THE FUNCTIONAL CHARACTERISTICS OF CULTURED RAT MESANGIAL CELL

TOSHIHiro TANAKA, M.D.* YOSHIHIRO FUJIWARA, M.D.*, YOSHIMASA ORITA, M.D.*
EIKI SASAKI, M.D.*, HAJIME KITAMURA, M.D.*, AND HIROSHI ABE, M.D.*

Glomerular mesangial cells were isolated from explants of normal rat whole glomeruli according to the differential growth capacities of the cultured cells. The cultured mesangial cells comprised almost all of the grown cells on day 21 of the primary culture when the subculture could be performed. They contained abundant cytoplasmic microfilaments and actin. The contractile response to vasoactive hormone of both primarily cultured and subcultured mesangial cells was quantified as a decrease in cell surface area. The maximum decrease in surface area in response to 1 nM of angiotensin II was about 30%. This magnitude of contraction is consistent with that observed in cultured rat vascular smooth muscle cells. The mesangial cells did not phagocytose horse-radish peroxidase or sensitized sheep red blood cells, nor did they have binding capacities for Fc or C3b, while the cells derived from explanted glomerulus and probably of blood-born monocyte origin did.

We conclude that the most important function of the glomerular mesangial cell is the control of glomerular blood flow and filtration by contraction.

GLOMERULAR mesangial cells are attributed with two major functions: the clearing of macromolecules from the mesangial region by phagocytosis, and the control of glomerular blood flow by contraction. The former function may be associated with the pathogenesis of immune-mediated glomerulonephritis and the latter one with the progressive fall in glomerular filtration rate in glomerular injuries.

Recently, doubt has been cast on the phagocytic activity since Striker, et al. demonstrated that marrow-derived monocytes other than mesangial cells, which had migrated into the mesangial region, were able to remove immune complexes from glomeruli by phagocytosis. At the same time, an increasing amount of evidence is accumulating on the contractile activity of the cells, using cell culture technique which allows the study of homogenous population of mesangial cells under controlled conditions without the influence of other glomerular cells and extraglomerular factors. Of special interest is the morphological evidence that cultured mesangial cells contract in response to vasoactive hormones such as angiotensin II and vasopressin and norepinephrine. In these studies, the subcultured mesangial cells which

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*Department of Pathology, **Department of Medicine, Osaka University Medical School, Osaka, Japan

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Mailing address: Toshihiro Tanaka, M.D., Department of Pathology, Osaka University Medical School, Nakanoshima 4-3-57, Kita-ku, Osaka 530, Japan

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firmly attached to the substratum of the culture dishes were incubated with the hormones and the subsequent changes in shape examined by electron or phase contrast microscopy. However, none of these studies were able to quantify the magnitude of the contraction.

In this study we isolated a culture of glomerular mesangial cells from explants of whole glomeruli in the normal rat, and reevaluated the immune-related functions of the cell. We also quantified the magnitude of contraction of the cell in response to angiotensin II using two new techniques.

MATERIALS AND METHODS

1. Isolation of glomeruli

Glomeruli were isolated from rat renal cortex by the sieving technique originally described by Krakower and Greenspon\(^\text{11}\) with minor modifications. Male Sprague-Dawley rats weighing 70 to 90g maintained on a normal sodium diet and water ad libitum were anesthetized with ethyl ether and the kidneys immediately removed. Eight kidneys, as a rule, were processed at one time. Following the removal of the renal capsule, the cortex was dissected and minced into a fine paste with scalp blades. After several washings with cold Hanks’ balanced salt solution (HBSS) (Osaka University, The Research Institute for Microbial Diseases (RIMD), Osaka), the minced cortex was mashed through a 120 mesh sterile stainless steel sieve with an angulated spatula, then passed through an intervening 60 mesh and a final 280 mesh in succession. The glomeruli retained above the final mesh were suspended in cold HBSS and centrifuged at 1000 rpm for about 5 min after which the supernatant was gently removed by suction. The pellet was resuspended in the same buffer, allowed to settle, and the supernatant was again drawn off by suction. This last procedure was repeated several times until the supernatant became clear. The numbers of glomeruli and nonglomerular fragments contained in the final suspension were determined microscopically, and a suspension containing over 90% decapsulated glomeruli (Fig. 1a, b) was used for the cell culture. All the
steps were carried out under sterile conditions.

2. Cell culture

A volume of glomerular suspension just adequate to cover the growth surface and containing about 5000 glomeruli was added to a 60 mm Lux culture dish (Miles Laboratories Inc., Naperville). The glomeruli were cultured at 37°C in an atmosphere of 95% air, 5% carbon dioxide, or a medium containing 5 ml of RPMI 1640, supplemented with 20% decomplemented fetal calf serum (FCS) (Irvine Scientific, Santa Ana), and insulin 1 µg/ml. The culture medium was not changed until the glomeruli firmly attached to the substratum of the dish, normally after 1 week. Thereafter, it was changed twice a week. On day 21 of the primary culture, confluent monolayers were rinsed with cold phosphate buffered saline without Ca²⁺ and Mg²⁺ (PBS (−)) and subcultures were taken using 0.02% EDTA solution (Osaka University, RIMD, Osaka) and warm 0.125% trypsin solution (Osaka University, RIMD, Osaka). The conditions of the subcultures were identical to those used for isolated glomeruli. Experiments were performed on days 10 and 21 of the primary culture and on day 10 of the 2nd subculture.

3. Immunofluorescent studies

Cells from days 10 and 21 of the primary culture grown on tissue culture chamber slides (Lab. Tek. Miles Laboratories Inc., Naperville) were used. The slides were washed twice by dipping in PBS (−) pH 7.5, fixed for 30 min in periodate-lysine-paraformaldehyde (PLP) at 4°C, washed in PBS (−) three times for 5 mins each, and air-dried. An adequate amount of either fluorescein isothiocyanate (FITC)-labeled rabbit antiserum for human fibronectin (Cappel Laboratories Inc., Cochraneville) or FITC-labeled, rabbit skeletal muscle heavy meromyosin (HMM) (a gift from Dr. N. Shibata, The Center for Adult Diseases, Osaka) was added, and staining allowed to proceed for 60 min in a moist atmosphere at room temperature. After staining, the slides were given three 5-min washes in PBS (−) then mounted in 50% glycerin. During this procedure, great care was taken to protect the cell layer from drying out. The slides were then examined using a Nikon fluorescent microscope equipped with an SH-200 mercury lamp.

4. Electron microscope studies

For transmission electron microscopic examination, the cells on day 10 of the 2nd subculture were used, prepared by two different methods. One preparation was a monolayer detached from the dish by the use of 0.02% EDTA alone. The other was dissociated cells scraped off the dish by the use of 0.02% EDTA, 380 IU/ml type I collagenase (Sigma Chemical Company, St. Louis) and 1 mg/ml trypsin inhibitor (Sigma Chemical Company, St. Louis) according to the method of Foidart, et al. The prepared cells were pelleted, fixed in 2.5% glutaraldehyde buffered with 0.1 M cacodylate buffer at 4°C for 1 hour, then rinsed overnight in the same buffer. They were postfixed in 1% osmic acid, washed several times, dehydrated in a graded ethanol series, and embedded in Epon 812. The ultrathin sections were doubly stained with uranyl acetate and lead citrate, and examined with a Hitachi H-300 electron microscope.

5. Determination of C3b and Fc receptors and phagocytosis in vitro

The cells on days 10 and 21 of the primary culture and those on day 10 of the 2nd subculture were used. To identify Fc receptors and phagocytic activity for immune complexes, the cells were incubated with a suspension containing sheep red blood cells (SRBC) sensitized with hemolysin at 37°C for 30 min. To identify C3b receptors, the cells were incubated with a suspension containing SRBC treated with hemolysin and C43. As controls, the cells were incubated with untreated SRBC and SRBC treated with hemolysin and C42. In each experiment, nonadherent SRBC were washed away and the preparation was examined for the presence of rosettes and intracellular uptake of SRBC by phase contrast microscopy (Olympus IM). As an additional experiment, cells from each culture were incubated with 0.2 mg/dl of horse-radish peroxidase (HRP) at 37°C for 1 hour, then fixed with PLP for 20 min. After washing with PBS, the preparation was allowed to react with substrate of diaminobenzidine for 15 min, counterstained with Carazzi's hematoxylin, and then finally examined by photomicroscopy (Nikon OPTIPHOTO XUW-21).

6. Analysis of cell contraction by a tablet digitizer

One hour before the experiment, the cells on day 21 of the primary culture had their growth medium changed to HBSS without FCS. They were then incubated in HBSS
Fig. 2. Phase contrast photomicrograph of A-cells (on day 10 of the primary culture). Note the typical cobblestone-like appearance of the cells. (×100).

Fig. 3. Phase contrast photomicrograph of B-cells (on day 21 of the primary culture). Note the prominent intracellular fibrillar structures and digitated projections. The cells overlap one another in culture, forming "hills and valleys". (×100).

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Fig.4. Transmission electron micrographs of B′-cells (on day 10 of the 2nd subculture). 
a: The cell dissociated from the dish as a monolayer shows large bundles of intracellular microfilaments (double arrows) and extracellular noncollagenous materials (short arrow). ×920. 
b: The cell dissociated from the dish and dispersed has short and blunt processes, abundant endoplasmic reticulum and few lysosomes. ×10600.
with 2 mM Ca\(^{2+}\), 2 mM Mg\(^{2+}\), and 1 nM angiotension II (Osaka University, Institute for Protein Research, Osaka). The movements of cells which had been freshly outgrown from the glomerulus and were attached only partially to the substratum of the dish were photographed every 10 sec for 10 min by a phase contrast microscope equipped with a carbon dioxide incubator conditioned at 37°C in an atmosphere of 90% air and 10% carbon dioxide. Photographs were magnified 5000 times using a projector (Olympus UP-350), and the surface outline of the cells was traced precisely and analyzed by a tablet digitizer (Mutoh Digigrammer Model G). In each experiment, 3 to 5 cells were analyzed every min, and the percentage of initial surface area (at time 0) was calculated for each cell.

7. Analysis of cell contraction by a particle counter
The cells on day 10 of the 2nd subculture were scraped off their dish, dissociated in the same way as described for electron microscope examination, and suspended in HBSS without FCS. We had preliminarily confirmed, by direct binding assay using \(^{125}\)I-angiotension II, that these cells had specific angiotension II receptors with an equilibrium dissociation constant in the order of 0.1 nM (unpublished data). Incubation with angiotension II was carried out as above and the diameters of \(10^4\) cells were analyzed within 10 sec by a particle counter (Micro-, and Computerized ELZONE, Particle Data, Inc., Chicago)

RESULTS
1. Characterization of cultured glomerular cells
Three types of cells were derived from whole glomerular explants. The 1st type (A-cell) grew from glomeruli within 1–2 days, reached a peak of growth on day 10, and disappeared by day 21. Under phase contrast microscopy, the A-cell was small, polygonal in shape and exhibited a pavement of cobblestone-like appearance (Fig. 2).

The 2nd type (B-cell) appeared on day 10, increased in number thereafter, and reached confluency on day 21 when the subculture could be taken. Under phase contrast microscopy, the B-cell was larger than the A-cell, flat and rectangular in shape, contained intracellular
Fig. 6. Direct immunofluorescence staining with FITC-HMM (×160). a: B-cells show strongly positive staining in a fibrillar pattern. b: The cytoplasm of A-cells is stained faintly in a stippled pattern. G represents an explanted glomerulus.

Fig. 7. Direct immunofluorescence staining for fibronectin (×160). a: The cell membrane, cytoskeleton, and extracellular material of B-cells show positive staining. b: A-cells show negative staining.
Fig. 8. Photomicrographs (×200) counterstained with Caracci's hematoxylin after incubation with HRP and reaction with substrate of diaminobenzidine (a, b). Phase contrast photomicrographs (×400) after incubation with sensitized SRBC (c, d). a: A-cells show negative staining. Note the positively stained cells (arrow, C-cells) with pseudopods. b: B'-cells show negative staining. c: C-cell shows phagocytosis for, and rosette formation of, sensitized SRBC. d: B'-cells show neither phagocytosis for, nor rosette formation of, sensitized SRBC.
Fig. 9. Phase contrast photomicrographs (×350) (a) and data for cell surface area determined by a tablet digitizer (b) showing the response to 1 nM angiotensin II in those B-cells which attach only partially to the substratum of the culture dish.

a: The number in the left lower field of each photograph represents minute(s) of incubation with the peptide. Note marked contractile response in cell shape at 7 min (large arrows) compared to the cell shape at 0 time (small arrows). b: The shadow area represents the mean ± 1 SE of the percentage of initial area in 10 cells when incubated with vehicle solution (control). The data points are the means ± SE of the percentage of initial value in 8 cells when incubated with 1 nM of angiotensin II. *p < 0.05 vs. 0 time.

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fibrillar structures, and overlapped one another in culture (Fig. 3). Under transmission electron microscopy, the 2nd subcultured B-cell (B'-cell) had large bundles of intracellular microfilaments and extracellular materials (Fig. 4a). The cell had a large nucleus with 2 or more nucleoli, short and blunt cytoplasmic extensions or pseudopods, abundant Golgi apparatus and rough endoplasmic reticulum, but few lysosomes or mitochondria (Fig. 4b). These features were quite different from those of the cultured rat skin fibroblast (offered by Dr. T. Tsujimura in our laboratory) which had an elongated central nucleus with dense peripheral chromatin and contained numerous vacuoles partially filled with electron dense materials (probably lysosome) (Fig. 5). In both B-cells (Fig. 6a, 7a) and B'-cells the intracellular fibrils were strongly stained by FITC-HMM, and intracellular fibrils, cell membranes and extracellular materials were strongly stained by FITC-antifibronecin antibody. By contrast, the A-cell structures were not stained, or stained only faintly by either of these agents (Fig. 6b, 7b).

The 3rd type (C-cell) appeared on day 1–2. However, these did not survive long in culture. They comprised less than 0.1% of total grown cells on day 21 of the primary culture and were absent or only scarcely present in the subcultures. C-cell was briskly motile, small, and round in shape.

From these morphological findings and the differential cell growth capacities, A-cell was thought to be of epithelial origin, while B- and B'-cell of mesangial origin. The origin of C-cell could not be identified by morphological examinations alone.

2. Immune-related functions of cultured glomerular cells

The C-cells phagocytosed both HRP and sensitized SRBC (Fig. 8a, 8c). They also contained the binding sites for Fc (Fig. 8c) and C3b on their cell surfaces. None of the A-, B-, or B'-cells exhibited phagocytosis for HRP (Fig. 8b) or SRBC (Fig. 8d), nor did they show binding capacities for Fc (Fig. 8d) and C3b. From these results, the origin of C-cell was thought to be circulating monocytes.12

3. Contractile response to angiotensin II in B- and B'-cell

The morphological responses of B-cell to 1 nM angiotensin II are shown in Fig. 9a. Immediately after the addition of the peptide, each cell responded and showed brisk movements. Distinct contraction began at 4 to 7 min when the cells lost their cytoplasmic processes, becoming rounded. The contraction continued up to the end of the incubation (10 min).

The surface area of the photographed B-cell decreased after the addition of the peptide (Fig. 9b). When compared with the initial value, the decrease was significant throughout from 2 to 10 min of the incubation. The maximum decrease in the surface area was observed at 7 min with a percentage of 70.2 ± 3.4% (mean ± SE of 8 cells) of original surface area.

The effect of 1 nM of angiotensin II upon the diameter of the B’-cells is shown in Fig. 10. As the incubation time increased, the peptide caused a shift to the left in the distribution of the cell diameters indicating that the B’-cells contracted. On the assumption that the cells were round in shape, the diameter measurements were transformed into surface area. Both the time course and the magnitude of the decrease (maximum 72.6 ± 0.1% of original value) in the B’-cell calculated surface area were consistent with those in the measured surface area of the B-cells (Fig. 11).

DISCUSSION

The cells on day 21 of our primary glomerular culture (B-cell) and the cells subcultured on that day (B’-cell) are thought to be of mesangial origin according to the following findings. First, B-cells began to grow after the growth peak of A-cells which were of apparent epithelial origin. These differential growth capacities of the 2 cell types are consistent with the findings by other investigators6–10. In addition, the B-cells grew in a swirl-like fashion and piled in culture, forming a “hills and valleys” pattern. This cell growth pattern, resembling that of vascular smooth muscle cells, has been reported as one of the unique properties of mesangial cells12. Second, both B-, and B’-cells showed numerous cytoplasmic bundles of microfilaments. Immunofluorescent studies with FITC-HMM showed that these contained abundant actin. This finding is similar to that of mesangial cells in situ13 and consistent with previous reports of cultured mesangial cells14,15. Third, immunofluorescent studies with FITC-labeled antifibronectin antiserum revealed that both B- and B’-cells produced fibronectin as an extracellular material. This agrees with the in situ finding that fibronectin is most abundant in the mesangial matrix in the glomerulus16,17 and also agrees with Linder, et al.18 who described the antibody against fibronectin as a useful marker for the glomerular mesangium.

We can thus conclude that both B- and B’-cells are of mesangial origin.

One of the most important functions of mesangial cells is phagocytosis19. However, we could not detect phagocytic activities for HRP or sensitized SRBC in the cultured mesangial cells. Nor did they show binding capacities for Fc or C3b. The lack of phagocytic activities in cultured mesangial cells was also reported by Kreisberg, et al.20 who demonstrated that the cultured rat mesangial cells did not phagocytose particles such as polystyrene, ferritin, zymosan or carbon. By contrast, C-cells showed highly phagocytic activities for HRP and sensitized SRBC and had both Fc and C3b receptors. The appearance of these phagocytic cells from glomerular explants was reported by other investigators19–21 and the existence of blood-born monocytes in normal and altered glomeruli was established19–22. Therefore, we agree with the hypothesis that the C-cell is a blood-born monocyte which travels into and out of the glomerular mesangium,12,23 and suggest that these monocytes, rather than the mesangial cells, have a major phagocytic role in the glomerulus.

Most importantly, we confirmed the contraction of cultured mesangial cells in response to angiotensin II on a quantitative basis. It has been reported24 and we confirmed, that the contractile response of the cultured cells largely depends on the distribution at the cell surface of zones firmly attached to the substratum of the culture dish. For this reason, the magnitude of contraction could hardly be quantified in previous studies6–10. Therefore, we used those B-cells which were freshly outgrown from the glomerulus and attached only partially to the substratum of the dish and dissociated B’-cells scraped off from the dish in such a way as to retain angiotension II-binding activity. Furthermore, we used two new techniques to quantify the change in cell shape: one utilized a computerized planimeter and the other used a computerized size-distribution analyzer. The data obtained from these two different cell preparations and determinations were consistent with each other, and the maximum decrease in cell surface area in response to 1 nM angiotensin II was about 30%. Gunther, et al.25 reported that isolated rat mesenteric artery smooth muscle cell in the primary culture shortened to 65% of its original length when incubated with a 10 times higher concentration (10 nM) of angiotensin II.
Since the width of the cell increased in association with shortening of the cell length, the decrease in cell surface area should be less than 65% of the original level.

These data indicate that cultured rat mesangial cells possess a highly contractile ability consistent with that of vascular smooth muscle cells. The contraction of mesangial cells may lead to glomerular capillary vasoconstriction and a decrease in ultrafiltration coefficient in the rat. In addition, we previously reported that mesangial cell contraction might be important in the regulation of glomerular filtration rate by angiotensin II in patients with mesangial proliferative glomerulonephritis. This evidence together with our present data, suggests that the most important function of the glomerular mesangial cell is the control of glomerular filtration by contraction.

REFERENCES
9. KREISBERG JJ: Insulin requirement for contraction of cultured rat glomerular mesangial cells in response to angiotensin II (AII): A possible role for insulin in modulating glomerular hemo-
11. KRAKOWER CA, GREEENSPON SA: Localization of the nephrotoxic antigen within isolated renal glomerulus. Arch Pathol 51: 629, 1951
25. GUNTHER S, ALEXANDER RW, ATKINS WJ,

