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Ischemia Induces Early Expression of a New Transcription Factor (6A3-5) in Kidney Vascular Smooth Muscle Cells

Studies in Rat and Human Renal Pathology

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Acute renal failure, characterized by rapid decline in glomerular filtration rate, is a major cause of morbidity and mortality. During the evolution of renal diseases chronic ischemia develops. Indeed, acute or chronic renal failure may occur as a result of renal ischemia, which induces cells to dedifferentiate, proliferate, or become apoptotic. In this study, we have investigated the expression of a newly identified transcription factor, 6A3-5, under *in vitro* and *in vivo* conditions. Proliferating vascular smooth muscle were investigated in response to different mitogenic agents. The 6A3-5 expression was then studied in ischemic rat kidney, induced by renal pedicle clamping, followed, or not, by reperfusion. Subsequently human renal biopsies from early kidney grafts and chronic renal diseases were also investigated for 6A3-5 protein expression by immunohistochemistry. *In vitro* study shows an over-expression of 6A3-5 following 2 to 4 hours stimulation by serum or Angiotensin II, of rat proliferating aortic smooth muscle cell. Moreover, *in vivo* study shows that this new protein is over expressed in rat kidney submitted to 45 minutes ischemia. An anti-6A3-5 antibody shows the protein to be expressed in smooth muscle cells of the arterioles and intermediate size arteries, in mesangial cells and interstitial myofibroblasts. In human biopsies of early kidney grafts and renal disease, the same up-regulation of 6A3-5, as in acute ischemic situation, is observed. This 6A3-5 expression is intimately associated with α-smooth muscle cell actin expression in mesangial cells, arteriolar smooth muscle cells as well as interstitial myofibroblasts. Transcription factor 6A3-5 could potentially be a novel early vascular marker of acute and chronic renal ischemic stress implicated in tissue remodeling. *(Am J Pathol 2003, 163:2485–2494)*

Acute renal failure, characterized by rapid decline in glomerular filtration rate (GFR) is a major cause of morbidity and mortality.[1,2] After transplantation, decreased GFR due to ischemia-reperfusion may lead to renal dysfunction and affect the long term prognostic of the kidney.[3,4] Such ischemia-reperfusion alterations induce a cascade of events leading to cellular damage. It is also important to note that chronic ischemic events take place during the evolution of most chronic renal diseases.[5] Increasing evidence is available to show that inflammatory reactions[6] and oxygen-derived free radical species[7,8] are implicated in this type of injury. The sequence of these events induce leukocyte migration, enhanced expression of adhesion molecules,[9,10] and inflammatory mediators such as platelet-derived growth factor (PDGF)[11] and Angiotensin II (AngII).[12] These inflammatory mediators activate a complex genetic program, which may induce cells to dedifferentiate, proliferate, and/or possibly undergo apoptosis.[1–4] Such events are preceded by transcription of several immediate early-

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activated genes and transcription factors, which may play a role in the differentiation, proliferation, and tissue repair.[13] Despite new insights into the pathogenesis of acute renal failure, neither the incidence nor the mortality rate has declined in decades. Discovering new factors implicated in acute renal failure may lead to improved strategies for preventing and treating such serious disorder.

Gene 6A3-5 expression was identified by differential display to be over expressed in proliferating rat aortic vascular smooth muscle cells (SMC).[14] This new gene, in a similar way to c-fos, was observed to be significantly up-regulated soon after mitogenic stimulation of vascular SMC by PDGF-BB, phorbol 12-myristate 13-acetate or fetal calf serum. We recently cloned the full-length cDNA of this gene in rat and identified four conserved motifs (Garin et al, unpublished). These four motifs are: a DNA binding motif called AT-rich interaction domain (ARID),[15–16] a bipartite nuclear localization signal (NLS) and two osa homology domain (OHD) motifs.[15] This new gene (6A3-5) is a member of a new transcription factor family (ARID family) that has been recently described to be involved in control of gene expression during cell growth, cell cycle, and organism development.[15–16] The human homolog of 6A3-5 (known as hELD/Osa1), recently cloned in human fetal brain,[15] also bears these motifs. Interestingly, Brahma related gene-1 (BRG-1), the partner of hELD/Osa1 in SWI/SNF-A chromatin remodeling complex, has been shown to modulate the transcription of a subset of genes (such as cyclin A, c-fos,[16] and CD44[17]) involved in proliferation or cellular adhesion.

In this study we investigated the expression of 6A3-5 in rat aortic vascular SMC stimulated by serum or AngII. We then looked at the *in vivo* expression of 6A3-5 in rat kidney undergoing ischemia followed by reperfusion over different periods of time. Renal biopsies taken from transplanted patients, immediately after completion of graft surgery were then investigated for 6A3-5 expression. Finally the expression of this new transcription factor was studied in different type of human renal diseases. Results show that 6A3-5 is an early gene responder that could be a specific marker of SMC activation in renal ischemia-reperfusion injury as well as chronic ischemic events during the evolution of human renal diseases.

Materials and Methods

Cell Culture

Primary aortic vascular SMC were obtained from explants of medial thoracic aortas from 7- to 8-week-old male Sprague-Dawley rats and cultured as previously described.[18] Vascular SMC at passage 9–12 were used in this study. For stimulation experiments, vascular SMC at 80% of confluence were serum-starved for 48 hours and stimulated by serum 10% for 0, 2, 4, 8, 16, and 24 hours or by AngII (100 nmol/L) for 0, 1, 2, 4, or 24 hours. Experiments were repeated three times.

Nuclear Extracts

Vascular SMC, at confluence, stimulated or not with serum were washed with 3 ml of cold phosphate-buffered saline (PBS) and then homogenized in 3 ml of cold PBS. Nuclear pellets were isolated as previously described.[19]

Animal Surgical Procedure

Adult male Wistar rats (220 to 240 g), obtained from Iffa Credo (Lyon, France), had free access to standard rat chow and tap water and were housed in a 12-hour light/dark cycle. Surgical procedures and care strictly conformed to the guidelines of the French National Institute of Health and Medical Research (INSERM). Rats were anesthetized (2% halothane in oxygen) and underwent a midline laparotomy to expose the right kidney and free it from the surrounding tissue. An atraumatic vascular clamp was placed across the right renal pedicle to induce ischemia. Reperfusion of the right kidney was achieved by releasing the clamp. Animal group 1 ($N = 12$) underwent 5, 20, or 45 minutes of ischemia without reperfusion. This group was used to determine the effects of ischemia, in absence of reperfusion, on 6A3-5 expression. Animal group 2 ($N = 12$) underwent 45 minutes of ischemia followed by 2, 4, and 24 hours of reperfusion. Group 2 was used to determine the additional impact of reperfusion on 6A3-5 expression. Animal group 3 ($N = 12$) underwent 5, 20, or 45 minutes of ischemia followed by 24 hours of reperfusion. Group 3 showed the impact of ischemia time on 6A3-5 expression following 24 hours of reperfusion. Left contralateral kidneys were excised at the end of ischemic operations performed on the right ones. Sham-operated kidneys were excised from animals ($N = 3$) that have been operated but not submitted to renal pedicle clamping. Cortical and medullar tissue were separated for both left and right kidneys. Isolated tissues were divided in three samples that were stored for 24 hours in RNAlater (Ambion, Austin, TX, USA), snap-frozen into liquid nitrogen, and fixed in formalin.

Human Renal Biopsies

Thirty-two human kidney biopsies were provided by the Pathology Department at the Edouard Herriot Hospital. Donor kidney biopsies were harvested immediately after graft surgery, time-zero biopsies, following a period of ischemia ranging between 13 to 28 hours with a mean of 13.5 hours. Frozen sections, from these biopsies, were labeled with monoclonal antibodies directed against 6A3-5 protein and α-SMA.

Northern Blot

Frozen tissue samples were ground to a powder in a mortar followed by homogenization at 0°C in 1 ml Trizol (Invitrogen, Carlsbad, CA, USA). Total RNA was isolated according to the Trizol procedure. RNA samples (20 μg) were separated on a formaldehyde-MOPS (3-[N-Morpholino]propanesulfonic acid)-agarose 1.2% gel, transferred

onto nylon membranes (Hybond-N$^+$, Amersham Biosciences, Arlington, IL, USA) and cross-linked by UV radiation. Probes, labeled by random priming method (High Prime; Boehringer Mannheim, Mannheim, Germany), were purified using G-sephadex columns. Prehybridization and hybridization were done in ExpressHyb solution at 68°C (BD Biosciences Clontech, Palo Alto, CA, USA). Membranes were then exposed to X-ray films (Biomax MS, Kodak, Rochester, NY, USA). The abundance of 6A3-5 mRNA was normalized with respect to 18S rRNA and the ratio are expressed in arbitrary units (au).

Western Blot

Kidney tissues were disrupted in a mortar then homogenized with a Polytron at 0°C in 50 mmol/L Tris-buffered saline (pH 7.6) containing 1% aprotinin, 2 mmol/L ϵ-aminocaproic acid, and 0.5 mmol/L phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 2000 $\times$ *g* for 5 minutes. The supernatant was then centrifuged at 2500 $\times$ *g* for 5 minutes and retained. Cultured cells were lysed in Triton lysis buffer (25 mmol/L Tris, pH 7.6, 150 mmol/L NaCl, 1% Triton-X 100, and 10 μg/μl each of aprotinin, leupeptine, ethylene diaminetetraacetic acid, and phenylmethylsulfonyl fluoride). Cell lysate was then incubated for 40 minutes at 4°C under agitation, centrifuged at 1200 $\times$ *g* for 5 minutes and the supernatant was stored at $-$20°C. Thirty micrograms of proteins extracts, denatured in Laemmli buffer and separated on sodium dodecyl sulfate (SDS) $-$7% polyacrylamide gels, were electrotransferred to a nitrocellulose membrane. The membrane, blocked for 4 hours at 37°C with Tris-buffered saline (TBS), 0.05% Tween20, 3% gelatin, was incubated overnight at 4°C with a rabbit anti-6A3-5 polyclonal antibody (2 μg/ml)[14] or with an anti-focal adhesion kinase (FAK) antibody (2 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). A swine anti-rabbit antibody, conjugated to horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA, USA), was then used with a chemiluminescent technique to reveal the labeled bands (ECL kit; Amersham Biosciences). Coomassie Blue was used to normalize the quantities of proteins loading onto the SDS-polyacrylamide gel electrophoresis (PAGE).

Immunohistochemistry

Immunohistochemistry was performed on frozen tissue sections (3 μm) fixed in acetone. Briefly, slide sections were microwaved for 12 minutes in a citrate buffer. Nonspecific sites were blocked and then incubated with 6H3,[15] an anti-6A3-5 monoclonal antibody, or an anti-α-smooth muscle actin (α-SMA) monoclonal antibody. The labeled slides were left overnight at room temperature in a humid chamber. Antibody binding was revealed using anti-mouse secondary antibodies conjugated to horseradish peroxidase and then revealed by 3-amino-9-ethylcarbazole (AEC; Dako, Glostrup, Denmark). Sections were counterstained using Harris hematoxylin solution. Negative controls were performed using a non-immune murine antibody of the same class.

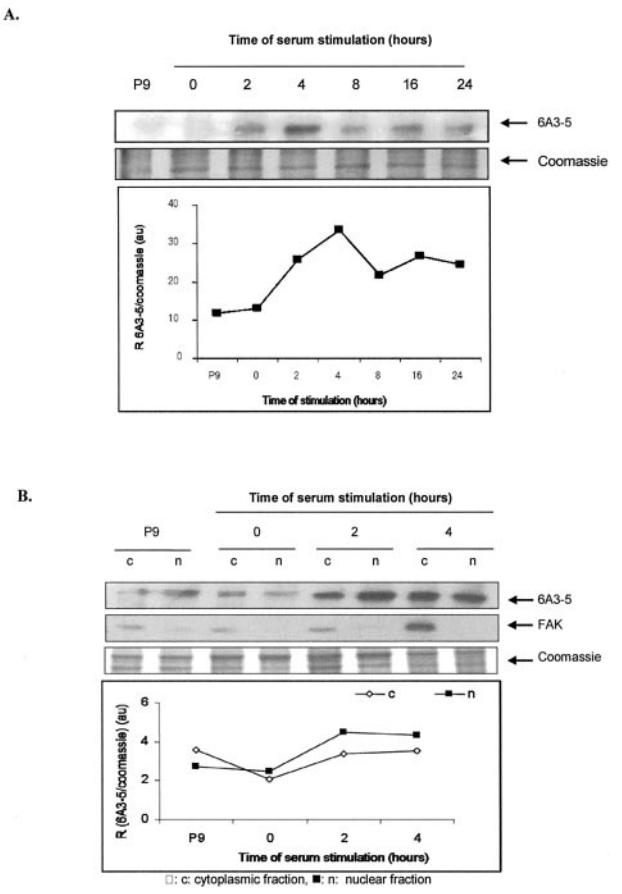

Figure 1. Response to serum. Rat aortic vascular SMC at passage 9 (P9) were serum-starved for 48 hours and then stimulated by 10% serum for 0, 2, 4, 8, 16, and 24 hours. **A**: Total protein lysate was resolved by SDS-PAGE and immunoblotted with anti-6A3-5 rabbit polyclonal antibody. Expression levels of 6A3-5 protein were estimated by Quantity One (Bio-Rad) and normalized by comparison with Coomassie Blue staining. **B:** Cellular fractions [cytoplasmic (c, ◇) and nuclear (n, ■)] were separated and compared for 6A3-5 expression following vascular SMC stimulation by serum for 0, 2, or 4 hours. Data represent mean of three individual experiments. Blot was reprobed by anti-FAK antibody, as a control to ascertain the purity of nuclear extract.

Statistical Analysis

All data are shown as mean $\pm$ SEM of measurements. The significance of differences of the data were determined with analysis by Student's *t*-test. $P < 0.05$ was considered statistically significant.

Results

6A3-5 Expression in Stimulated Vascular SMC under in Vitro *Conditions*

Protein expression for 6A3-5 was observed to peak by three-fold increments, in comparison to non-stimulated vascular SMC, following 2 to 4 hours of serum stimulation. Interestingly, 6A3-5 is overexpressed in nuclear, in comparison to cytoplasmic, fractions of serum-stimulated rat aortic vascular SMC (Figure 1b). Moreover, a 2-hour stimulation by AngII (100 nmol/L) of serum-starved vascular SMC, induced a rapid 1.5-fold increase of 6A3-5 mRNA in comparison to basal level (Figure 2a). Western blot

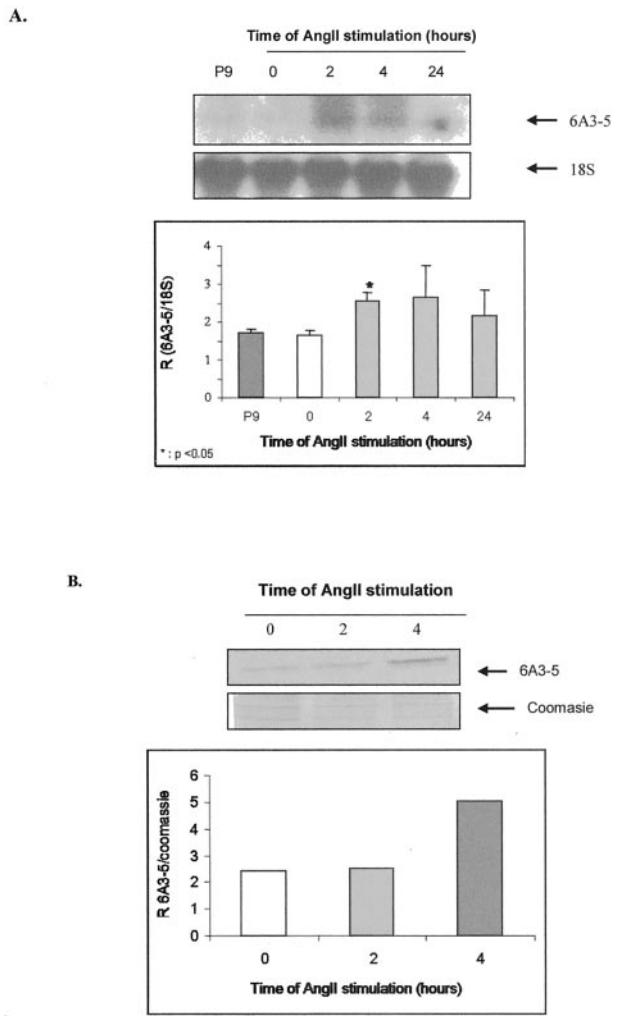

Figure 2. Response to AngII. Rat aortic vascular SMC at passage 9 (P9) were serum-starved for 48 hours and then stimulated by AngII (100 nmol/L) for 0, 1, 2, 4, and 24 hours. **A:** 6A3-5 mRNA was analyzed by Northern blot and normalized by 18S rRNA level. Northern blots were quantified by Quantity One tool (Bio-Rad). Data, showing representative results of three independent experiments, are presented as means ± SEM. * $P < 0.05$ *versus* non-stimulated control cell. **B:** 6A3-5 protein levels were investigated by Western blot. Expression level of 6A3-5 protein was estimated by Quantity One (Bio-Rad) and normalized by comparison with Coomassie Blue staining.

showed increased 6A3-5 protein levels, after 4 hours of AngII stimulation (Figure 2b).

6A3-5 Expression in Ischemic Kidney with No Reperfusion

Rats were subjected to 0 (sham-operated kidneys), 5, 20, or 45 minutes of unilateral renal ischemia without reperfusion. In sham-operated kidneys, basal 6A3-5 mRNA is very low and levels are similar in cortical and medullar tissue. A significant up-regulation of 6A3-5 gene expression is observed after 45 minutes of ischemia, compared to sham-operated kidneys. Such enhanced expression is more pronounced in medulla (3.5-fold) compared to cortex (twofold) (Figure 3a). Western

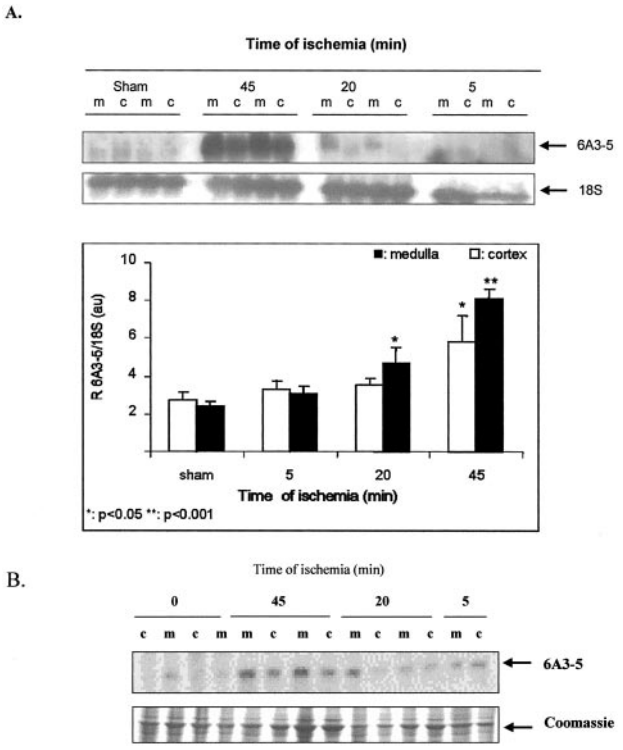

Figure 3. Kidney tissue subjected to ischemia for different periods of time without reperfusion. Analysis of the 6A3-5 expression after different periods of ischemia: 0 (sham-operated kidneys), 5, 20, or 45 minutes without reperfusion. Medulla (m, ■) and cortex (c, □) were compared at each point. **A:** Northern blots were quantified by Quantity One (Bio-Rad). Data are presented as means ± SEM. * $P < 0.05$ *versus* sham-operated kidneys. ** $P < 0.001$ *versus* sham-operated kidneys. **B:** Western blot analysis of the 6A3-5 expression. Expression level of 6A3-5 protein was estimated by Quantity One and normalized by comparison to Coomassie Blue staining.

blots showed that 6A3-5 protein levels correlated with mRNA levels (Figure 3b).

6A3-5 Expression in Ischemic Kidney (45 Minutes) Followed by Reperfusion (0, 2, 4, and 24 Hours)

Following clamping of the pedicle for 45 minutes we investigated postischemic 6A3-5 gene expression levels in reperfused kidney medulla and cortex at 0, 2, 4, and 24 hours. Gene 6A3-5 expression level progressively dropped during the reperfusion period (Figure 4a). However, after 24 hours of reperfusion, medullar and cortical 6A3-5 expression remained significantly (1.5-fold) higher than in sham-operated kidneys. Protein expression levels of 6A3-5, in medulla or cortex, followed a similar pattern to those observed in Northern blots (Figure 4b).

6A3-5 Expression in Ischemic Kidney (0, 5, 20, and 45 Minutes) Followed by Reperfusion (24 Hours)

Following clamping of the pedicle for 0 (sham-operated kidneys), 5, 20, and 45 minutes we investigated postischemic 6A3-5 gene expression levels after 24 hours of

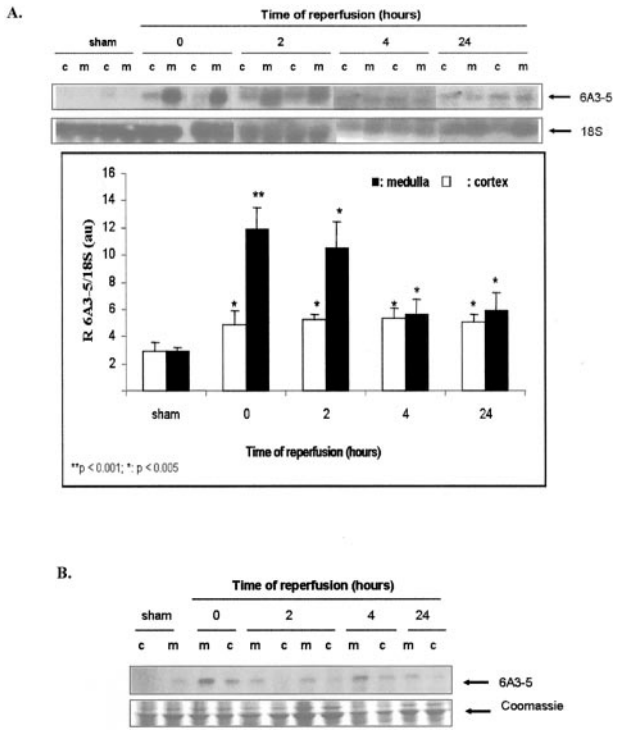

Figure 4. Analysis of the 6A3-5 expression after 45 minutes of ischemia followed for different periods of time of reperfusion (0, 2, 4, 24 hours) rat kidney. Medulla (m, ■) and cortex (c, □) were compared at each point. **A:** Northern blots were quantified by densitometry (Bio-Rad). Data are presented as means ± SEM. * $P < 0.05$ *versus* sham-operated kidneys. **B:** Western blot analysis of the 6A3-5 expression. Coomassie Blue was used to normalize the quantity of proteins resolved by SDS-PAGE.

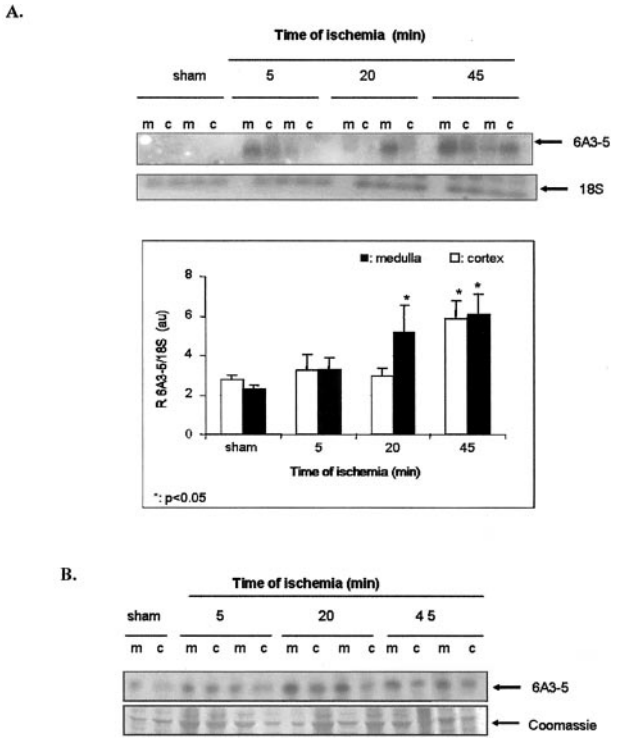

Figure 5. Analysis of the 6A3-5 expression after different periods of ischemia: 0 (sham-operated kidneys), 5, 20, or 45 minutes and reperfusion for 24 hours. Medulla (m, ■) and cortex (c, □) were compared at each point. **A:**. Data are presented as means ± SEM. * $P < 0.05$ *versus* sham-operated kidneys. **B:** Western blot analysis of the 6A3-5 expression. Coomassie Blue was used to normalize the quantity of proteins loading on the SDS-PAGE.

reperfusion of kidney. Five minutes of ischemia followed by reperfusion did not induce any significant changes, compared to sham-operated kidney, in 6A3-5 gene expression levels. In contrast, 20 and 45 minutes of ischemia induced a significant change in 6A3-5 gene expression levels, particularly in the medulla, which did not return to basal value after reperfusion (Figure 5a). Finally, 45 minutes of ischemia, as previously indicated, induced the most significant changes in medulla and cortex levels of 6A3-5 gene expression. Protein expression levels followed quite closely those observed on Northern blots (Figure 5b).

6A3-5 Expression in Contralateral Kidney

An increase of 6A3-5 gene expression in contralateral tissue was observed following 45 minutes of ischemia (on the right kidney, Figure 6a) compared to sham-operated kidneys. One should note that the 6A3-5 mRNA and protein levels were lower in contralateral compared to ischemic kidney (Figure 6, a and b).

Localization of the 6A3-5 Protein in Rat Kidney

Immunohistochemical staining, following 45 minutes of ischemia, with or without reperfusion, showed the expression of 6A3-5 protein in mesangial cells and SMC of arterioles and medium-sized arteries in renal cortex. In the medulla, the 6A3-5 protein is also expressed in the SMC of arterioles so called the vasa recta of the vascular bundles (Figure 7, A and B). Cellular staining shows 6A3-5 to be present in the cytoplasm, predominantly around the nucleus. α-SMA labeling shows the same pattern of expression as 6A3-5, albeit more weakly for mesangial cells. Sham-operated kidneys and antibody-negative control show no 6A3-5 labeling (Figure 7, D and E). In contralateral kidney, in contrast to ischemic kidneys, 6A3-5 protein and α-SMA are rarely labeled in mesangial cells, but present in arterioles and medium-sized arteries (Figures 7, F and G).

Localization of 6A3-5 Protein in Human Kidney Graft: Six Cases of Human Kidney Graft Studied at Time-Zero

The same pattern of expression of the 6A3-5 protein is observed in kidney graft as in rat ischemic kidney specimens. Immunohistochemical staining of 6A3-5 protein is observed in mesangial cells, vascular SMC of arterioles and medium-sized arteries (Figure 8, A to C). In addition, interstitial myofibroblasts are also focally labeled in certain cases (Figure 8A). Results obtained from the various patients clearly indicate that the intensity and number of vascular SMC, mesangial cells, and myofibroblasts labeled with a 6A3-5 antibody vary according to cases.

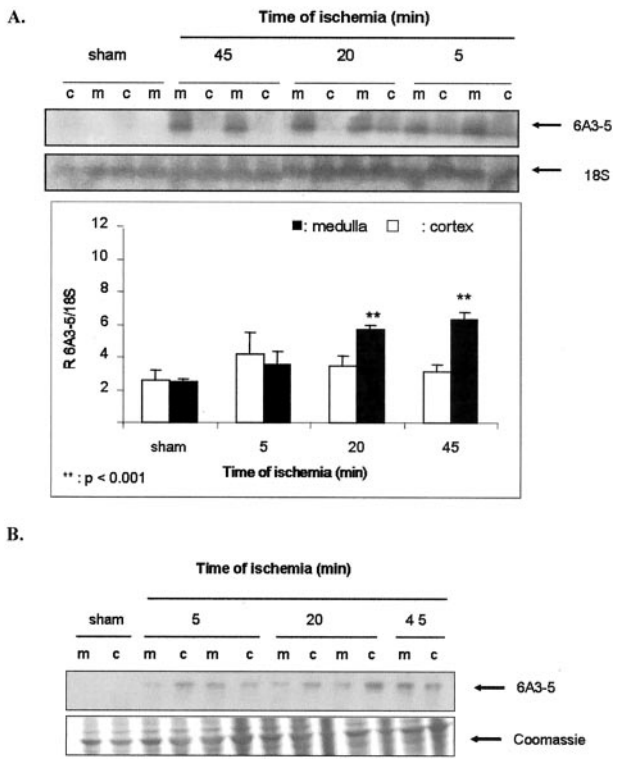

Figure 6. Analysis of the 6A3-5 expression after different periods of ischemia: 0 (sham-operated kidney), 5, 20, or 45 minutes in contralateral kidney. Medulla (m, ■) and cortex (c, □) were compared at each point. **A:** Data are presented as means ± SEM. * $P < 0.05$ *versus* sham-operated kidneys. ** $P < 0.01$ *versus* sham-operated kidneys. **B:** Western blot analysis of the 6A3-5 expression. Coomassie Blue was used to normalize the quantity of proteins loading on the SDS-PAGE.

Localization of the 6A3-5 Protein in Human Renal Disease

It is again of considerable interest to note that the same pattern of expression of the 6A3-5 protein that is observed in rat ischemic kidney specimens, is observed in human renal disease. A total of 26 renal biopsies were studied that included minimal change disease (MCD) (six cases), IgA nephropathy (seven cases), C3 mesangial nephropathy (three cases), vasculitis (two cases), and acute graft kidney rejection (eight cases). In MCD, a diffuse human renal disease, labeling of the 6A3-5 protein is observed diffusely in mesangial cells, and focally in arteriolar SMC and interstitial myofibroblasts (Figure 8D). Labeling of SMC with α actin shows identical pattern of staining as the 6A3-5 protein but with enhanced intensity (Figure 8E). In IgA nephropathy, a focal and segmental human renal disease, labeling of the 6A3-5 protein is observed focally in mesangial cells, arteriolar SMC, and interstitial myofibroblasts depending on the severity and length of evolution of the disease (Figure 8, G to I). Indeed, mesangial cells are intensely labeled in patient A (76-year-old, 4 g/day proteinuria, and 260 μmol/L creatininemia) (Figure 8G), moderately labeled in patient B (39-year-old, 1.8 g/day proteinuria, and 100 μmol/L creatininemia) (Figure 8H) and very weakly in patient C (36-year-old, 0.8 g/day proteinuria, and 64 μmol/L creatininemia) (Figure 8I). In C3 mesangial nephropathy labeling is very similar to IgA nephropathy (results not shown). In vasculitis, labeling of the 6A3-5 protein is mostly limited to arterioles (Figure 8J). In acute graft kidney rejection, labeling of the 6A3-5 protein is observed focally in mesangial cells, arteriolar SMC, and interstitial myofibroblasts depending on the severity/chronicity of the graft rejection. Whenever, the medulla is present in biopsy specimens, the expression of the 6A3-5 protein in arteriolar SMC and in interstitial myofibroblasts is shown as well (Figure 8L). In summary, in renal diseases, the 6A3-5 protein is expressed by cells of the α-actin-expressing cell family, namely the SMC of arteriolar/medium-sized arteries, the mesangial cells, and the interstitial myofibroblasts.

Discussion

This study investigates for the first time a new transcription factor, 6A3-5, and shows that it is a specific early marker of SMC activation in renal ischemia-reperfusion injury, as well as chronic ischemic events during the evolution of renal diseases. Several lines of evidence back the above statement: 1) *in vitro* studies show that 6A3-5 mRNA and protein expression are significantly increased in vascular SMC following serum or AngII stimulation in comparison to non-stimulated cells; 2) significant 6A3-5 overexpression is observed in ischemic rat kidneys compared to sham-operated kidneys; 3) 6A3-5 up-regulation is sustained during reperfusion; 4) blood pressure variation on rat contralateral kidney induces 6A3-5 expression, albeit with reduced intensity; 5) human graft kidney biopsies, immediately after transplantation, show a significant expression of 6A3-5; 6) expression and localization of 6A3-5 in human renal diseases is related to the type and the severity of the disease; 7) expression of 6A3-5 in both human and rat tissues shows a similar labeling pattern as α-SMA.

Bioinformatic analysis, following the cloning of 6A3-5, shows the presence of four conserved motifs (1 ARID, 2 OHD, 1 NLS) (Garin et al, unpublished). Proteins bearing an ARID motif are present in a variety of eukaryotic organisms and have been shown to participate in several biologically significant processes including embryonic development, cell lineage gene regulation, differentiation, and cell cycle control.[15–16] The OHD motifs define a new family of transcription factors known as "OSA Family". Indeed, OSA proteins are member of the chromatin-remodeling complex SWI/SNF. The OSA proteins participate in the targeting of transcription factors to specific promoters for selectively promoting or repressing the expression of target genes.[15–17] Chromatin remodeling complexes such as the SWI/SNF complex make DNA accessible to transcription factors by disrupting nucleosomes. It's interesting to note that BRG-1, a partner of the human homolog of 6A3-5 (known as hELD/osa1), inhibits the transcription of genes, such as cyclin A, c-fos,[16] and

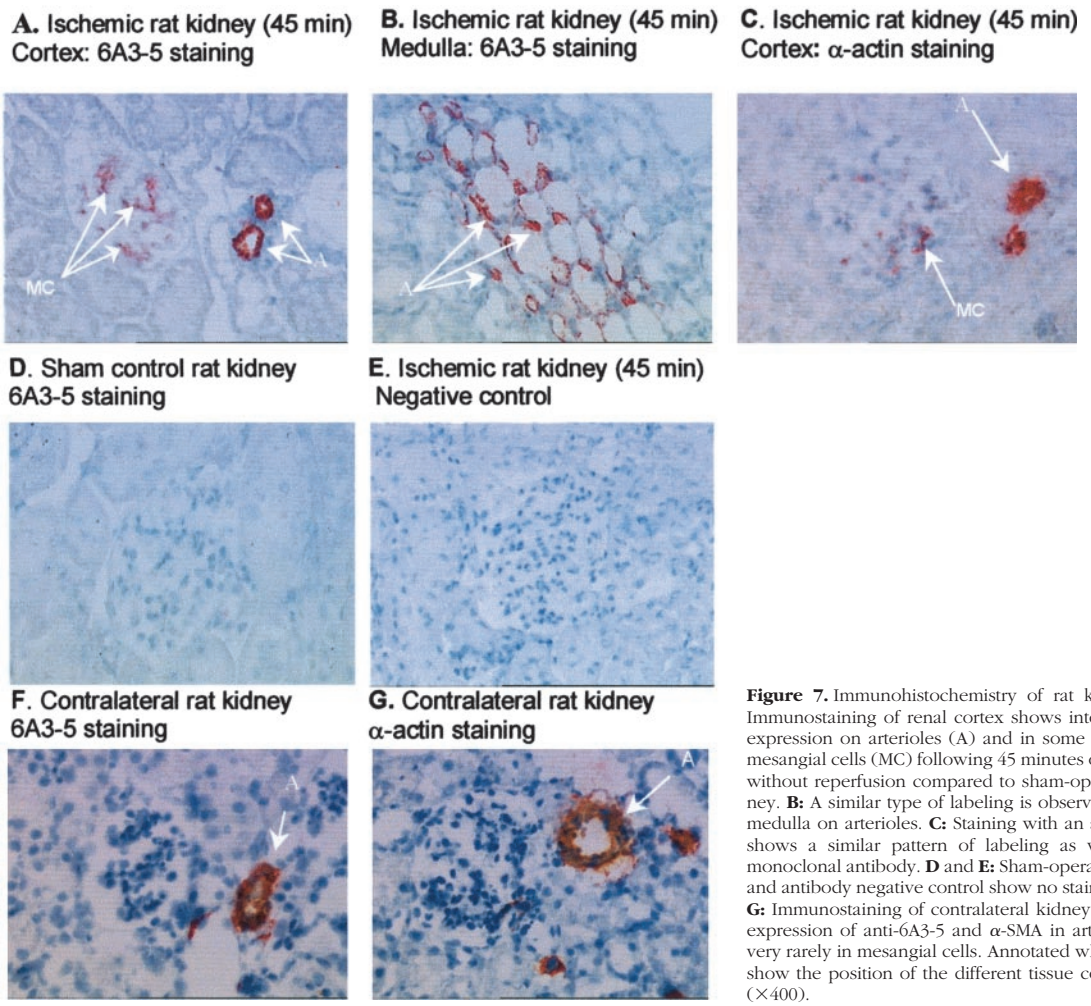

Figure 7. Immunohistochemistry of rat kidneys. **A:** Immunostaining of renal cortex shows intense 6A3-5 expression on arterioles (A) and in some glomerular mesangial cells (MC) following 45 minutes of ischemia without reperfusion compared to sham-operated kidney. **B:** A similar type of labeling is observed in renal medulla on arterioles. **C:** Staining with an anti-α-SMA shows a similar pattern of labeling as with 6A3-5 monoclonal antibody. **D** and **E:** Sham-operated kidney and antibody negative control show no staining. **F** and **G:** Immunostaining of contralateral kidney shows the expression of anti-6A3-5 and α-SMA in arterioles but very rarely in mesangial cells. Annotated white arrows show the position of the different tissue components (×400).

CD44,[17] involved in proliferation or cellular adhesion. Moreover, in cultured mammalian cells, hELD/osa1 stimulates glucocorticoid receptor-dependent transcriptional activation.[20] Interestingly, modulation of glucocorticoid receptor activity, by a glucocorticoid ligand such as Dexamethasone, down-regulates ICAM-1 expression. Such down-regulation of ICAM-1 reduces neutrophil infiltration in rat kidneys following renal ischemia/reperfusion.[21] It is conceivable that 6A3-5 could modulate glucocorticoid transcription and affect ischemia/reperfusion injury observed in kidneys. The fourth functional motif on 6A3-5, NLS, suggests a nuclear localization for this transcription factor. In fact, Western blots show the presence of 6A3-5 in the nucleus but also in the cytoplasm. This is confirmed by immunofluorescence studies (Garin et al, unpublished).

Ischemia induces a very significant expression of 6A3-5 protein in arterioles and mesangial cells. A similar type of 6A3-5 labeling in human kidney grafts is also observed immediately after transplantation. Vascular ischemia induces hypoxia and the release of certain agents, such as tumor necrosis factor-α (TNF-α),[22] endothelin-1 (ET-1),[23] vascular endothelial growth factor,[24] and AngII,[25] which influence gene expression in vascular cells. TNF-α or ET-1 stimulate vascular SMC proliferation,[26,27] differentiation, and enhanced expression of genes such as c-fos[27] and Ets-1.[28] In similar ways to hypoxic inducible factor-1α (HIF1α), 6A3-5 is overexpressed during AngII vascular SMC stimulation and during renal ischemia.[29,30] It is conceivable that the increase of 6A3-5 expression in contralateral kidney arterioles is due to increased blood pressure and/or Angiotensin II released by the ischemic kidneys.[31] Our *in vitro* studies on vascular SMC show that 6A3-5 expression is significantly increased following AngII stimulation. In addition, one should note that inflammation in contralateral kidneys, observed in this model, can affect 6A3-5 expression.[32] 6A3-5 remains significantly higher in ischemic kidneys (45 minutes), during reperfusion (2 to 24 hours), compared to sham-operated kidneys. This is in contrast to c-fos, for which no expression is detected after 24 hours of reperfusion. During renal ischemia reperfusion, mesangial cells, and myofibroblasts are submitted to various mitogenic stimuli (eg, cytokines, growth factors). Indeed, one should note that interleukin-4 co-stimulates the PDGF-BB and fibroblast growth factor-mediated proliferation of mesangial cells.[33] The presence in arterioles and mesangial cells of high levels of 6A3-5 expression, after 24 hours of reperfusion, may indicate that this gene is also involved in prolonged tissue injury.

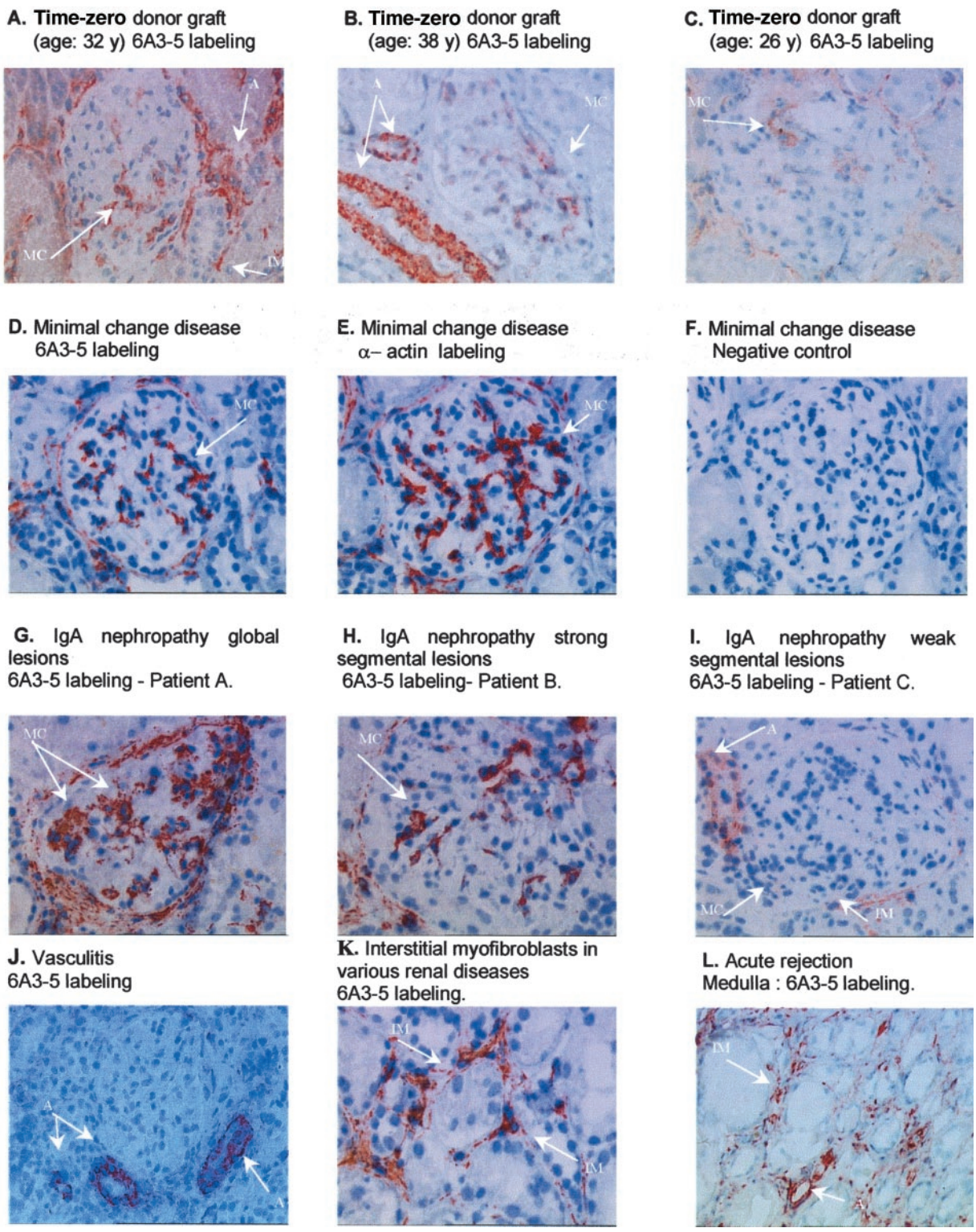

Figure 8. Immunohistochemistry of human kidney graft and renal diseases. **A** to **C:** Immunostaining of grafts kidneys (time-zero biopsies) from donors of various ages (32, 38, and 26 years old, respectively) show various intensity of 6A3-5 labeling of mesangial cells (MC), arteriolar SMC (A), and interstitial myofibroblasts (IM). **D** and **E:** Labeling of MCD biopsies with 6A3-5 and an anti-α-SMA. Both antibodies, albeit α-SMA at higher intensity, label mesangial cells diffusely. **F:** Antibody-negative control shows no staining. **G** to **I:** Labeling of IgA nephropathy with 6A3-5 shows that mesangial cells are intensely labeled in patient A (76-year-old, 4 g/day proteinuria, and 260 μmol/L creatininemia) (**G**) compared to moderate labeling in patient B (39-year-old, 1.8 g/day proteinuria, and 100 μmol/L creatininemia) (**H**) and very weak in patient C (36-year-old, 0.8 g/day proteinuria, and 64 μmol/L creatininemia) (**I**). **J:** Labeling of vasculitis shows intense expression of 6A3-5 on arteriolar SMC but not mesangial cells. **K:** Labeling of interstitial myofibroblasts, in different renal diseases, with 6A3-5 antibody. **L:** Labeling of renal medulla in acute graft kidney rejection shows 6A3-5 expression on arterioles and interstitial myofibroblasts. Annotated white arrows show the position of the different tissue components (×400).

In human renal diseases such as MCD and IgA nephropathy, the same up-regulation of 6A3-5 as in acute ischemic situation, is observed. This suggests that under chronic insults, such as ischemia, hypertension, high proteinuria, a similar mechanism of mitogenic stimuli may be present. In diffuse diseases such as MCD the glomerular expression of 6A3-5 is homogeneous. In contrast, in IgA nephropathy glomerular lesions tend to be focal and segmental and 6A3-5 expression is likewise present on sites of lesions. Moreover, with increasing clinical presentation (high proteinuria and severe chronic renal failure) the 6A3-5 expression is enhanced. This new transcription factor (6A3-5) is also focally observed in interstitial myofibroblasts with an enhanced expression related to the severity of inflammatory interstitial lesions. One should note that 6A3-5 expression is intimately associated with α-SMA expression in glomerular lesions as well as in interstitial myofibroblasts. Interstitial expression of α-SMA has been shown as an early marker of chronic renal allograft dysfunction[34] and of severe evolution in membranous human renal disease.[35] This would suggest that 6A3-5 may take part in key events implicated in tissue remodeling and fibrogenesis. In vasculitis, the overexpression of the 6A3-5 protein by vascular SMC could conceivably be a sign of arteriolar remodeling.

Transcription factor 6A3-5 could potentially be a novel early vascular marker of acute and chronic renal ischemic stress and implicated in tissue remodeling.

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