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Human Mesangial Cell Production of Macrophage Cheuoactic protein-1: Modulation by Lovastatin

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INTRODUCTION

Hyperlipidemia that complicates various progressive and proteinuric renal diseases has been considered an important modulator of renal injury[1]. In several experimental models of renal disease, lipid-lowering agents such as 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors reduced circulating lipids and ameliorated glomerular injury[1]. We have recently shown that HMG-CoA reductase inhibition also reduced serum-stimulated mesangial cell proliferation[2]. This effect was independent of the availability of exogenous cholesterol. Moreover, experimental data indicated that this antiproliferative effect was due to depletion of farnesyl, a nonsterol isoprenoid metabolite of mevalonate[2]. These results suggested that HMG-CoA Reductase inhibitors may have direct beneficial effects on the glomerulus by modifying the proliferation of mesangial cells.

The development of focal glomerulosclerosis in experimental models of progressive renal disease is frequently characterized by the presence of monocytes (M ∅) and monocyte-derived foam cells[3~7]. Recently, we have also demonstrated an increased glomerular influx of M ∅ after initiating a 4% cholesterol diet in normal rats[8,9]. Over a four week interval, glomerular foam cells were detected and this was associated with expansion of mesangial matrix[9]. These data suggested that lipids may be a modulator of glmerular macrophage recruitment.

The factors that govern migration of monocytes into the glomerular mesangium and their subsequent activation have not been characterized. Mesangial cells are known to produce a variety of chemotactic substances, including leukotrienes, interleukins and cytokines such as monocyte chemotactic protein-1 (MCP-1)[10~13]. In the present experiments, we demonstrated that serum-stimulated mesangial cells produced a chemotactic factor exclusively for monocytes. This factor was identified as MCP-1. Importantly, inhibition of mesangial cell HMG-CoA reductase by lovastatin resulted in a reduction of a secreted chemotactic protein as well as a marked reduction in mesangial cell expression of MCP-1 mRNA. In vivo, the importance of these observations was tested by demonstrating that cholesterol-fed rats treated with lovastatin did not demonstrate an increased glomerular macrophage influx despite hypercholesterolemia.

METHODS

1. Human Mesangial Cell Studies

1) Mesangial Cell Cultures

Human fetal mesangial cells were obtained from an aborted human fetus. Permission to use this kidney tissue was obtained from the Committee on Use of Human Subjects in Research, University of Minnesota. Glomerular cores were seeded on plastic tissue culture flasks and incubated at 37°C in an atmosphere of 95% air and 5% CO_2. The culture medium was RMPI 1640 supplemented with 20% fetal bovine serum (FBS). Fresh media was added every 3-4 days. For selective passage of mesangial

cells, ridges were removed and replated in the same media. Details of the mesangial cell isolation and culture techniques used in our laboratory as well as their morphological characteristics have been reported[2,14].

2) Experimental Design

In all experiments $2\text{-}3\times10^4$ mesangial cells from the 4th-6th passage were seeded onto a 24-well culture plate and allowed to attach and grow to a semiconfluent state over 3-4 days. Mesangial cells were then synchronized to quiescence in serum-free RPMI 1640 for 72 hours. After synchronization, mesangial cells were exposed to 10% FBS as a mitogen in the presence or absence of the HMG-CoA reductase inhibitor lovastatin, a gift from Merck, Inc. (West Point, PA). Supernates were harvested at specific times for evaluation of polymorphonuclear (PMN) cell and MØ chemotactic activity. In some experiments, mevalonate was added to assess the reversibility of HMG-CoA reductase inhibition as previously described[2].

2. Evaluation of Chemotaxis

1) Leukocyte Preparation

Heparinized blood obtained from healthy donors was mixed with dextran, and red blood cells were allowed to sediment to produce a leukocyte-rich supernatant. Isolation of PMNs was performed as we have previously described[15]. Briefly, the dextan-sedimented, leukocyte-rich supernatant was centrifuged on discontinuous Ficoll-Hypaque (equal volumes of specific gravity 1.080 and 1.120) and the cells were collected at the interface of the bottom layer. Monocytes were obtained by a modification of the Recalde method[16]. After recovering the white cell fraction by centrifugation, they were incubated in a slightly hypertonic solution for approximately 30 minutes at 37°C. The MØ were then separated using Ficoll-Hypaque and density centrifugation[16].

2) Chemotaxis Assay

Assessments of mesangial cell supernates for chemotactic activity were performed in 48-well Neuroprobe® chemotaxis chambers (Neuro probe Inc., Cabin John, MD). The lower wells were loaded with 28 ul of the test solution and covered with a 5 um pore size polycarbonate filter. Upper wells were loaded with 50 ul of the cell suspension containing 5×10^4 MØ or PMNs. The chambers were incubated at 37°C for 1 or 1 1/2 hours to assess chemotaxis of PMNs and MØ, respectively. The filters were then removed, fixed in methanol, and stained with Diff-Quick (Baxter Healthcare Inc., Miami, FL). Migrating cells were evaluated by dividing a well into four quadrants and adding the counts from one 200× field in each quadrant. A total of 3-5 replicate wells were counted and used for determination of the mean value of each data point. Formyl-methionyl-leucyl-phenylalanine (FMLP; Sigma, St. Louis, MO) at a concentration of 1×10^{-8} M/l was used as the standard chemoattractant for MØ and PMN.

Incertain experiments, the supernate chemotactic activity was assayed after treatment with an affinity-purified mouse monoclonal IgG antibody against human MCP-1 which was kindly provided by T. Yoshimura, M.D., National Cancer Institute, Frederick, MD[17]. Mesangial cell supernates were incubated with varying dilutions of the MCP-1 antibody for one hour at 37°C. Antibody and complexes were than removed from the sample by ultrafiltration using an Amicon Centricon 100 filter (Amicon Inc., Beverly, MA) with an average molecular cut-off of 100,000 daltons. As a control, similar protein concentrations of an irrelevant mouse monoclonal IgG antibody were added to supernates and processed as described above.

3) In Vivo Evaluation of Chemotaxis

To assess the effect of HMG-CoA reductase inhibition on glomerular MØ chemotaxis we used dietary-induced hypercholesterolemic rat model[8,9]. Sprague-Dawley rats (Harlan Sprague Dawley, Indianapolis, IN) that weighed approximately 200 g were fed either normal chow (n=6) or a 4% (n=12) cholesterol

enriched chow (Ralston-Purina, Inc.), as previously described[8]. One-half of the 4% cholesterol chow group received daily subcutaneous injections of lovastatin (4 mg/kg) in a saline: ethanol (92 : 8) diluent, while the other one-half received vehicle alone. After two weeks of the diets, rats were sacrificed under light ether anesthesia and serum was obtained for measurement of cholesterol and triglycerides, as previously described[8]. Renal tissue was also obtained for light and immunocytochemical studies as previously described[8]. Specifically, using a monoclonal mouse IgG antibody (Serotec, Oxford, UK) against rat monocytes and macrophages (ED1) the number of ED1 positive cells was quantitated in a random population of ten glomeruli from each of the groups[8].

3. Molecular Techniques

1) cDNA Probes

The cDNA for MCP-1 was a kind gift from. T. Yoshimura, M.D., and has been previously characterized[18]. The full length cDNA insert contains 739 base pairs and has been shown to recognize a ~0.8 kb mRNA from human mononuclear and smooth muscle cells[18]. The cDNA insert was cloned in an ECOR 1 site in p Bluescript SK (-) plasmid. In addition, a cDNA for human HMG-CoA reductase was obtained from American Type Culture Collection (Rockville, MD). The cDNA insert contains 4.3 kb, the full sequence for human HMG-CoA reductase that was obtained from a fetal adrenal cDNA library[19]. The full length cDNA was cloned in a pcDV 1 plasmid. After digestion with BgL II, a fragment of about 2.5 kb was isolated and used as a template for radiolabelling. This fragment recognizes a mRNA of ~4.2 kb from human mesangial cells. Between 10-50 ng of a cDNA template were labeled with 50 microcuries of ^{32}[P]-dCTP using Prime it II, a random primer DNA labeling system (Stratagene Cloning System, La Jolla, CA). The cDNAs were purified on a Nuctrap probe purification column (Stratagene Cloning System). The specific activity varied between $1\text{-}2\times10^8$ cpm/ug cDNA.

2) Northern Blot Analysis

Total RNA was isolated from mesangial cells by a modification of the single step guanidium-thiocyanate-pheno-chloroform extraction method[20]. The RNA was quantitated by absorbance at 260 nm and its purity assessed by the ratio of absorbance at 260 : 280 being greater than 1.8-2.0. Twenty ug samples of denatured mesangial cell RNA together with 10 μg of a RNA ladder (BRL Inc., Gaithersburg, MD) were electrophoresed in a 1.0% agarose gel containing 2.2 M formaldehyde, 20 mM MOPS, 8 mM sodium acetate and 1 mM EDTA stained with ethidium bromide and photographed to assess degradation and molecular size. The electrophoresed RNA was transferred to a Zeta Probe membrane (Bio-Rad, Richmond, CA). The membrane was air dried and baked at 80°C for 30-60 minutes in a vacuum oven and stored at room temperature until needed. Blotted membranes were incubated with 1.5×10^6 cpm/ml of a labeled cDNA probe in standard hybridization solution for 12-24 hours, subjected to a series of stringency washes and exposed on Kodak X-OMAT XAR5 at -70°C. Autoradiograms of the probed RNA were scanned by densitometry.

4. Statistical Analysis

Results were expressed as mean±SEM. The significance of differences between the means of two groups was tested using the unpaired Student's t test. When more than two groups were compared, significance was tested using analysis of variance with the Bonferroni method for comparing multiple groups. These analyses were performed using the Statistical Package for Social Sciences[21]. Differences were considered significant for $p<0.05$.

RESULTS

1. Mesangial Cell Production of a Chemotactic Factor

Quiescent human mesangial cells, when exposed to 10% FBS for 24 hours, secreted a factor that stimulated chemotaxis of normal human peripheral blood MØ. This effect was quantitatively similar to that seen when the chemotactic peptide FMLP was used to stimulate MØ chemotaxis (Fig. 1). In contrast, no effect on chemotaxis of PMNs was seen when samples of supernates obtained from serum-stimulated mesangial cells were studied (data not shown). Quiescent mesangial cells maintained in serum-free RPMI did not secrete detectable amounts of chemotactic factor during the 24-hour incubation (Fig. 1). Similarly, 10% FBS alone in the chemotaxis chamber did not demonstrate any chemotactic activity (data not shown). Incubation of mesangial cell supernates with a 1 : 500 dilution of 1.4 mg/ml solution of an IgG mouse monoclonal antibody against human MCP-1 completely inhibited the supernate chemotactic activity (Fig. 1). Addition of a comparable concentration of an irrelevant IgG mouse monoclonal antibody (von Willebrand factor) had no effect on MØ chemotactic activity of the mesangial cell supernates (data not shown).

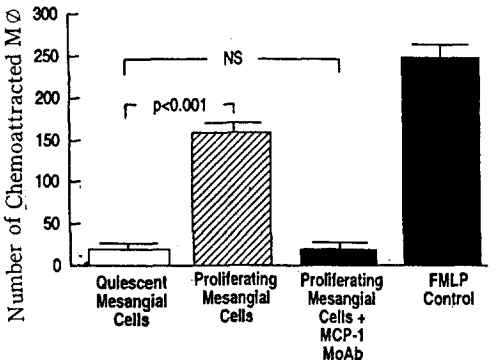

Fig. 1. Quiescent human mesangial cells when stimulated to proliferate with 10% fetal bovine serum secreted a factor that stimulated chemotaxis of normal peripheral blood monocytes (hatched bar). Quiescent mesangial cells maintained in serum-free RPMI (open bar) did not secrete a significant amount of this factor. Incubation of the supernates from proliferating mesangial cells with a monoclonal antibody to MCP-1 completely removed all chemotactic activity (stippled bar). As a positive control the effect of 10^{-8}M FMLP is also displayed (solid bar). (MØ, monocytes; MCP-1, monocyte chemotactic protein-1; MoAb, mooclonal antibody; FMLP, formyl-methionyl-leucyl-phenylalanine)

2. Effect of Inhibition of HMG-CoA Reductase on Production of Mesangial Cell MCP-1

Lovastatin, in a concentration dependent manner, inhibited mesangial cell production of a MØ chemotactic factor (Fig. 2). Between 20 and 40% of chemotactic activity in the suppernates was inhibited by 1-20 μM lovastatin. The addition of the MCP-1 monoclonal antibody to these supernates completely abolished the remaining chemotactic activity (data not shown). The mesangial cell numbers after the 24-hour incubation with lovastatin were unchanged (data not shown). In separate experiments, the addition of lovastatin to the chemotaxis chambers did not alter the chemotaxis of monocytes to FMLP nor did it affect the chemotactic response

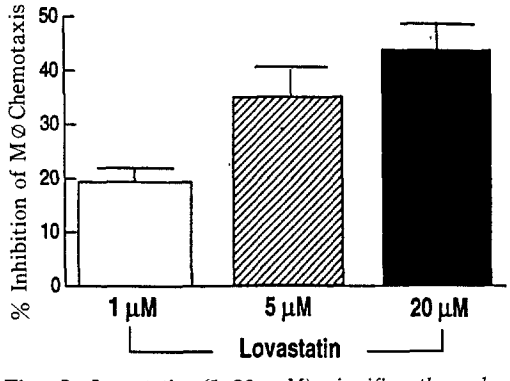

Fig. 2. Lovastatin (1-20 μM) significantly reduced production of a chemotactic factor by serum-stimulated human mesangial cells over a 24 hour incubation. (MØ, monocytes)

to the mesangial cell-derived chemotactic factor (data not shown). The addition of mevalonate to the mesangial cell incubation medium completely reversed the inhibitory effect of lovastatin and restored mesangial cell production of the chemotactic factor (Fig. 3).

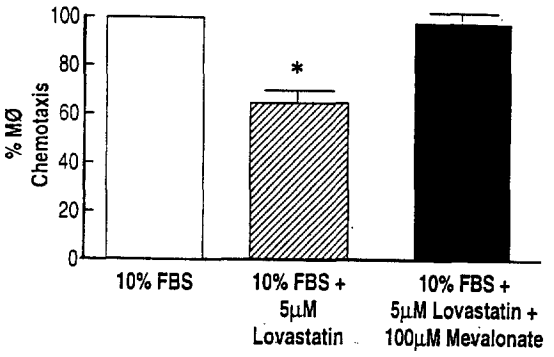

Fig. 3. *In these experiments 5 μM lovastatin significantly reduced (hatched bar) serumstimulated human mesangial cell production of a chemotactic factor for monocytes (MØ). This was completely restored by the addition of 100 μM mevalonate (solid bar). (*p < .001; FBS, fetal bovine serum)*

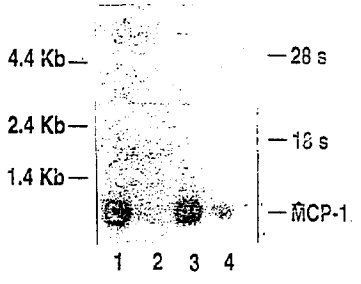

Fig. 4. *Northern analysis of MCP-1 mRNA in serum-stimulated human mesangial cells (lane 1). The presence of 5 μM lovastatin dramatically reduced MCP-1 mRNA expression in these cells (lane 2). The presence of 100 μM mevalonate completely reversed the inhibitory effect of lovastatin (lane 3). Quiescent mesangial cells maintained in serum-free RPMI demonstrated minimal constitutive expression of MCP-1 (lane 4). (MCP-1, monocyte chemotactic protein-1)*

3. Effect of HMG-CoA Reductase Inhibition on MCP-1 mRNA Expression

To evaluate whether the effect of lovastatin influenced steady state mRNA for MCP-1 after 24 hours, total RNA was obtained from serum-stimulated mesangial cells in the presence of lovastatin with or without mevalonate. In these experiments, total RNA was also probed for HMG-CoA reductase mRNA to ascertain the effectiveness of lovastatin inhibition of HMG-CoA reductase. Quiescent mesangial cells had minimal constitiutive expression of MCP-1 (Fig. 4). This was consistent with our results demonstrating virtually no detectable chemotactic activity in the supernates from quiescent mesangial cells (Fig. 1). In contrast, serum-stimulation of mesangial cells resulted in a significantly increased expression of MCP-1 mRNA (Fig. 4). This effect was markedly reduced by lovastatin, while mevalonate completely reversed the effects of HMG-CoA reductase inhibition (Fig. 4). Consistent with the effect of lovastatin to inhibit HMG-CoA reductase, lovastatin markedly up-regulated mRNA expression for this enzyme, while the addition of

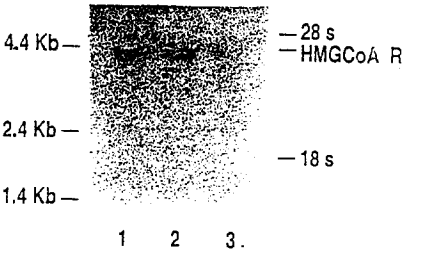

Fig. 5. *Northern analysis of HMG-CoA reductase mRNA in serum-stimulated human mesangial cells (lane 1). In accordane with the metabolic effects of lovastatin and mevalonate, HMG-CoA reductase mRNA expression was significantly up-regulated by 5 μM lovastatin (lane 2) and markedly down-regulated after the addition of 100 μM mevalonate (lane 3). (HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A reductase)*

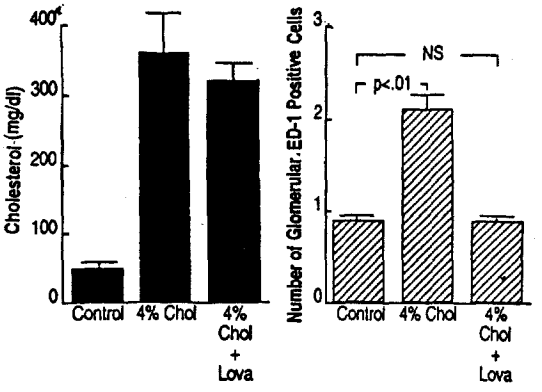

Fig. 6. The effect of 4% cholesterol diet on serum cholesterol (left) and the number of glomerular ED-1 positive cells (right). Therapy with lovastatin (Lova) did not significantly influence cholesterol levels but significantly reduced the number of glomerular ED-1 cells to control levels.

mevalonate significantly down-regulated the expression of this mRNA (Fig. 5).

4. Effect of Lovastatin on Glomerular Macrophage Recruitment in Hypercholesterolemic Rats

A 4% cholesterol diet was associated with a marked increase in serum cholesterol. Lovastatin treatment had no significant effect on serum cholesterol (Fig. 6). Cholesterol-fed rats injected with vehicle demonstrated a significant increase in glomerular ED1 positive cells. Lovastatin therapy completely prevented the increased glomerular MØ influx (Fig. 6). This effect was independent of serum cholesterol level. Light microscopy failed to reveal any detectable glomerular or interstitial changes in control or experimental rats. Thus, these results suggest that HMG-CoA reductase inhibition, in vivo, influenced glomerular MØ recruitment and that this effect was independent of circulating cholesterol levels.

DISCUSSION

Many in vivo studies have demonstrate that increased mesangial cellularity and matrix expansion are early, characteristic changes seen in progressive glomerular injury[22]. One critical cell that is frequently increased in glomeruli of experimental animals with progressive renal injury is the macrophage[3~8]. Moreover, macrophage depletion has been shown to reduce the degree of renal injury in some experimental models suggesting an important role for this cell in initiating glomerular injury[6,7]. The mechanisms involved in MØ-mediated injury are incompletely understood but increased production of cytokines, reactive oxygen molecules, and products of arachidonic acid metbolism have been implicated[6,7]. Recently, it has been demonstrated that a variety of human and rodent cells produce MCP-1, a potent chemotactic peptide that has a molecular weight of approximately 8700 daltons[10~12,18,23]. A number of growth promoting and inflammatory cytokines have been shown to increase expression of MCP-1 mRNA as well as the secreted peptide[10~12,23]. Experimental studies have also demonstrated that low density lipoprotein (LDL), and particularly modified LDL is a stimulus for production of MCP-1 by endothelial and vascular smooth muscle cells[24,25]. Indeed, MCP-1 has been proposed as playing an early role in the pathogenesis of atherosclerosis[26,27]. Glomerular mesangial cells have also been shown to produce MCP-1 in response to a variety of stimuli[10~12] and increased glomerular expression of MCP-1 has been described within minutes of induction of anti-Thy 1.1-induced glomerulonephritis[28]. This model of mesangial cell injury is also associated with an early infiltration of MØ in the mesangial region[28]. Recently, it has been demonstrated that mesangial cells have receptors for LDL and LDL has been shown to stimulate expression of MCP-1 in human mesangial cells[29].

Several experimental studies have suggested that the benefits of antilipemic therapy in models of progressive renal disease are associated with a reduction in circulating cholesterol[1]. In the current

experiments, we demonstrated that serum stimulation of mesangial cells was associated with the production of an unique monocyte chemotactic factor. This factor had chemotactic activity exclusively for MØ. Since we were able to completely inhibit this activity by a monoclonal antibody to MCP-1, we can reasonably conculde that this chemotactic factor was indeed MCP-1. It has been previously established that mesangial cells can produce MCP-1 and that various cytokines, including PDGF (the main mitogen in serum), can induce mesangial cells to produce MCP-1 as well as to increase mRNA expression for this peptide[10,11]. Our data are consistent with these results. However, what is unique about our findings is that this effect was modulated by inhibition of HMG-CoA reductase. The reduction in mesangial cell MCP-1 protein production by HMG-CoA reductase inhibition was also associated with a marked decrease in the expression of MCP-1 mRNA and this was reversed by the addition of mevalonate.

To determine if HMG-CoA reductase inhibition in vitro, and its consequent decrease in MCP-1, could have an in vivo correlate we studied cholesterol-fed rats. In this model, MØ have been observed to accumulate in the glomerulus prior to the development of proteinuria or glomerular damage[8,9]. Our results demonstrated that lovastatin prevented MØ from localizing in the glomerulus, independent of any reduction in serum lipid levels. Whether this effect could be attributed exclusively to reduction in mesangial cell production of MCP-1 cannot be concluded from these experiments, particularly since endothelial cells may also produce thia chemotactic peptide [23,25]. Nonetheless, these data suggest an important mechanism whereby lovastatin, in vivo, may exert a beneficial effect in modifying glomerular response to cholesterol-induced injury.

In summary, our studies demonstrated that mesangial cell expression and production of MCP-1 can be reduced by lovastatin inhibition of HMG-CoA reductase. This effect could be extrapolated to an in vivo model of cholesterol-induced glmerular injury in which glomerular influx of macrophages was prevented by lovastatin, independent of any action on serum cholesterol levels.

= 국문초록 =

인간 메산지움 세포의 단핵구 주화성 펩타이드 (MCP-1) 생성 : Lovastatin에 의한 조절

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저자들은 인간 메산지움세포의 배양시 혈청의 존재하에서 강력한 단핵구 주화성 펩타이드(MCP-1)가 생성됨을 밝혔으며 이때 lovastatin의 첨가에 의하여 3-hydroxy3-methylgutaryl coenzymeA (HMG CoA)를 억제함으로써 MCP-1의 생성이 현저히 감소됨을 볼 수 있었는데 이는 lovastatin 첨가 용량에 비례하였다. MCP-1 생성의 감소는 MCP-1 mRMA expression 감소를 증명함으로써 더욱 확인되었다. 이러한 lovastatin에 의한 효과는 mevalonate을 첨가하므로써 상쇄(역전)되었는데 이러한 점은 MCP-1 생성을 조절하는데에 있어 mevalonate의 비스테롤 isoprenoid대사물질이 관여함을 의미한다.

In vivo 실험으로서 2주간 고콜레스테롤 식이를 투여받은 백서에서 정상 식이를 투여받은 군보다 혈청 콜레스테롤이 7배 상승하였고 신 사구체에서 단핵구와 대식세포의 침윤이 두배 증가하였다. 이 고콜레스테롤 식이 백서중 lovastatin을 함께 투여받은 군에서는 혈청 콜레스테롤치는 높았으나 사구체의 ED-1 양성 세포의 침윤은 현저히 감소되었다. 따라서 이상의 실험 결과는 in vivo에서 HMG CoA reductase의 억제는 콜레스테롤에 의한 사구체의 대식세포 증가를 억제할 수 있고 이러한 것은 적어도 부분적으로는 메산지움 세포의 MCP-1 생성과 분비를 직접 억제함으로써 가능하다는 것을 나타내고 있다.

Key Words: Mesangial cell, MCP-1, HMG-CoA Reductase, Lovastatin, Chemoattractant

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