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Induction of Heat Shock Protein 70 Inhibits Tumor Necrosis Factorα-Induced Lipid Peroxidation in Rat Mesangial Cells

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ABSTRACT

Monocyte/macrophage infiltration is the well known initial features associated with the development of glomerular disease including non-immune mediated nephropathy. Tumor necrosis factor α(TNFα), a cytokine produced primarily by monocyte/macrophage, exhibits similar effects as observed at the initial stages and during the progression of glomerular injury. Because the mesangial cells are target cells for glomerular injury, the present study examined the effect of TNFα on glomerular mesangial cell membrane lipid peroxidation as an index of cytotoxicity attributing to TNFα. Primary culture of rat mesangial cell was established by incubation of glomeruli isolated from male Sprague-Dawley rat kidneys utilizing a standard sieving method. The levels of lipid peroxides in the mesangial cells were quantitated by malondialdehyde- thiobarbituric acid adduct formation. During an 8 hour incubation at 37℃, TNFα at 10 to 10,000 units/ml increased the levels of lipid peroxides dose dependently. Western blot analysis demonstrated that a short thermal stress induced heat shock response and the synthesis of heat shock protein 70(hsp70) in this mesangial cells. Further, this induction of hsp 70 prevented increase of lipid peroxides in the mesangial cells exposed to TNFα. These data suggest that TNFα-induced lipid peroxidation in the mesangial cells may have pathophysiological relevance to glomerular injury and prior induction of heat shock response may play a role in the cellular resistance against TNFα-induced glomerular injury.

Key Words: Primary cultured rat mesangial cells, Tumor necrosis factor, Heat shock proteins, Lipid peroxidation

INTRODUCTION

The mechanisms involved in the initial injury of the kidney and the progression to glomerulosclerosis are still not completely understood. However, the presence of monocytes and monocyte-derived foam cells (Fong and Lowrey, 1990; Saito and Atkins, 1990; Robin and Schreiner, 1991; Schreiner, 1991; Baud *et al.*, 1992; Pesek-Diamond, 1992; Wolthuis *et al.*, 1993; Baud and Adaillou, 1994) in the glomeruli is the prominent feature associated with the development of focal glomerulosclerosis in both human and experimental models of progressive renal disease(Saito and Athkins, 1990; Rovin and Schreiner, 1991; Schreiner, 1991; Pesek-Dia-

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mond *et al.*, 1992; Wolthuis *et al.*, 1993; Baud & Ardaillou, 1994). Tumor necrosis factor α(TNF α), a multifunctional cytokine with a crucial role in immune and inflammatory reactions, endotoxin shock, cachexia, and cell differentiation (Fong and Lowry, 1990), is produced mainly by monocytes and/or macrophages but also by intrinsic glomerular cells and other cells (Diamond and Pesek, 1991; Baud *et al.*, 1992). TNFα also displays a number of effects in vitro that are similar to the manifestations observed at the initial stage and during the progression of glomerular injury (Baud & Ardaillou, 1994 and refs therein). In addition, increased expression of TNFα has been observed both in animal models of renal disease (Diamond and Pesek, 1991; Tipping *et al.*, 1991) and in the patients with renal allograft rejection (Maury and Teppo, 1987) and the nephrotic syndrome (Suranyi *et al.*, 1993). Baud & Ardaillou (1994), therefore, proposed TNFα as an important autocrine and paracrine factor in glomerular injury.

The cytotoxic and cytostatic potential of TNFα against some transformed cell lines has been well documented. The mechanism whereby TNFα exerts its toxic effects, however, are not completely understood. In order to evaluate the possible relevance of TNFα-mediated cytotoxicity to the mechanism of glomerular injury, we have established the primary culture of rat mesangial cells, a target tissue involved in glomerular injury (Davies, 1994), in the present study . We then measured the levels of lipid peroxides in cultured mesangial cells in response to exogenous TNFα, as an index of TNFα-mediated cytotoxicity. Radeke *et al.* (1990) suggested the possible role of oxidative stress in TNFα-induced cytotoxicity in mesangial cells.

On the other hand, prior heat treatment and various other stresses rendered TNFα-sensitive cells resistant to it (Gromgowski, 1989; Jattela *et al.*, 1989; Jattela, 1990; Kusher *et al.*, 1990). It is well known that mammalian cells respond to stresses by preferentially synthesizing a set of proteins called heat shock proteins (Lindquist, 1986; Lindquist and Craig, 1988). Heat shock protein (hsp), especially of 70 kD (hsp70), has been implicated in the cellular resistance against a variety of stress conditions (Jaattela *et al.*, 1992; Jaattela and Wissing, 1993). Consequently, a possible role for heat shock proteins was suggested in protecting cells against cellular insults caused by TNFα. We, therefore, evaluated this hypothesis in cultured mesangial cells by examining the effect of hsp70 induction on TNFα-induced lipid peroxidation.

METHODS

Animals

Male Sprague-Dawley rats (ca. 200 g body weight) from the animal facility at Yonsei University College of Medicine were used for this study.

Reagents and media

Dulbecco's modified eagle's medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were obtained from GIBCO (Grand Island, New York, USA). Monoclonal anti-vimentin antibody and FITC conjugated antimouse IgG were purchased from Boehringer Mannheim (Indianapolis, IN, USA) and Cappel (Durham, NC, USA), respectively. 4, 6-dihydroxy-2-mercaptopyrimidine (2-thiobarbituric acid), sodium dodecyl sulfate and tetraethoxypropane were obtained from Sigma Chemical (St. Louis, MO, USA). Recombinant human TNFα was a gift from the Cetus Corporation (Emeryville, CA, USA) as a lyophilized sterile powder. Each vial had a bioactivity of 6.5 million units and a protein content of 0.29 mg. The purity before lyophilization was 95 % and the endotoxin level was 0.058 ng per vial. TNFα was reconstituted and further diluted in PBS. ^{35}S-Methionine (specific activity>1,000 Ci/mmol) was obtained from Amersham Corporation (Arlington Height, IL, USA). All other chemicals used were of analytical grade.

Rat mesangial cell culture

Primary culture of rat mesangial cells was established using collagenase-treated rat glomeruli as described by others (Kriesburg, 1983; Haper *et al.*, 1984; Striker and Striker, 1986). In brief, glomeruli were isolated from finely minced

renal cortical tissue by passing through successively a series of sieves of decreasing pore size (200, 150, 75 μm). The glomerular preparation retained on the 75 μm sieve was treated with 0.1% collagenase and trypsin at 37℃ for 30 min, and cultured in DMEM containing 10% heat inactivated FBS in a 37℃ humidified incubator. Confluent mesangial cells were subcultured by trypsinization and used for experiments between four and seven passages.

On phase-contrast microscopy, the identity of mesangial cells was confirmed by their elongated, stellate or fusiform appearance. Mesangial cells were further characterized by positive immunofluoresence staining of vimentin. Twenty thousand to 40,000 cells were plated in each well of 4 well Lab Tek chamber slides in DMEM with 10% FBS. Slides were fixed with 4 % paraformaldehyde in 20 mM PBS, pH 7.3, and permiabilized in 95% ethanol for 5~10 min at room temperature. The slides were then successively incubated with 1% BSA at 37℃ for 30 min followed by incubation with mouse anti-vimentin antibody (1:200). The slides were washed three times with DMEM and incubated with the FITC-conjugated goat anti-mouse IgG (1:50) for 45 min.

Measurement of lipid peroxides (LPO)

The thiobarbituric acid method of Ohkawa *et al.* (1979) was used to measure the level of LPO. Each 100 μl of sonicated mesangial cells was mixed with 100 μl of 8% sodium dodecyl sulfate, and then a reaction mixture consisting of 200 μl of 0.8% 2-thiobarbituric acid and 200 μl of 20% acetic acid was added. This solution was placed in a water bath and kept at 95℃ for 60 min. After stopping the reaction by cooling with tap water, the mixture was centrifuged at 15,000 g for 5 min to precipitate any interfering particulate materials. The amount of LPO formed was measured by spectrofluorometry (SPF-500 C; SLM instruments, Inc., Urbana, IL, USA) at emission wavelength 553 nm with excitation wavelength 515 nm.

Thiobarbituric acid solution (0.8%) was made just prior to use, on a hot plate, by stirring in less than the desired amount of distilled water. After the solution cooled, the exact volume was reached by the addition of distilled water.

For the preparation of LPO standard solution, tetraethoxypropane was diluted with ethanol at a concentration of 10 mM and stored in small aliquots at −70℃. At the time of use, 0.1 to 2.5 nmol/ml were prepared by dilution with PBS.

The values of LPO for samples were expressed as pmole per μg of tissue protein. Protein content was measured by micro Bradford method (1976) using bovine serum albumin as a standard.

Western blot analysis

Immunoblot analyses were performed to determine the synthesis of hsp70 in the mesangial cell using the method described by Towbin *et al.* (1979). Briefly, after heat shock at 45℃ for 30 min mesangial cells were solubilized in SDS-sample buffer and were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) with 10 % acrylamide for separating gel. Immediately after electrophoresis, the proteins were transferred from the polyacrylamide gel to nitrocellulose membrane by electroblotting (E.C. Apparatus, St. Petersburg, Fl, USA). Proteins transferred to the membrane were probed with a mouse monoclonal antibody, N6 F3-5 (a gift from Dr. William Welur, UCSF, USA), which recognizes both the constitute and inducible forms of hsp70. Binding of the antibody to hsp70 was detected by incubation of the blot with alkaline phosphatase conjugated goat antimouse IgG. 3-hydroxy-2-naphthonic acid 2, 4-dimethyanizide/Fast Red TK dye were used as substrates for alkaline phosphatase reaction.

In addition, the newly synthesized heat shock proteins after subjecting mesangial cells to heat treatment were metabolically labelled with 20 μCi of L-[^{35}S]-methionine per ml of media. Cellular proteins were then solubilized in SDS-sample buffer and were subjected to SDS-PAGE, with 10% acrylamide for separating gel. Autoradiograms were prepared using Kodak XAR-2 film.

Experimental protocols

For the study of the effects of TNFα on formation of LPO, confluent mesangial cells were

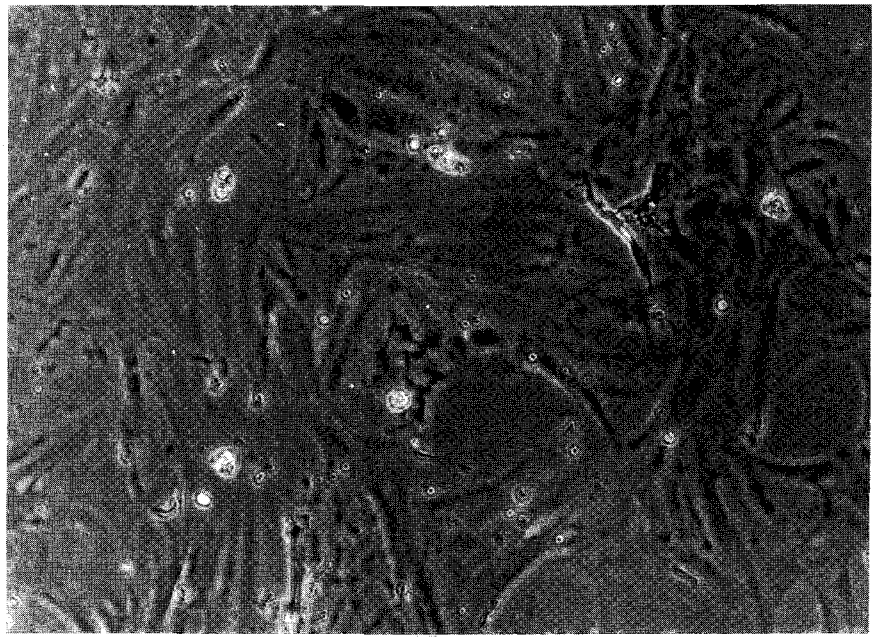

Fig. 1. Photomicrograph of primary cultured rat mesangial cells, subconfluent state. These cells are elongated, stellate or fusiform shape with distinct cell borders (×200).

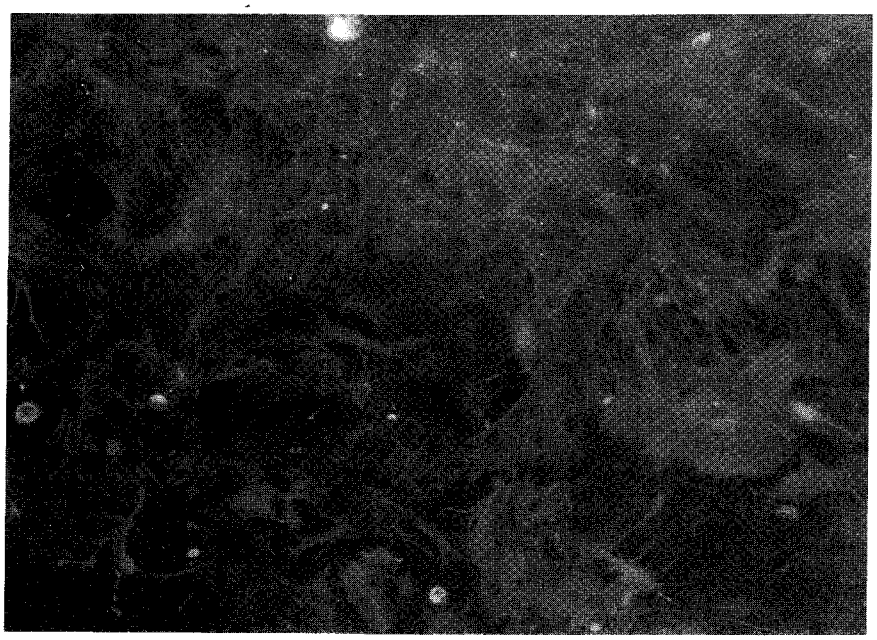

Fig. 2. Immunofluorescence study of primary cultured rat mesangial cells. Stained with anti-vimentin antibody and FITC-conjugated goat anti-mouse IgG (×200).

treated with different concentrations of TNFα for 8 hours.

In order to investigate the effects of heat treatment on cultured mesangial cells, confluent cells were heated at 45℃ for 30 min in a temperature- controlled, circulating water bath. The medium was changed before and after heat treatment. The cells were returned and remained ar 37℃ in at atmosphere of 5% CO_2-95 % air for 12~14 hours. Our pilot study revealed that at least 30 min of heating at 45℃ was necessary to induce heat shock response and the synthesis of heat shock proteins in primary cultured rat mesangial cells. To evaluate the effect of heat shock protein synthesis on TNFα induced lipid peroxidation, fresh medium containing TNFα was added after heating. Control cells were treated identically except for the priming heat shock.

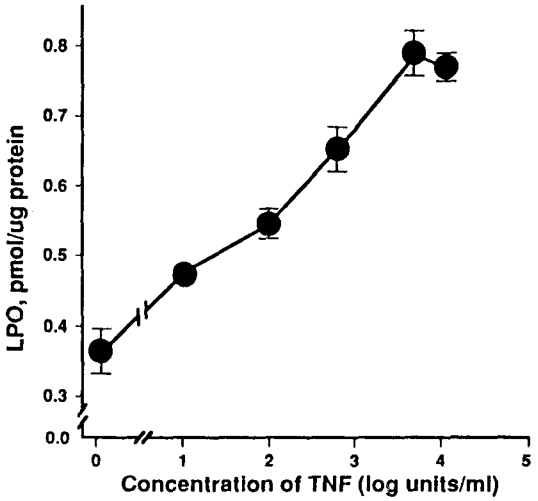

Fig. 3. Effects of various concentratrions of tumor necrosis factor α (TNFα) on lipid peroxidation in cultured rat mesangial cells. Values are expressed in terms of pmol lipid peroxides (LPO)/μg cellular protein. LPO in response to 100 units/ml TNFα is significantly higher ($p<0.05$; Student's t-test) than basal level. Values are means of a triplicate experiment±SE.

RESULTS

Rat mesangial cell culture

Cultured mesangial cells displayed the typical spindle or stellate morphology as shown in figure 1. Immunofluorescent staining of these cells displayed prominent cytoskeletal staining for vimentin with abundant parallel fibrils throughout the cytoplasm(fig. 2), and the cells were capable of growth in D-valine substituted medium.

Effects of TNFα on mesangial cellular lipid peroxidation

As summarized in figure 3, TNFα increased

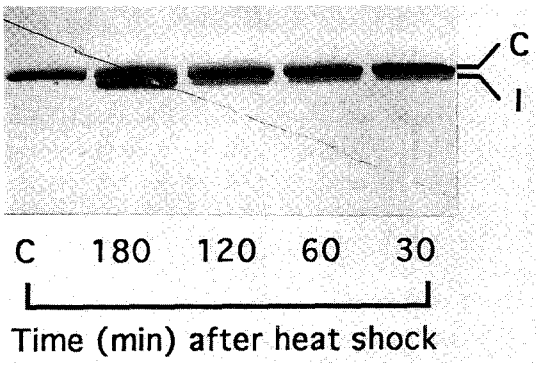

Fig. 4. Effects of heat treatment on mesangial cell heat shock protein 70 synthesis by Western blot analysis. Confluent mesangial cells were heated at 45℃ for 30min and moved to and remained in a 37℃ incubator in an atmosphere of 5% CO_2 and 95% air for increasing time intervals. Thirty μg of cellular proteins were electrophoresed and transferred onto a nitrocellulose membrane. Western blots were incubated with a mouse monoclonal antibody (1:1,000 dilution), N6 F3-5, which recognizes both the constitutive (c) and inducible (I) forms of hsp70. Binding of the antibody to hsp70 was detected by incubation of the blot with an alkaline phosphatase-conjugated goat antimouse IgG (1:1,000 dilution). 3-hydroxy-2-naphtholic acid 2, 4-dimethyanizide/Fast Red TQ dye were used as substrate for alkaline phosphatase reaction.

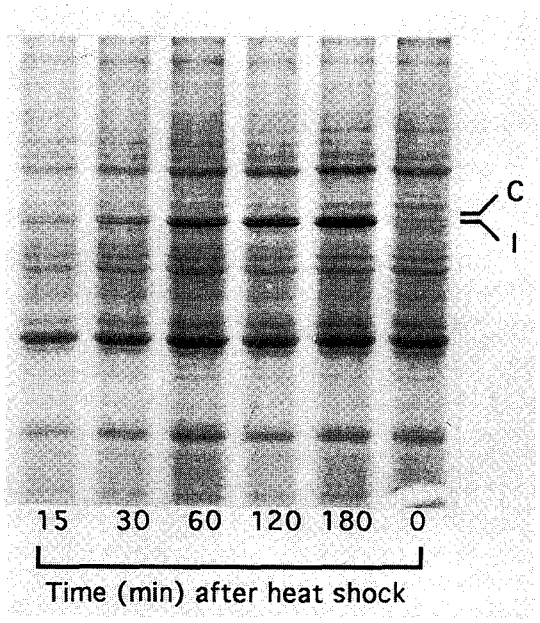

Fig. 5. Effects of heat treatment on newly synthesized heat shock protein 70 by metabolic labelling. During the recovery period, the newly synthesized proteins were labelled with 20 μCi of L-[^{35}S]-methionine per ml of media. Cellular proteins were solubilized in SDS sample buffer and subjected to SDS-PAGE with 10% acrylamide for separating gels. Autoradiograms were prepared using Kodak XAR-2 film.

lipid peroxidation dose dependently in primary rat mesangial cells. The level of lipid peroxides (LPO) stimulated by TNFα reached a plateau by 10^4 units/ml.

Induction of heat shock protein in mesangial cells by heat treatment

Western analysis demonstrated that heat treatment at 45℃ for 30 min induced hsp70 synthesis in cultured mesangial cells as shown in figure 4. Metabolic labelling for the newly synthesized proteins using ^{35}S-methionine also revealed increased hsp 70 synthesis in rat mesangial cells after heat treatment (Fig. 5).

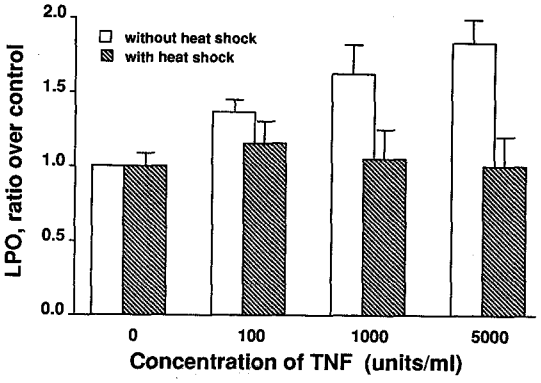

Fig 6. Effects of heat treatment on basal and TNF α-induced lipid peroxie (LPO) in cultured mesangial cells. Data are means±SE (n=3). Heat shock was induced by incubating cells at 45 ℃ for 30 min.

Effects of heat treatment on TNFα-induced lipid peroxidation in mesangial cells

To elucidate whether induction of hsp70 protects mesangial cells from TNFα-induced cytotoxicity, cells were treated with TNFα after heat treatment. Prior heat treatment totally blocked the generation of LPO stimulated by TNFα at a concentration of 100, 1,000, and 5,000 units/ml without any significant effect on basal level of LPO in mesangial cells, as depicted in figure 6.

DISCUSSION

Although the role of TNFα in the development of glomerular disease has been generally accepted (Baud & Ardaillou, 1994 and refs therein), the mechanisms of TNFα-mediated glomerular injury are yet poorly understood.

The use of cultured mesangial cells, a target cells in glomerular injury, has permitted speculation as to possible mechanisms that contribute to cytokine associated pathogenesis of glomerular disease (Davies, 1994). In the present study, primary cultured rat mesangial cells are established by adopting enzymatic isolation

(Haper *et al.*, 1984) in which isolated glomeruli by a standard graded sieving method are preincubated with collagenase and trypsin to generate a glomerular 'core' devoid of endothelial cells and epithelial cells.

These cultured mesangial cells increased the levels of lipid peroxidation in response to TNF α. Increased levels of lipid peroxides in a given tissue have been considered as index of increased production of reactive oxygen metabolites and a subsequent cytotoxic mechanism. Release of reactive oxygen metabolites is a proposed way where by TNFα might affect the function of mesangial cells (Radeke *et al.*, 1990). In addition, release of arachidonic acid or its metabolites as a result of increased phospholipase A_2 activity has been shown to be required for the TNFα-mediated cytotoxicity in TNF-sensitive cell lines (Jaattela, 1993). Increased metabolism of arachidonate could clearly be linked to TNFα-induced changes in mesangial lipid peroxidation. The actual biochemical mechanisms by which TNFα governs lipid peroxidation in mesangial cells remain a matter of future investigation.

This TNFα-induced lipid peroxidation did not alter cell viability estimated by LDH release (data not shown). However, peroxidation of lipid molecules influencing cellular growth-regulatory proteins normally dependent on membrane lipid integrity (Rice-Evans and Bundon, 1993) still could have some pathophysiological relevance associated with glomerular injury.

In this study, we clearly demonstrated that primary cultured rat mesangial cells also responded to heat shock by preferentially synthesizing heat shock proteins like other mammalian cells (Lindquist, 1986; Lidquist and Craig, 1988). Further, this induction of heat shock proteins ameliorated TNFα-induced lipid peroxidation in mesangial cells like in TNF-sensitive cell lines (Gromgowski, 1989; Jaattela *et al.*, 1989; Jaattela, 1990; Kusher *et al.*, 1990; Jaattela, 1993). If activation of phospholipase A_2 is inhibited by heat shock proteins like in TNF-sensitive cell line (Jaattela M, 1993), this inhibition of phospholipase A_2 may have some role in ameliorating lipid peroxidation in mesangial cells. Increased lipoxygenase and/or cyclooxygenase activity could be linked to generation of reactive oxygen free radicals leading to increase lipid peroxidation

Taken together, these results suggest that increased levels of lipid peroxides triggered by TNFα in mesangial cells may have pathophysiological relevance to the mechanisms of glomerular injury and that the induction of heat shock proteins may help ameliorating the progress of glomerular injury by reducing the toxic effect of TNFα in mesangial cells.

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=국문초록=

Heat Shock Protein 70이 흰쥐 배양 혈관간 세포에서 관찰되는 TNFα에 의한 지질과산화에 미치는 보호 효과

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사구체내 단핵구의 침윤은 면역학적뿐 아니라 비면역학적 사구체 질환 발생 초기에 특징적으로 관찰된다. 단핵구에서 합성되는 대표적인 사이토 카인인 tumor necrosis factor (TNF)α의 합성이 각종 사구체 질환과 관련되어 증가할 뿐 아니라 외부에서 투여한 TNFα는 사구체 질환의 발생과 진행에 수반된 유사한 증세를 초래한다. 따라서 본 연구에서는 사구체 질환의 표적세포인 혈관간 세포를 이용하여 TNFα에 의한 세포독성 기전을 검색하고자 하였다.

표준화된 채걸름법을 이용하여 사구체를 분리한후 collagenase로 처리하여 배양하므로써 혈관간 세포의 특징을 지닌 일차 배양 혈관간 세포계를 수립하였다. 세포독성의 지표로서 지질과산화물을 측정했을때, TNFα는 용량의존적으로 배양 혈관간 세포의 지질과산화를 증가시켰다. 배양 혈관간 세포를 45℃에서 30분간 처리했을 때 heat shock protein 70의 합성이 증가함을 western 분석으로 확인하였을 뿐 아니라, TNFα에 의한 지질과산화 증가를 효과적으로 억제함을 관찰하였다. 이상의 결과는 TNFα에 의한 지질과산화 증가가 사구체 질환의 발생이나 진행에 관여할 수 있음과 고온 전처리에 의해서 heat shock 반응을 초래하므로써 TNFα에 의한 사구체 손상을 보호할 수 있음을 시사하였다.