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Subcellular localization of glucocorticoid receptor protein in the human kidney glomerulus

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Background. The detailed mechanisms of glucocorticoid action in idiopathic nephrotic syndrome and progressive glomerulonephritides have not been clearly elucidated. The pharmacological actions of glucocorticoids are mediated by their binding to an intracellular protein, the glucocorticoid receptor (GR). The determination of GR localization in normal glomerular cells is essential to elucidate the mechanisms of glucocorticoid action in various glomerular diseases.

Methods. We carried out an immunoblot examination using antihuman GR-specific antibody and homogenates of isolated normal human glomeruli and mesangial cells in culture. Immunohistochemical examinations were also performed on normal human kidney specimens at light and electron microscopic levels. The nuclear translocation of GRs elicited by ligand binding was further investigated by confocal laser-scanning microscopic inspection of freshly isolated glomeruli and mesangial cells cultured with dexamethasone.

Results. An immunoblot examination demonstrated the presence of a 94 kDa protein, a molecular weight consistent with that of GRs, in the homogenates of glomeruli and cultured mesangial cells. By light microscopic examination, GRs were strongly detected in the nucleus and moderately in the cytoplasm of all glomerular cells, parietal and visceral epithelial cells, endothelial cells, and mesangial cells. By electron microscopic examination, the nuclear GRs of all glomerular cells were found to be diffusely distributed in the euchromatin. Additionally, the immunofluorescence intensities of nuclear GRs in isolated glomeruli and mesangial cells in culture became more intense by the addition of dexamethasone.

Conclusions. Our findings suggest that all subsets of human glomerular cells definitely express the GR protein, which potentially undergoes translocation by glucocorticoids.

Key words: mesangial cell, nephrotic syndrome, progressive glomerulonephritis, cell injury, glucocorticoid action, ligand binding.

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Glucocorticoids modulate a large number of metabolic, cardiovascular, and immune functions [1]. The action of glucocorticoids is initiated by its binding to an intracellular 94 kDa protein, termed the glucocorticoid receptor (GR), which results in the formation of glucocorticoid-GR complexes that are subsequently translocated to the nucleus, where they bind to the DNA to regulate the transcription of specific genes [2]. In idiopathic nephrotic syndrome and various types of progressive glomerulonephritis, synthetic glucocorticoids have been widely used therapeutically [3–7]. However, the detailed mechanisms of glucocorticoid action in these diseases have not been clearly elucidated. Such glomerular diseases are initiated by an immune response, which may be humoral or cell mediated against either exogenous or native antigens and results in glomerular cell injury [8, 9]. Thus, the target cells of glucocorticoids in those glomerular diseases can be principally divided into the injured glomerular cells themselves and cells derived from the peripheral blood that are involved in glomerular cell injury. The expression of the GR protein has been investigated in monocytes [10], macrophages [11], and lymphoid cells [12, 13], but not in the glomerulus. Some authors reported that isotope-labeled glucocorticoids showed a specific binding to isolated normal glomerulus [14] and to the cytosol of glomerular preparations [15]. Furthermore, a recent report demonstrated by the use of the competitive polymerase chain reaction method the existence of GR mRNA in the normal glomeruli of rat [16]. These reports suggest that the GR protein would also exist in glomerular cells. The detection of GR protein expression and localization in normal glomerular cells is essential to elucidate the mechanisms of glucocorticoid action in various glomerular diseases. In our study, to clarify the existence of the GR protein, we performed immunoblot and immunohistochemical

examination in the human kidney glomeruli. Additionally, to determine whether the nuclear translocation of GRs in the glomerular cells is provoked by a ligand, we treated freshly isolated glomeruli and mesangial cells in primary culture with dexamethasone.

METHODS

Materials and antibodies

Antihuman GR polyclonal antibody (PA1-512) and a synthetic peptide were purchased from Affinity Bioreagents Inc. (Nashenic Station, NJ, USA). This antibody was raised in rabbits after immunization of them with a synthetic peptide spanning human GR amino acids 245 to 259 and recognizes all known forms, such as liganded and unliganded, of the human GR protein [17]. The specificity of this antibody was documented in another report [18]. Additionally, as a positive control cell to detect human GR protein expression, we employed the GR-expressing Chinese hamster ovary cell (CHOpMTGR; kindly provided by Dr. Stefan Nilsson, Karo Bio, Sweden) cultured in Ham's F-12 medium (Life Technologies, Inc., Grand Island, NY, USA) supplemented with antibiotics and 10% fetal calf serum (FCS; Life Technologies, Inc.) in a humidified atmosphere at 37°C and containing 5% CO_2. These CHOpMTGR cells were transfected with a full-length GR expression vector and stably expressed the human GR protein [19]. The Chinese hamster ovary cell (CHO-K1), which is not transfected with a GR expression vector, was purchased from the Health Science Research Resources Bank (Osaka, Japan) and used for negative control experiments. Serum-free medium (AIM-V medium) and RPMI-1640 medium were also purchased from Life Technologies. Horseradish peroxidase (HRP)-labeled dextran polymer-conjugated goat antirabbit antibody, biotinylated goat antirabbit immunoglobulin, and fluorescein-conjugated streptavidin were purchased from Dako Corporation (Kyoto, Japan). Dexamethasone, phenylmethylsulfonyl fluoride, pepstatin, leupeptin, and $CoCl_2$ were purchased from Wako Pure Chemical Ltd. (Osaka, Japan). Deoxyribonuclease, ribonuclease A, 1,4-diazabicyclo (2.2.2.) octane (DABCO), and 3,3′diaminobentizine tetrahydrochloride (DAB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Isolation of glomeruli and mesangial cells

Renal cortex was obtained from the intact part of kidneys that had been surgically removed because of renal cancer or ureter cancer in patients from Kyorin University Hospital. Glomeruli were isolated by a differential sieving technique [20]. The purity of the preparation, as assessed by phase contrast microscopy, was approximately 95%. Additionally, human mesangial cell cultures in RPMI-1640 medium supplemented with antibiotics and 10% FCS were prepared as previously described [21].

Glomeruli and mesangial cell culture with dexamethasone

Passage 5 mesangial cells from primary cultures on glass slides and freshly isolated glomeruli in 24-well plates (Iwaki Glass, Funabashi, Japan) were cultured for 24 hours with 2 ml of AIM-V medium in a humidified atmosphere at 37°C that contained 5% CO_2 in order to dissociate any endogenous ligand bound to GRs and to avoid further GR occupancy by exogenous hormone [22]. Thereafter, the glomeruli were further cultured for three hours, and mesangial cells for one and three hours, in the presence or absence of 10 nM dexamethasone. After having been washed with phosphate-buffered saline (PBS; pH 7.4), glomeruli were fixed with 4% formaldehyde-0.1 M phosphate buffer (PB; pH 7.4) at 4°C for one hour and immersed in 5, 10, and 20% sucrose-PBS. Subsequently, they were embedded in OCT compound, and frozen serial 10-mm thick sections were prepared for indirect immunofluorescence examination. Mesangial cells were washed with PBS and then fixed with 4% formaldehyde-0.1 M PB at 4°C for 30 minutes. After having been washed with PBS, these cells were also prepared for indirect immunofluorescence examination.

Indirect immunofluorescence examination

Frozen sections of glomeruli and cultured mesangial cells on the slides were immersed in 5% normal goat serum and 0.05% Triton-X 100 in PBS for 20 minutes, reacted with PA1-512 (5 μg/ml) for two hours, and then washed with PBS. After incubation with biotinylated goat antirabbit immunoglobulin (100-fold dilution) for one hour, the slides were washed with PBS and incubated with fluorescein-conjugated streptavidin (100-fold dilution) for 30 minutes. Only glomeruli were further stained in a PI staining reagent (PBS, pH 7.4, with 50 μg/ml ribonuclease A and 50 μg/ml propidium iodide) for six minutes in order to counterstain the nucleus. All of these reactions were performed at room temperature. After a final washing with PBS, the slides were mounted in 10 mg/ml DABCO in PBS-glycerol (1:3) to retard laser bleaching and were examined by fluorescence microscopy using a confocal laser scanning microscope (GB 200; Olympus, Tokyo, Japan). Serial optical sections were obtained at 1 μm increments.

Light microscopic examination

Kidney cortex samples were fixed by immersing each sample in either (*a*) 10% neutralized formalin for 24 hours at 4°C, (*b*) 10% neutralized formalin for 6 hours at 4°C, or (*c*) 4% formaldehyde-0.1 M PB for 6 hours at 4°C. No significant differences in GR antigen preservation were noted among the three fixation regimens (data

not shown). Therefore, light microscopic examination was carried out on tissues fixed in 10% neutralized formalin for 24 hours at 4°C. Two kidney cortex samples were embedded in paraffin, and serial 3-μm thick sections were prepared for immunohistochemistry. Routine histological examination was performed with periodic acid-Schiff staining. Immunohistochemical staining of GRs in kidney cortex specimens was performed by the indirect immunoperoxidase method. Sections were deparaffinized at 55°C for 30 minutes, placed in xylene, and hydrated in 100, 90, 80, and then 70% ethanol; thereafter, endogenous peroxidase activity was quenched with a 0.6% H_2O_2/methanol solution for 30 minutes at room temperature. After having been washed with PBS, the slides were immersed in 5% normal goat serum and 0.05% Triton-X 100 in PBS for inhibition of nonspecific binding. They were then incubated with PA1-512 (5 μg/ml) or with PA1-512 preabsorbed with synthetic peptide (preabsorbed PA1-512; 5 mg/ml) diluted in 5% normal goat serum and 0.05% Triton-X 100 in PBS as primary antibodies for two hours at room temperature. After the slides had been washed with PBS, HRP-labeled dextran polymer-conjugated goat antirabbit antibody (Envision system) was applied as a second antibody for one hour at room temperature. The slides were washed with PBS and developed by immersion in 1.4 mM DAB, 1% $CoCl_2$, 0.1% H_2O_2 in PBS (DAB-Co-H_2O_2) for eight minutes. After a final washing in distilled water, the slides were dehydrated in ethanol and xylene and mounted in Permount.

Immunoelectron microscopic examination

Kidney cortex samples were fixed in 4% formaldehyde-0.1 M PBS for six hours at 4°C, immersed in 5, 10, and 20% sucrose-PBS, and embedded in OCT compound. Immunostaining of GRs in frozen sections was performed as mentioned earlier here and was further prepared for electron microscopic observation by employing the pre-embedding procedure [23]. Briefly, after DAB-Co-H_2O_2 treatment, the sections were treated with 1% osmium tetroxide in 0.1 M PB for 10 minutes, dehydrated by passage through a series of graded ethanols, and embedded in Epon on glass slides. Ultrathin sections were made, stained with 0.1% lead citrate for seven minutes, and examined at 80 kV with a transmission electron microscope (JEM-1010; JEOL, Tokyo, Japan).

Immunoblotting

Kidney cortex samples, freshly isolated glomeruli, cultured passage 5 mesangial cells, cultured CHOpMTGR cells, and cultured CHO-K1 cells were washed with PBS and were homogenized on ice in homogenizing buffer [20 mM Tris-HCl (pH 8.6), 5 mM ethylenediaminetetraacetic acid (EDTA), 50 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1% Nonidet P-40, 150 mM NaCl, 100 μg/ml deoxyribonuclease, 50 μg/ml ribonuclease A] with a Teflon glass tissue homogenizer (Eyela, Tokyo, Japan). Homogenates were centrifuged at 100,000 × *g* in a Beckman 50 Ti rotor (Palo Alto, CA, USA), and the supernatants were then collected, mixed with an equal volume of sample buffer [125 mM Tris-HCl, 1% sodium dodecyl sulfate (SDS), 5% sucrose, and 0.1% bromophenol blue], boiled for five minutes, and stored at −80°C. Samples containing 100 μg protein were resolved by electrophoresis through 8% SDS polyacrylamide gels and electrophoretically transferred to nitrocellulose membranes in Tris-glycine buffer [25 mM Tris-HCl (pH 8.3), 192 mM glycine, and 20% methanol]. Membranes were blocked for 60 minutes at 37°C in blocking buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.05% Tween-20, 5% nonfat dry milk, 1% bovine serum albumin (BSA)] and then incubated overnight at 4°C with PA1-512 or preabsorbed PA1-512 diluted to 5 μg/ml with Tris-buffered saline [TBS; 10 mM Tris-HCl (pH 7.4), 150 mM NaCl] containing 0.05% Tween-20 and 1% nonfat dry milk (TBS-Tween). Subsequently, the membranes were washed with TBS-Tween, reacted with HRP-labeled dextran polymer-conjugated goat antirabbit second antibody (1:100 in TBS-Tween) for one hour at room temperature, washed in TBS-Tween, reacted with chemiluminescent reagents (Life Science Products, Boston, MA, USA), and then processed for autoradiography.

RESULTS

To demonstrate the presence of GR protein in human kidney tissue, we started an immunoblot study using PA1-512. To confirm the specificity of PA1-512 antibody against human GRs, either 0.5 and 5 μg total protein extracts of CHOpMTGR cells or CHO-K1 cells were studied with immunoblotting. As shown in Figure 1A, immunoblots of CHOpMTGR cells showed a band corresponding to a molecular weight of 94 kDa, but those of CHO-K1 cells did not demonstrate anything at all. These results indicate that PA1-512 has the specific reactivity to human GR protein. Accordingly, homogenates of freshly isolated glomeruli, cortex tissue, and cultured mesangial cells were further subjected to immunoblotting with PA1-512 antibody. Figure 1B showed that GR protein was expressed in all kidney samples examined. These protein bands were abolished when preabsorbed PA1-512 was used as a probe (Fig. 1C). These results prove the presence of the GR protein in human glomeruli and cultured mesangial cells. Therefore, by using this PA1-512 antibody, we performed immunohistochemical studies on GRs in kidney specimens, cultured glomeruli, and cultured mesangial cells.

As shown in Figure 2A, by light microscopic examination, immunoreactivity of GRs was intense in distal con-

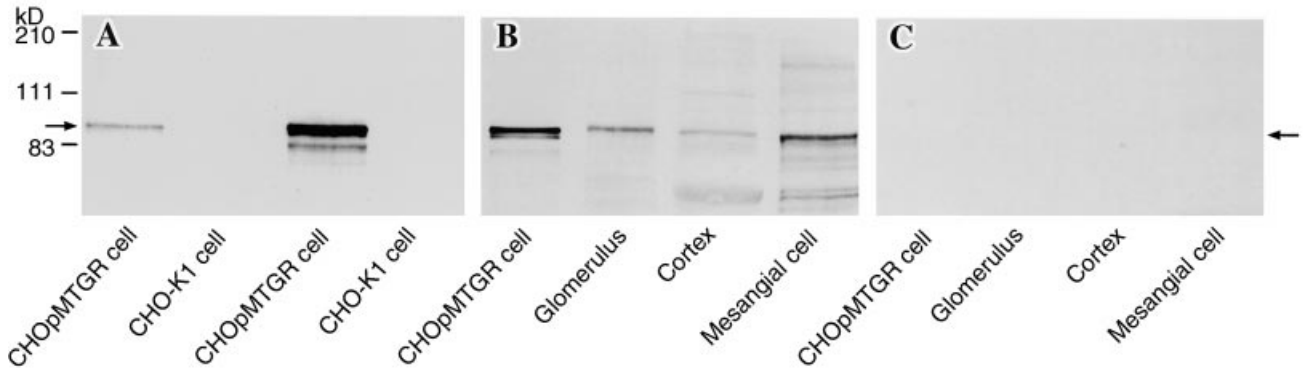

Fig. 1. Identification of glucocorticoid receptor (GR) immunoreactivity by immunoblotting in isolated glomerulus, renal cortex, and primary cultured mesangial cell. Protein extracts (0.5 and 5 μg, respectively) of CHOpMTGR cells and CHO-K1 cells were prepared and subjected to immunoblot analysis reacted with PA1-512 to confirm the specificity of this antibody (*A*). Protein extracts (100 μg) of glomerulus, cortex, and mesangial cells were also analyzed with PA1-512 in the absence (*B*) or presence (*C*) of the peptide antigen, and the band position was compared with that of CHOpMTGR cells (2 μg protein extract). The positions of molecular weight markers are shown at the left. The arrows indicate the band of 94 kDa protein.

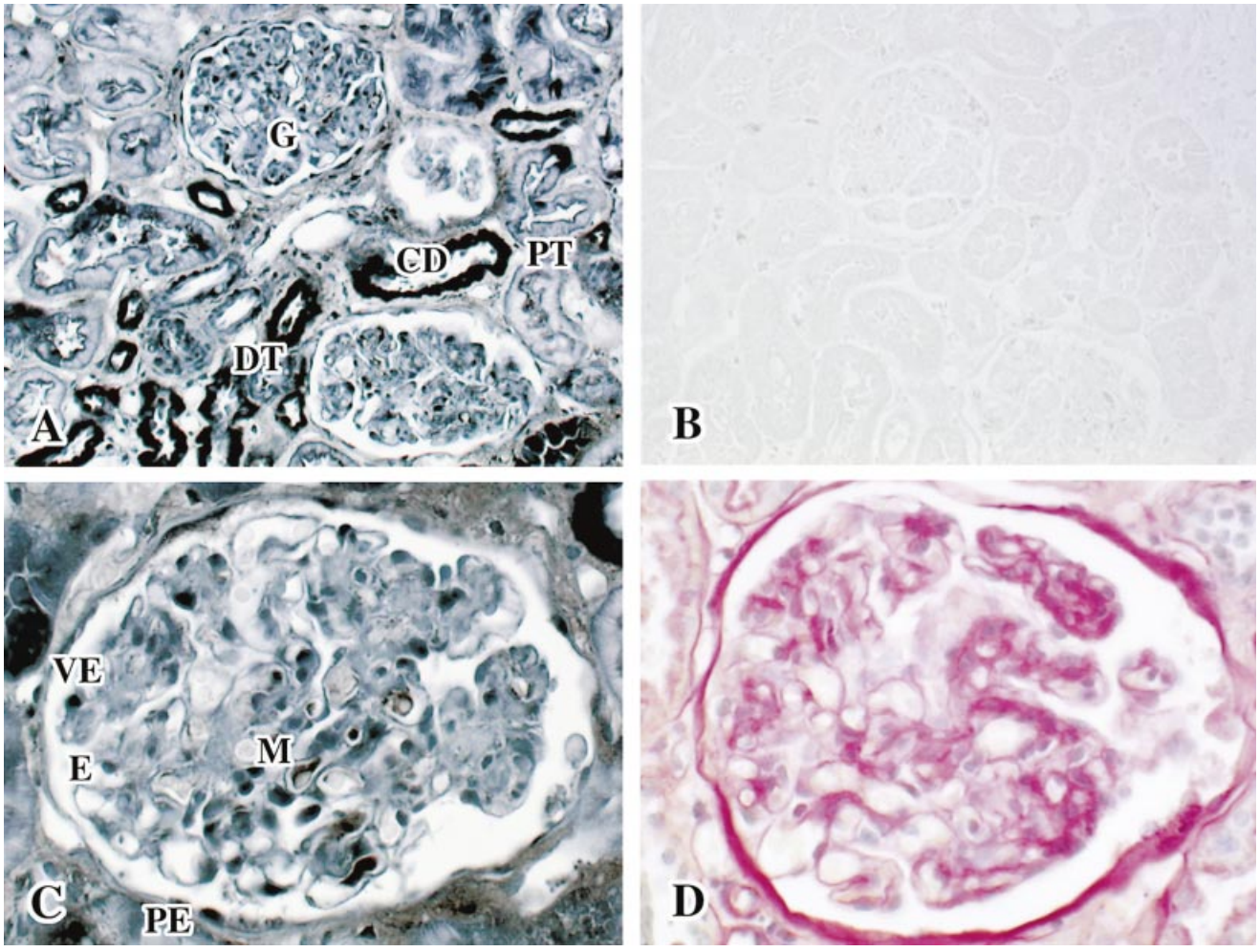

Fig. 2. Light microscopic observation of glucocorticoid receptor (GR) protein localization in normal kidney cortex. (*A*) Kidney cortex staining with PA1-512 (×200). (*B*) Abolition of cortical staining by preabsorption of PA1-512 with antigen peptide (×200). (*C*) Higher magnification of glomerulus staining with PA1-512 (×400). (*D*) PAS staining of adjacent serial section of glomerulus shown in C (×400). Abbreviations are: G, glomerulus; PT, proximal convoluted tubule; DT, distal convoluted tubule; CD, collecting duct; PE, parietal epithelial cell; VE, visceral epithelial cell; E, endothelial cell; M, mesangial cell.

voluted tubules and collecting ducts, moderate in glomeruli, and faint in proximal tubules. In the tubules, immunoreactive GRs were observed in both the cytoplasm and nucleus. This immunostaining pattern of GRs in the tubules is consistent with previous reports [24, 25]. In addition, in the glomerulus, we found that the GR protein was definitively expressed in all subsets of glomerular cells (Fig. 2C). Glomerular parietal and visceral epithelial cells, endothelial cells, and mesangial cells exhibited intense nuclear staining and moderate cytoplasmic staining. Immunoreactivity observed in the previously mentioned tissues was not present in the sections incubated with preabsorbed PA1-512 (Fig. 2B) or with nonimmune rabbit serum diluted to an equal γ globulin concentration as PA1-512 (data not shown).

After electron microscopic examination, immunostaining positive for GRs was detected in all of the subsets of glomerular cells, in agreement with light microscopic observations (Fig. 3A), and no immunostaining was observed in the specimens reacted with preabsorbed PA1-512 (Fig. 3 B, D). In all glomerular cells, the reaction products representing GRs were diffusely distributed within the nucleus and the cytoplasm. The immunoreactivity of epithelial cells (Fig. 3C) and endothelial cells (Fig. 3E) was more intense compared with that of the mesangial cells (Fig. 3F). The ultrastructural location of GRs in the nuclei was the euchromatin, and no GRs were detected in the marginated heterochromatin. The receptors in the cytoplasm were diffusely distributed irrespective of the location of specific organella.

To investigate whether the localization of the GR protein in glomerular cells and mesangial cells might be altered by glucocorticoids, we cultured freshly isolated glomeruli and mesangial cells in serum-free medium in either the presence or absence of dexamethasone and then conducted an indirect immunohistochemical analysis on them using a confocal laser scanning microscope. The GR protein of cultured glomeruli without dexamethasone was found in both nuclei and cytoplasm, as in the case of kidney specimens (Fig. 4 A, B). However, the immunoreactivity of GRs in the nuclei in the treatment with 10 nM dexamethasone was more intense compared with that of the receptor in the dexamethasone-free condition (Fig. 4 C, D). Moreover, in cultured mesangial cells, the localization of GRs was also demonstrated in the cytoplasm and in the nuclei in agreement with the *in vivo* observations (Fig. 5A). When the cells were treated with 10 nM dexamethasone for one or three hours, the intensity of nuclear GRs tended to increase time dependently compared with the intensity seen in the dexamethasone-free condition (Fig. 5 B, C).

DISCUSSION

This is the first report, to our knowledge, concerning the localization of GR protein in the human kidney. In this study, we started the examination with an immunoblot study using antihuman GR-specific antibody and human kidney samples. The specificity of this antibody was demonstrated earlier [17, 18] and was further confirmed in this immunoblot study, as shown in Figure 1. Thus, light microscopic examination using this antibody was subsequently performed to clarify the precise cellular location of GRs in the kidney. These results demonstrated that GRs were widely distributed with intense expression in distal convoluted tubules and collecting ducts, faint expression in proximal tubules, and moderate expression in glomerular cells.

There are two reports concerning the localization of GR protein in the kidney, one involving the rabbit protein and the other the rat receptor; however, both results demonstrated positive staining in only the tubules, not in the glomerular cells [24, 25]. The reason for this different staining of GR protein in the glomerular cells is obscure. More recently, in the rat kidney, it was demonstrated by the use of the competitive polymerase chain reaction method that GR mRNA was present in glomeruli derived from microdissected nephron segments [16]. Thus, the lower specificity or sensitivity of their anti-GR antibody and second antibody to detect the protein or lower protein levels of GRs may account for the negative staining of glomerular cells.

With regard to the subcellular localization of GR protein, our study showed that this protein exists in both nuclei and cytoplasm of all glomerular cells in the human kidney. The *in vivo* studies on rats also demonstrated that the immunostaining for GRs in various tissues except the glomeruli was found in both the nuclei and cytoplasm [26–28]. In adrenalectomized rats, nuclear staining of GRs in hepatocytes was markedly reduced, whereas in adrenalectomized rats treated with synthetic glucocorticoids, the density of nuclear staining was comparable to and often slightly higher than that in intact animals [28, 29], suggesting that the localization of GRs in the nuclei may be provoked by translocation from the cytoplasm in response to endogenous glucocorticoids.

In this study, we also investigated whether the localization of GR protein in freshly isolated glomerular cells can be altered after ligand treatment. The results demonstrated that the immunostaining of nuclear GRs in isolated glomerular cells, following the treatment with glucocorticoid drastically increased, compared with that in the absence of the ligand. In addition, the intensity of immunostaining of the nuclear GRs in cultured mesangial cells showed a tendency to increase in a time-dependent manner under the presence of ligand. Previous studies suggested that these changes of the localization of GRs by ligand may support the nuclear translocation of the unliganded cytoplasmic GRs in response to ligand binding [18, 29, 30]. Thus, we speculate that endogenous and synthetic glucocorticoids bind the cytoplasmic GRs

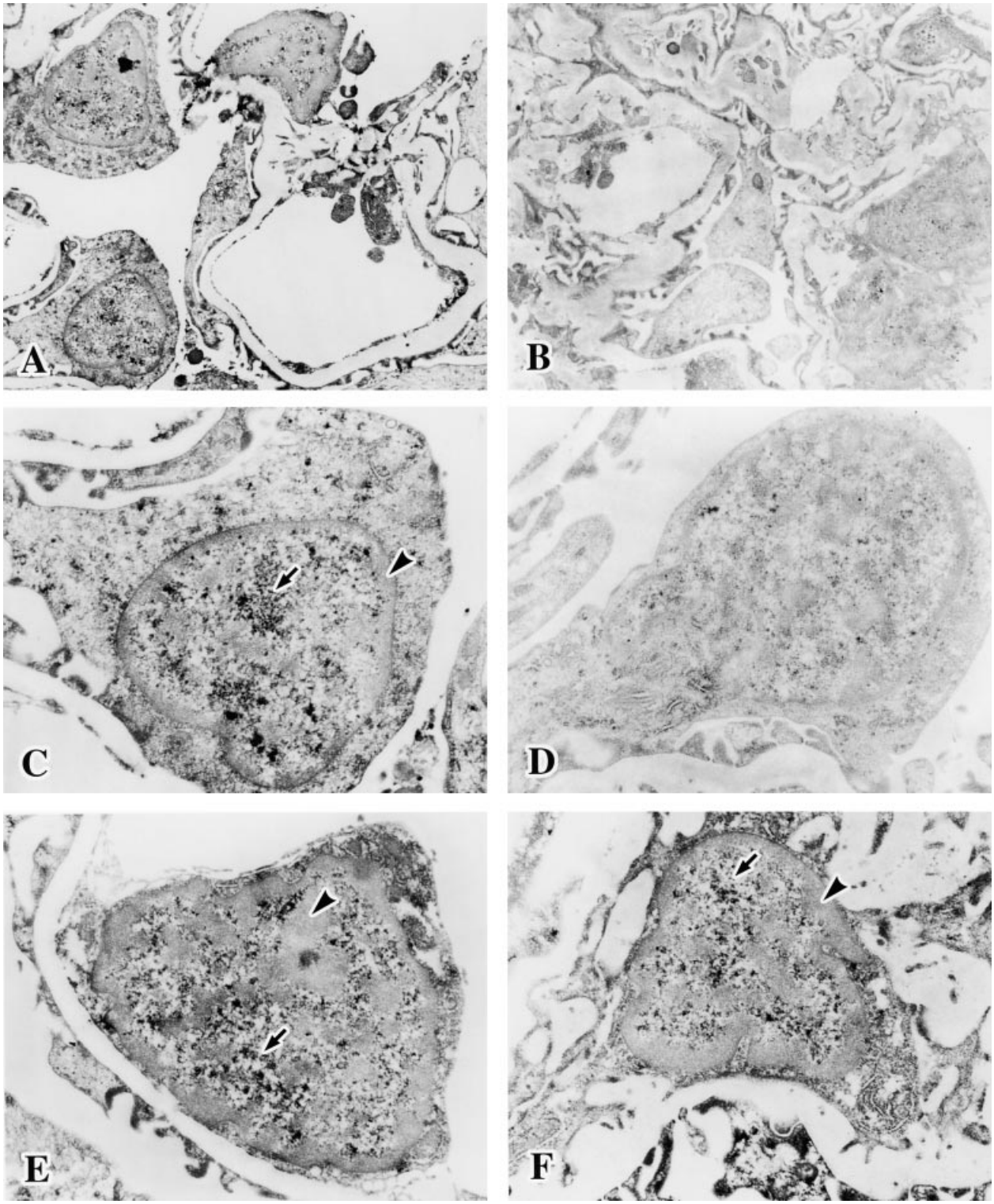

Fig. 3. Immunoelectron microscopic localization of glucocorticoid receptor (GR) protein in normal glomerulus. Low magnification of glomerulus reacted with PA1-512 (×4800; *A*). Abolition of GR staining of glomerulus at a low magnification of (×4600; *B*) and of visceral epithelial cell (×10,000; *D*) reacted with PA1-512 preabsorbed with antigen peptide. GR-positive immunostaining in visceral epithelial cell (×13,000; *C*), endothelial cell (×10,000; *E*), and mesangial cell (×11,000; *F*) indicates a sparse distribution in the cytoplasm and nucleus. GRs of these nuclei are observed ultrastructurally in the euchromatin (arrow), but not in the heterochromatin (arrowhead).

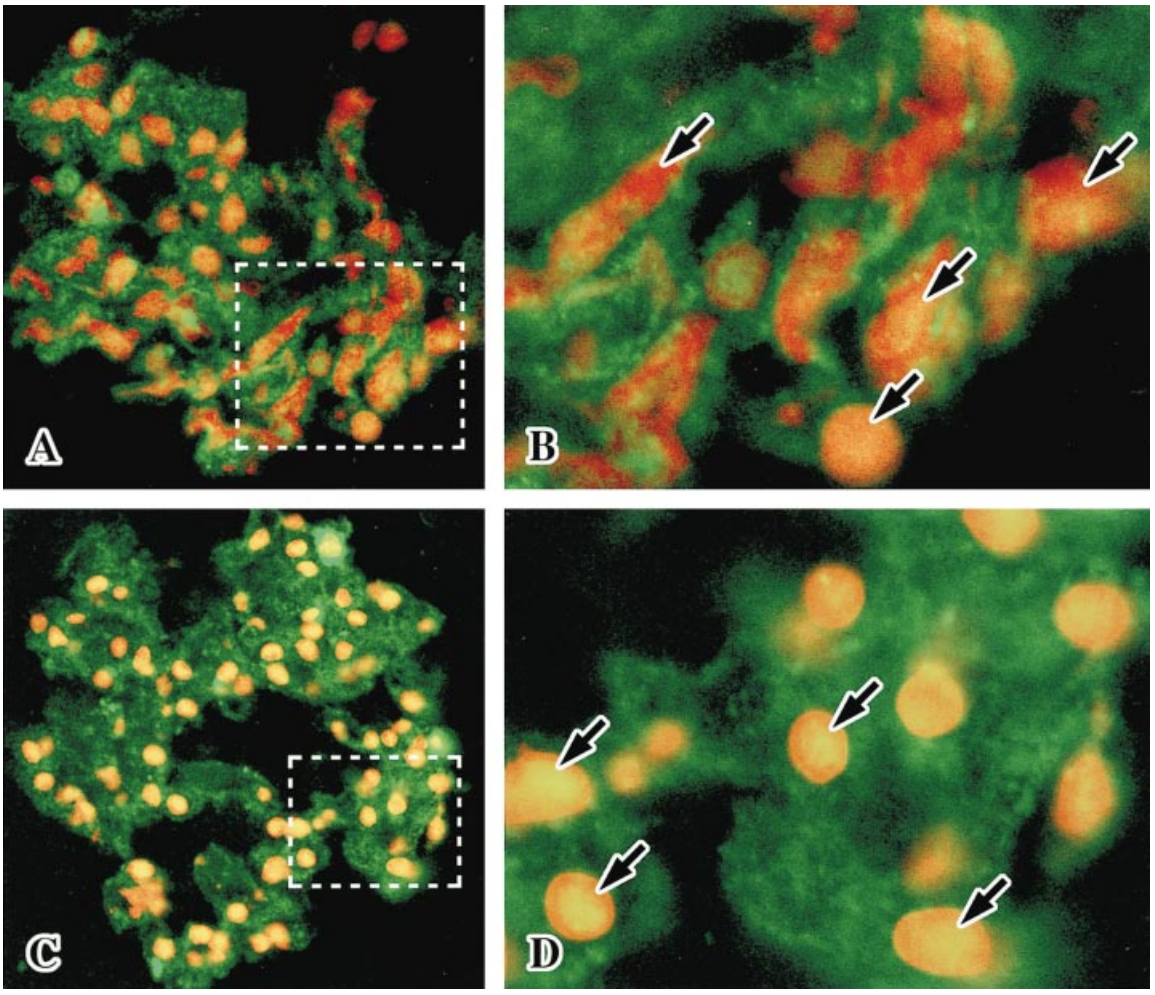

Fig. 4. Change of localization of glucocorticoid receptor (GR) in isolated glomerulus with dexamethasone. The glomerulus is incubated with the absence (*A, B*) or presence (*C, D*) of 10 nM dexamethasone in a serum-free medium for three hours. (B) and (D) are showed the scale of enlargement at a square area of each (A) and (C). Glomeruli are stained for GRs with fluorescein-conjugated streptavidin (green color), and nuclei are stained with PI (red color). GR staining of nuclei appears as a yellow color (magnification of A and C ×700; B ×2000; D ×2800).

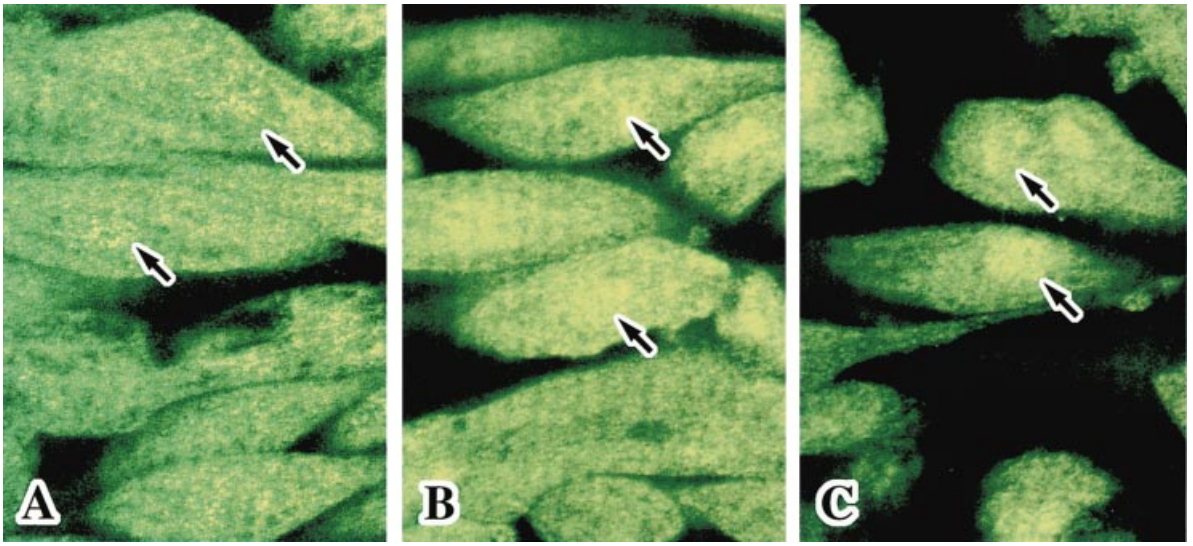

Fig. 5. Change of localization of glucocorticoid receptor (GR) in primary cultured mesangial cells with dexamethasone. Mesangial cells are incubated with 10 nM dexamethasone with serum-free medium for one hour (*B*) and three hours (*C*). Cells are stained for GRs with fluorescein-conjugated streptavidin. GRs in the absence of dexamethasone are observed in both the cytoplasm and nucleus (*A*), but treatment with dexamethasone drastically promoted nuclear translocation in a time-dependent manner (B, C). The arrow points to GR staining in the nucleus (magnification ×1400).

of glomerular cells, resulting in the formation of a hormone-receptor complex, which may interact with the genome and regulate transcription of selected genes by nuclear translocation. This speculation is supported by our immunoelectron microscopic examination demonstrating that GRs in the nucleus were associated with euchromatin, not with heterochromatin. Other investigators also demonstrated the same intranuclear localization of estrogen receptor [31] and androgen receptor [32]. Heterochromatin is inactive in DNA transcription, whereas euchromatin contains transcriptionally active regions [33]. Therefore, the GR localization within euchromatin may also suggest an association of GRs with specific DNA-regulatory elements, subsequently leading to the regulation of gene expression.

In summary, our findings demonstrate that GR protein is localized in all subsets of normal glomerular cells and potentially mediates the pharmacological effects of synthetic glucocorticoids. The knowledge of the existence and localization of GR protein in normal glomerular cells may be useful for the evaluation of sensitivity to synthetic glucocorticoids in various glomerular diseases. Because the reasons for the changes of GR protein expression in diseased glomeruli, proliferation of mesangial cells, and the permeability of isolated glomeruli with and without ligands remain unknown, further studies should be performed to elucidate the molecular mechanism of glucocorticoid action in various glomerular diseases.

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