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Research Article

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Evidence for Immunoglobulin Fc Receptor-mediated Prostaglandin$_2$ and Platelet-activating Factor Formation by Cultured Rat Mesangial Cells

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Abstract

The possibility of Fc-dependent uptake of IgG immune complexes was examined in subcultured rat mesangial cells free of monocytes. ^{195}Au-labeled colloidal gold particles were coated either with BSA only or with BSA followed by rabbit anti-BSA-IgG or the F(ab')$_2$ fragment of the IgG. Mesangial cells preferentially took up ^{195}Au particles covered with BSA-anti-BSA-IgG over those covered with BSA or the F(ab')$_2$ fragment. This uptake was a time-dependent and saturable process inhibitable by sodium azide or cytochalasin B. Using phase-contrast microscopy in the light reflectance mode, it was established that essentially all mesangial cells took up IgG-coated gold particles. By electron microscopy the process was shown to consist of vesicular uptake with delivery to endosomes. Mesangial binding-uptake of the IgG-covered particles was associated with stimulation of PGE$_2$ synthesis and production of platelet-activating factor, a lipid mediator of inflammation. To characterize the potential Fc receptor for IgG we used the rosetting technique with sheep red blood cells coated with IgG subclass-specific mouse monoclonal antibodies. 50% of mesangial cells exhibited rosetting with red cells coated with mouse IgG2a, whereas negligible rosetting was observed with IgG2b or IgG1. Competition experiments confirmed the specificity of IgG2a binding. We conclude that cultured rat mesangial cells exhibit specific receptors for IgG and that occupancy of Fc receptors results in endocytosis and is associated with generation of PGE$_2$ and platelet-activating factor. These observations may be of significance for immune-mediated glomerular diseases.

Introduction

Macromolecular uptake by mesangial cells was demonstrated by Farquhar and Palade (1) in their initial studies identifying the glomerular mesangial cell. They also established that vasoactive agents and glomerular injury could alter the amount of the macromolecules localizing to the mesangium (1). These pioneering observations have since been confirmed in numerous in vivo studies (2–5). The mesangium is only separated from the glomerular capillary blood stream by a fenestrated, leaky endothelium without an intervening basement membrane (1, 2, 6). The mesangium is therefore constantly bathed by plasma, so that mesangial localization of macromolecules could just represent exposure to the glomerular filtrate. Clearly, however, immune complex formation and deposition in the mesangium is observed in many forms of glomerulonephritis. This had led to the proposal that mesangial cells represent specialized vascular pericytes that may contribute to the pathophysiology of certain forms of glomerular diseases (7, 8). In this context it has been debated whether mesangial cells are capable of actively taking up macromolecular complexes and specifically immunoglobulins. One view is that only bone marrow-derived macrophages that reside in the glomerulus and represent a small percentage of glomerular cells are capable of phagocytosis of immune complexes (9). An argument in favor of this interpretation has been the failure to demonstrate Fc receptors for immunoglobulins on mesangial cells by use of immunofluorescence microscopy (10). Although the glomerular resident macrophages may play a major role in the handling of macromolecules and immune complexes, it has been proposed that the mesangial cell proper may also have specific uptake mechanisms for such molecules.

In vitro macromolecular uptake has been examined in cells isolated from glomeruli (11) and in primary cultures of rat mesangial cells. Baud et al. (12) first described uptake of serum-treated zymosan particles by cultured mesangial cells. Studies from several groups have shown that this is associated with generation of prostaglandins, lipoxygenase products of arachidonic acid, and release of reactive oxygen species (12–14). Recently we demonstrated uptake of serum-coated gold particles by cultured mesangial cells that proceeded in a typical coated pit, coated vesicle, endosome, lysosome pathway (15). Again, this was associated with stimulation of PGE$_2$ synthesis by the mesangial cells. The stimulation of PGE$_2$ production only required surface binding of the serum-coated gold particles, and not the actual endocytosis, as demonstrated by experiments with cytochalasin B (15). These observations would indicate that a ligand in the serum coat of the particles bound to a specific surface receptor triggering the subsequent events. We therefore considered the possibility that one of the ligands present on serum-coated gold particles might be an IgG. This possibility also received support from simultaneous studies by Sedor et al. (16) who showed stimulation of reactive oxygen production by mesangial cells exposed to IgG immune complexes. In these studies, surface binding was also sufficient to trigger the cellular response. Using ^{125}I-labeled IgG, these authors further provided evidence that mesangial cells in culture express Fc receptors.

In the present study we examined Fc-dependent uptake of [^{195}Au] colloidal gold particles coated with IgG-BSA immune complexes. In parallel, we determined generation of PGE$_2$ and platelet-activating factor (PAF).[1] PAF is a lipid mediator of

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1. *Abbreviations used in this paper:* KR, Krebs-Ringer (buffer); PAF, platelet-activating factor.

inflammation that can be generated by a number of white cells upon stimulation (17, 18). It is of interest that macrophages will generate PAF during phagocytosis (19) and that mesangial cells can produce PAF (20). In addition, we attempted to characterize a potential Fc receptor by evaluating rosette formation with sheep red blood cells (SRBC) coated with IgG subclass-specific mouse monoclonal antibodies. Our results provide evidence that cultured rat mesangial cells preferentially take up IgG-coated [^{195}Au] gold particles and contain IgG subclass-specific receptors. Furthermore, uptake of the IgG immune complexes is associated with generation of PGE_2 and PAF, findings that may be of significance for immune-mediated glomerular diseases.

Methods

Culture of glomerular mesangial cells

The culture of rat mesangial cells was carried out as previously described (20). After 3 wk in primary culture the mesangial cells were detached from the flask by adding a 0.25% trypsin-EDTA solution (Gibco, Grand Island, NY) and transferred to a plastic flask containing 5 ml of growth medium including RPMI 1640 (Gibco), 10% fetal calf serum, penicillin (50 U/ml), and streptomycin sulfate (50 μg/ml) (Gibco). Culture flasks were kept in a 95% air, 5% CO_2 environment at 37°C. Within 7–10 d the mesangial cells grew to confluence. Subsequent subcultures were at 7–10-d intervals by the above method. Only third- to fifth-passage cells were utilized for our experiments. The mesangial cells used represent an apparently uniform cell population as evaluated by the following criteria: (*a*) morphology, (*b*) uniform fluorescence with FITC-phallacidin for F-actin, (*c*) uniformly positive immunofluorescence with the monoclonal antibody Thy 1.1, (*d*) pattern of binding for specific lectins, (*e*) absence of immunofluorescence with factor VIII antibody, with antibodies for Ia or common rat leucocyte antigen (21). For uptake and morphologic studies, mesangial cells were seeded onto round, 13-mm diam sterile plastic tissue culture coverslips (Miles Laboratories, Inc., Naperville, IL) kept in six-well tissue culture plastic dishes (35 × 10 mm), (Vanguard International Inc., Neptune, NJ). Cells were seeded at a density of 50,000 cells per well. Cells were used after having reached confluence (usually 8–10 d). For the various conditions in any given experiment, coverslips or individual wells from the same plate were used. Within the aliquots of an experimental series these varied by < 10% as evaluated by cell count or determination of cellular protein.

Preparation of gold particles

Colloidal gold particles, ranging in diameter from 10 to 40 nm were prepared by the method of Horisberger et al. (22), except that [^{195}Au] (783 mCi/mg; New England Nuclear, Boston, MA) was included. The [^{195}Au] colloidal gold particles were then coated with BSA by incubating them in Krebs-Ringer's (KR) buffer with 20 mg/ml of fatty acid-free BSA for 60 min with stirring at room temperature. The gold particles were sedimented at 20,000 *g* for 10 min and washed three times with KR buffer. The gold particles were then resuspended in KR and divided into three aliquots. The first one was left as such, i.e., [^{195}Au] gold particles covered with BSA only. The second and third aliquots were incubated for 2 h with either the $F(ab')_2$ fragment of a rabbit anti–BSA-IgG (150 μg/ml); Cappel Laboratories, Cochranville, PA), or intact rabbit anti–BSA-IgG (150 μg/ml; Cappel Laboratories). The three preparations of coated [^{195}Au] gold particles were again sedimented at 20,000 *g*, washed three times with KR buffer, and then utilized for experiments. Comparability of the number of [^{195}Au] gold particles in the three different solutions were verified by determination of radioactivity. The different preparations were labeled as BSA-gold, $F(ab')_2$-gold and IgG-gold, respectively.

In two experimental series, a standard curve for ^{195}Au radioactivity per IgG-gold particle was established. This was achieved by submitting aliquots of serial dilutions of the [^{195}Au]IgG-gold to (*a*) radioactivity determination and (*b*) to counting of gold particles deposited on an electron-microscopic grid. These were examined by electron microscopy at 10,000-fold final magnification. This resulted in a standard curve for ^{195}Au radioactivity versus the number of gold particles, thus allowing an extrapolation from the radioactivity to the number of particles taken up by mesangial cells.

Incubation of cells with [^{195}Au] gold particles for determination of uptake and PGE_2 generation

The mesangial cells grown on coverslips were transferred onto 24-well culture plates so that each well contained one coverslip. They were washed thrice very gently with KR buffer at 37°C and then allowed to equilibrate for 30 min at 37°C in 0.5 ml of KR buffer. Aliquots of the appropriate [^{195}Au] gold suspension (0.5 ml) were then added to the wells and left to incubate for the times indicated in Results. The amount of ^{195}Au added ranged from 100,000 to 400,000 cpm of ^{195}Au in different series of experiments but varied by < 5% within each experiment. Incubations were terminated by removal of buffer, five rapid washings with cold KR buffer, and fixing the coverslips in 1% glutaraldehyde for 12 h at room temperature. The number of cells per coverslip ranged from 100 to 300 between different experimental series, but varied by < 20% within each experimental series. Blank experiments were always performed in parallel in an identical fashion, except that coverslips without cells were used. Radioactivity on the coverslips was determined in a gamma counter. Counts were corrected for those from the blank incubations, which amounted to < 10% of the experimental values.

For evaluation of PGE_2 production, aliquots (0.2 ml) of buffer were removed from the incubations after 15 min and immediately extracted twice with 1 ml of ethylacetate. Recoveries were monitored by the addition of 500 cpm of [^{3}H]PGE_2 (140 mCi/mmol; Amersham Corp., Arlington Heights, IL) and were > 90%. After drying under a stream of nitrogen samples were stored at −20°C until determination of PGE_2 by enzyme immunoassay. Enzyme immunoassay for PGE_2 was performed in duplicate and usually at two different dilutions by the method of Pradelles et al. (23) as previously reported (24).

Determination of PAF generation

For PAF generation mesangial cells (third subcultures) were grown to confluence in six-well plates (Inter-Med NUNC, Roskilde, Denmark). After removal of culture medium, the cells were washed three times with KR buffer and left to equilibrate for 30 min in KR buffer at 37°C. Aliquots of the three different gold preparations were then added to duplicate wells and incubations continued for the times indicated in Results. Incubations were terminated by removal of the buffer into cold methanol, the cells were washed once with cold buffer which was added to the buffer/methanol mixture. 2 ml of cold methanol was added to the cells, and the cells were scraped with a rubber policeman and collected into a glass extraction tube. The wells were washed with an additional 2 ml of methanol, which was added to the cell extract. Both incubation buffer and cell extract were spiked with 2,000 cpm of [^{3}H]PAF (179 mCi/mg; Amersham Corp.) for monitoring of recovery. Incubation buffer and cells were extracted, purified and assayed separately for PAF.

Methods of lipid extraction

All samples (buffer or cell fractions) in methanol were homogenized in a Polytron (Brinkmann Instruments Co., Westbury, NY) on power output setting no. 6 for 30 s, then centrifuged at 1,300 *g* for 5 min. The pellet was saved for protein determination by the Bio-Rad Laboratories (Richmond, CA) kit. The supernate samples were extracted by the methods of Bligh and Dyer (25) as described previously in detail (20). Extracted samples were further purified by either of two methods: (*a*) samples were spotted on TLC plates (LK6D silica gel plates, Whatman, Inc., Clifton, NJ) that had previously been heated to 180°C for 1 h, allowed to cool and run in a system of chloroform/methanol/water (165:35:6). In other lanes of the TLC plates ^{14}C-labeled PAF, lyso-PAF,

and phosphatidylcholine standards were run. Positions of the ^{14}C standards were visualized by autoradiography and areas corresponding to the [^{14}C]PAF standard were scraped off the sample lanes, and extracted from the TLC silica by incubation with methanol/water (95:5). Alternatively, the samples were purified over an open silicic acid column containing 500 mg of silicic acid (40 μm, J. T. Baker Chemical, Phillipsburg, NJ) as described (20). In brief, the dried samples were resuspended in 0.5 ml of chloroform/methanol (3:1) and applied to the column, followed by another 0.5 ml of the same to rinse the sample tube. The column was eluted sequentially with 10 ml of chloroform/methanol (1:1), 5 ml of methanol, and 5 ml of methanol/water (3:1). This last fraction containing PAF was collected and dried under nitrogen.

Bioassay for PAF activity

For PAF bioassay we used aggretagion of rabbit platelets, as previously described (20). Platelet aggregation was determined in a four-channel aggregometer (Bio/Data Platelet Aggregation Profiler-4, Bio/Data Corp., Hatboro, PA) using 0.45 ml of the platelet suspension, and adding standards or samples resuspended in the Tris-Tyrode's buffer with calcium and magnesium. A standard curve of PAF bioactivity was obtained using this method, from 10^{-10} to 10^{-7} M PAF. In some experiments half of the sample was subjected to alkaline hydrolysis (15 min in 0.5 M KOH at 45°C) followed by reextraction and bioassay, a treatment that destroys the platelet aggregating ability of standard PAF (17). Results extrapolated from the PAF standard curve were expressed as picomoles of PAF bioactivity generated per milligram of mesangial cell protein.

Mass spectrometry for PAF

The lipid extract of experiments that tested positive for PAF bioactivity was further analyzed as the pentafluorbenzoyl ester by negative ion chemical ionization mass spectrometry (26). By this method, the hexadecyl PAF species yields an ion peak at m/z 552, the octadecyl PAF species at m/z 580, and the C18:1 species at m/z 578. Approximately 8 ng of deuterated PAF (D3) was added to each sample before analysis, as this gives an m/z ion at 555, clearly separated from the hexadecyl PAF, and allows standardization (26).

Preparation of cells for electron microscopy

In some experiments endocytosis of [^{195}Au] BSA-IgG gold particles was verified by transmission electron microscopy as previously described in detail (15). In brief cells on the coverslips were fixed at the end of the experimental incubation in PBS with 1% glutaraldehyde and 0.2% tannic acid for 1 h at room temperature. Sequential dehydration was carried out in ethanol. The cells were embedded in Epon 812 (Polysciences, Inc., Warrington, PA). Thin sections were made with an ultramicrotome (LKB Instruments, Inc., Gaithersburg, MD) and observed with a 1200EX electron microscope (JEOL USA, Peabody, MA) after being counterstained by uranyl acetate and lead citrate.

Evaluation of uptake by polarized light reflectance microscopy

Mesangial cells, grown on glass coverslips, were washed thrice with KR buffer and preincubated in 1 ml of KR with 100 mg% of glucose for 1 h at 37°C. Aliquots (20 μl) of the three different types of gold particles were then added to the buffer and incubations continued for 0, 10, 20, or 30 min. Incubations were terminated by three washes with cold PBS and cells fixed with 1% glutaraldehyde for 1 h. The cells on the coverslips were then evaluated under a microscope (Carl Zeiss, Inc., Thornwood, NY) in the polarized light reflectance mode and photographs were taken.

Determination of Fc receptor by rosetting with SRBC

Preparation of Ig-coated SRBC. 1 ml of a 5% (vol/vol) solution of SRBC was incubated for 12 h at 4°C with 10 μl of ascitic fluid containing BALB/c mouse monoclonal anti-SRBC antibodies of different IgG subclasses directed against SRBC. The amounts of antibodies used saturated binding sites on SRBC (see below). The IgG subclasses were IgG1, IgG2a, and IgG2b as previously described (27). After three washes in PBS the SRBC were tested to assure that they did not spontaneously agglutinate. The actual amount of mouse monoclonal antibody absorbed on the SRBC was determined by radioimmunoassay using a mixture of two [^{35}S]methionine-radiolabeled rat monoclonal antibodies directed against the mouse κ light chain as reported (27). The amount of ^{35}S-labeled antibodies binding to the coated SRBC was comparable for SRBC-IgG2a (21,000 cpm) and SRBC-IgG2b (23,000 cpm), but less for SRBC-IgG1 (12,000 cpm). However, this represented saturating amounts of subclass IgG binding to the SRBC as coating with double the amount of the respective monoclonal antibodies did not increase the amount bound to SRBC.

Fc rosetting of SRBC on mesangial cells. Mesangial cells (third to fifth subculture) on their culture dishes had their medium removed and were washed three times with PBS. They were then incubated with PBS containing 4 mg/ml of type IV collagenase (Worthington Biochemical Corp., Freehold, NJ) for 1 h at 37°C, resulting in lifting off of the cells from the culture dish. Cells were pelleted, washed three times in PBS, and then resuspended in PBS to yield approximately 50,000 cells/ml. Aliquots of 0.5 ml of mesangial cell suspensions were then mixed with 10 μl of the different IgG coated SRBC and incubated for 1 h at 4°C. Rosetting was then evaluated by counting 100 mesangial cells in a hemocytometer under a microscope. Mesangial cells associated with at least three SRBC were considered as rosette-positive.

Results

Uptake of [^{195}Au] gold particles. Initially we examined the effect of different concentrations of [^{195}Au]IgG gold particles on uptake by cultured mesangial cells grown on plastic culture coverslips. Serial dilutions of the [^{195}Au]IgG and of [^{195}Au] F(ab')$_2$ were prepared and incubated with mesangial cells for 15 min, and uptake was determined by counting of the radioactivity incorporated into the mesangial cells on the coverslips. As shown in Fig. 1 uptake of [^{195}Au]IgG-covered particles proceeded in a concentration-dependent and saturable manner. [^{195}Au] F(ab')$_2$ particle uptake was much less and nonsaturable. In this series of experiments the specific activity of the

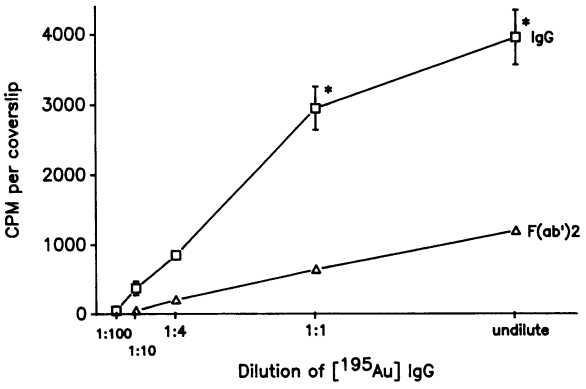

Figure 1. Uptake of [^{195}Au] colloidal gold particles covered with BSA and anti-BSA-IgG or its F(ab')$_2$ fragment by rat mesangial cells. Mesangial cells were grown on coverslips to confluency and then exposed to the gold particles in serial dilutions. Uptake was determined after 15 min of incubation at 37°C. Results are expressed as ^{195}Au uptake in counts per minute by the mesangial cells on the coverslips, and represent the means±SEM from three series of experiments. Where SEM are not apparent they fall within the limits of the symbols used. For details refer to Methods. *$P < 0.05$ or better compared to [^{195}Au] F(ab')$_2$.

[^{195}Au]IgG particles corresponded to 100 cpm per 30,000 particles, so that each cell took up about 10,000 particles of [^{195}Au]IgG at the 1:1 dilution. For all subsequent experiments a 1:1 dilution of the respective colloidal gold particles was used.

In the next series of experiments we evaluated the time course for uptake of [^{195}Au]IgG particles and compared it to that for [^{195}Au] BSA and [^{195}Au] F(ab')$_2$. As shown in Fig. 2 [^{195}Au]IgG uptake increased during the initial 30 min and then leveled off. ^{195}Au particles coated with F(ab')$_2$ (Fig. 2) were incorporated by mesangial cells to a lesser extent. Uptake of [^{195}Au] BSA after 30 min was also significantly lower (560±90 cpm per coverslip $n = 4$; $P < 0.02$).

To compare the effect of different coatings of ^{195}Au particles, mesangial cells were exposed to equal concentrations of either [^{195}Au]gold, [^{195}Au]F(ab')$_2$ gold, or [^{195}Au]IgG gold for 30 min and uptake was determined together with PGE_2 synthesis (see below). As shown in the bottom panel of Fig. 3 mesangial cells took up significantly more [^{195}Au]IgG-coated particles (IgG-gold) than either ^{195}Au particles coated with BSA only (BSA gold) or with BSA F(ab')$_2$ fragment (F(ab')$_2$-gold). This would be consistent with an Fc receptor-mediated uptake process. The slight and statistically insignificant increase of [^{195}Au] F(ab')$_2$ gold over [^{195}Au] BSA-gold uptake may be related to some intact IgG remaining in the F(ab')$_2$ preparation. The amounts of [^{195}Au]BSA and [^{195}Au] BSA-F(ab')$_2$ associated with mesangial cells may represent nonspecific, non-receptor-mediated binding or endocytosis of macromolecules by mesangial cells (15) or uptake by albumin or albumin associated receptors.

In order to assure that [^{195}Au]IgG associated with mesangial cells represented active uptake, cells were pretreated with either sodium azide (10^{-5} M) or cytochalasin B (10^{-5} M) for 15 min before a 30-min exposure to the [^{195}Au]IgG. This pretreatment did not result in detachment of cells from the coverslips. As shown in Table I, pretreatment with sodium azide markedly reduced uptake of [^{195}Au]BSA-IgG by mesangial cells, indicating that uptake is an energy-requiring process. Similarly, pretreatment with cytochalasin B significantly diminished [^{195}Au]BSA-IgG uptake, consistent with a requirement for an intact cytoskeleton in order to incorporate the

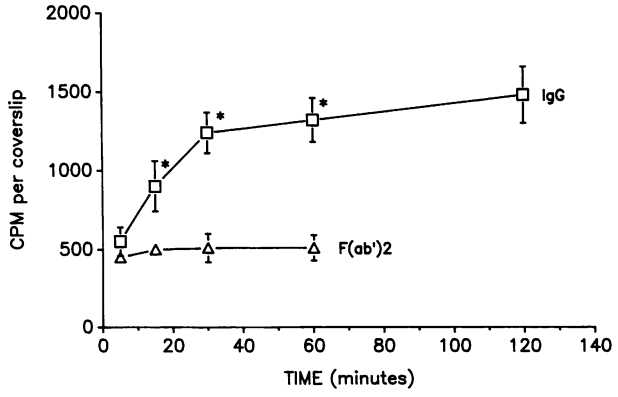

Figure 2. Time course of uptake of [^{195}Au]IgG and [^{195}Au] F(ab')$_2$ particles by mesangial cells grown on coverslips. Results are means±SEM of three series of experiments. Where standard errors are not apparent they fall within the limits of the used symbols. *$P < 0.05$ or better as compared to [^{195}Au] F(ab')$_2$.

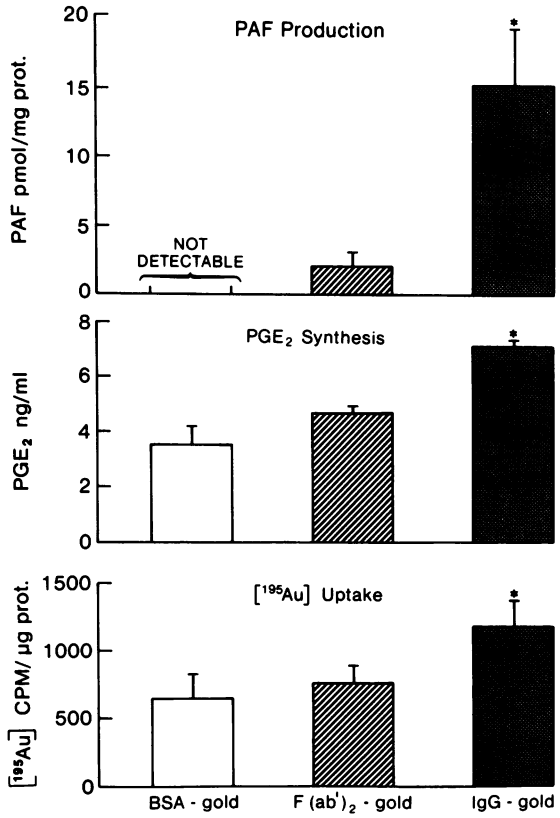

Figure 3. Comparison of mesangial cell uptake of ^{195}Au-labeled colloidal gold particles covered with BSA (BSA-gold), BSA and anti-BSA-IgG (IgG-gold), or BSA and F(ab')$_2$ fragment of the IgG (F(ab')$_2$-gold) and the concomitant synthesis of PGE_2 or PAF. All incubations were carried out for 30 min. Results are means±SEM from 12 series of experiments for ^{195}Au particle uptake, 6 series for PGE_2 synthesis, and 4 series for PAF generation. For comparison PGE_2 synthesis by mesangial cells under control conditions (i.e., in absence of any ^{195}Au particles) was 2.6±0.5 ng/ml. *$P < 0.05$ or better as compared to BSA-gold or (F(ab')$_2$ gold using Bonferoni modification for paired data.

[^{195}Au]IgG particles into mesangial cells, as previously described for serum-coated gold particles (15). For comparison results with [^{195}Au] BSA particles were also performed (Table

Table I. Effect of Pretreatment with Sodium Azide or Cytochalasin B on [^{195}Au]IgG Uptake by Mesangial Cells

Preincubation	Buffer only	Buffer±Cyto B	Buffer±Na^+ azide
Incubation	[^{195}Au]IgG	[^{195}Au]IgG	[^{195}Au]IgG
(*cpm/coverslip*)	1310±60	70±30*	130±60*
Incubation	[^{195}Au]BSA	[^{195}Au]BSA	[^{195}Au]BSA
(*cpm/coverslip*)	280±30	100±20*	50±20*

Mesangial cells cultured on coverslips were preincubated for 15 min with either buffer only, or with buffer containing cytochalasin B (10^{-5} M) or sodium azide (10^{-5} M). Subsequently cells were incubated with buffer containing [^{195}Au]IgG or [^{195}Au]BSA for 30 min at 37°C. After washing and fixation radioactivity incorporated by the mesangial cells on the coverslips was determined. Results are means±SEM of four series of experiments.
* $P < 0.01$ or better by Student's t test for unpaired data compared to respective controls (i.e., preincubation with buffer only).

Table II. Comparison of [^{195}Au]IgG Uptake by Mesangial Cells and Peritoneal Macrophages

Time of incubation (*min*)	5	15	30	60
Mesangial cells (*cpm/500 cells*)	500±100	750±300	900±100	1,150±250
Peritoneal macrophages (*cpm/500 cells*)	14,800±2,600	23,600±2,950	34,200±2,100	53,200±6,900

Mesangial cells grown on coverslips and rat peritoneal macrophages adherent to coverslips were incubated with buffer containing [^{195}Au]IgG for the times indicated at 37°C. After washing and fixation radioactivity incorporated was determined and expressed per 500 cells. Results represent means±SEM of three sets of experiments.

I). Again uptake of these particles was significantly lower ($P < 0.02$) than [^{195}Au] BSA-IgG particles, but was also inhibited by cytochalasine B or sodium azide. These results also confirm our previous observations (15), that mesangial cells will take up gold particles to some extent by ligand-independent endocytossis, though to a much smaller degree than by receptor-mediated endocytosis. Uptake of ^{195}Au particles by mesangial cells was also compared with that by rat peritoneal macrophages. Peritoneal macrophages were obtained by lavage of the peritoneal cavity of Sprague Dawley rats with buffer. After washing, the peritoneal macrophages were left to adhere to culture coverslips for 6 h and were then washed and handled in parallel with the mesangial cells grown on coverslips. As shown in Table II peritoneal macrophages incorporated 40 times more [^{195}Au]IgG than mesangial cells over a 60-min incubation. Both macrophages and mesangial cells showed a 50% decrease in incorporation of [^{195}Au]IgG when incubated with cytochalasin B (four experiments each).

In the next series of experiments, we evaluated whether preaddition of heat-aggregated rabbit IgG would influence subsequent handling of [^{195}Au]IgG. Heat-aggregated IgG was prepared by incubating 2 mg of rabbit IgG (Sigma Chemical Co., St. Louis, MO) in 4 ml of KR buffer at 63°C for 30 min, clearing it of precipate by centrifugation, and, after cooling, adding 0.5 ml to the mesangial cells. After 30 min of preincubation [^{195}Au]IgG was added and uptake evaluated after 15 min. Preincubation with heat-aggregated rabbit IgG reduced ^{195}Au uptake/binding from 670±30 cpm per coverslip ($n = 4$) to background. These results indicate that the preincubation with aggregated IgG blocked subsequent binding and uptake of [^{195}Au]IgG, most likely by occupancy of receptor sites on mesangial cells. To confirm this finding, studies were performed at 4°C to prevent endocytosis of the gold particles. Mesangial cells were preincubated for 2 h at 4°C with either buffer only, buffer with rabbit IgG, buffer with heat-aggregated rabbit IgG, or buffer with [^{195}Au]IgG. They were then extensively washed with cold KR buffer. Subsequently, they were incubated for 1 h at 4°C with [^{195}Au]IgG, except for the cells which had been preincubated with [^{195}Au]IgG. The latter were exposed to buffer with heat-aggregated IgG, to see if previously bound [^{195}Au]IgG could be displaced by heat-aggregated IgG. As shown in Table III, preincubation of mesangial cells with either monomeric IgG or heat-aggregated IgG reduced subsequent binding of [^{195}Au]IgG by 50% or more. Furthermore, prebound [^{195}Au]IgG could be displaced to 50% by incubation with aggregated IgG for 1 h. These results are consistent with competition for common binding sites by [^{195}Au]IgG with both monomeric and aggregated IgG.

Some murine Fc receptors can be removed by limited trypsinization (27, 28). Limited trypsinization was, therefore, performed on mesangial cells before subsequent binding studies. In four experiments mesangial cells were first exposed to trypsin in the cold for 30 min, then washed and incubated with [^{195}Au]IgG for 2 h at 4°C. Under those conditions [^{195}Au]IgG binding was reduced by 20–30% but that of [^{195}Au]BSA remained unaffected. Thus it appears that the binding sites for [^{195}Au]IgG on mesangial cells are partially sensitive to trypsin, similar to some murine Fc receptors (27, 28).

Light and electron microscopic studies. In order to evaluate what percentage of mesangial cells take up [^{195}Au]IgG we employed the technique of light microscopy in the mode of polarized light reflectance (29). This allows observation of the cells directly by phase contrast and of [^{195}Au] particles taken up by light reflectance. By comparing these two, the percentage of cells having incorporated gold particles can be evaluated. It should be noted though that the size of the colloidal [^{195}Au] gold particles employed in our studies (10–40-nm diam) is probably too small to give a discernible light reflectance for individual gold particles. However, the gold particles conglomerate in endosomes and lysosomes (see below) and these conglomerates should be big enough to provide for a clear reflectance signal. As shown in Fig. 4 incubation of mesangial cells with [^{195}Au]IgG showed a time-dependent increase of light reflectant particles in mesangial cells. Both the number of reflectant particles per cell and their size appeared to in-

Table III. Effect of IgG on Binding (4°C) of [^{195}Au]IgG to Mesangial Cells

First addition	Buffer	Heat-aggregated IgG (0.5 mg/ml)	Monomeric IgG (0.5 mg/ml)	[^{195}Au]IgG
Second addition	[^{195}Au]IgG	[^{195}Au]IgG	[^{195}Au]IgG	Heat-aggregated IgG
(*cpm/coverslip*)	500±45	220±15*	160±35*	250±50*

Mesangial cells cultured on coverslips were incubated for 2 h at 4°C with the first additions as indicated. Cells were then washed ten times with cold buffer, followed by a second incubation at 4°C for 1 h with the additions indicated. After washing and fixation radioactivity bound to the mesangial cells was determined. Results are means±SEM of four series of experiments. * $P < 0.01$ or better by Student's t test for unpaired data.

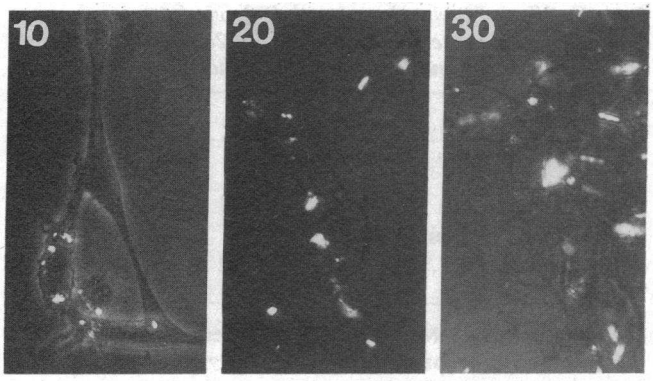

Figure 4. Photomicrographs taken in the mode of polarized light reflectance microscopy of mesangial cells incubated with IgG-gold particles. The cell outlines appear as dark contours and particle aggregates as bright spots overlying the cells. The numbers indicate the time of incubation with IgG-gold in minutes.

crease. In two sets of experiments essentially all mesangial cells (involving a total of about 600 cells) took up [^{195}Au]IgG as evaluated by light reflectance microscopy. In contrast no light reflectance-positive particles could be observed when mesangial cells were incubated up to 30 min with either [^{195}Au]BSA or [^{195}Au]F(ab')$_2$ particles. Possibly the absence of light reflectance-positive signals with these gold particles is related to less uptake and perhaps also to lesser conglomeration into endosomes.

To verify that the reflectance-positive signals do indeed represent cellular uptake of gold particles transmission electron microscopy was performed after 30 min of incubation with [^{195}Au]IgG. As shown in Fig. 5 the mesangial cells had incorporated the gold particles into vesicles and endosomes, as previously described in detail for serum-coated gold particles (15). Thus these experiments demonstrate that essentially all mesangial cells can take up [^{195}Au]IgG particles.

PGE_2 and PAF formation by mesangial cells incubated with [^{195}Au]gold particles. In previous experiments we had observed that incubation of mesangial cells with serum-treated zymosan (14) or serum-treated gold particles (15) stimulated PGE_2 production. We therefore evaluated whether uptake of [^{195}Au]BSA, [^{195}Au]F(ab')$_2$, and [^{195}Au]IgG resulted in PGE_2 synthesis. As shown in the middle panel of Fig. 3, PGE_2 production was significantly stimulated by [^{195}Au]IgG over [^{195}Au]BSA or [^{195}Au]F(ab')$_2$, similar to the pattern of uptake for these differently coated gold particles. Synthesis of PGE_2 by mesangial cells incubated under control conditions (i.e., without addition of any gold particles) was 2.6±0.5 ng/ml per 30 min, a value not significantly different from PGE_2 synthesis with [^{195}Au]BSA or [^{195}Au]F(ab')$_2$.

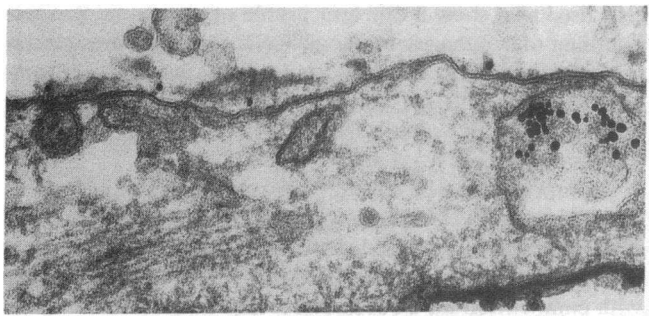

Figure 5. Transmission electron micrograph of mesangial cell that had been incubated with IgG-gold particles for 30 min. Gold particles are apparent on the surface, in a coated pit forming a vesicle (on the left), and conglomerated in an endosome (on the right). Initial magnification 33,000, final 90,000.

During phagocytosis macrophages can generate PAF (19). We had previously demonstrated the capacity of stimulated mesangial cells to produce PAF (20). We, therefore, investigated the possibility that uptake of the coated gold particles by mesangial cells might result in PAF formation. As shown in the top panel of Fig. 3, no bioactivity for PAF could be detected after exposure of mesangial cells to BSA-gold and very little after F(ab')$_2$-gold. In contrast, after exposure to IgG-gold considerable amounts of PAF were measured in partially purified lipid extracts from the mesangial cells by the platelet aggregation bioassay. Due to the limited sensitivity of the bioassay no bioactive PAF could be determined in the extracts of the incubation buffer. Identity of the bioactive material as PAF was verified by loss of bioactivity after alkaline hydrolysis (17) and by identification with negative ion chemical ionization mode of mass spectrometry (26). A single species of PAF with a 16-carbon saturated alkyl group in position one was found. By comparison with the deuterated PAF standard 40 pmol of PAF per mg mesangial cell protein had been generated after exposure to IgG-gold (Fig. 6). This exceeds the PAF values obtained by bioassay (Fig. 3) which may represent an underestimation due to the limits of the bioassay. With the negative ion chemical ionization method PAF could also be determined in incubation buffer. 5 pmol of PAF per mg protein of mesangial cells was detected in the buffer after incubation with IgG gold (Fig. 6). No PAF was detectable in the buffer after incubation with either BSA gold or F(ab')$_2$ gold.

Characterization of Fc receptors on mesangial cells. The experiments with [^{195}Au]IgG particle binding to and uptake by mesangial cells indicated the potential presence of Fc receptors on these cells. In order to confirm the presence of Fc receptors on mesangial cells and to characterize their specificity we employed the rosetting technique with SRBC coated with subclass specific mouse monoclonal IgGs. As shown in Table IV ~ 50% of mesangial cells in suspension showed rosetting with SRBC coated with mouse IgG2a, whereas essentially no rosetting was observed with IgG2b- or IgG1-coated SRBC.

To validate this finding competition experiments were performed with IgG2a, IgG2b, or IgG1 before exposure to coated SRBC. As shown in Table V preincubation of mesangial cells with IgG2a abolished subsequent rosetting of IgG2a-coated SRBC to these cells. In contrast, neither IgG2b nor IgG1 preincubation diminished the percentage of IgG2a rosette-positive mesangial cells, showing that mesangial receptors bind mouse IgG2a to the exclusion of IgG2b or IgG1. We also compared rosetting of the differently coated SRBC to rat peritoneal macrophages (Table V). 85% of rat peritoneal macrophages were rosette-positive with IgG2a-coated SRBC. In contrast to the results obtained with mesangial cells, the percentage of IgG2a rosette-positive macrophages was considerably diminished by preincubation with either IgG2b or IgG1. Thus the Fc receptors on rat mesangial cells and peritoneal macrophages differ in their specificity for binding of mouse IgG subclasses. We next evaluated the effect of trypsinization of mesangial cells on subsequent rosetting with IgG2a-coated

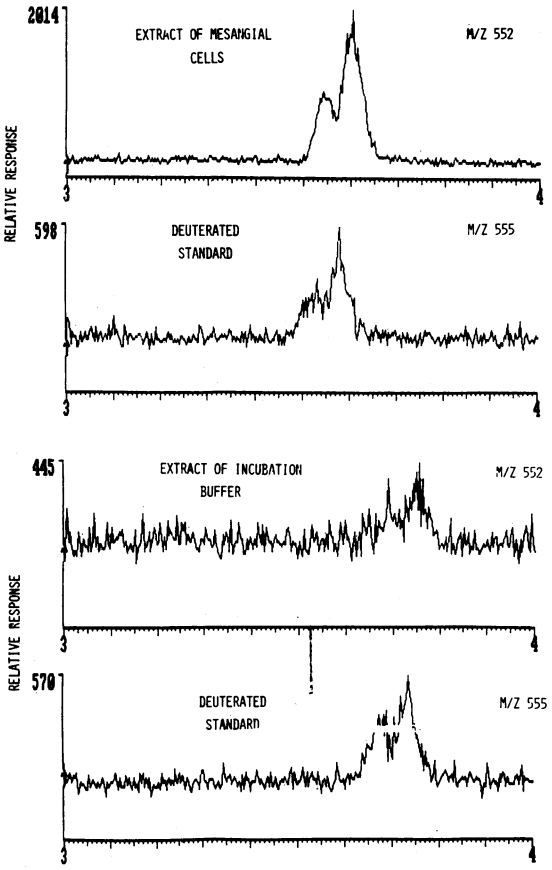

Figure 6. Mass fragmentograms of PAF synthesized by mesangial cells incubated with IgG-gold particles. PAF was extracted separately from the incubation buffer and the mesangial cells, purified, derivatized, and analyzed by gas chromatography-negative ion chemical ionization mass spectrometry as described under Methods. The m/z ions represent the molecular anion of the individual PAF species analyzed, i.e., m/z 552 corresponding to the hexadecyl PAF species and m/z 555 to the deuterated standard added (8 ng). There are two peaks in each ion channel because of isomerization of the diglycerides occurring during derivatization. Note the different scales for the relative responses in the four ion channels recorded on the ordinate which are due to the different sensitivities adjusted to the amounts of PAF in the samples.

SRBC. Mesangial cells were either released from their culture support by 30 min of incubation at 37°C with collagenase (the routine method of suspending mesangial cells for rosetting experiments), or by 30 min of incubation with 0.25% trypsin. Collagenase or trypsin were subsequently removed by extensive washings and rosetting assays were then performed. In two sets of experiments 52% and 46% of mesangial cells detached by collagenase were positive for rosetting with IgG2a-coated SRBC, whereas only 3% and 4% of cells detached by trypsin showed rosetting. These results indicate that the receptor on mesangial cells responsible for binding of mouse IgG2a-coated SRBC is sensitive to trypsinization.

Table IV. Percentage of Mesangial Cells Showing Rosette Formation with SRBC Coated with IgG2a, IgG2b, or IgG1

IgG2a	IgG2b	IgG1
	%	
45±5	1±1	2±2

Results are means±SEM of four series of experiments.

Table V. Effect of Preincubation with Different IgGs on Subsequent Rosette Formation with IgG2a-coated SRBC on Mesangial Cells and Peritoneal Macrophages

Preincubation	Buffer	IgG2a	IgG2b	IgG1
		%		
Mesangial cells (n = 3)	53±1	9±6	43±3	51±7
Peritoneal macrophages (n = 2)	85±4	36±2	72±6	44±5

Results are means±SEM of three series of experiments for mesangial cells and two for peritoneal macrophages. They are given as percentage of the respective cells with rosettes.

Discussion

The present results support the existence of a specific Fc receptor for IgG on an apparently homogenous population of cultured rat mesangial cells. Also, receptor-mediated binding/uptake of IgG immune complexes by the mesangial cells was associated with production of PGE_2 and PAF. These observations support the hypothesis that the contractile mesangial cell may represent a specialized pericyte with paraimmunologic potentials. The cells used in our experiments were smooth muscle-derived mesangial cells and not bone marrow-derived resident macrophages. All mesangial cells used in our studies were of third to fifth subculture after the initial glomerular explant. Under our culture conditions rat peritoneal macrophages did not survive in subculture. Furthermore, the mesangial cells used were uniformly positive for (*a*) stress fibers demonstrated by FITC-phallacidin labeling, (*b*) a mesangial cell-specific lectin-binding pattern; (*c*) immunofluorescence with Thy 1.1 antibody. All of these markers have also been shown on mesangial cells in kidney sections and this pattern can be used to identify mesangial cells in culture (21). The mesangial cells used in our studies were uniformly negative for Ia antigen or common leukocyte antigen which further distinguishes them from resident macrophages. The subcultured mesangial cells used also show a different profile of eicosanoid production than macrophages. Mesangial cells produce almost exclusively PGE_2 whereas macrophages in addition produce thromboxane and leukotrienes (30). Finally, contraction in response to angiotensin II, vasopressin, and leukotrienes distinguishes the mesangial cells from macrophages (6, 7, 9).

In support of an Fc receptor are our observations of preferential uptake of [^{195}Au] colloidal gold covered with BSA-anti-BSA IgG complexes. Also both uptake and surface binding at 4°C of [^{195}Au]IgG could be blocked by excess IgG consistent with competition for a common receptor. Based on the correlation between [^{195}Au]IgG binding and particle number determined by electron microscopy we estimate that about from 1,000 to 10,000 particles were taken up per mesangial cell. This represents, however, a 40-fold lower degree of phagocytosis than that observed for peritoneal macrophages (see Table

II). The electron microscopic studies confirm the uptake of the immune complex-coated gold particles into vesicles and endosomes of mesangial cells. The use of light microscopy in the polarized light reflectance mode allowed us to evaluate the percentage of mesangial cells involved in phagocytosis. By this method essentially 100% of the mesangial cells ingested gold particles covered with BSA-anti-BSA IgG, ruling out that only a subpopulation of cells was responsible for the uptake of [^{195}Au]IgG. In the experiment using reflectance light microscopy none of the cells incubated with BSA- or BSA-F(ab')$_2$-coated gold particles showed evidence for uptake, while in experiments using [^{195}Au]BSA or [^{195}Au]F(ab')$_2$, some uptake always occurred. A possible explanation for this discrepancy may relate to the particle size requirement for reflectance mode microscopy in order to give a positive signal. Most of the colloidal gold particles employed were of 10–20-nm diam. The resolution limit for polarized light reflectance microscopy is between 50 and 100 nm. Thus aggregation of colloidal gold particles in endosomes, as occurs with BSA-anti-BSA IgG-coated gold particles, is required in order to detect the uptake. It is possible that gold particles taken up by a mechanism not involving Fc receptors (i.e., the BSA- or F(ab')$_2$-gold) are handled differently by the mesangial cell. Also the possibility exists that some of the BSA- or F(ab')$_2$-gold particles were bound nonspecifically to cell surface or matrix. This would be detected by ^{195}Au experiments, but not by reflectance microscopy. The fact that cells became positive after 10-min incubations with BSA-anti-BSA IgG-coated colloidal gold indicates that at this time the particles are already collecting in endosomes, a time course supported by our earlier studies with serum-coated gold particles (15).

The presence of Fc receptors on mesangial cells suggested by the preferential uptake of BSA-anti-BSA IgG-coated gold particles was further demonstrated by the rosetting studies. In these experiments we took advantage of IgG subclass-specific monoclonal mouse antibodies directed against SRBC (27). While this study uses mouse antibodies and rat mesangial cells, it nonetheless allows some characterization of the Fc receptor. Mesangial cells showed positive rosetting only with mouse IgG2a-coated SRBC. The specificity of this rosetting was further supported by the ability of IgG2a to inhibit IgG2a SRBC rosettes and the failure of either mouse IgG2b or IgG1 to inhibit IgG2a rosette formation. Rosetting of IgG2a-coated SRBC on rat peritoneal macrophages was, however, reduced by preincubation with either mouse IgG2a or IgG1, confirming that rat mesangial cells have different Fc receptors than rat macrophages. It is also of interest that in our studies the rat mesangial Fc receptor responsible for rosetting with mouse IgG2a-coated SRBC was lost after trypsinization, a finding similar to the mouse macrophage IgG2a receptor (27). The presence of Fc receptors on cultured rat mesangial cells is also in agreement with recent studies by Sedor et al. (16). These authors reported Fc receptor-dependent binding of radioiodinated immune complexes to cultured rat mesangial cells. The immune complexes consisting of BSA- and anti-BSA-IgG from rabbit were closely comparable to the ones we used. Interestingly, binding of the radioiodinated immune complexes in the studies by Sedor et al. (31) was associated with generation of reactive oxygen species by mesangial cells. In our previous studies with serum-coated gold we had observed that stimulation of PGE_2 synthesis only required surface binding of the serum-treated gold but not actual uptake. These observations would indicate that, similarly to leukocytes (31) in mesangial cells, binding of specific ligands to surface receptors triggers intracellular events that stimulate PGE_2 synthesis and reactive oxygen generation.

The stimulation of PGE_2 synthesis requires activation of phospholipase A_2 (32), which is also the initial step in PAF formation (17). We have previously reported that mesangial cells can produce PAF (20) and PAF formation occurs during phagocytosis by macrophages (31). It is therefore of interest that mesangial cells generated considerable amounts of PAF when exposed to [^{195}Au]IgG, but not when exposed to [^{195}Au]-BSA or [^{195}Au]F(ab')$_2$. This finding again implicates an Fc-mediated trigger event on mesangial cells. The PAF formed was authenticated by loss of bioactivity after alkaline hydrolysis and by gas chromatograph-mass spectrometry. The PAF generated was exclusively of the hexadecyl species, i.e., containing a 16-carbon saturated alkyl group in position 1. This is similar to the results we had obtained with ionophore-stimulated PAF generation (20). About 80–90% of the PAF generated remained cell associated and 10–20% was released into the incubation buffer. These findings are also similar to those obtained with PAF generation by cultured endothelial cells (33, 34). The significance of intracellular PAF remains unclear but could change surface adherence of cells (34). Formation of PAF by mesangial cells during exposure to immune complexes could contribute to glomerular immune injury. For example, PAF can reduce glomerular filtration rate and can alter ultrafiltration characteristics and glomerular capillary permselectivity (17, 18). Furthermore, blockage of PAF by specific receptor antagonists has been reported to improve experimental glomerulonephritis (17, 18).

Taken together it appears that exposure of cultured mesangial cells to serum-treated zymosan or colloidal gold particles or to IgG immune complexes triggers various cellular responses that include phagocytosis, release of reactive oxygen metabolites, formation of lipoxygenase products of arachidonic acid, prostaglandin synthesis, and generation of PAF. These findings are highly reminiscent of Fc-receptor triggered events in neutrophils and macrophages (31, 35, 36). Thus mesangial cells may represent a specialized pericyte that, while clearly distinct from monocyte-macrophages, exhibits a number of characteristics that may allow it to participate in immunologic and inflammatory processes within the glomerulus.

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