BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **233,** 50–55 (1997)
ARTICLE NO. RC976406

Metal-Catalyzed Oxidation of Extracellular Matrix Proteins Disrupts Integrin-Mediated Adhesion of Mesangial Cells

Joseph Mattana, Linda Margiloff, and Pravin C. Singhal
Department of Medicine, Long Island Jewish Medical Center, New Hyde Park, New York 11040; and Long Island Campus for the Albert Einstein College of Medicine, Bronx, New York 10461

Received February 25, 1997

We undertook the present study to determine whether oxidation of extracellular matrix could alter RGD (arginine-glycine-aspartic acid)-integrin interaction in mesangial cells. Mesangial cells demonstrated significantly less adhesion to matrix oxidized using a metal-catalyzed oxidation system and lost their typical spindle-shaped morphology. N-*tert*-butyl-α-phenylnitrone reversed in part both oxidation and impaired adhesion to matrix. Mesangial cells adhered to plates coated with GRGDSP but demonstrated impaired adhesion to oxidized GRGDSP. Oxidation of this peptide was demonstrated using immunoblot analysis with an antibody to dinitrophenylhydrazine bound to carbonyl groups on oxidized amino acid residues. This represents the first report demonstrating that oxidative modification of extracellular matrix impairs integrin-mediated adhesion and suggests that the mechanism may be oxidative modification of one or more amino acids in the RGD sequence. These data suggest a new mechanism by which cell-matrix interaction may be altered in disease states characterized by enhanced oxidative stress.

Extracellular matrix (ECM) is an important regulator of cellular function (1-7). While the physicochemical interactions between normal ECM and integrins as well as the signal transduction pathway through which integrin-ECM interaction modulates cellular function continue to be delineated in increasing detail (8-10), little is known regarding how biochemical modification of the ECM can modulate its interaction with cells. Nonenzymatic glycation of matrix, for example has been shown to alter proliferation and matrix synthesis by mesangial cells (11-12) and adhesion of macrophages (13). However, oxidation may be an important modification of ECM as well. It is known that with aging there is an accumulation of oxidized proteins which may play at least some role in the pathophysiology of aging itself (14). A number of disease states other than aging are associated with enhanced oxidative stress and a number of proteins have been shown to be susceptible to oxidation (15-18). We have shown that mesangial ECM is susceptible to metal catalyzed oxidation (MCO) and that this augments adhesion of macrophages and nitric oxide generation (19,20). Mesangial cell adhesion to ECM takes place principally through integrin binding to ligand in the ECM (21). The best described integrin ligand consists of the sequence arginine-glycine-aspartic acid (RGD, 8-10). In the present study we attempted to determine whether MCO of RGD-containing proteins in the ECM could alter integrin-mediated adhesion of glomerular mesangial cells.

METHODS

Oxidation of ECM using a MCO system and measurement of protein carbonyl content. Matrigel (Collaborative Biomedical Products, Becton-Dickinson, Bedford, MA), a murine ECM derived from the Engelbreth-Holm-Swarm (EHS) tumor, was diluted in serum-free RPMI 1640 at 4°C, added onto 24-well plastic tissue culture plates and incubated at room temperature to produce an adherent coating of ECM. Wells were washed with PBS, pH 7.4, and selected wells were incubated with a MCO system consisting of 2 mM $FeCl_3$, 2.4 mM EDTA and 25 mM ascorbate or EDTA alone at 37°C and then washed as described (19). Carbonyl content as a measure of protein oxidation was then determined spectrophotometrically using 2,4-dinitrophenylhydrazine (DNPH) as described elsewhere in detail (19). Protein content was determined using the method of Bradford (22).

Incubation of mesangial cells with oxidized and unmodified EHS ECM and measurement of adhesion. Mouse mesangial cells transfected with non-capsid forming, nonreplicating SV-40 virus were generously provided by Dr. Eric Neilson (University of Pennsylvania, Philadelphia, PA). Hence in all experiments both ECM and mesangial cells were of murine origin. The cells were grown in tissue culture flasks in Dulbecco's MEM (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 50 U/ml penicillin and 50 μg/ml streptomycin in a humidified 95% air, 5% CO_2 environment at 37°C. For experiments mesangial cells were washed 3 times with calcium-free PBS and were detached by a brief exposure to 0.25% trypsin/EDTA solution. Cells were resuspended in DMEM containing 2.5% FCS, seeded onto unmodified or oxidized ECM and incubated at 37°C for 24 h. Wells were gently washed with PBS containing 1 mM $CaCl_2$ and

0006-291X/97 $25.00

adherent cells counted using phase contrast microscopy using an inverted microscope fitted with an eyepiece grid as described (19).

Effect of the radical spin trap N-tert-butyl-α-phenylnitrone (PBN) on carbonyl content of MCO-treated ECM and effect on mesangial cell adhesion. It is plausible that adhesion to ECM treated with the MCO system could be due to effects other than oxidative modification. In an attempt to exclude this possibility the radical spin trap PBN was used (16). In these experiments selected wells coated with ECM were preincubated with PBN (100 μg/ml) before addition of the MCO system (also containing PBN 100 μg/ml) as described above and the agents incubated with ECM for 1 h. Other wells were treated with EDTA alone or the MCO system alone. ECM was then washed, mesangial cells were seeded onto the wells and adhesion measured as above. ECM from selected wells on each culture plate was not seeded with mesangial cells but was instead used for carbonyl assay.

Mesangial cell adhesion to oxidized and unmodified GRGDSP-coated plates. If RGD-containing ECM proteins play a significant role in mesangial cell adhesion to ECM then mesangial cells should be able to adhere to GRGDSP-coated plates. To test this hypothesis 96-well plastic ELISA plates (non-tissue culture treated) were incubated with 50 μg of GRGDSP (Peninsula Laboratories, Belmont, CA) in dH_2O and evaporated to dryness overnight under a laminar flow hood. One set of wells was then treated with EDTA alone while another set was incubated with the MCO system described above. After 1 h plates were washed with PBS and then all wells were incubated in 3% BSA in PBS for 1 h at 37°C to block remaining sites (23). Other investigators have shown that mesangial cells adhere only minimally to BSA-coated plates (23). Wells were then washed with PBS and were seeded with mesangial cells in 2.5% FCS as described in the experiments above. After 1 h wells were washed with PBS and adherent cells counted as above.

Immunoblot analysis of oxidized GRGDSP using antibodies to DNPH. To determine whether GRGDSP is susceptible to oxidative modification we carried out immunoblotting using the OxyBlot oxidized protein detection kit (Oncor, Gaithesburg, MD) which utilizes a highly specific antibody to DNPH bound to oxidized proteins. GRGDSP was incubated for 1 h with the MCO system or EDTA alone at 37°C. At the end of the incubation period the samples were incubated with 10 mM DNPH or derivitization control for 15 min and were then incubated with neutralization solution according to the manufacturer's instructions. Samples were then dot-blotted onto nitrocellulose membranes prewetted with PBS pH 7.2 using a Bio-Rad dot-blot apparatus. Wells were washed with PBS and the membrane was blocked in 1% BSA/PBS pH 7.2/0.05% Tween-20 for 1 h. The membrane was then incubated with a 1:150 dilution of anti-DNPH antibody for 1 h at 25°C rinsed twice with PBS/Tween, then washed once for 15 minutes then twice for 5 minutes each. Membranes were then incubated with secondary antibody (HRP-conjugated goat anti-rabbit IgG) at 1:300 dilution for 1 h. Membranes were then washed, incubated with chemiluminescent reagent and autoradiographs produced using Kodak X-OMAT AR film.

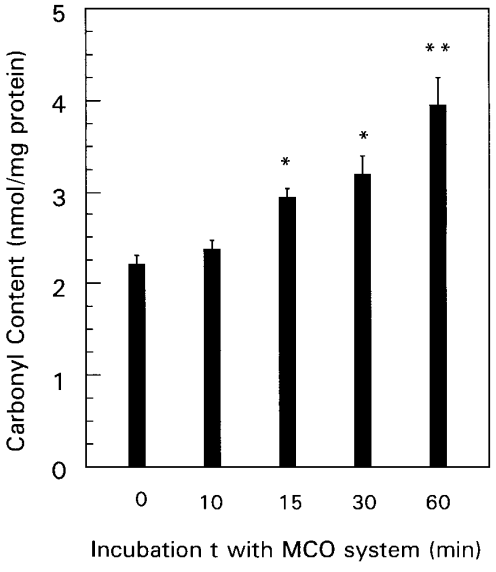

FIG. 1. Time course relationship between duration of MCO system treatment and EHS ECM carbonyl content. ECM was incubated for varying time periods with a MCO system ($FeCl_3$/EDTA/ascorbate) and carbonyl content measured using 2,4-DNPH as described in Methods. Results are from 3 experiments carried out in triplicate. Statistical analysis carried out using ANOVA with Newman-Keuls multiple range testing. *p <0.05 compared to control; **p <0.01 compared to control.

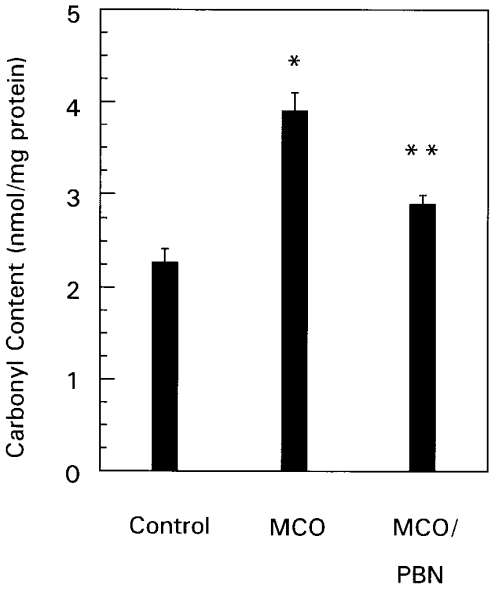

FIG. 2. Effect of radical spin trapping on ECM carbonyl content. ECM was incubated under control conditions, with $FeCl_3$/EDTA/ascorbate alone (MCO) or the MCO system plus 100 μg/ml N-*tert*-butyl-α-phenylnitrone (PBN) for 60 min. Carbonyl content was then measured as described in Methods. Results are from 4 experiments carried out in triplicate. Statistical analysis carried out using ANOVA with Newman-Keuls multiple range testing. *p <0.01 compared to control; **p <0.01 compared to MCO without PBN.

RESULTS

Carbonyl content of oxidized EHS ECM. EHS ECM which was incubated with a MCO system consisting of $FeCl_3$, EDTA and ascorbate demonstrated a significant time-dependent increase in protein carbonyl content as illustrated in Fig. 1. For ECM treated under control conditions carbonyl content was 2.2 ± 0.1 nmol/mg protein whereas for ECM oxidized for 1h carbonyl content was 3.95 ± 0.3 nmol/mg protein ($p < 0.01$, n=3 experiments carried out in triplicate). These results demon-

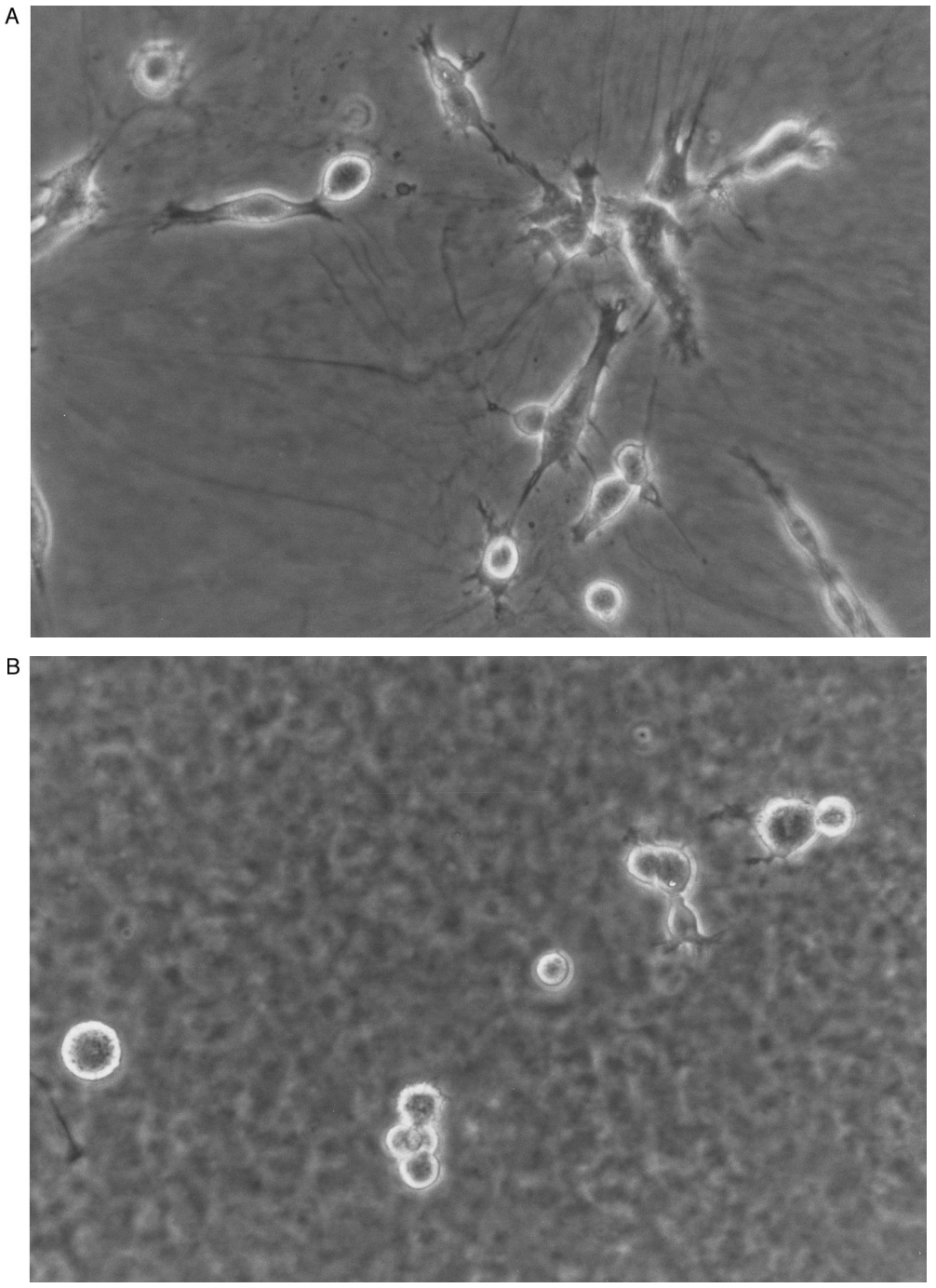

FIG. 3. Phase contrast photomicrographs of mesangial cells on unmodified and oxidized ECM. Mouse mesangial cells were seeded onto ECM that was either unmodified (control, A) or ECM oxidized with a MCO system (B) as described in Methods. After 24 h wells were washed and cells examined via phase contrast microscopy.

strate that EHS ECM is susceptible to oxidation as is mesangial ECM.

Effect of the radical spin trap PBN on carbonyl content of ECM. It is important to exclude the possibility that changes in properties of ECM as a consequence of treatment with $FeCl_3$/EDTA/ascorbate could be due to modifications of ECM other than oxidation. This can be determined by use of a radical spin trap such as PBN. As illustrated in Fig. 2 coincubation with PBN was able to significantly attenuate the increase in car-

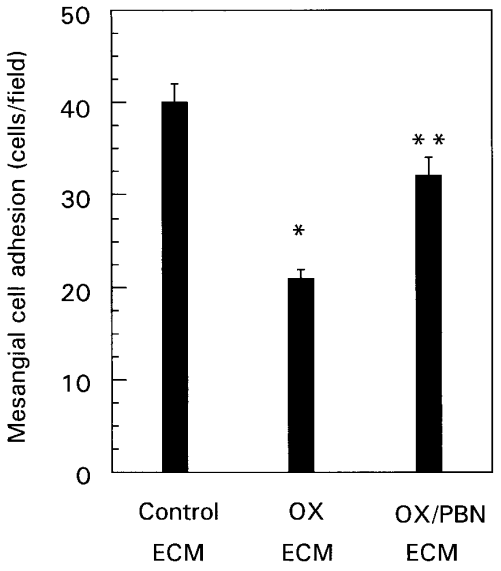

FIG. 4. Mesangial cell adhesion to unmodified and oxidized ECM and specificity of oxidative modification of ECM. ECM was kept under control conditions, was oxidized via a 1 h incubation with the MCO system, or coincubated with the MCO system plus 100 μg/ml PBN as described in Methods. Mouse mesangial cells were then seeded onto these matrices and incubated at 37°C for 24 hours. Wells were then washed with PBS containing 1 mM $CaCl_2$ and adherent cells counted using phase contrast microscopy as described in Methods. Data represent means ± SEM cells/field from 4 experiments each carried out in triplicate. Statistical comparisons carried out using ANOVA with Newman-Keuls multiple range testing. *p <0.01 compared to control; **p <0.01 compared to MCO treatment alone.

bonyl content with MCO treatment (control, 2.26 ± 0.1 nmol/mg protein; MCO treatment, 3.90 ± 0.2, p <0.01; MCO/PBN treatment, 2.89 ± 0.1, p <0.01 compared to MCO treatment, n=4 experiments in triplicate). Hence PBN appears to be able to significantly antagonize the ability of the MCO system to oxidize ECM.

Mesangial cell adhesion to oxidized and unmodified ECM and effect of PBN. As shown in Fig. 3A, mesangial cells incubated on unmodified ECM-coated wells adhered and acquired a spindle-like structure which represents the typical morphology of mesangial cells on EHS ECM (24). By contrast, mesangial cells on oxidized ECM were largely rounded up and poorly adherent (Fig 3B). Fig. 4 compares mesangial cell adhesion between unmodified ECM, oxidized ECM and ECM coincubated with the MCO system and PBN. As this figure illustrates the decrease in mesangial cell adhesion to oxidized ECM was attenuated when ECM was coincubated with PBN plus the MCO system (control, 40 ± 2 cells/field; MCO treated ECM, 21 ± 1, p <0.01; MCO/PBN treatment, 32 ± 2 cells/field, p <0.01 compared with MCO alone, n=4 experiments in triplicate). These data demonstrate that mesangial cell adhesion to oxidized ECM is diminished and that this is not due to nonspecific effects of MCO treatment on the ECM.

Adhesion of mesangial cells to oxidized and unmodified GRGDSP. Mesangial cells demonstrated significantly less adhesion to plates coated with oxidized GRGDSP compared to nonoxidized GRGDSP (nonoxidized GRGDSP, 43 ± 2 cells/field; oxidized GRGDSP, 15 ± 2, p <0.01; BSA alone [control], 11 ± 3, p NS compared with oxidized GRGDSP, n=4 experiments carried out in triplicate). These data, which are illustrated in Fig. 5, demonstrate that adhesion of mesangial cells to the integrin ligand-containing peptide GRGDSP which has been oxidized is significantly diminished.

Immunoblot analysis of GRGDSP treated with a MCO system. Fig. 6 illustrates the results of immunoblot analysis of GRGDSP treated under control conditions or with a MCO system using an antibody to DNPH bound to carbonyl groups in oxidized proteins. As this figure illustrates there is enhanced antibody binding to the MCO system-treated peptide compared to control in which comparatively very little antibody binding can be seen. This increase is evident at both 1 and 4 μg of peptide. These results demonstrate that GRGDSP is susceptible to oxidative modification when incubated with a MCO system.

DISCUSSION

While once thought to provide little more than an inert structural support for cells, the ECM is now recog-

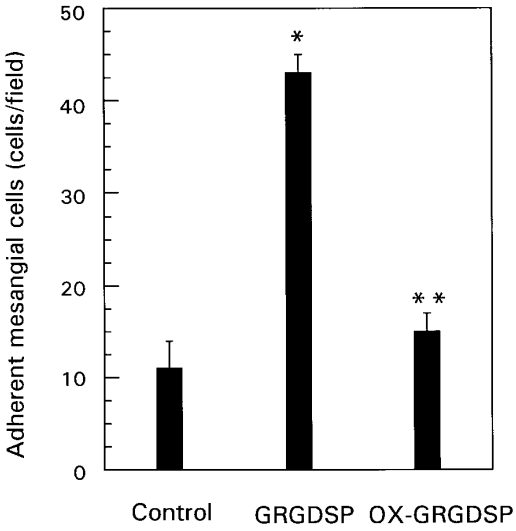

FIG. 5. Mesangial cell adhesion to oxidized GRGDSP. Plastic 96-well plates were coated with GRGDSP and then treated under control conditions or oxidized using a MCO system as described in Methods. After blocking with 3% BSA mesangial cells were seeded onto the plates and adhesion was measured as described in Methods. Data are from 4 experiments carried out in triplicate. Statistical comparisons carried out using ANOVA and Newman-Keuls multiple range testing. Control: BSA coating alone. *p <0.01 compared to control (BSA alone); **p NS compared to control, p <0.01 compared to oxidized GRGDSP.

nized to be an important regulator of cellular function through interaction of cell surface receptors with ligands in the ECM. In recent years great progress has been made in elucidating the signal transduction pathway from integrin-ECM interaction to altered cellular function as reviewed elsewhere (8,9). However, less is known regarding how modification of ECM as may occur in inflammatory disorders and aging may alter the interaction of the ECM with cells and alter their function.

Enhanced oxidative stress is believed to play an important role in a number of renal diseases (25-30) as well as atherosclerosis (31) though most such studies have evaluated lipid peroxidation. We have previously reported that mesangial ECM protein is susceptible to oxidation (19) and that oxidation increases adhesion of macrophages, possibly through scavenger receptor interaction with oxidized moieties in the ECM (19). We have also recently reported that macrophage interaction with oxidized ECM enhances nitric oxide generation (20). In the present study we have shown for the first time that oxidation of ECM significantly impairs the ability of mesangial cells to adhere to this substrate and that this appears at least in part to be due to modification of one or more amino acids of the integrin ligand RGD. This was suggested by the observation that while mesangial cells adhered to plates coated with the integrin ligand-containing peptide GRGDSP, adhesion to oxidized GRGDSP was impaired. Using immunoblot analysis, we demonstrated that GRGDSP underwent oxidation as a result of exposure to the MCO system. Hence it appears that oxidative modification of this peptide impairs its ability to bind to mesangial cell integrins though it is plausible that one or more amino acids other than those of the RGD sequence itself might have been modified and that these changes could result in altered integrin binding as well. While in contrast we have reported that oxidation of ECM enhances adhesion of macrophages, this appeared to have been due to interaction of scavenger receptors, which are expressed in relatively high density on macrophages (32), with oxidized moieties in the ECM. While mesangial cells do display scavenger receptor functions such as uptake of oxidized LDL they adhere principally through integrins. Hence modifications of ECM which affect integrin ligands are likely to have a significant impact upon mesangial cell adhesion.

It is unclear what the physiological consequences of altered mesangial cell integrin-ECM interaction might be. Other investigators have shown that the ECM plays an important role in modulating mesangial cell morphology to a spindle-type (23) characteristic of smooth muscle cells. Indeed, the mesangial cell is regarded as a modified smooth muscle cell but in disease states may take on more of a fibroblast-like morphology which may be important in the development of glomerulosclerosis

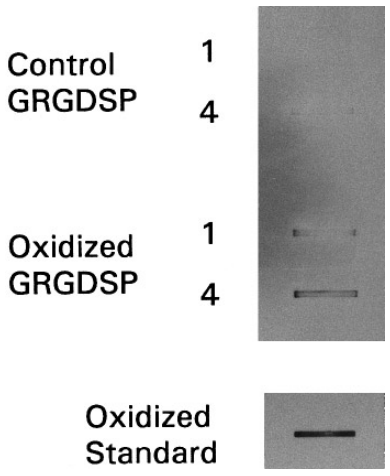

FIG. 6. Immunoblot analysis of GRGDSP treated with a MCO system. GRGDSP was incubated with EDTA alone or a MCO system consisting of $FeCl_3$/EDTA/ascorbate as detailed in Methods. After labelling with DNPH samples were dot blotted onto nitrocellulose membranes and then incubated with antibodies to DNPH bound to carbonyl groups in oxidized protein as described in Methods. A significant increase in binding can be seen for the oxidized GRGDSP at 1 and 4 μg compared to control treated (unmodified) GRGDSP. An oxidized protein standard is shown as well.

(33). Disruption of mesangial cell integrin-ECM interaction through oxidation of integrin ligand might allow greater expression of a fibroblast-like phenotype (33) with a resultant increase in ECM synthesis and eventual glomerulosclerosis (34), a mechanism which could account for the development of glomerulosclerosis in renal diseases characterized by oxidant injury. However, at present this is only speculative.

In summary, ECM-mesangial cell integrin interaction appears to be disrupted by MCO of the ECM. This may result from oxidation of one or more amino acids in the sequence RGD or perhaps other amino acids which contribute to integrin-ECM interaction. These data suggest a new mechanism to account for altered cell-matrix interaction in disorders characterized by enhanced oxidative stress.

ACKNOWLEDGMENTS

This work was supported by NIH Grant 2RO1 DA 06753 (P.C.S.) and by a Young Investigator Award (J.M.) and a research fellowship grant (L.M.) from the National Kidney Foundation of NY/NJ.

REFERENCES

1. Madri, J. A., and Marx, M. (1992) *Kidney Int.* **41,** 560–565.
2. Mark, K. V. D., Mark, H. V. D., and Goodman, S. (1992) *Kidney Int.* **41,** 632–640.
3. Raghow, R. (1994) *FASEB J.* **8,** 823–831.
4. Teti, A. (1992) *J. Am Soc. Nephrol.* **2,** S83–S87.

5. Kashgarian, M., and Sterzel, R. B. (1992) *Kidney Int.* **41,** 524–529.
6. Akiyama, S. K., Nagata, K., and Yamada, K. M. (1990) *Biochim. Biophys. Acta* **1031,** 91–110.
7. Newman, S. L., and Tucci, M. A. (1990) *J. Clin. Invest.* **86,** 703–714.
8. Schwartz, M. A., Schaller, M. D., and Ginsberg, M. H. (1995) *Annu. Rev. Cell Dev. Biol.* **11,** 549–599.
9. Loftus, J. C., Smith, J. W., and Ginsberg, M. H. (1994) *J. Biol. Chem.* **269,** 25235–25238.
10. Ruoslahti, E., and Pierschbacher, M. D. (1987) *Science* **238,** 491–497.
11. Crowley, S. T., Brownlee, M., Edelstein, D., Satriano, J. A., Mori, T., Singhal, P. C., and Schlondorff, D. O. (1991) *Diabetes* **40,** 540–547.
12. Silbiger, S., Crowley, S., Shan, Z., Brownlee, M., Satriano, J., and Schlondorff, D. (1993) *Kidney Int.* **43,** 853–864.
13. El Khoury, J., Thomas, C. A., Loike, J. D., Hickman, S. E., Cao, L., Silverstein, S. C. (1994) *J. Biol. Chem.* **269,** 10197–10200.
14. Oliver, C. N., Ahn, B., Moerman, E. J., Goldstein, S., and Stadtman, E. R. (1987) *J. Biol. Chem.* **262,** 5488–5491.
15. Levine, R. L., Garland, D., Oliver, C. N., Amici, A., Climent, I., Lenz, A.-G., Ahn, B.-W., Shaltiel, S., and Stadtman, E. R. (1990) *Methods Enzymol.* **186,** 464–478.
16. Oliver, C. N., Starke-Reed, P. E., Stadtman, E. R., Liu G. J., Carney, J. M., and Floyd, R. A. (1990) *Proc. Nat. Acad. Sci. USA* **87,** 5144–5147.
17. Oliver, C. N. (1987) *Arch. Biochem. Biophys.* **253,** 62–72.
18. Lenz, A.-G., Ulrich, C., Shaltiel, S., and Levine, R. L. (1989) *Anal. Biochem.* **177,** 419–425.
19. Mattana, J., Margiloff, L., and Singhal, P. C. (1995) *Biochem. Biophys. Res. Commun.* **212,** 63–69.
20. Mattana, J., Margiloff, L., and Singhal, P. C. (1994) *J. Am. Soc. Nephrol.* **7,** 1740A.
21. Adler, S. (1992) *Am. J. Physiol.* **262,** F697–F704.
22. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
23. Weeks, B. S., Kopp, J. B., Horikoshi, S., Cannon, F. B., Garrett, M., Kleinman, H. K., and Klotman, P. E. (1991) *Am. J. Physiol.* **261,** F688–F695.
24. Kitamura, M., Mitarai, T., Maruyama, N., Nagasawa, R., Yoshida, H., and Sakai, O. (1991) *Kidney Int.* **40,** 653–661.
25. Diamond, J. R. (1992) *Am. J. Kidney Dis.* **19,** 292–300.
26. Shah, S. V. (1988) *Am. J. Physiol.* **254,** F337–F344.
27. Thakur, V., Walker, P. D., and Shah, S. V. (1988) *Kidney Int.* **34,** 494–499.
28. Oberle, G. P., Niemeyer, J., Thaiss, F., Schoeppe, W., and Stahl, R. A. K. (1992) *Kidney Int.* **42,** 69–74.
29. Rahman, M. A., Emancipator, S. S., and Sedor, J. R. (1988) *J. Lab. Clin. Med.* **112,** 619–626.
30. Neale, T. J., Ojha, P. P., Exner, M., Poczewski, H., Rüger, B., Witzum, J. J., Davis, P., and Kerjaschki, D. (1994) *J. Clin. Invest.* **94,** 1577–1584.
31. Steinberg, D., Parthasarathy, S., Carew, T. E., Khoo, J. C., and Witzum, J. L. (1989) *N. Engl. J. Med.* **320,** 915–924.
32. Krieger, M., Acton, S., Ashkenas, J., Pearson, A., Penman, M., and Resnick, D. (1993) *J. Biol. Chem.* **268,** 4569–4572.
33. Johnson, R. J., Floege, J., Yoshimura, A., Iida, H., Couser, W. G., and Alpers, C. E. (1992) *J. Am. Soc. Nephrol.* **2,** S190–S197.
34. Fogo, A., Hawkins, E. P., Berry, P. L., Glick, A. D., Chiang, M. L., MacDonell, R. C., Jr., and Ichikawa, I. (1990) *Kidney Int.* **38,** 115–123.