ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Aug; 22 (8): 756 - 760
http://www.chinaphar.com

Effects of *L*-arginine on proliferation of human renal mesangial cells and production of extracellular matrix[1]

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KEY WORDS arginine; glomerular mesangium; extracellular matrix; collagen

ABSTRACT

AIM: To investigate the effect of *L*-arginine (*L*-arg) on the proliferation of human mesangial cells and production of collagen. **METHODS**: The influence of *L*-arg on the cell proliferation was determined by MTT assay, immunocytochemical detection of expression of proliferative cell nuclear antigen (PCNA), and flow cytometrical analysis of cell cycle. Procollagen Ⅲ and total collagen level in the supernatant and expression of collagen Ⅳ mRNA in human mesangial cells were determined by radioimmunoassay, hydroxyproline colorimetric assay, and reverse transcription polymerase chain reaction (RT-PCR). **RESULTS**: *L*-Arg induced inhibition of human mesangial cell lines (HMCL) in a concentration- and time-dependent manner. Immunocytochemical study for PCNA showed the number of cells was decreased, though the percentage of PCNA positive cells was increased in *L*-arg-treated group. Flow cytometrical analysis showed that cells in S and G_2-M phases were markedly increased in *L*-arg-treated group compared with those in control group. Furthermore, *L*-arg significantly inhibited the production of procollagen Ⅲ and total collagen in the supernatants determined by radioimmunoassay and hydroxyproline colorimetric assay ($P<0.05$ and 0.01, respectively) and inhibited the expression of collagen Ⅳ mRNA determined by RT-PCR ($P<0.01$). **CONCLUSION**: *L*-arg could exert an inhibitory effect on the proliferation of human mesangial cells and production of extracellular components, which strongly suggested its potential therapeutic role in the chronic renal scarring.

INTRODUCTION

Chronic renal failure is a self-perpetuating and progressive disease, leading to end-stage renal failure, even if primary insult to the kidneys was removed. Progressive course of disease is caused by glomerular sclerosis, tubular atrophy, and chronic interstitial inflammation[1]. The proliferation of mesangial cells is a feature of many glomerular diseases. Increased cellularity, coupled with the deposition of excessive extracellular matrix, may lead to progressive glomerulosclerosis and, subsequently, end-stage renal failure[2]. *L*-Arginine (*L*-arg) is classified as semi-essential amino acid. Kidney is the major site of endogenous *L*-arg synthesis. Recently, many interests have been raised on the role of *L*-arg on the progression of chronic renal diseases since the discovery of *L*-arg/nitric oxide (NO) metabolic pathway[3]. However, the exact mechanisms remain to be clarified. Our present study was to investigate the influence of *L*-arg on the proliferation of human renal mesangial cells (HMC) and production of extracellular components (ECM), so as to clarify the potential role of *L*-arg in renal fibrosis.

MATERIALS AND METHODS

Materials An established stable human mesangial cell line (HMCL, kindly provided by Renal Unit, Royal Free Hospital, UK) was used in the experiments. Human mesangial cells (HMC) were immortalized by transfection with *T-SV40* and *H-ras* oncogene. It remained many morphological features of normal HMC. *L*-Arg and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma, and proliferating cell nuclear antigen (PCNA) immunohistochemical reagent was purchased from Wuhan Boster Biological Engineering Ltd (China), the kits for procollagen

[1] Project supported by the Railway Scientific Research Grant, No J97Z 027.
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Received 2000-11-20 Accepted 2001-03-26

Ⅲ radioimmunoassay (Institute of Chongqing Tumor Research, China), and thermal imaging system FTL-500 (Pharmacia Biotech, USA).

Cell culture HMC were cultured in growth medium containing RPMI-1640, 10 % fetal calf serum (FCS), glutamine 2 mmol/L, benzylpenicillin 100 kU/L, streptomycin 0.1 g/L, amphotericin 2.5 kg/L, insulin 5 mg/L, human transferrin 5 mg/L, and sodium selenite 5 μg/L. The medium was renewed every day. Cells reached confluence in approximately 3 d and then were passaged every 3 – 4 d.

MTT assay MTT (5 μg/L, 20 μL) was added into each well. After 4-h incubation, 100 μL of acidic isopropanol was used to solubilize the formanzan crystals. The absorbance was calculated at 570 nm (A_{570}) using a scaning multiwell spectrophotometer.

Reversible test of removing *L*-arg The experiment included three groups: control, L-arg$_1$ group, and L-arg$_2$ group. HMC in L-arg$_1$ group and L-arg$_2$ group were incubated in the medium treated by L-arg 30 mmol/L, whereas HMC of control group were incubated in the medium without L-arg. Cells in control and L-arg$_1$ group were carried out MTT assay after cultured for 48 h, while cells in L-arg$_2$ group were continued to incubate with RPMI-1640 for 48 h without addition of L-arg, then carried out MTT assay.

Analysis of cell immunocytochemistry Proliferation was expressed as the number of mesangial cells that were reactive with an antibody recognizing PCNA as determined by immunocytochemistry. Cells were incubated with RPMI-1640 medium treated by L-arg 30 mmol/L for 24 h, followed by methanol fixative for 10 min. After being incubated with 0.25 % of Triton X-100 and 5 % Me_2SO in PBS (NaCl 8.0, KCl 0.2, $Na_2HPO_4 \cdot 12H_2O$ 3.58, and KH_2PO_4 0.24 g/L) for 10 min and blocked in 4 % goat serum, sections were incubated with monoclonal PCNA antiserum of mouse at 4 ℃ overnight. The sections were washed and incubated with anti-mouse IgG conjugated to biotin at 20 ℃ for 20 min. After washing, the substrate solution containing DAB (0.5 g/L, 0.03 % H_2O_2 in Tris-HCl 50 mmol/L, pH 7.6) was added. The reaction was stopped by removal of the substrate and rinsing with PBS. Negative control sections received identical treatment except for the primary antibody. Select randomly five fields of vision (×400) per section and count the number of cells in positive immunostaining for PCNA.

Cell cycle distribution analysis Cells were seeded at the density of 1×10^8/L in 6 well plastic plates, then incubated in RPMI-1640 medium for 24 h, supplemented with L-arg with different concentrations (0, 20, and 30 mmol/L). After 48 h, cells were isolated by 0.25 % trypsin, washed and fixed in 70 % ice-cold ethanol solution for 30 min, spun and washed twice in PBS, resuspended in PBS 0.5 mL, and RNase 150 U was added. After incubation at 37 ℃ for 30 min, 1.5 mL of propidium iodide (PI) 50 mg/L was added and incubated for 40 min. Using a FACS440 instrument, PI fluorescence-activated cell sorting, single parameter frequency histograms were generated. With the help of computer, cell cycle stages were estimated.

Hydroxyproline colorimetric assay Cells were incubated with the medium treated with L-arg (0, 1, 10, 20, and 30 mmol/L) for 4 d. Supernatant of each well (150 μL) was pipetted into Nalgene tubes and dried in an oven at 100 ℃. NaOH (4 mol/L, 150 μL) was added to the tubes with gentle shaking. Then kept them in boiling water for 90 min. Citric acid (150 μL, 1.4 mol/L) was pipetted into each tube to bring the pH of the hydrolysate to 6.0. Chloramine-T solution 1 mL was mixed with each sample and the mixture was incubated at room temperature. After 20 min, 1.0 mL of the aldehyde/perchloric acid solution was added, and the samples were vortexed and incubated in a water bath at 65 ℃. Full color development occured by 20 min and the fractions were read at 560 nm.

Radioimmunoassay of procollagen Ⅲ in the supernatants Cells were incubated in RPMI-1640 medium treated with L-arg 30 mmol/L. After 72 h, procollagen Ⅲ in the supernatants was determinted by the kits of radioimmunoassay according to the procedures shown in the kits.

Reverse transcription polymerase chain reaction (RT-PCR) Total RNA was isolated with the Trizol one step method from human mesangial cells, dissolved in DEPC treated water and quantitated by spectrometry at 260 nm.

Total RNA 1 μg was heated at 70 ℃ for 10 min and reversely transcribed using random primer and reverse transcriptase 200 U (Promega). The mixture was incubated at 37 ℃ for 60 min, heated at 95 ℃ for 10 min, and stored at – 20 ℃ until use. Selected sequences in 5 μL aliquots of cDNA were amplified by PCR for 30 cycles using primers for collagen Ⅳ and β-actin as an internal standard in the same tube. Mixtures were cycled at 94 ℃ for 1 min, 55 ℃ for 45 s, 72 ℃ for 1 min, and 72 ℃ for 7 min. The primer of collagen Ⅳ was de-

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Aug; 22 (8)
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signed by our group and synthesized by Genosys Biotech Ltd (London). The sequence of the primers were: collagen Ⅳ, 5′ CTCCGGGGCTCCAAGGTGTTC and 5′ TGGGCAGGGGGCATCTAATCC, amplified fragment 377 bp; β-actin, 5′ GGTCAGAAGGATTCATATGTG and 5′ ATTGCCAATGGTGATGACCTG, amplified fragment 615 bp. PCR reactants were resolved in 1.7 % agarose gel and stained with ethidium bromide and photographed. The film was developed and examined with a densitometer CS-9000 (Shimadzu). The identity of each fragment was confirmed by restriction digestion and sequencing of the fragments. The ratio of collagen Ⅳ mRNA to β-actin mRNA (collagen Ⅳ/β-actin) was referred to the relative level of collagen Ⅳ mRNA transcript.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and compared by unpaired t-test and χ^2-test. $P < 0.05$ was accepted as statistical significance.

RESULTS

Effect of *L*-arg on the proliferation of human mesangial cells Cells in logarithmic phase of growth were seeded at a density of 1×10^4 onto 96-well microtitre plates. Once adherent, they were treated with 200 μL fresh medium (containing 10 % FCS) supplemented with *L*-arg at concentrations of 0, 10, 20, 30, and 40 mmol/L for 72 h. The results by MTT assay showed that the proliferation of HMC was significantly inhibited in a concentration-dependent manner ($P < 0.01$, Fig 1). Furthermore, the antiproliferative effect of *L*-arg 25 mmol/L was demonstrated in a time-dependent manner as well (Tab 1).

Reversible test of removing *L*-arg We performed the recovery test to exclude the possibility of cytotoxicity of *L*-arg at the above mentioned concentrations. The results showed that *L*-arg significantly inhibited the proliferation of human mesangial cells (control 1.71 ± 0.14 *vs* $L\text{-arg}_1$ group 0.81 ± 0.09, $P < 0.01$). The cells in $L\text{-arg}_2$ group were continued to incubate for 48 h with *L*-arg-free media, and the results showed that the growth of cells were partly recovered 48 h later ($L\text{-arg}_2$ group 1.12 ± 0.16 compared with $L\text{-arg}_1$ group 0.81 ± 0.09, $P < 0.01$).

Effect of *L*-arg on the expression of PCNA of human mesangial cells It clearly showed that the cell number was reduced after cells treated by *L*-arg 30 mmol/L for 48 h, although the ratio of positive immunostaining for PCNA significantly increased (*L*-arg group 28.1 % *vs* control 17.4 %, $P < 0.05$).

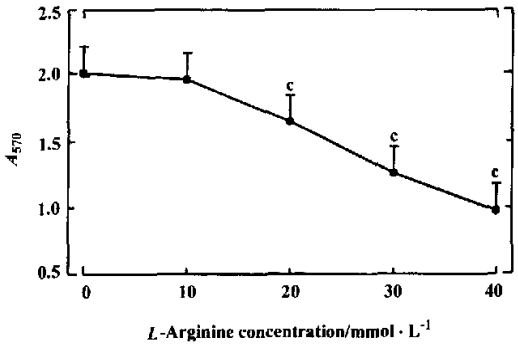

Fig 1. Effect of *L*-arginine on the proliferation of human mesangial cells (72 h). $n = 8$ wells. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Tab 1. Effect of the time course of *L*-arginine on the proliferation of human mesangial cells. $n = 8$ wells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Time/h	A_{570} Control	*L*-arg group
24	1.23 ± 0.15	0.99 ± 0.13^c
48	1.36 ± 0.12	1.02 ± 0.14^c
72	1.66 ± 0.16	1.45 ± 0.12^b

Effect of *L*-arg on DNA distribution of cell cycle Flow cytometrical study showed that the ratio of cells in S and G_2-M phases was increased compared with the control group with the increasing of *L*-arg concentration in the medium (Tab 2).

Tab 2. Effect of *L*-arginine on DNA distribution of cell cycle (48 h).

Group	$G_0 - G_1$ phase/%	S phase/%	G_2-M phase/%
Control	56.9	33.8	9.3
$L\text{-arg}_1$ 20 mmol/L	40.7	35.8	23.5
$L\text{-arg}_2$ 30 mmol/L	20.6	50.8	28.5

Effect of *L*-arg on the production of total collagen in the supernatants of cultured human mesangial cells *L*-Arg significantly inhibited the production of total collagen in a concentration-dependent manner compared with that of control group when the concentration of *L*-arg in the media was higher than 1 mmol/L (0.62 ± 0.04 *vs* control 0.51 ± 0.03, $P < 0.01$, Fig 2).

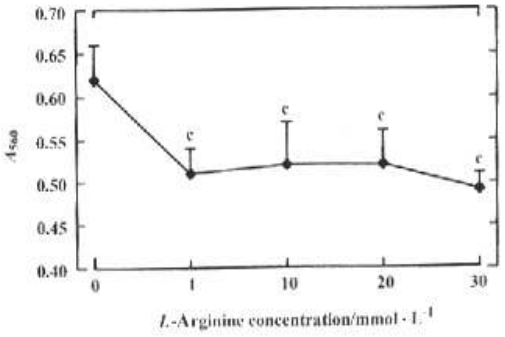

Fig 2. Effect of *L*-arg on the production of total collagen in the supernatants of human mesangial cells. $n = 5$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

Effect of *L*-arg on the production of procollagen Ⅲ in the supernatants of human mesangial cells *L*-Arginine significantly inhibited the production of procollagen Ⅲ in the supernatants of human mesangial cells compared with control group (288 ± 12 *vs* 224 ± 24, $P < 0.05$).

Effect of *L*-arg on the collagen Ⅳ gene expression of human mesangial cells It was shown that collagen Ⅳ mRNA expression significantly decreased in *L*-arg-treated group compared with that of control group ($P < 0.01$, Fig 3 and 4).

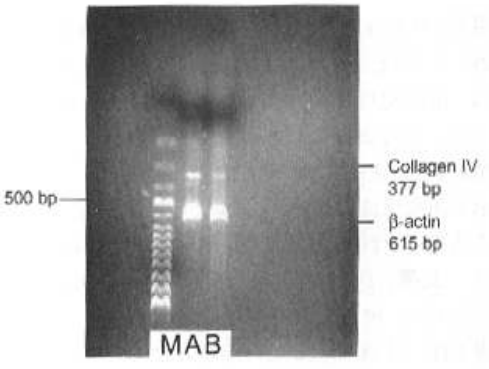

Fig 3. Expression of collagen Ⅳ mRNA and β-actin mRNA by human mesangial cells incubated with *L*-arg 20 mmol/L for 12 h. Lane M: Molecular marker; Lane A: control; Lane B: *L*-arg group.

DISCUSSION

The present study clearly showed that *L*-arg could inhibit the proliferation of human mesangial cells and production of collagen with its gene expression, which suggested that *L*-arg might play an important role in modifying the progression of glomerulosclerosis.

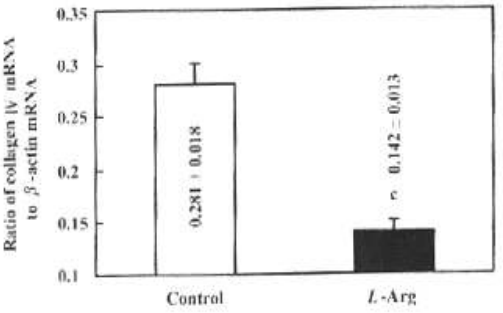

Fig 4. Collagen Ⅳ mRNA expression of human mesangial cells treated with *L*-arg as quantitated by UVP densitometry. $n = 3$. $\bar{x} \pm s$. $^cP < 0.01$ *vs* control.

L-Arg is a semi-essential amino acid, which provides molecular substrate for generation of NO, polyamines, *L*-proline, and agmatine. All of these *L*-arg metabolites have been involved in renal pathophysiology. Reyes[4] showed that dietary supplementation with 1 % *L*-arg ameliorated the decline of renal function and worsening of structure in rats with subtotal nephrectomy or obstructive nephropathy models. Ingram's work suggested that therapeutic effect of *L*-arg to glomerular sclerosis may be associated with its inhibiting production of endothelin and expression of PCNA[5]. However, it remains quite controversial whether or not the effects of *L*-arg are related with its product, NO. Mohaupt *et al*[6] found that nitrite, the major stable end-product of NO, was significantly increased after incubation of mesangial cells with interleukin-1β or lipopolysaccharide, while the uptake of [^{3}H]-thymidine in resting or proliferating mesangial cells were not altered when compared with control group. More interestingly, Rupprecht *et al*[7] recently reported that NO could inhibit the proliferation of MC partly by inhibiting its transcription factor EGR-1. Hence, the exact effect of NO on mesangial cell proliferation is still uncertain.

Our results showed that the cell number was reduced after cells treated with *L*-arg regardless of the increase of positive immunostaining for PCNA. It is well known that PCNA is an accessory protein of DNA polymerase delta, whose expression increases from the late G_1 phase and reaches its maximum during the S-phase, declines during G_2-M phases of the cell cycle. Kurki *et al*[8] showed that drugs which affected the early phase of G_1 inhibited PCNA expression; whereas drugs which affected

ISSN 0253-9756 Acta Pharmacol Sin 中国药理学报 2001 Aug; 22 (8)
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S-phase and prevented DNA synthesis did not block PCNA expression. Our flow cytometrical study suggested that numerous cells in *L*-arg treated group were retarded at S-phase and G_2-M phase of the cell cycle, which was consistent with our result that *L*-arg increased the expression of PCNA. Taken together, our results, therefore, suggested that the antiproliferative effect of *L*-arg might be related with its inhibition on the cell mitogenesis.

To further address the effect of *L*-arg on renal fibrosis, we measured the level of total collagen, procollagen Ⅲ, in the supernatants and mRNA expression of collagen Ⅳ in mesangial cells as well. The consistent inhibition of these components strongly suggested that *L*-arg might contribute to the inhibition of deposition of ECM, and subsequently delay the progression of chronic renal diseases. Although the exact mechanism is unknown so far, Trachtman *et al*[9] demonstrated that NO, a product of *L*-arg/NO synthase pathway, could decrease synthesis and increase degradation of extracellular matrix by stimulating the activity of a neutral matrix metalloproteinase (M_r72 000). Moreover, Morrissey *et al*[10] also suggested that it could be associated with the inhibition of *L*-arg on the expression of tissue inhibitor of metalloproteinase-1 mRNA and collagen Ⅳ mRNA.

In summary, our pilot *in vitro* study clearly demonstrated that *L*-arg could exert an inhibitory role on the proliferation of human mesangial cells and the production of ECM, which strongly suggested its potential therapeutic role to the chronic renal diseases.

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L-精氨酸对人肾系膜细胞增生及胞外基质产生的影响[1]

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关键词 精氨酸；肾小球系膜；细胞外基质；胶原

目的：研究 *L*-精氨酸(*L*-arg)对人肾系膜细胞增生和胞外基质成分胶原产生的影响. **方法**：采用 MTT 法、细胞免疫化学检测增殖细胞核抗原(PCNA)表达以及流式细胞仪等方法观察 *L*-arg 对系膜细胞增殖的影响；*L*-arg 对系膜细胞胞外基质产生的影响分别采用放免法、羟化脯氨酸比色法以及 RT-PCR 等方法测定前胶原Ⅲ、总胶原以及胶原Ⅳ mRNA 的表达. **结果**：*L*-arg 呈剂量和时间依赖性抑制人系膜细胞增殖；细胞免疫化学法显示 *L*-arg 导致细胞总数下降，但 PCNA 阳性比例相对增高，用流式细胞仪进一步证实 *L*-arg 治疗组细胞主要处于细胞周期的 S 及 G_2-M 期. 另外，*L*-arg 显著抑制培养的系膜细胞上清液中总胶原和前胶原Ⅲ合成(分别为 $P<0.05$ 和 $P<0.01$)以及细胞对胶原Ⅳ mRNA 的表达($P<0.01$). **结论**：*L*-arg 可抑制人肾系膜细胞增生和胞外基质成分产生，此提示 *L*-arg 对慢性肾脏纤维化可能有潜在的治疗价值.

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