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OXIDATION OF MESANGIAL MATRIX USING A MIXED FUNCTION OXIDASE SYSTEM AUGMENTS ADHESION OF MACROPHAGES: POSSIBLE ROLE OF MACROPHAGE SCAVENGER RECEPTORS

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SUMMARY: Oxygen radicals are believed to play a role in the pathogenesis of aging and glomerular injury. Changes in cellular function may result from modification of the extracellular matrix with which they interact. We produced oxidized mesangial matrix protein using a mixed function oxidase system. Carbonyl content of the oxidized matrix was significantly increased. Adhesion of macrophages to the oxidized mesangial matrix was significantly enhanced, an effect which was significantly abrogated by scavenger receptor blockade with polyinosinic acid or dextran sulfate. Neither polyinosinic acid nor dextran sulfate diminished macrophage adhesion to unmodified matrix. These data demonstrate for the first time that mesangial matrix can undergo oxidation and that such oxidation may promote accumulation of macrophages in the mesangium via interaction of macrophage scavenger receptors with oxidized matrix proteins.

Accumulation of macrophages at sites of tissue injury is regarded as a key event in the pathogenesis of glomerulosclerosis (1) and atherosclerosis as well (2). In animal models of glomerular injury measures that diminish macrophage accumulation in the glomerular mesangium such as x-irradiation, use of antimacrophage serum and induction of essential fatty acid deficiency have all been shown to ameliorate the development of glomerulosclerosis (3-5). Mesangial expansion, consisting of increased mesangial cell proliferation and matrix accumulation, is regarded as a precursor to the development of glomerulosclerosis (6-8), a common final pathway for progressive renal injury regardless of the etiology of the initial insult. Macrophages, a normal component of the glomerular mesangium, are significantly increased in number following glomerular injury and have been strongly implicated in promoting mesangial expansion most likely via their rich secretory array of mitogens and fibrogenic cytokines (9). Macrophage adhesion to extracellular matrix may play an important role in accumulation of these

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cells in the mesangium. Whereas the extracellular matrix had once been thought to simply serve as a scaffolding for cells, it has been increasingly recognized that the extracellular environment can profoundly influence cellular function (10-12). It has been shown, for example, that modifying the mesangial matrix such as by nonenzymatic glycation as occurs in diabetes mellitus significantly alters mesangial cell function compared to unmodified matrix (13,14).

Oxygen radicals have been implicated in the pathogenesis of aging as well as glomerular injury (15-29). For example, oxidation of membrane-associated lipids may occur with subsequent adverse effects of these products on the glomerulus (18). While oxygen radicals can cause lipid peroxidation, oxygen radicals are known to modify proteins as well (15,16). Increased protein oxidation has been demonstrated to occur in aging (15, 27) and has been shown to alter function of enzymes such as erythrocyte glucose 6-phosphate dehydrogenase (16). Presumably, mesangial matrix proteins may also be susceptible to oxidation as are other proteins but measurements of oxidation of mesangial matrix and effects on cellular function including adhesion have never been reported. In light of the potential role of oxygen radicals as well as macrophage accumulation in the mesangium in glomerular disease, we attempted to oxidize mesangial matrix in vitro, quantitate the degree of oxidation, and determine whether oxidative modification of matrix proteins could modulate adhesion of macrophages.

MATERIALS AND METHODS

Mesangial cell culture. Mesangial cell culture was carried out as described (30,31). In brief, kidneys were removed from pentobarbital anesthetized Sprague-Dawley rats and glomeruli isolated by differential sieving. Glomeruli were seeded into plastic culture flasks in RPMI 1640 (Gibco) containing penicillin 50U/ml, streptomycin 50 μg/ml and 10% fetal calf serum (FCS, Gibco) at 37°C in a humidified 95% air 5% CO_2 environment. After 3 weeks in primary culture mesangial cells were subcultured by incubating with 0.25% trypsin/EDTA solution (Gibco) and seeded in fresh media. Mesangial cells were subcultured at 7-10 day intervals and were used for experiments from passages 4 through 8. These mesangial cells represent an apparently uniform cell population as described (30).

Generation of mesangial matrix. Mesangial cells were seeded at 10,000 cells/ml onto 6-well plastic culture plates in RPMI 1640 containing penicillin/streptomycin solution and 10% FCS and incubated at 37°C. Media was changed every 3 days. After 3 weeks cells were incubated with 2.5 mM ammonium hydroxide and 0.1% Triton X-100 for 15 minutes to remove mesangial cells (13,14). Each well was then washed 3 times with phosphate buffered saline (PBS) followed by 1 wash with distilled water. Absence of cells was confirmed by examination of matrix by phase contrast microscopy. As described elsewhere this protocol has been shown to remove mesangial cells leaving behind insoluble mesangial matrix (13).

Oxidation of mesangial matrix using a mixed function oxidase system. After preparing acellular insoluble mesangial matrix, matrix in selected wells was oxidized by incubating for 90 minutes with a mixed function oxidase system consisting of 25 mM ascorbate, 2mM $FeCl_3$, and 2.4 mM EDTA at 30°C. Wells in control plates were incubated with 2.4 mM EDTA solution without ascorbate or $FeCl_3$. At the end of the incubation period matrix in all wells was washed 5 times with phosphate buffered saline and used for experiments.

Determination of matrix protein carbonyl content using 2,4-dinitrophenylhydrazine. Oxidative modification of proteins by mixed function oxidase systems results in the production of protein carbonyl derivatives which can be quantitated by incubation with 2,4-dinitrophenylhydrazine (2,4-DNPH) to form stable protein hydrazones (15,16,32). This method has been used to quantitate protein oxidation as a consequence of aging and other processes (15,16,32). To determine whether incubation of mesangial matrix with a mixed function oxidase system as described above resulted in oxidation of matrix, mesangial matrix was scraped from untreated and mixed function oxidase-treated wells and pipetted into 1.5 ml microcentrifuge tubes and proteins precipitated in 10% trichloroacetic acid at 4°C. After microcentrifuging at 3,000 rpm for 15 minutes the supernatant was discarded and the precipitate resuspended in 500 μl of 10mM 2,4-DNPH in 2N HCl. Control samples of untreated and mixed function oxidase-treated matrix were incubated with 2N HCl without 2,4-DNPH as well. After room temperature incubation for 60 minutes with vortexing every 10 minutes 500 μl 20% trichloroacetic acid was added to each sample and microcentrifuged at 11,000g for 3 minutes. The supernatant was discarded and samples washed 3 times with 1 ml 1:1 ethanol-ethylacetate. The precipitate was redissolved in 6M guanidine solution in 20mM potassium phosphate adjusted to pH 2.3 with 0.05% trifluoroacetic acid, any remaining insoluble material removed by microcentrifuging and absorbance measured at 374 nm. Carbonyl content was calculated using the molar absorption coefficient of 22,000 $M^{-1}cm^{-1}$ (32). Protein content of samples was determined using the Biorad method (33) and results expressed as nmol carbonyl per mg matrix protein.

Measurement of adhesion of macrophages to mesangial matrix. To determine whether adhesion of macrophages to mesangial matrix is modulated by oxidation of matrix, J774.16 macrophages (31) grown in plastic culture dishes at 37°C in a humidified 95% air 5% CO_2 in Dulbecco's MEM containing 50 U/ml penicillin, 50 μg/ml streptomycin, 1% nonessential amino acid solution and 10% FCS were detached by gentle pipetting and suspended at 300,000 cells per ml in fresh media. The cell suspensions were added to wells containing unmodified or oxidized matrix and incubated at 37°C for 3 hours. At the end of the incubation period nonadherent cells were removed by gentle washing with PBS and adherent cells counted using phase contrast microscopy. An eyepiece grid was used and the mean number of adherent cells within the borders of the grid was determined.

Role of scavenger receptors in adhesion of macrophages to mesangial matrix. Macrophages are known to possess scavenger receptors which can bind oxidized proteins (34). To determine whether interaction of macrophage scavenger receptors with oxidized matrix proteins could modulate adhesion of cells to this substrate, J774.16 macrophages were suspended at 300,000 cells/ml in media alone or media containing the scavenger receptor binding agents polyinosinic acid (10 μg/ml,Sigma) or dextran sulfate (50 μg/ml, Sigma) for 15 minutes (34). Cell suspensions were then added to either unmodified or oxidized mesangial matrix and after 3 hours incubation at 37°C nonadherent cells were removed by washing gently with PBS and adherent cells recorded as above.

RESULTS

Carbonyl content of mesangial matrix incubated with a MFO system. As illustrated in figure 1, carbonyl content, a measure of protein oxidation, was significantly enhanced by incubation with a mixed function oxidase system. This data suggests that mesangial matrix underwent oxidative modification under these experimental conditions.

Adhesion of macrophages to unmodified and oxidized matrix. Macrophage adhesion to oxidized mesangial matrix was found to be significantly enhanced compared to matrix that was unmodified (unmodified, 54 $\pm$ 5 cells/field; oxidized, 97 $\pm$ 4 cells/field, $p < 0.001$, 4

experiments performed in triplicate). This data is illustrated in figure 2 and suggests that oxidative modification of mesangial matrix augments the adhesion of macrophages.

Role of scavenger receptors in adhesion of macrophages to oxidized matrix. Macrophages that were preincubated with polyinosinic acid (10 μg/ml) and dextran sulfate (50 μg/ml), agents which bind to scavenger receptors (34), demonstrated significantly less adhesion to oxidized matrix compared to macrophages preincubated in media alone as illustrated in figure 3 (unmodified, 52 $\pm$ 4 cells/field; oxidized, 91 $\pm$ 5 cells/field, $p < 0.001$ compared to unmodified; oxidized plus polyinosinic acid, 54 $\pm$ 4 cells/field, $p < 0.001$ compared to oxidized; oxidized plus dextran sulfate, 50 $\pm$ 4 cells/field, $p < 0.001$ compared to oxidized, n=4 experiments performed in triplicate). However, neither of these agents significantly modified adhesion of macrophages to unmodified matrix as also illustrated in figure 3. These data suggest that enhanced adhesion of macrophages to oxidized mesangial matrix may be mediated via scavenger receptor binding to oxidized matrix proteins.

DISCUSSION

Previously the extracellular matrix had been conceptualized as simply a static framework or scaffolding for cells, providing structural support but otherwise having no significant interaction with the cells. The mesangial matrix as well as extracellular matrix of other cells is a dynamic structure, capable of growth factor-like effects and capable of altering cellular function via interaction of cell surface receptors such as integrins with extracellular matrix components (35,36). The mechanism for mesangial expansion has been under intense investigation and in

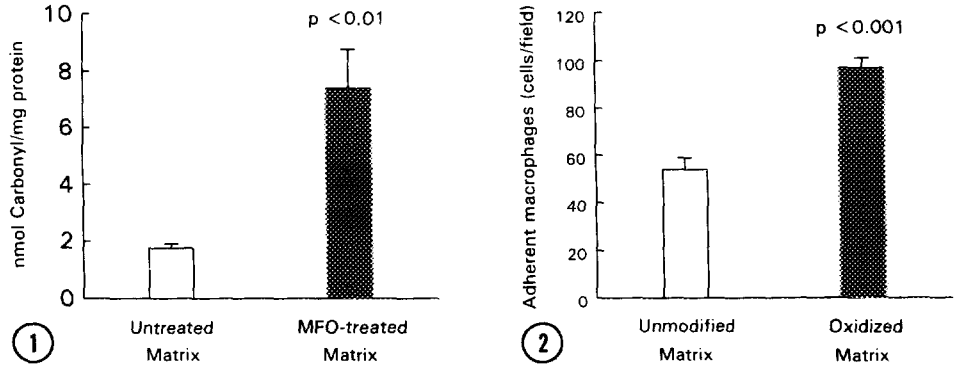

Fig. 1. Carbonyl content of mesangial matrix oxidized using a mixed function oxidase system (MFO) versus unmodified matrix. Results are from 4 experiments carried out in triplicate. Data compared using Student's unpaired t test.

Fig. 2. Adhesion of macrophages to unmodified and oxidized matrix. Adhesion of macrophages was measured after a 3-hour incubation with unmodified or oxidized matrix as detailed in *Methods*. Results represent mean $\pm$ SEM adherent macrophages per field from 4 experiments carried out in triplicate. Data compared using Student's t test.

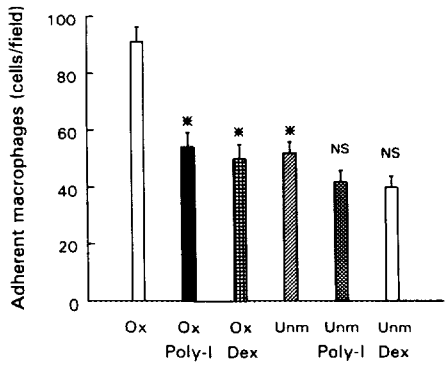

Fig. 3. Role of scavenger receptors in macrophage adhesion to oxidized matrix. Macrophages were preincubated with the scavenger receptor binders polyinosinic acid (Poly-I, 10 μg/ml) or dextran sulfate (Dex, 50 μg/ml) for 15 minutes and then adhesion to unmodified (Unm) or oxidized (Ox) matrix measured. Data are mean $\pm$ SEM from 4 experiments performed in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. *p <0.001 compared to oxidized matrix; NS: not significant compared to unmodified matrix.

recent years it has been recognized that changing the quality as well as the quantity of mesangial matrix, for example by nonenzymatic glycation, may have significant consequences on cellular function including alterations in cell proliferation and matrix synthesis. While proteins including those of the extracellular matrix are susceptible to glycation and formation of adducts with lipid peroxidation products it is plausible that the mesangial matrix can be oxidized as can other proteins. In the present study we have reported for the first time that mesangial matrix proteins can be oxidized and such oxidative modification appears to enhance adhesion of macrophages to this surface.

Increased adhesion of macrophages to oxidized mesangial matrix in the present study appeared at least in part to possibly be due to scavenger receptor binding as this enhanced adhesion could be attenuated to control levels (adhesion to unmodified matrix) by agents which bind to scavenger receptors. As other oxidized proteins are known to bind to macrophage scavenger receptors (34), it is plausible that the scavenger receptor may bind to oxidized matrix proteins as well. However, our evidence is indirect and we have not identified at this time which component of the modified mesangial matrix might be recognized by the macrophage scavenger receptor. Increased accumulation of macrophages in the mesangium might result in increased exposure of the mesangium to macrophage secretory products which may promote mesangial expansion and perhaps cause further matrix oxidation as they also produce oxygen radicals. While there are many mechanisms by which antioxidant therapy may exert a salutary effect on glomerular injury as has been shown by other investigators (19), one possible contributing mechanism might be diminished oxidation of the mesangial matrix and hence diminished macrophage accumulation in the mesangium although this must be regarded as highly speculative.

In summary, the present study demonstrates that mesangial matrix proteins can undergo oxidative modification in vitro using a mixed function oxidase system as evidenced by increased carbonyl content. Adhesion of macrophages to mesangial matrix appears to be enhanced by oxidation of this substrate, an effect that may be mediated via interaction of macrophage scavenger receptors with oxidized matrix proteins. Oxidative modification of mesangial matrix may play a role in the pathogenesis of glomerulosclerosis by promoting accumulation of macrophages in the mesangium.

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