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ENHANCED EXPRESSION OF SELENOCYSTEINE LYASE IN ACUTE GLOMERULONEPHRITIS AND ITS REGULATION BY AP-1

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Abstract: Acute glomerulonephritis can lead to chronic glomerulonephritis or resolve without permanent damage to the kidneys. Differential gene expression was studied in a model of acute and chronic glomerulonephritis to identify factors influencing the course of glomerulonephritis towards healing or chronification. One of the differentially expressed genes was identified as SCL, encoding selenocysteine lyase. Its expression was higher in acute glomerulonephritis and lower in chronic glomerulonephritis. The transcriptional regulation of SCL was studied *in vitro* in rat mesangial cells (MC). SCL RNA expression increased eight-fold compared to the baseline after stimulation with interleukin-1β (IL-1β) for three hours. Luciferase expression and gel shift experiments revealed an enhancer element between bp -152 and -298 of the SCL 5'-regulatory region, with protein binding to an AP-1 binding site that may be involved in the regulation of SCL-RNA *in vivo* in an endogenous feedback mechanism to the inflammatory reaction in acute glomerulonephritis, leading to resolution of this disease.

Key words: Selenocysteine lyase, Glomerulonephritis, Differential display, Inflammation, Mesangial cells, AP-1, Transcription

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Abbreviations used: ATS – anti-rat thymocyte serum; ESRD – end-stage renal disease; IL-1β – interleukin-1β; LUC – luciferase; MC – mesangial cells; PAS – periodic acid-Schiff; PCR – polymerase chain reaction; SCL – selenocysteine lyase

INTRODUCTION

Chronic glomerulonephritis is one of the major causes of end-stage renal disease (ESRD). The clinical course of glomerulonephritis can vary from mild disease with stable renal function to severe disease with progression to ESRD. The mechanisms responsible for disease resolution or chronification are largely unknown. Different animal models of glomerulonephritis have been created to investigate the molecular mechanisms involved in glomerulonephritis. One of the best-characterized models is the anti-Thy-1 model in rats [1]. Intravenous injection of a rabbit antibody directed against the Thy-1 epitope on mesangial cells leads to complement activation and mesangiolysis, followed by an influx of monocytes into the glomerulus and mesangial proliferation. These changes are similar to those seen in human glomerulonephritis. Acute glomerulonephritis, which resolves after several weeks, can be induced by a single intravenous injection of an anti-Thy-1 antiserum following uninephrectomy. Chronic glomerulonephritis with scarification and progression to ESRD develops after a second injection of the anti-Thy-1 antiserum [2]. There are several described factors which play a role or reveal different patterns of expression in acute and chronic nephritis [3-7].

In our search for other factors differentially expressed in acute and chronic glomerulonephritis, we identified selenocysteine lyase. This enzyme was first described in 1982 [8]; it is predominantly expressed in the liver and kidneys, whereas its activity has not been found in the serum. Selenocysteine lyase specifically decomposes cytotoxic selenocysteine into alanine and elemental selenium, which is reused for selenoprotein synthesis. Selenocysteine is produced during the degradation of selenoproteins like thioredoxin reductase [9] and glutathione peroxidase [10], which have antioxidative properties. Since oxidative stress and monocyte activation play a role in glomerulonephritis [5, 11], a study of the expression and regulation of selenocysteine lyase promised to shed some light on the pathophysiological processes involved in this disease. To the best of our knowledge, no other data on the expression and function of selenocysteine lyase in kidney diseases is currently available.

MATERIALS AND METHODS

Induction of nephritis and experimental protocol

Thirty male Sprague-Dawley rats (average body weight 100 g) were divided into three equal-sized groups. Acute immune-mediated mesangial injury was induced in the rats of one group after uninephrectomy by a single intravenous injection of 0.9 ml of a rabbit anti-rat thymocyte serum (ATS). Chronic immune-mediated mesangial injury was induced in the rats of the second group after uninephrectomy by two intravenous ATS-injections one week apart. The ATS had been generated in New Zealand rabbits by repeated immunization with $2x10^8$ thymocytes from Lewis rats combined with Hunter's Titer Max, as described earlier [12]. The control rats (the third group) were

uninephrectomized. The kidneys were removed one week after the last serum injection. Tissue samples were taken, fixed in formalin, embedded in paraffin, and stained with periodic acid-Schiff (PAS). Glomeruli were isolated from the remaining, non-fixed tissue using a fractional sieving technique [13, 14].

RNA isolation and differential display

Total glomerular RNA was isolated via the guanidinium isothiocyanate technique [15]. The RNAimage kit (GeneHunter, Nashville, Tennessee) was used for differential display. Reverse transcription and subsequent PCR using degenerated anchored oligo(dT)primer was performed according to the manufacturer's recommendations. The PCR reactions were performed with [α-^{35}S]dATP (1200 Ci/mmole; Amersham, Braunschweig, Germany) for 40 cycles with an annealing temperature of 40ºC for 2 minutes, an extension step at 72ºC for 30 seconds, and a denaturation step at 94ºC for 30 seconds. The PCR products were separated on a 6% denaturing polyacrylamide gel. The DNA bands of interest were cut out of the vacuum dried gel and boiled, and DNA was precipitated. The DNA was reamplified and subcloned into the pGEM-T Easy vector (Promega, Madison, Wisconsin). Inserts were sequenced on an ABI 377 sequencer and compared with available sequences from the NCBI data base for homologies to identify differentially expressed genes.

Northern blot

Twenty micrograms of total glomerular RNA were electrophoresed on a 1.2% denaturing agarose gel containing 2.2 M formaldehyde, and vacuum blotted onto a nylon membrane (Hybond-N, Amersham). A 320-bp SCL cDNA insert corresponding to the band in the differential display was used as a probe. The cDNA insert was labelled with [α-^{32}P]dCTP using hexamer primers. As a control for small loading and transfer variabilities, a fragment of 18 S rRNA was used. Hybridization was performed for 18 hours at 42ºC. The membrane was washed twice in 2 x SSC/0.5% SDS for 30 minutes at room temperature, and subsequently in 0.4 x SSC/0.5% SDS at 65ºC for 30 minutes. Autoradiography was performed for 24 to 72 hours at -80ºC with intensifying screens. Exposed films were scanned with a Flour-S multi-imager (Bio-Rad, Hercules, California), and the data was analyzed with MultiAnalyst (Bio-Rad). The ratio between the intensities of the SCL and 18 S bands was calculated, and the control group was arbitrarily assigned a relative value of 1.0.

SCL RNA expression in mesangial cells after stimulation with IL-1β

Rat glomerular mesangial cells were maintained in RPMI medium supplemented with 1% non-essential amino acids, 2 mM glutamine, 100 µg/ml streptomycin, 100 U/ml penicillin and 10 % fetal bovine serum. Before stimulation, the cells were incubated for 24 hours without fetal bovine serum. Afterwards, the cells were stimulated with IL-1β (1 ng/ml) for 1, 3, 16, and 24 hours. Non-stimulated cells were used as a control. RNA was isolated using the Nucleo Spin II RNA-Isolation Kit (Machery & Nagel, Düren, Germany).

Real-time PCR

RNA was reversely transcribed into cDNA and Real-time PCR was performed with 6.25 µl SYBr Green (Invitrogen, Karlsruhe, Germany), 1.25 µl of an SCL-specific forward primer ($^{5'}$GAGGTGGACAAGAGCGGAATT$^{3'}$), 1.25 µl of an SCL-specific reverse primer ($^{5'}$GCAATCATGGGCGTGTTCT$^{3'}$), and 1.5 µl cDNA in a final volume of 10 µl. GAPDH was used as a housekeeping gene, and the results were normalized to the control level.

Western blot analysis of SCL

Cells were centrifuged in 1 x PBS. Pellets were resuspended in 50 µl of Laemmli buffer 1 (33% 0.5 mM Tris-HCl, pH 6.8, 66% SDS 10%). The samples were boiled for 10 minutes and centrifuged afterwards. Protein concentrations were determined with a Protein DC-Assay (Bio-Rad, Munich, Germany). To equal amounts of protein (25 µg), ½ vol% loading buffer (187.5 mM TrisHCl, pH 6.8, 6% SDS, 30% Glycerol, 150 mM DTT, 0.3% bromphenolblue) was added. Samples were electrophoresed on a 12% Nu Page gel (Invitrogen, Karlsruhe, Germany). Proteins were transferred electrophoretically to a nitrocellulose membrane (Hybond P, Amersham, Freiburg, Germany) at 40 V for 1 hour using a Bio-Rad Mini Trans-Blot Apparatus. The membrane was blocked with 7.5% non-fat dry milk in a washing buffer (1 x PBS, 0.1 % Tween) overnight at room temperature, and then incubated for 1 hour at room temperature with a monoclonal anti-mouse SCL antibody (Transduction Laboratories, Bruxelles, Belgium) at a concentration of 1:1000. After rinsing the membrane in washing buffer for 3 x 15 minutes, the secondary antibody, a goat-anti-mouse IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Bruxelles, Belgium) was added at a concentration of 1:5000 for 1 hour at room temperature. After washing the membrane again for 3 x 15 minutes, the luminescence detection of peroxidase was performed with the ECL system according to the manufacturer's recommandations (Amersham, Freiburg, Germany). Films were exposed for 1-10 minutes at room temperature. As a control for small loading variabilities, an antibody against β-actin was used, and the ratio between the SCL and β-actin intensities was calculated.

Generation of SCL promoter constructs

The 5' regulatory region of the rat SCL gene was identified from the rat genome in the NCBI data base. Mesangial cDNA served as a template for the PCR of four promoter fragments of different lengths. This set of 5' deletion constructs was directionally subcloned into the promoterless luciferase expression vector, pGL3-Basic (Promega), using the *Kpn I-Hind III* sites. The constructs were terminated at bp 152, 298, 865 and 1556 relative to the translational start site, and are respectively denoted pT4-Luc152, pT4-Luc298, pT4-Luc865 and pT4-Luc1556.

Transient transfections

Transient transfections of MCs were performed with PEI (Polyethylenimin) according to Boussif *et al.* [16]. The respective pT4-Luc expression plasmids and a normalizing pCMV-β-galactosidase plasmid were used at concentrations of 1 μg/well. The total incubation time after transfection was 24 hours. All the experiments were carried out in triplicate, and independently performed at least three times. Luciferase and β-galactosidase assays of the cell lysates were performed as described previously [17, 18]. The results are expressed as the ratio of luciferase activity to β-galactosidase activity.

Electrophoretic mobility shift assays

Nuclear extracts were prepared according to Dignam [19] from rat mesangial cells. The DNA fragments were labelled with [γ-^{32}P]dATP. The labelled DNA fragments were incubated for 30 minutes with 5 μg nuclear extracts and 2 μg of poly(dI-dC) in a total volume of 20 μl. The samples were loaded onto a 4% polyacrylamide gel and electrophoresed for 2.5 hours. The gels were air-dried and autoradiographed. For antibody depletion experiments, antibodies against c-Fos and c-Jun were added to the incubation mix.

Statistical analysis

The results are given as means ± SD. The statistical analysis was performed with the unpaired Student *t* test with a correction for multiple comparisons when applicable [20]. A value of $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Renal histologies

Renal histological findings showed a high number of proliferating cells in the glomeruli from rats with acute nephritis (one intravenous injection of ATS) and a mild increase in the volume of the extracellular matrix compared with the controls (Fig. 1). Rats with chronic nephritis (two intravenous injections of ATS) revealed a major increase in the volume of the extracellular matrix, leading to a compression of the capillary loops in the glomeruli.

Selenocysteine lyase mRNA is upregulated in acute anti-Thy-1 nephritis

In a search for genes involved in the resolution or chronification of glomerulonephritis, gene expression was studied by differential display in acute and chronic anti-Thy-1 nephritis in Sprague-Dawley rats. One of the differentially expressed genes (data not shown) was identified as that for selenocysteine lyase. Compared to the levels in the controls, SCL mRNA was upregulated in the glomeruli of rats with acute glomerulonephritis, while its expression was downregulated in the glomeruli of rats with chronic glomerulonephritis. These results were confirmed by Northern blot analysis (Fig. 2). When the level of expression in the control group was arbitrarily assigned a value of 1.0, the relative expression of SCL was 1.65 in rats with acute

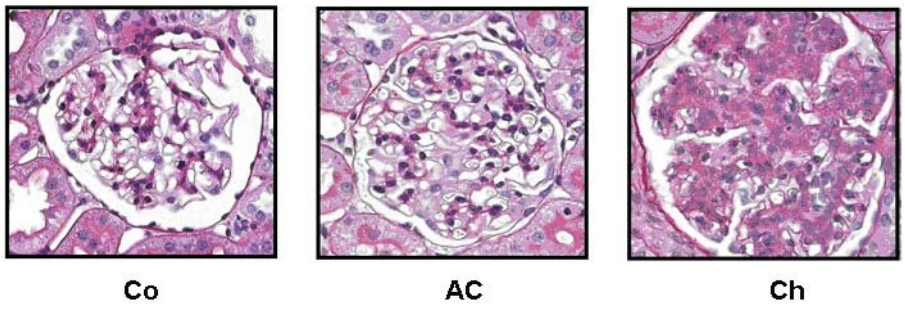

Fig. 1. The renal histologies (PAS staining) show a high increase in the number of proliferating cells and a mild increase in the volume of the extracellular matrix in rats with acute nephritis (Ac). In those with chronic nephritis (Ch), an extensive increase in the volume of the extracellular matrix is observed, with compression of the glomerular capillary loops.

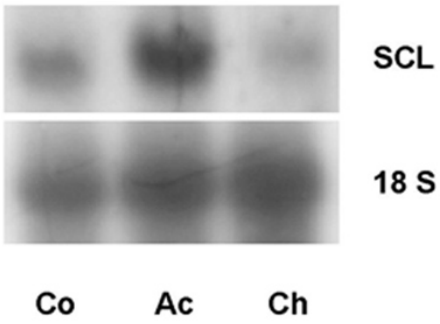

Fig. 2. The Northern Blot analysis shows a significant ($p < 0.05$) increase in the level of SCL mRNA (1.65-fold by densitometry) in rats with acute nephritis (Ac), while the SCL mRNA in rats with chronic nephritis (Ch) decreased (0.65-fold by densitometry), compared with the levels for the controls (Co).

nephritis and 0.65 in those with chronic nephritis. We also studied SCL activity in renal tissue from all three groups using the method of Esaki [8]. A slight but not significant increase was seen in tissue from rats with acute nephritis and a major, but also not statistically significant decrease was observed in tissue from rats with chronic nephritis (data not shown). The literature provides evidence that this SCL activity assay is very difficult to perform, and that the data obtained is not really valid for precise quantitative analysis [21]. Furthermore, the question remains whether post-mortem SCL activity measurements can be exact. However, since interleukin-1 (IL-1) has been identified as one of the major factors for mesangial cell proliferation and matrix deposition in experimental mesangioproliferative nephritis [22], we studied the influence of IL-1 on the expression of SCL in mesangial cells *in vitro*.

Stimulating rat mesangial cells with IL-1β upregulates SCL expression

To further characterize SCL regulation, the course of SCL expression in MC after stimulation with IL-1β was examined via real-time PCR. One hour of stimulation led to an increase in SCL mRNA expression by up to 2.7-fold, while after 3 hours of stimulation with IL-1β, SCL mRNA expression was significantly increased (8-fold) compared to the baseline level (Fig. 3). After 16 hours of stimulation, SCL mRNA expression decreased to the baseline level.

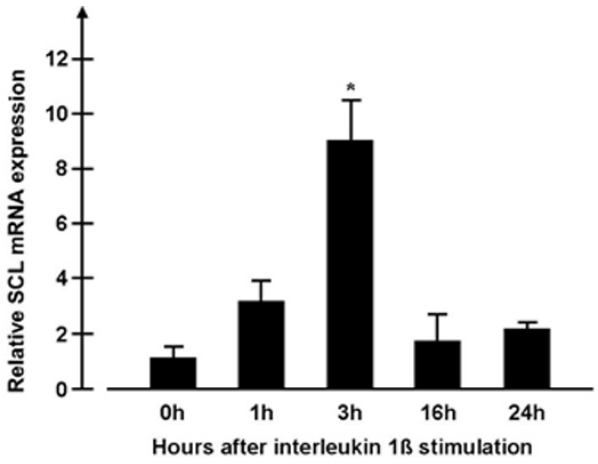

Fig. 3. Real-time PCR shows a marked increase ($p < 0.05$) in the expression of SCL mRNA after 3 hours of IL-1β stimulation of MC *in vitro*, while mRNA expression decreased again after 16 and 24 hours of stimulation.

SCL protein expression in rat mesangial cells after stimulation with IL-1β was also analyzed. Western Blot analysis revealed a steady increase in the level of SCL protein over time: 1.8-fold after 3 hours, 2.8-fold after 6 hours and 3.4-fold after 8 hours of IL-1β stimulation (Fig. 4).

Given that SCL decomposes cytotoxic selenocysteine which is produced during the degradation of selenoproteins and provides elemental selenium for the synthesis of new selenoproteins, the upregulation of SCL by IL-1 produced by mesangial cells in the inflammatory process [22] might be one mechanism involved in the course of acute glomerulonephritis. Investigations by Gaertner *et al.* [23] showed that oxygen radicals and the expression of different antioxidative enzymes are upregulated initially in experimental mesangioproliferative glomerulonephritis. Since SCL mRNA is upregulated in acute glomerulonephritis, as shown above, the provision of more elemental selenium might be a possible mechanism to increase the amount of glutathione peroxidase in this nephritis model. Downregulation of SCL in chronic glomerulonephritis could thus lead to an increase in glomerular damage via inflammatory processes.

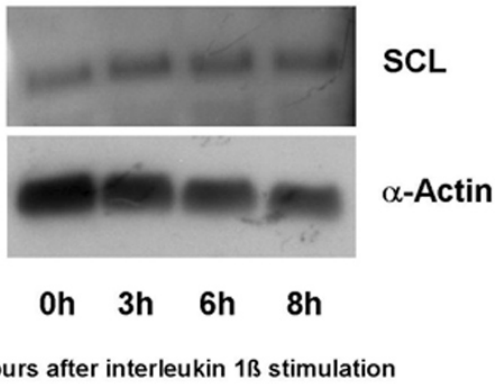

Fig. 4. SCL protein expression in MC increases significantly ($p < 0.05$) after stimulation with IL-1β for 3 hours and remains at a high level after 6 and 8 hours of IL-1β stimulation. The control group was assigned a value of 1.0.

The discovery of an enhancer element in the 5'-regulatory region of the SCL gene between bp -152 and -298

Mesangial cells were transiently transfected with a set of reporter gene constructs containing variable lengths of the SCL 5'-regulatory region subcloned into a promoterless luciferase expression vector. Co-transfection with a β-galactosidase-expressing plasmid was performed to normalize fordifferences in transfection efficiency. Construct pT4-Luc298 revealed an almost 4-fold increase in luciferase activity compared to construct pT4-Luc152 (Fig. 5), suggesting the presence of an enhancer element between bp -152 and -298.

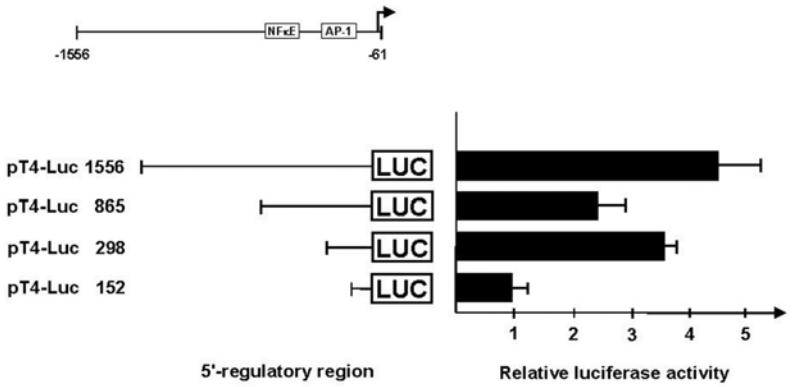

Fig. 5. Transient transfection of MC with deletion constructs of the 5'-regulatory region of the SCL gene demonstrating a significant increase in luciferase activity with construct pT4-Luc298 ($p < 0.05$). The data is presented as the ratios of luciferase (LUC) *versus* β-galactosidase activities with the construct pT4-Luc152 assigned a value of 1. The results are the means of three independent transfection experiments.

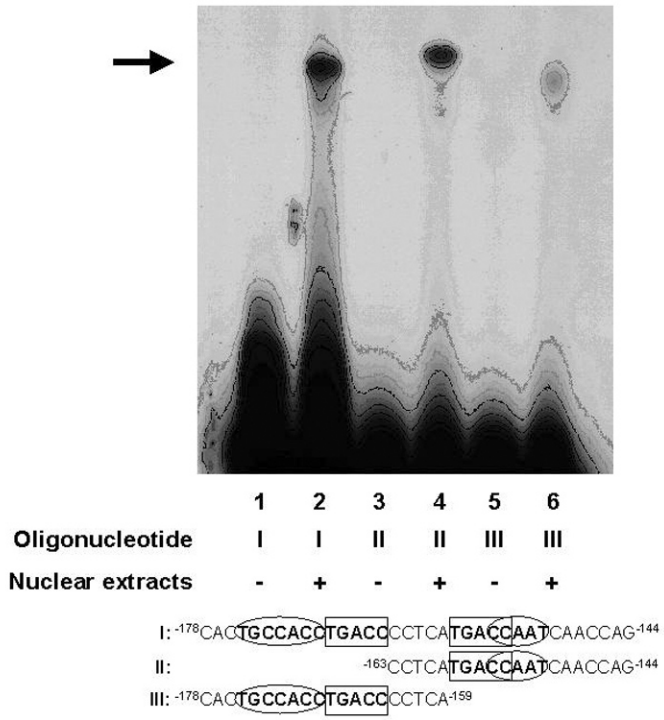

Fig. 6. Gel shift with three oligonucleotides containing putative transcription factor-binding sites within the region revealing enhancer activity in the luciferase experiments. NFκE-site: TGCCACC, AP-1 site: TGACC, CCAAT-box. The binding activity of the nuclear proteins from MC is mostly found within bp -178 and -159, while the region between bp -163 and -144 shows hardly any nuclear protein binding.

Screening of this region with the TRANSFAC database [24] revealed several putative transcription factor binding sites: two AP-1 sites, one NF-κE site [25] and a CCAAT-box. To identify whether one or several of these elements demonstrate binding of MC nuclear proteins, three DNA fragments for gel shift analysis containing the different binding sites were constructed. Fragment I, containing all four binding sites, showed strong protein binding, as did fragment II, containing one AP-1 site and the CCAAT-box, while fragment III, with the second AP-1 site and the NFκE site, revealed almost no protein binding (Fig. 6). The specificity of DNA/protein binding could be demonstrated for all three DNA fragments by incubation with the respective unlabelled fragment (50-fold excess), whereas incubation with a non-specific DNA fragment did not interfere with DNA/protein binding (data not shown).

The AP-1 binding site between bp -158 and -154 of the 5'-regulatory region of the SCL gene displays specific nuclear protein binding of c-Fos and transcriptional activity

To further characterize the specificity of nuclear protein binding to fragment I, four mutants of fragment I were used as unlabelled competitors in a gel shift experiment. In fragment M1, the putative NFκE binding site was mutated. Fragment M2 contains a mutated AP-1 site between bp -168 and -164, fragment M3 has a mutated AP-1 site between bp -158 and -154, and fragment M4 contains mutated bases instead of the CCAAT-box. While fragments M1, 2 and 4 did not show any competition for nuclear protein binding, competition with fragment M3 almost completely blocked the binding of nuclear proteins to fragment I, underscoring the specificity of nuclear protein binding to this site (Fig. 7).

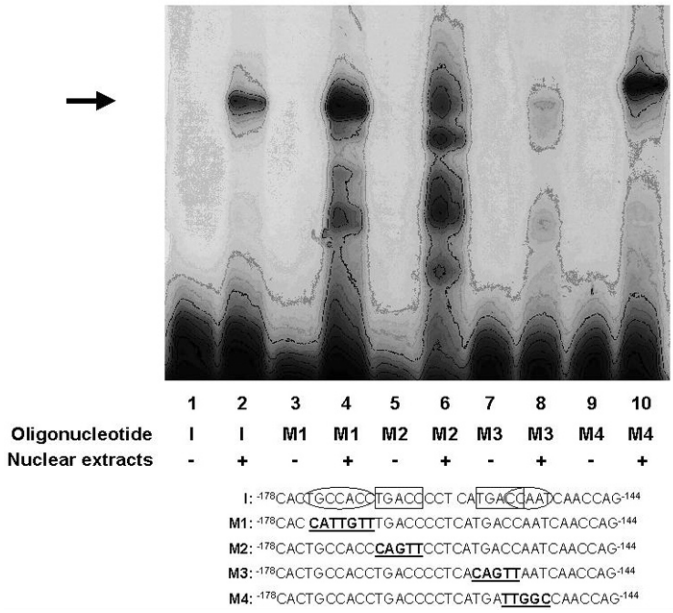

Fig. 7. Gel shift with mutated oligonucleotides. Fragment I as shown in Fig. 6. M1: mutation of NFκE-site. M2: mutation of AP-1 site. M3: mutation of AP-1 site. M4: mutation of CCAAT-box. Nuclear protein binding disappears almost completely with mutation of the AP-1 site in fragment M3.

To investigate whether nuclear protein binding to this AP-1 site was also important in enhancing transcriptional activity, luciferase construct pT4-Luc 298M was generated; it has the same length as construct pT4-Luc298 but contains a mutated AP-1 site between bp -158 and -154. Transfection of

mesangial cells with the mutated construct led to a significant decrease in luciferase activity ($p < 0.05$) compared to pT4-Luc298, suggesting that the AP-1 site between bp -158 and -154 is responsible for the original enhancing activity seen with this construct (Fig. 8).

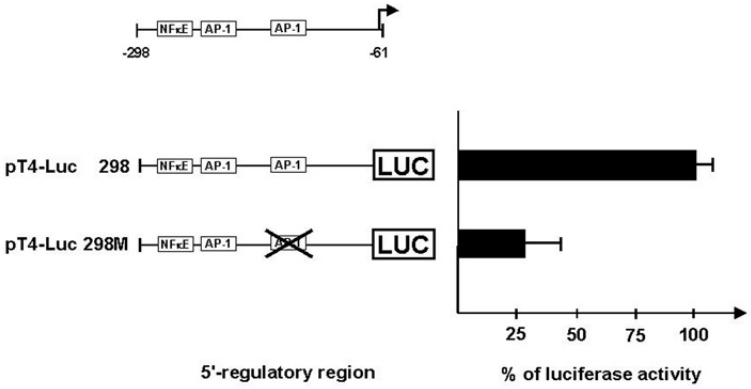

Fig. 8. Transient transfection of MC with luciferase construct pT4-Luc298 compared with construct pT4-Luc298M containing a mutated AP-1 site in position -158 and -154. The mutated construct shows a significant decrease in luciferase activity compared to the non-mutated construct with construct pT4-Luc298 being assigned a luciferase activity of 100%.

Since AP-1 is a collective term referring to dimeric transcription factors composed of Jun and Fos subunits that bind to a common DNA site, the AP-1 binding site, the antibody depletion analysis was performed with antibodies against the c-Fos and the c-Jun subunit of AP-1 (Fig. 9).

Pre-incubation with the c-Fos antibody yielded a significant decrease in nuclear protein binding, whereas pre-incubation with the c-Jun antibody did not show any difference in protein binding, suggesting that the c-Fos subunit is involved in enhancing the transcriptional activity of SCL.

AP-1 is a redox-sensitive transcription factor that is upregulated in cultured mesangial cells by H_2O_2 [26]. It has also been demonstrated that the c-Fos subunit of AP-1 is activated by IL-1 [27]. Furthermore, earlier studies revealed an upregulation of AP-1 in acute anti-Thy-1 nephritis [28]. Considering these studies and regarding the data presented in this study, we would like to propose the following mechanism of activation of SCL in anti-Thy-1 nephritis: IL-1 expression by mesangial cells is increased in anti Thy-1 nephritis [22], which leads to an upregulation of AP-1 [28]. This upregulation might lead to an enhanced transcriptional expression of SCL *in vivo*, as has been demonstrated in *in vitro* luciferase experiments in this study.

According to our data, the following feedback loop can be postulated: interleukin-1β is induced by the initial inflammatory process in acute nephritis, and oxygen radicals are produced by infiltrating leukocytes, leading to glomerular damage.

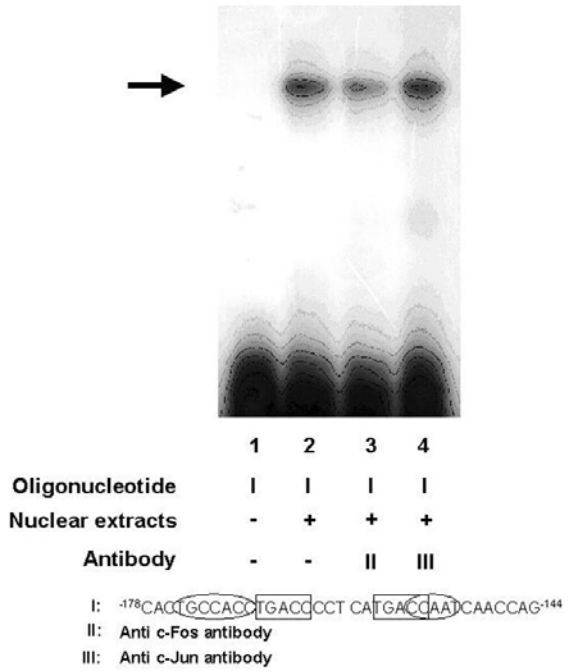

Fig. 9. The results of a gel shift experiment with nuclear extracts from MC and the radiolabelled fragment I. Antibody depletion analysis was performed by preincubating nuclear extracts with antibodies against the c-Fos (lane 3) and c-Jun (lane 4) subunits of the AP-1 complex. Only preincubation with an antibody against c-Fos reveals a reduction in nuclear protein binding.

Both mechanisms result in the upregulation of the transcription factor AP-1, which induces SCL transcription. Increased SCL enzymatic activity will induce the synthesis of selenoproteins, which act as oxygen radical scavengers leading to the limitation and resolution of the acute glomerular inflammation. This feedback loop may be interrupted by the repetitive injection of ATS, resulting in chronic glomerulonephritis. For further investigation of this hypothesized mechanism, more *in vivo* studies will be necessary.

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