.6%), GM1 (23.4%), GD1a (24.7%), GD1b (13.7%), GT1b (15.2%), and GQ1b (2.9%). Application of exogenous gangliosides to human GMCs biphasically modulated cell proliferation. Specifically, they stimulated proliferation at low concentrations and inhibited proliferation at high concentrations (Fig. 1A). Although there is no direct information to explain the cellular mechanisms involved in the biphasic proliferation of human GMCs by exogenous gangliosides, we considered that these cellular responses may be related with a great structural complexity and functional heterogeneity of gangliosides, because gangliosides express a variety of biological functions with cell-specific patterns (Hakomori, 1990). The inhibitory effects of exogenous gangliosides on mesangial cell proliferation seen in this study are consistent with previous findings (Tsuboi *et al.*, 2001), suggesting that mesangial cells constitutively shed gangliosides and then possibly suppress the proliferation of these cells through an apoptotic mechanism. The results of this study may also support the idea that the wide spectrum of cellular effects exerted by gangliosides can be explained by interaction of gangliosides with functional molecules (lipids and proteins) distributed in lipid rafts of the plasma membrane.

It is generally accepted that [$^3$H]leucine incorporation and the ratio of total protein to the number of cells are useful methods to measure cellular hypertrophy (Fujino *et al.*, 2002; Monkawa *et al.*, 2002). Consequently, we examined the effects of exogenous gangliosides and TGF-β1 on the hypertrophy of human GMCs using these reliable techniques. Application of exogenous gangliosides to human GMCs caused a significant increase in [$^3$H]leucine incorporation in the serum-free condition, whereas a decrease in [$^3$H]leucine incorporation was observed in 10% serum-supplemented condition. TGF-β1 also displayed a different effect on the proliferation and hypertrophy in human GMCs cultured in the serum-free and 10% serum-supplemented conditions. These results were corresponded well with the ratio of total protein to the number of cells (data not shown). Interestingly, flow cytometric analysis showed that the application of exogenous gangliosides to human GMCs produced a significant increase in cell population with granules. Several reports have suggested that gangliosides play a major role in cell-cell and cell-matrix recognition *via* interactions with cell adhesion molecules such as integrins, extracellular matrix proteins and other glycosphingolipids (Hakomori, 1990) and are also known to regulate transmembrane signaling by modulating functional membrane proteins (Mirkin *et al.*, 2002). Thus, they have been described to regulate proliferation

of different cell types. With regards to cellular interaction and transmembrane architecture, we believe that mitogenic factors contained in the serum may directly and/or indirectly regulate the ganglioside- and TGF-β1-induced mesangial proliferation and hypertrophy through modulation of cell adhesion molecules and growth factor receptors, which may interact with gangliosides in the cellular transmembrane (Hakomori, 1990; Simons *et al.*, 1999).

In addition, growth of GMCs during pathological states consists of an early, transient and limited cellular proliferation, followed by growth-arrest and hypertrophy. Specifically, it has been shown in streptozotocin-induced diabetic glomeruli that GMCs arrest at the $G_1$ phase and undergo sustained hypertrophy associated with an increase in the synthesis and deposition of extracellular matrix proteins (Wolf, 2000). These findings may be supported by the results of this study, demonstrating that exogenous gangliosides modulate the proliferation and hypertrophy of human GMCs. Recently, Masson *et al.* suggested that glucosamine induced cell cycle arrest and hypertrophy through an increase in the expression of gangliosides GM2 and GM1 and cyclin-dependent kinase inhibitor p21 in mesangial cells (Masson *et al.*, 2005). These suggestions may be comparable with our results. Although this study did not explain the cellular mechanisms involved in the exogenous ganglioside-induced hypertrophy of human GMCs, we believe that this may be related with the apoptotic pathway for human GMCs, because GMCs during pathological states undergo an early, transient and limited cellular proliferation, followed by growth-arrest at $G_1$ phase and hypertrophy, and the emerging role of gangliosides as apoptosis regulators are increasingly recognized (Morales *et al.*, 2004). If this is correct, the granules observed in the human GMCs treated with exogenous gangliosides may be related to the apoptotic fragments in the nuclei of cells.

In conclusion, the results of this study show that the application of exogenous gangliosides to human GMCs modulates cellular proliferation and hypertrophy in serum-free and 10% serum-supplemented conditions, and that these cellular responses were regulated differently based on whether the media contained serum or not. In addition, the results from this study raise new possibilities about the potential involvement of gangliosides in the development of mesangial cell proliferation and hypertrophy. It would be of interest to investigate the cellular and molecular roles of each ganglioside in the modulation of mesangial cell proliferation and hypertrophy caused by pathological conditions such as hyperglycemia, and this effort may help in the development of a pharmacological strategy to prevent and/or treat progressive glomerular sclerosis. This is currently under investigation.

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