Extracellular Matrix Component mRNA Expression in Glomeruli in Experimental Focal Glomerulosclerosis\textsuperscript{1}

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ABSTRACT

This study was designed to assess how the expression of genes for components of the extracellular matrix is altered in a model of focal glomerular sclerosis. In this model, a unilateral nephrectomy combined with injections of puromycin aminonucleoside induces a much higher incidence of focal glomerular sclerosis. Rats received puromycin aminonucleoside on days 0, 27, 34, and 41 and underwent unilateral nephrectomy on day 22. Control rats received physiologic saline injections with and without unilateral nephrectomy. Rats from each group were killed on days 48, 60, and 80. The steady-state levels of glomerular mRNA encoding type IV collagen, the BI and B2 chains of laminin, heparan sulfate proteoglycan, and type I and type III collagens were compared in both the puromycin aminonucleoside-treated and the control glomeruli. The mRNA levels encoding type IV collagen and laminin BI and B2 were increased three-, two-, and twofold, respectively, on day 48 of focal glomerular sclerosis. These transcripts were further increased eight-, seven-, and eightfold, respectively, on day 80 compared with the control glomeruli ($P < 0.01$). In contrast, heparan sulfate proteoglycan mRNA levels were not increased on day 48 when the animals had marked proteinuria. However, the heparan sulfate proteoglycan mRNA levels did become elevated by day 60 and remained elevated thereafter. The expression of type I and type III collagen mRNA was increased 12- and 7-fold, respectively ($P < 0.01$), on day 80 in focal glomerular sclerosis rats compared with the controls. An immunofluorescence study revealed the accumulation of immunoglobulin M, C3, type IV collagen, laminin, heparan sulfate proteoglycan, and type I and type III collagens in the sclerotic area. These data indicate that changes in the mRNA levels for components of the basement membrane and interstitial collagen are associated with the development of glomerular sclerosis.

Key Words: Type IV collagen, laminin, heparan sulfate proteoglycan, interstitial collagen, puromycin aminonucleoside

Focal glomerular sclerosis (FGS) is the histologic description of a form of glomerular injury that is usually associated with proteinuria and progressive loss of renal function. Although the pathogenesis of this disease remains unclear (1), immunologic, hemodynamic, genetic, and metabolic factors may be involved in its development (2–5).

Recent interest has focused on extracellular matrix (ECM) accumulation in the areas of mesangial sclerosis (6–8), and both interstitial and basement membrane collagens have been shown to be present (9–11). However, less is known regarding the expression of the genes for the basement membrane and interstitial collagens in glomerular sclerosis (12,13). Rats injected with puromycin aminonucleoside (PAN) after the removal of one kidney develop marked glomerular sclerosis in the remnant kidney (14). This model is characterized by heavy proteinuria and by the expansion of the mesangium due to an increase in periodic acid-Schiff-positive materials. Because of the difficulty in solubilizing and isolating ECM components from glomeruli, we have used specific cDNA probes to quantitate the levels of the mRNA encoding some basement membrane proteins and interstitial collagens (type I and type III) in isolated glomeruli during the development of glomerular sclerosis.

METHODS

Model of FGS

The method used to produce FGS in rats has been described previously (14). Briefly, 60 male Sprague-Dawley rats, weighing 200 to 250 g, were divided into three groups as described in Table 1. PAN (Sigma Chemical Co., St. Louis, MO), 15 mg/100 g body wt in 1.0 mL of physiologic saline, was injected ip into...
the group III rats on day 0. Group I and II rats received an injection of 1.0 mL of physiologic saline. On day 22, the right kidney was removed from the rats in groups II and III and a sham nephrectomy was performed on the rats in the group I. The nephrectomized rats in group III received ip injections of 5 mg/100 g body wt of PAN in physiologic saline on days 27, 34, and 41. The rats in groups I and II received 1.0 mL of physiologic saline on the same days. Five animals from each group were killed 48, 60, and 80 days after the initial dose of PAN or saline under ether anesthesia, and then the left kidney was removed and processed for glomerular RNA isolation and for histopathologic analysis. The animals were allowed free access to regular rat chow and tap water during the experiments.

**RNA Extraction, Northern Blot, and Dot Blot**

Glomeruli were isolated by a serial sieving procedure with 75-, 106-, and 125-μm-pore-size meshes. All experimental details were as reported previously (18). To determine whether these mRNA changes are a direct effect of PAN, we isolated glomeruli only 2 days after PAN administration, before the onset of proteinuria. To ensure that the glomerular isolation technique did not lead to a selective enrichment of a subpopulation of glomeruli in the diseased animals, 100 μL of isolated glomeruli were evaluated. The isolated glomeruli were centrifuged, fixed with formalin, and then embedded in paraffin. The sections were cut and stained with periodic acid-Schiff and methylene blue-G250 method with a commercial standard (Tonin Kita: Ohtsukka Pharmacy, Tokyo, Japan).

**Histologic Analysis of Renal Tissue**

Portions of the kidney removed at the time of nephrectomy and at the time of death were fixed in buffered formalin (pH 7.4) for light microscopy or frozen in liquid nitrogen for immunohistochemical studies (15). One hundred glomeruli from each kidney were examined for the prevalence of glomerular sclerosis, and the percentage of sclerotic glomeruli was obtained by dividing the number of glomeruli with sclerotic lesions by the total number of glomeruli examined. Glomerular sclerosis was defined as capillary collapse, increased mesangial matrix, and capillary adhesion to Bowman’s capsule. Renal tissues for the immunofluorescence study were sectioned by cryostat and stained with antibodies against matrix components or with fluorescently labeled antibodies specific for immunoglobulin M (IgM) and C3 (Cappel, Malvern, PA). Immunofluorescence analyses for rat collagen IV, laminin, heparan sulfate proteoglycan (HSPG), and collagens I and III were performed by incubating the tissue with an appropriate dilution of rabbit antibody for 30 min, followed by staining with fluoresceinated goat anti-rabbit IgG (Cappel). Antibodies for collagen IV, laminin, and HSPG were obtained from Drs. Hynda Kleinman, John Hassel, and Yoshihiko Yamada (NIH, Bethesda, MD) (15,16). The monoclonal antibodies for rat collagens I and III were obtained from Dr. Akira Ooshima (Wakayama Medical College, Wakayama, Japan), and their specificities were checked by an ELISA (17). The intensity and distribution of the immunofluorescence were evaluated by two observers who were unacquainted with the pertinent data. The intensity of fluorescence was graded from 0 to 3+ (none, normal, moderate, prominent) on the basis of the intensity of the control glomeruli at the same time point. For each specimen, 40 glomeruli were evaluated and the mean value per specimen was calculated.

**Measurement of Urinary Protein**

A 24-h urine sample was collected from each rat every 5 days during the course of the experiments. Urinary protein was measured by the Coomassie brilliant blue-G250 method with a commercial standard (Tonein Kita: Ohtsuka Pharmacy, Tokyo, Japan).

**Histologic and Immunohistochemical Assays**

Sections of the kidney were collected and stained with periodic acid-Schiff and methylene blue-G250 method with a commercial standard (Tonein Kita: Ohtsuka Pharmacy, Tokyo, Japan).

**Northern Blot Analysis**

Total RNA was extracted by a direct effect of PAN. We isolated glomeruli only 2 days after PAN administration, before the onset of proteinuria. To ensure that the glomerular isolation technique did not lead to a selective enrichment of a subpopulation of glomeruli in the diseased animals, 100 μL of isolated glomeruli were evaluated. The isolated glomeruli were centrifuged, fixed with formalin, and then embedded in paraffin. The sections were cut and stained with periodic acid-Schiff and assessed by light microscopy. The F9 cells were induced to differentiate by the addition of retinoic acid and dibutyryl cAMP to the media (19). NIH 3T3 cells were obtained from the American Type Culture Collection (Rockville, MD). Total RNA was extracted by acid guanidinium thiocyanate phenol chloroform extraction (20). The RNA concentration was determined spectrophotometrically at 260 nm. The purity of the RNA preparation was assessed by measuring the optical density ratio at 260 and 280 nm. A series of four dilutions of each RNA sample (1.0, 0.5, 0.25, and 0.125 μg) were dotted onto nylon filters (Gene Screen; DuPont, NEN Research Products, Boston, MA) with a 96-well manifold. For Northern blot analysis, 10 μg of the RNA samples were denatured and electrophoresed through 0.7% agarose gels as previously reported (15). Before transfer to nylon filters, the ethidium bromide-stained gels were
checked under ultraviolet illumination to determine the position of the 28S and 18S ribosomal RNA bands to assess the integrity of the RNA and to verify that equal amounts of RNA were loaded. The filters were irradiated by ultraviolet light for 5 min to fix the RNA.

cDNA Probes and Hybridization

The filters were hybridized with the specific cDNA probes as previously described (15). Details of the cDNA for the type IV collagen and laminin used in this study were previously published (15,21). The cDNA for the core protein of the basement membrane HSPG (clone 7) was kindly provided by Dr. D.M. Noonan (22). The cDNA for collagens I and III were kindly provided by Dr. Y. Yamada (23). The cDNA probes were labeled with a random primed cDNA labeling kit (Boehringer-Mannheim-Yamanouchi, Tokyo, Japan) with [32P]deoxynucleotide (DuPont, NEN Research Products) to a specific activity of 10⁶ cpm/μg. The hybridizations were performed, and the filters were then washed three times in 5% sodium dodecyl sulfate (SDS), 0.5% bovine albumin, 1 mM EDTA, and 40 mM NaH2PO4 at 65°C, followed by three washes in 1% SDS, 1 mM EDTA, and 40 mM NaH2PO4 at 65°C, by the method of Church and Gilbert (24). The filters were dried and exposed at −70°C to Kodak X-OMAT-AR film (Eastman-Kodak Co., Rochester, NY). The autoradiograms were scanned with a densitometer (Shimazu Manufacturing, Kyoto, Japan). Multiple exposures were taken to obtain the linear range of exposure. After exposure, the membranes were stripped by being washed twice in 0.1× SSC/1% SDS at 100°C, followed by being rinsed in 0.1× SSC, and then being dried. The filters were exposed to Kodak X-OMAT-AR film to ensure that the stripping was complete. The stripped membranes were rescreened with β-actin.

Statistics

All data were analyzed by one-way analysis of variance with replication by Fisher's Protected Least Significant Difference for multiple comparisons. The Spearman rank correlation coefficient was also used in some instances. Significance was assigned at the P < 0.05 level.

RESULTS

Urinary Protein Excretion

The initial urinary protein excretion in the three groups of rats was 15 ± 4.4 mg/24 h. The rats in group III developed significant proteinuria within 5 days after the initial injection of PAN (Figure 1). Proteinuria reached a peak by day 8 and then declined (Figure 1). Significant proteinuria again developed in the group III rats on day 48 after the right nephrectomy and the second series of PAN injections (Figure 1). The rats in group II showed a gradual increase in protein excretion 30 days after nephrectomy. It remained higher than that in the group I rats. The urinary protein excretion in the group III rats, but not in the group I or II rats, correlated with the occurrence of FGS (P < 0.05).

Occurrence of FGS

Group 1. Histologic examination of glomeruli from five normal control rats revealed no evidence of FGS (Table 1). The mesangial matrix and mesangial cells were not increased, and no deposits were present.

Group II. FGS was observed in two of the five rats killed on day 60, and the percentage of FGS increased throughout the experiment (Table 1).

Group III. FGS was observed in five of five animals, and 16.6 ± 8.4% of the glomeruli showed FGS on day 48 (Table 1; Figure 2). The percentage of glomeruli with sclerosis increased markedly thereafter and, by day 80 involved 72.8 ± 20.4% of the glomeruli in this group. IgM and C3 were stained in a focal segmental pattern in the glomeruli on day 80 that corresponded to the areas of glomerular sclerosis (data not shown).
Immunohistochemical Study

On day 0, laminin (Figure 3), type IV collagen, and HSPG (data not shown) were localized in the mesangium, glomerular basement membrane, and tubular basement membrane as previously reported. Collagen I (Figure 4) and collagen III (data not shown) were stained in the interstitium but not in the glomeruli. However, as FGS developed over time, both the basement membrane components and collagens I and III accumulated in the sclerotic areas.

Isolation of Glomeruli

All glomerular preparations used were over 90% free of extraglomerular elements. Glomeruli were assessed for glomerular sclerosis, which was defined as increased mesangial matrix. For the isolated glo-

![Image of immunohistochemical study results]

Figure 2. Periodic acid-Schiff-stained glomeruli from a group III rat on days 0 (A), 48 (B), 60 (C), and 80 (D). Magnification, x400 (B). Note the increase in the mesangial matrix by day 48. (C) Glomerulus showing increased mesangial matrix. (D) Glomerulus showing marked mesangial matrix enlargement with collapse of the capillary lumens. Bar, 5 μm.

![Image of immunofluorescence results]

Figure 3. Immunofluorescence for laminin in a glomerulus from a rat in group III on days 0 (A), 48 (B), 60 (C), and 80 (D). On day 0, the mesangium and glomerular basement membrane react with the antibody. On day 48, glomerular architecture is distorted as assessed by the antibody. Note that by day 80 immunofluorescence for laminin is significantly increased in the sclerotic area. Bar, 5 μm.
Figure 4. Immunofluorescence for type I collagen in glomeruli from rats in group III on days 0 (A), 48 (B), 60 (C), and 80 (D). By day 48, the interstitium, but not the glomeruli, reacts with the antibody. On day 80, the sclerotic area contains large amounts of type I collagen. Bar, 5 μm.

meruli, the percentages of glomeruli with sclerosis in the group III rats were 17.3 ± 1.2, 42.4 ± 0.8, and 59.7 ± 1.5% on days 48, 60, and 80, respectively (Table 1). These are not statistically different from the percentages found on histologic sections. In our isolated glomerular preparation, severely sclerotic glomeruli were also observed by light microscopy. This indicates that we could isolate representative glomeruli on day 80 of the disease model by a sieving procedure and that the isolated glomeruli were neither biased towards more normal nor more affected glomeruli. Thus, the severely injured glomeruli that were isolated are representative of the disease process and might be involved in the altered steady-state levels of the mRNA of the ECM components.

Northern and Dot Blot Analysis of mRNA Levels

The results of the Northern blots for α1(IV) collagen, the B1 and B2 chains of laminin, the core protein of HSPG, and β-actin are shown in Figure 5. mRNA isolated from differentiated F9 teratocarcinoma cells were used as a control. The individual mRNA in the group I, II (data not shown), and III rats were similar in size to those of the control specimens, suggesting that there were no changes in the sizes of the mRNA over the course of the experiment. Figure 6 shows the Northern blot results for the α1(I) and α1(III) collagens, with RNA isolated from rat calvaria and fibroblasts serving as controls. Again, no change in the size of the individual transcripts was seen over the course of the experiment and the size was similar to that observed in the controls. Because the cDNA probes used showed no cross-hybridization with the other species under the hybridization conditions used, we could quantitate the mRNA species by dot blot hybridization.

In glomeruli obtained from the group I rats, the steady-state levels of mRNA for α1(IV) collagen, laminin B1 and B2 chains, and HSPG decreased over time, as previously reported by us and others (15,25) (Figure 7). On day 80, the expression of these mRNA was 20 to 30% that of day 0. The mRNA for the α(I) and α1(III) chains were expressed even on day 0 and showed the same pattern as the mRNA for the basement membrane components. Although the levels of the mRNA for β-actin showed little change (Figure 8C) among the three groups throughout the experiment, the radiodensity of the specific probes and the β-actin were corrected for inaccuracies in loading or transfer.

A different expression pattern was observed in glomeruli from the rats in group III (Figure 7A). The α1(IV) transcripts increased threefold by day 48 (P < 0.01) and eightfold by day 80 (P < 0.01) as compared with that in the control. Laminin B1 (Figure 7B) and B2 (Figure 7C) chain mRNA showed a similar pattern. However, the level of HSPG mRNA was decreased somewhat (80% of day 0 level but not statistically significant) but remained significantly elevated over that in controls (P < 0.01) on day 48 and thereafter increased ninefold (P < 0.01) by day 80 as compared with that in the controls (Figure 7D). In the glomeruli of the group III rats, the mRNA levels for α1(I) and...
Figure 5. Northern blot analysis of glomerular RNA (10 μg/lane) for α1(IV) collagen (A), laminin B1 (B), laminin B2 (C), HSPG (D), and β-actin (E) from rats in group III. The membranes were stripped and rehybridized with a β-actin probe as described in Methods. Lane 1, day 0; lane 2, day 48; lane 3, day 60; lane 4, day 80; lane 5, induced F9 cells.

α1(III) increased in a way similar to that of α1(IV) and laminin (Figure 8). On day 80, they were increased 12- (P < 0.01) and 7 (P < 0.01)-fold, respectively, over control levels. On day 2, before the animals developed proteinuria, the mRNA levels for the α1(IV) chain and laminin B1 and B2 chains were already increased and the mRNA levels for HSPG were decreased. However, the mRNA levels for β-actin and for the α1(I)
and α1(III) chains showed little change (data not shown).

Spearman rank correlations were significant for the relationship between the mRNA levels of the basement membrane components (P < 0.01) or the interstitial collagens (P < 0.01) and the grade of immunohistochemical changes (Figures 3 and 4).

In the group II rats, the mRNA levels showed the same pattern as those in group I, except for that of α1(I) and α1(III) (Figure 8). The mRNA levels for the α1(I) and α1(III) chains decreased by day 48. On day 60, both type I and type III mRNA levels were increased 1.5-fold (P < 0.01) over the control levels. On day 80, the type I and type III mRNA levels were increased 3.5- (P < 0.01) and 3 (P < 0.01)-fold, respectively, over the control levels.

**DISCUSSION**

The PAN-treated, unilateral nephrectomized rat provides a well-characterized model of glomerular sclerosis that results in the development of massive proteinuria and renal failure. These lesions, by light microscopy, consist of an expanded mesangium, hypercellularity, collapsed capillaries with accumula-
tions of periodic acid-Schiff-positive material, and adhesions of capillary loops to Bowman's capsule. In this study, the mRNA levels for α1(IV) collagen, B1 and B2 chains of laminin, and HSPG in the glomeruli of rats with PAN-induced FGS were higher than those in age-matched controls or those in uninephrectomized rats. This increase reflects the increased synthesis of basement membrane component proteins in the glomerulus. By immunohistochemical study, we found an accumulation of laminin, collagen IV, and HSPG in the sclerotic areas. These results are supported by the findings of Lombet et al. (12), who reported a correlation between glomerular α1(IV) mRNA levels and glomerular sclerosis in subtotal nephrectomized rats. In this study, we found significant relationships between the mRNA levels and the protein accumulations.

Unlike the mRNA for the collagens and laminin, that for HSPG exhibited a different pattern. On day 48, when the animals developed maximal urinary protein excretion, the glomerular HSPG mRNA level remained at the day 0 level, whereas the other ECM mRNA levels had increased. We have shown previously that, in PAN nephrosis, the mRNA levels for HSPG were abnormally low when the animals had heavy proteinuria and that, when the abnormal mRNA levels for HSPG were ameliorated by treatment with methylprednisolone, it was accompanied by decreased proteinuria (18,26). Thus, it is likely that glomerular sclerosis may attenuate the reduced HSPG mRNA level in experimental FGS on day 48. Another possibility is that the increased gene expression for HSPG occurred later than that for the other basement membrane components in the FGS rats.

Figure 8. Quantitative comparison of mRNA for α1(I) (A), α1(III) (B) procollagen, and β-actin (C) in rat glomeruli over time. Data were normalized for equivalent amounts of β-actin RNA, and the mean control value was designated 100%. Error bars indicate the SD. The inset shows dot blots (0.5 μg of RNA), which were quantitated by densitometric scanning. N = 4.
It has been postulated that glomerular epithelial cells (GEC) and endothelial cells are the main source of HSPG (27). PAN is known to be toxic to rat GEC both in vivo and in vitro (28) and to cause a reduction in the HSPG core protein content of GEC in vitro (29). Accordingly, we have determined whether these mRNA changes are due to a direct effect of PAN. We measured the mRNA levels of several basement membrane genes only 2 days after PAN administration and before the development of proteinuria. We found that the steady-state mRNA levels for these basement membrane genes had already changed. However, we also have shown that the mRNA levels for β-actin, α1(I), and α1(III) undergo little change (26). Therefore, it appears that the changes in basement membrane gene expression are not due to proteinuria but rather are a specific and direct effect of PAN administration. Furthermore, our results also suggest that the altered basement membrane gene expression is not a result of nonspecific alterations in total mRNA expression (18). However, it is difficult to study the effects of PAN on the expression of basement membrane genes in the chronic form of renal disease that occurs after uninephrectomy and repeated injections of PAN. It has been shown that a single ip injection dose of PAN can affect the renal cells for at least 14 days (30). We have demonstrated previously the sequential induction of proto-oncogene and ECM component gene expression in the renal cortex after a unilateral nephrectomy (31). The removal of one kidney from a normal rat followed by a high protein intake leads to proteinuria and the development of nephrosclerosis, suggesting that increased RBF and hyperfiltration may contribute to the development of FGS. Thus, different mechanisms may be involved in the process of glomerular sclerosis.

In this study, we demonstrated an increased expression of the interstitial collagen genes in FGS. Type III collagen is expressed in many tissues, as is type I collagen, and these two collagens may be co-regulated. We have demonstrated previously that the mRNA expression of both collagens was increased and coregulated in lupus nephritis (32). The relative synthesis of these collagens appears to be under stringent biologic control except for disorders such as keloid formation (33). However, Nast et al. have demonstrated that α1(I) but not α1(III) mRNA levels are increased in the renal cortex in cyclosporine-induced renal scarring (34). They suggest that there are cyclosporine-related events that stimulate the specific increase in α1(I) mRNA levels in the renal cortex. In this context, it is possible that there are different mechanisms involved in the pathogenesis of cyclosporine-induced renal scarring and FGS. Although we have not identified the cell populations in the glomeruli that are the source of the elevated mRNA, the resident glomerular cells may be the main source. Mesangial cells in culture were reported to produce interstitial collagens in vitro (35–37), although we could not detect these collagens in normal glomeruli in vivo. We cannot exclude the possibility that the immunofluorescence technique was not sensitive enough to detect small amounts of interstitial collagens that may be present in normal glomeruli.

In this study, we could detect type I collagen mRNA in normal glomeruli. However, others have been unable to detect this transcript in normal animals (13,38). This discrepancy may result from the probes or the hybridization conditions used. It has been postulated that in small synechiae, the accumulation of ECM is produced by cells intrinsic to the glomerulus, whereas in large synechiae, the interstitial cells may be the source (8). There may be at least three mechanisms by which the intrinsic glomerular cells undergo a phenotypic change to up-regulate interstitial proteins: (1) control of the steady-state RNA levels, probably through a combination of transcription and degradation; (2) altered RNA processing; and (3) increased "translatability" of the interstitial collagen mRNA, which may also be influenced by factors specific for glomerular cells. Our Northern blot study showed no difference in the size of the type I collagen mRNA obtained between days 0 and 48, 60, and 80, indicating that there was no difference in the RNA processing of this transcript between normal glomeruli and those with FGS. It has been shown that type I collagen mRNA initiate protein synthesis less effectively than do many other messages (39). The control of translation may be regulated by various specific factors, including transforming growth factor-β (TGF-β), that cause an increase in the steady-state amounts of collagens I and III mRNA (40,41).

It is very likely that several soluble regulators are involved in the development of FGS. These include platelet-derived growth factor, TGF-β, interleukin-1, tumor necrosis factor, or endothelin. In cultured mesangial cells, we have demonstrated that peptide growth factors have the ability to regulate ECM production (42). In this model of FGS, the steady-state level of TGF-β mRNA was increased (unpublished data), suggesting that this growth factor may play a role in these processes. An increase in both TGF-β mRNA and renal collagen synthesis was reported recently in a rabbit model of anti–glomerular basement membrane disease (43). These mediators may induce the intrinsic glomerular cells to increase collagen synthesis via the activation or transduction of some cytoplasmic factors that have not been identified in glomerular cells. Some previous studies have also suggested that macrophages may participate in the initiation and development of the glomerular injury in FGS. The increased production of TGF-β or interleukin-1 by infiltrating macrophages appears to be one of the molecular mediators of the FGS (44,45). In addition, the infiltrating macrophages secrete sol-
uble regulators that stimulate ECM production by the resident glomerular cells. The cytokines and growth factors involved in the regulation of the molecular events that occur in this FGS model are likely to be numerous and involve complex interactions.

The chronic PAN model was used to study the molecular events associated with the development of glomerular sclerosis. The regulatory effects on ECM components described here shall help us to understand the pathogenesis of glomerular sclerosis.

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