Effects of Macrophage Supernatants on Mesangial Cell Migration and Hillock Formation (43855)

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Abstract. **There is considerable evidence suggesting a role for the macrophage (Mϕ) in the development of glomerulosclerosis (GS) and atherosclerosis, lesions which appear to be analogous. Migration of mesangial cells (MC), which are modified smooth muscle cells, may play a role in the pathogenesis of glomerular injury, and smooth muscle migration may play a role in the pathogenesis of atherosclerosis as well. We undertook the present study to determine the effects of Mϕ supernatants (MϕSN) on MC migration and formation of MC hillocks, which are considered an *in vitro* model of GS. By means of a migration assay using wounded cultures of confluent, growth-arrested MC, MC migration was found to be significantly enhanced by incubation with MϕSN at 24 hr (migration score: MϕSN, 24.3 ± 1.3; control, 11.6 ± 1.0, $P < 0.001$) as well as 48 hr incubation (migration score: MϕSN, 34.0 ± 1.4; control, 15.4 ± 1.4, $P < 0.001$). Enhanced MC migration following prolonged incubation with MϕSN was also shown using phase contrast microscopy and scanning electron microscopy. MC hillock formation was enhanced by MϕSN in a concentration-related manner as was hillock size. These data demonstrate that MϕSN can directly enhance MC migration and hillock formation, processes that may in part account for the observed role for the Mϕ in the development of mesangial expansion and GS as well as atherosclerosis.**

[P.S.E.B.M. 1995, Vol 208]

Considerable *in vivo* evidence suggests that macrophage (Mϕ) infiltration of the masangium may play an important role in the modulation of glomerular injury and glomerulosclerosis (GS) (1–8). In addition, we and other investigators have shown *in vitro* that Mϕ supernatants (MϕSN) can modulate mesangial cell (MC) proliferation and matrix synthesis (9–12), a process regarded as a precursor to the development of GS (1). As Mϕ represent up to 15% of cells in the mesangium under normal conditions (7) and are present in increased numbers following renal injury, these cells are in a unique position to modulate mesangial expansion via their vast array of secretory products (13).

Migration of MC, which are modified smooth muscle cells, is believed to play an important role in the pathogenesis of certain glomerular diseases (14). Of note, migration of smooth muscle cells is believed to play a role in the pathogenesis of atherosclerosis as well (15). Also notable is the fact that the Mϕ is believed to play a key role in the development of atherosclerosis as well as GS (16), perhaps via its secretory products. These findings are of particular interest since there are significant parallels between atherosclerosis and GS (17), although it is unknown whether Mϕ might participate in the development of these lesions by modulating MC migration. In the present study, we have attempted to evaluate the *in vitro* effect of MϕSN on MC migration as well as the effect on formation of MC hillocks, which are considered an *in vitro* model of focal GS (18).

Materials and Methods

MC Culture. MC culture was carried out as previously described (11, 12, 19, 20). In brief, glomer-

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Received May 4, 1994. [P.S.E.B.M. 1995, Vol 208]
Accepted September 13, 1994.

0037-9727/95/2083-0271$10.50/0

uli were isolated from pentobarbital-anesthetized Sprague-Dawley rats by differential sieving and seeded into plastic culture flasks (Becton-Dickinson, Lincoln Park, NJ) in RPMI 1640 (GIBCO, Grand Island NY) containing 50 U/ml penicillin (GIBCO), 50 μg/ml streptomycin (GIBCO), and 10% fetal calf serum (FCS) (GIBCO), and incubated at 37°C in a 95% air/5% CO_2 environment. After 3 weeks in primary culture, MC were detached from the flask using a 0.25% trypsin-EDTA solution (GIBCO) and subcultured at 7- to 10-day intervals. For experiments, MC from the fifth to eighth subculture were utilized. As discussed previously, these MC represent an apparently uniform cell population (19).

Mϕ Culture and Preparation of MϕSN. The Mϕ cell line J774.16 was utilized as previously described (11–12, 21, 22). In brief, Mϕ were grown in Dulbecco's modified Eagle's Medium (GIBCO, Grand Island, NY) containing 4500 mg/l glucose, 10% fetal calf serum, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 mg/l L-alanine (GIBCO), 75 mg/l L-glutamine (GIBCO) on 100 mm-diameter plastic culture dishes (Corning, NY) at 37°C in a 95% air/5% CO_2 environment and for experiments were detached from the dish by gentle pipetting and seeded onto 6-well culture plates (Becton-Dickinson) at 3×10^5 cells/ml in fresh media. MϕSN were then prepared as described previously (11–12). After Mϕ were grown to confluence on 6-well plates cells were washed five times with phosphate buffered saline (PBS) (GIBCO) and then incubated with 2 ml/well serum-free RPMI for 24 hr at 37°C in a 95% air/5% CO_2 environment. The supernatant was then collected, centrifuged at 1500 rpm for 10 min and filtered using a 0.2-μm pore size filter (Acrodisc; Gelman, Ann Arbor, MI) as described (11, 12). To confirm the absence of cells the supernatant was examined with a hemacytometer.

Incubation of MC with MϕSN and Performance of Migration Assay. The effect of MϕSN on MC migration was determined using a modification of the method of Kartha and Toback (23). MC were seeded at 20,000/ml onto 35 mm-diameter plastic culture dishes (Becton-Dickinson) and after reaching confluence were washed twice with PBS and growth-starved by incubating with serum-free RPMI containing 0.5% BSA and 1% insulin/transferrin/selenium solution (GIBCO) for 72 hr (11, 12, 19, 20). At this point, wounds were created by using a sterile rubber policeman to gently denude a 1 cm-wide area of cells on the dish. Both edges of this denuded area were then labeled with lines drawn on the underside of the dish as a reference point. Dishes were then thoroughly washed to remove all nonadherent cells which was confirmed by phase contrast microscopy. Cells were then incubated with serum-free RPMI alone or serum-free RPMI containing varying concentrations of MϕSN (10%, 25%, 50%, 75%, or 100%) and incubated for 24 hr at 37°C. MC migration was then assessed by means of phase contrast microscopy using an eyepiece grid containing 100 squares to view the denuded area on the dishes. With the grid over the denuded area and the outer border of the grid was aligned with the line marking the edge of the denuded strip and the migration score calculated by counting the number of squares within the grid that were occupied by cells. For convention, a square that was at least 50% covered by a cell was counted as occupied and less than 50% was counted as not occupied. A numerical score was then obtained for that field which represented the total number of squares occupied by cells. The grid was then moved to an adjacent area and the assay repeated for a total of five measurements in each experiment. Eight such experiments were carried out. Phase contrast photomicrographs were taken at 24 hr as detailed previously (19).

To determine the effect of 48 versus 24 hr incubation on MC migration confluent MC were prepared for the migration assay in identical fashion. After growth-arresting and denuded an area on each dish as above cells were incubated with serum-free RPMI alone or serum-free RPMI containing 50% MϕSN. Cells were incubated at 37°C and at 24 and 48 hr the migration score was determined. Eight such experiments, each carried out five times, were performed.

Scanning Electron Microscopy of MC Migration. To further evaluate the effect of MϕSN on MC migration, MC treated in identical fashion as above for the migration assay were incubated for 24 hr with or without MϕSN and prepared for scanning electron microscopy as described (19). In brief, cells were post-fixed with 1% OsO_4 followed by dehydration with progressive concentrations of ethanol and air dried. Samples were then passed through critical point dehydration, coated with gold, and photographed on a JEOL JSM 25S scanning electron microscope.

MC Hillock Formation in Prolonged Culture with MϕSN. MC in prolonged culture are well-known to form hillocks which are focal accumulations of cells and matrix. These nodular foci are regarded as an *in vitro* model of focal GS (18). The effect of MϕSN on formation of MC hillocks was determined as described previously (19). In brief, MC were seeded at 2×10^4 cells/ml onto 24-well plastic culture plates (Becton-Dickinson) in RPMI containing 10% FCS and varying concentrations of MϕSN (0%, 10%, 25%, 50%, 75%, or 90%) and incubated at 37°C. Cells were examined daily by phase contrast microscopy and media was changed every 3 days. After 3 weeks in culture the total number of hillocks in each well were counted. To determine the effect of MϕSN on MC hillock size, MC were incubated with standard media alone (control) or media containing 10% or 30% MϕSN. Media were

changed every 3 days, cells examined daily by phase contrast microscopy, and after 2 and 4 weeks, hillocks in each well were categorized as small, medium, or large by means of an inverted microscope containing a built-in eyepiece grid with rectangles of three different sizes as described (19). Hillocks were defined as small if they fit within the borders of the smallest rectangle on the grid, medium if they fit inside the next largest size rectangle in the grid and large if they extended beyond the borders of this second rectangle. All hillocks in each well were examined and the percentage of hillocks falling into the medium or large size categories was calculated.

Statistical Analysis. For comparison between control and MϕSN-treated groups in the migration assay Student's unpaired T test was used. For comparison of number of hillocks between groups in prolonged culture with varying concentrations of MϕSN, analysis of variance was applied and Neuman-Keuls multiple range testing utilized. To compare the proportion of medium and large hillocks between groups the Chi-square test was applied. All values are reported as mean ± SEM. Statistical significance was defined as P value less than 0.05.

Results

Effect of MϕSN on MC Migration. Figure 1 illustrates the concentration-response effect of MϕSN on MC migration. As this figure shows, MC migration was significantly enhanced in a concentration-related manner with a peak effect seen at 50% concentration but a decline seen with incubation with very high concentrations of MϕSN (75% and 90%). When MC were incubated under control conditions or with 50% MϕSN, MC migration was found to be significantly enhanced at both 24 and 48 hr for MC incubated with MϕSN. These data, which are illustrated in Figure 2, suggest that MϕSN can significantly enhance MC migration except at extremely high concentrations of MϕSN in which MC migration is decreased.

Phase Contrast and Scanning Electron Microscopy of MC Migration. Representation phase contrast photomicrographs of MC migration following incubation under control conditions or with MϕSN-supplemented media are shown in Figure 3, A and B, respectively. These photomicrographs illustrate greater migration of MC when treated with MϕSN compared with control as evidenced by their migrating a further distance from the original border of the denuded area (bottom of each photomicrograph). Similar findings are evident in the scanning electron photomicrographs shown in Figure 4, A and B in which MC migration appears greater for MϕSN-treated MC (Fig. 4, B) compared with control (Fig. 4A). These data further suggest that MϕSN can significantly enhance MC migration.

Effect of MϕSN on MC Hillock Number. MC in prolonged culture with MϕSN were found to form hillocks in a concentration-related manner as illustrated in Figure 5. These data demonstrate that MC hillock formation is significantly enhanced by incubation with MϕSN at 10%, 25%, and 50% concentrations. A scanning electron photomicrograph of a representative MC hillock is shown in Figure 6. These data demonstrate that MϕSN can increase MC hillock formation in a concentration-related manner.

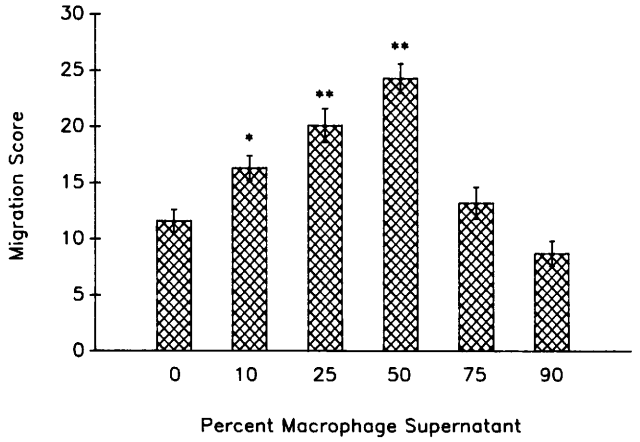

Figure 1. Concentration-response effect of MϕSN on MC migration. MC were grown to confluence on 35 mm-diameter plastic culture dishes and growth arrested; then a 1 cm-wide area of the plate was gently denuded of cells using a rubber policeman. After thorough washing to remove all nonadherent cells MC were incubated with serum-free medium alone (control) or serum-free medium containing varying concentrations of MϕSN. After 24 hr migration assay was performed as described in Materials and Methods. Results are from eight experiments each carried out five times. *$P < 0.01$ compared with control (o or no supernatant); **$P < 0.001$ compared with control.

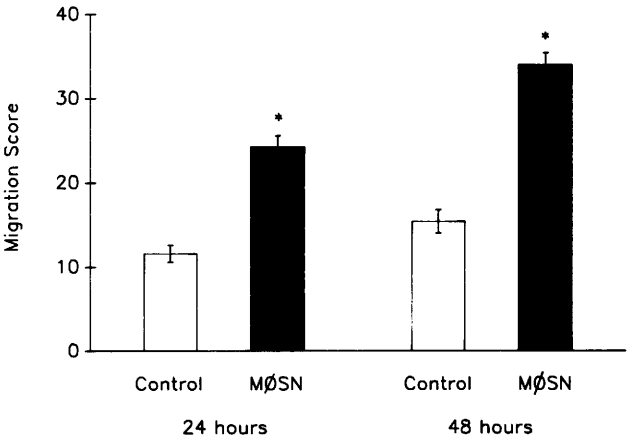

Figure 2. Effect of MϕSN on MC migration. MC were grown to confluence on 35 mm-diameter plastic culture dishes and growth arrested for 72 hr. A 1 cm-wide area was then gently denuded of cells, and after thoroughly washing cells were incubated with serum-free media alone or serum-free media supplemented with MϕSN (50%). Cell migration (graded on a numerical scale from 0 to 100) was then determined at 24 and 48 hr as described in Materials and Methods. Results are expressed as mean ± SEM of eight experiments each repeated five times. *$P < 0.001$ compared with control.

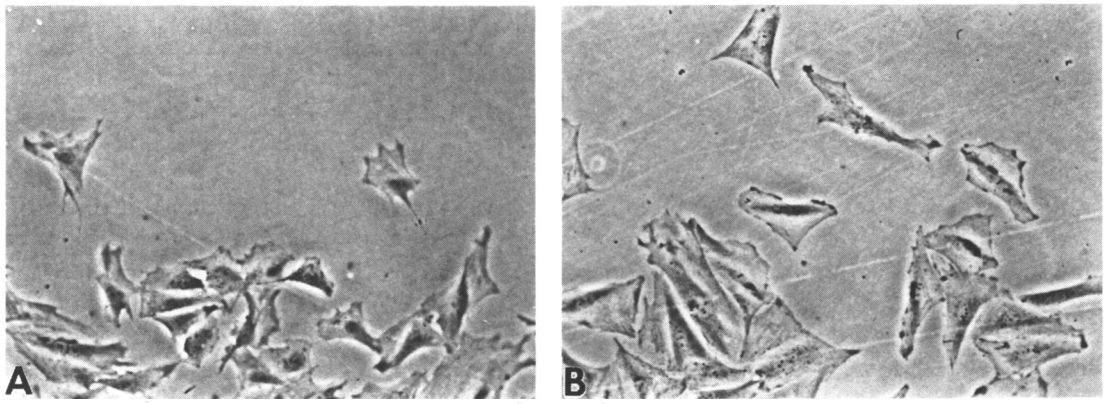

Figure 3. Phase contrast photomicrograph of MC migrating into denuded area following incubation with serum-free media alone (control) (A) or with media supplemented with MϕSN (B) for 24 hr. Cells were prepared as described in Materials and Methods and photographed at ×1000 using an inverted microscope. The lower border of the photomicrograph corresponds with the original border of the denuded area.

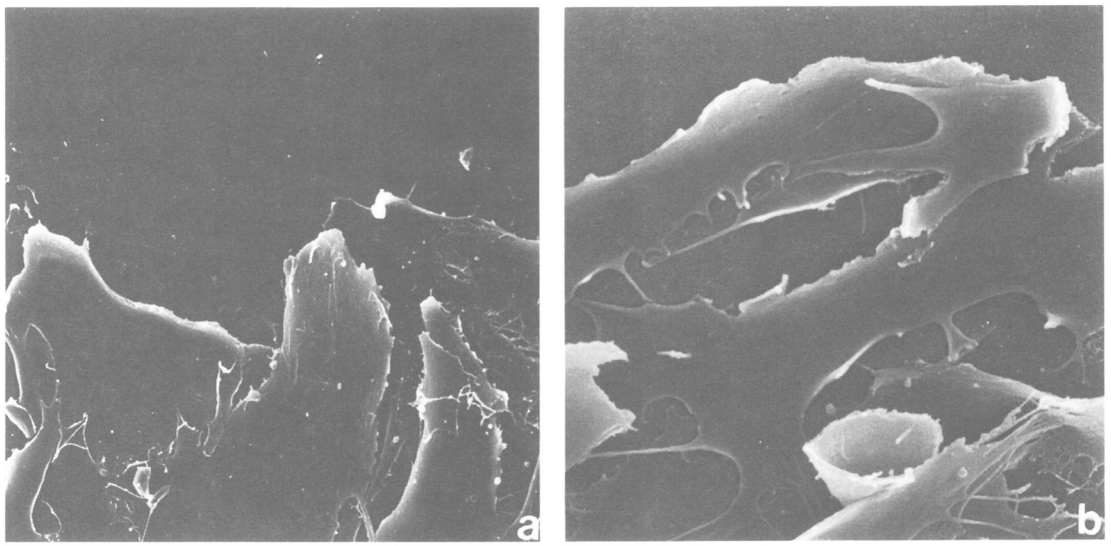

Figure 4. Scanning electron photomicrograph of MC migrating under control (A) and MϕSN (B) treated conditions after 24 hr. Cells were prepared as described in Materials and Methods. The lower border of the photomicrograph corresponds with the original border of the denuded area.

Effect of MϕSN on MC Hillock Size. After 2 weeks incubation MC incubated with 10% and 30% MϕSN demonstrated significantly greater proportion of hillocks falling into the medium or large size categories compared with control as shown in Table I. After 4 weeks incubation the percentage of medium or large hillocks was significantly greater for cells incubated with 30% MϕSN compared with control as also shown in Table I. These data suggest that while MϕSN can increase the number of hillocks in prolonged culture in a concentration-related manner, they can also increase hillock size as well.

Discussion

The present study demonstrates that MϕSN can directly enhance MC migration and hillock formation, effects which are consistent with the suggested role for the Mϕ in the development of GS and atherosclerosis and which suggest some possible mechanisms by which Mϕ may participate in these analogous pathologic processes. As noted above, migration of these modified smooth muscle cells has been demonstrated in glomerular disease (14), and smooth muscle cell migration appears to play a role in the pathogenesis of atherosclerosis as well (15). Although smooth muscle cell migration has been demonstrated in these lesions as has infiltration of Mϕ, no data has been previously available to support the hypothesis that it is the infiltrating Mϕ themselves which are playing a role in enhancing or modulating smooth muscle migration rather than representing simply an epiphenomenon. The present study suggests that Mϕ infiltration of the glomerular mesangium and the vascular wall could play a role in promoting smooth muscle cell migration by

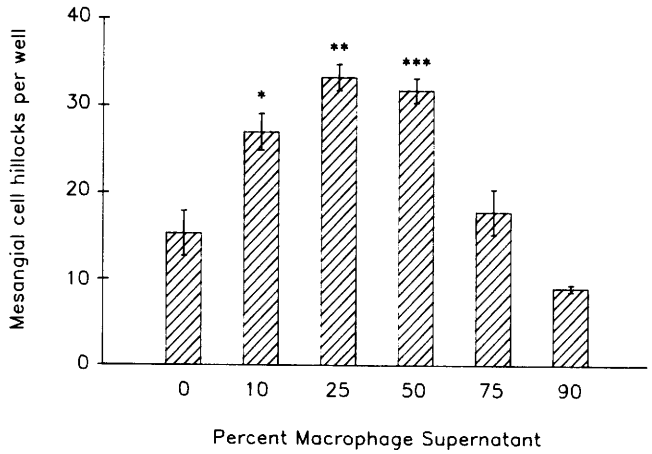

Figure 5. Effect of incubation of MC with MϕSN on hillock formation. MC were seeded onto 24-well plates and grown at 37°C in RPMI containing 10% FCS with or without varying concentrations of MϕSN. Media was replaced twice weekly. After 3 weeks incubation the number of hillocks per well were counted using an inverted microscope. Results are expressed as mean ± SEM ($n = 4$). *$P < 0.02$ compared with control; **$P < 0.001$ compared with control; ***$P < 0.01$ compared with control.

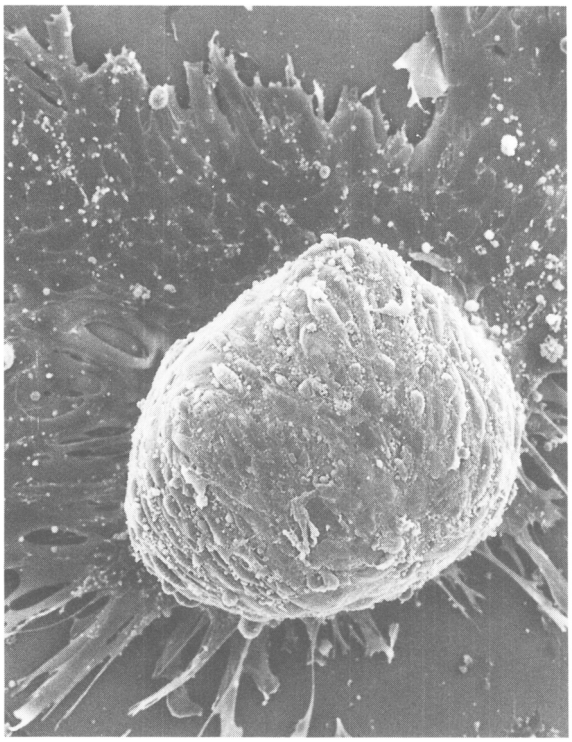

Figure 6. Scanning electron photomicrograph of a MC hillock. MC were maintained for 2 weeks in standard media alone or standard media supplemented with MϕSN and prepared for scanning electron microscopy as described in Materials and Methods. This photomicrograph demonstrates a focal accumulation of MC and matrix which characterizes a MC hillock.

means of MϕSN and by this mechanism may perhaps play a role in the development of GS and atherosclerosis.

It must be emphasized that the findings in the present study are all *in vitro* and it is unclear to what extent they can be applied to the *in vitro* state. In particular, it is unclear whether one can infer from these findings any potential link with glomerulosclerosis. In addition, the ability of MϕSN to induce MC proliferation raises the possibility that to a large extent the ability of MϕSN to enhance MC migration could be due to a mitogenic effect. However, as these cells were studied in the growth-arrested state, a significant increase in cell mass to account for the approximate doubling in MC migration with MϕSN compared with control appears unlikely though some contribution of cellular proliferation cannot be excluded.

Table I. Effect of Prolonged Incubation of MC with MϕSN on MC Hillock Size

	Control	10% MϕSN	30% MϕSN
2 weeks	9.8%	22.2%[a]	26.8%[b]
4 weeks	26.0%	30.5%	41.4%[a]

Note. MC were seeded onto 24-well plates and incubated with RPMI containing 10% FCS plus 0, 10%, or 30% MϕSN collected from unstimulated Mϕ. Media was changed twice weekly and after 2 and 4 weeks incubation hillocks were examined and classified as small, medium, or large as described in Materials and Methods. Results are expressed as the percentage of hillocks falling into the medium or large size category. Comparison carried out via the Chi-square test.
[a] $P < 0.05$ compared with control.
[b] $P < 0.01$ compared with control.

MC are well known to form hillocks, focal accumulations of cells and matrix, in prolonged culture (18), and as discussed above hillocks have been regarded as an *in vitro* model of GS. In the present study, MϕSN were demonstrated to enhance both the number and the size of MC hillocks in prolonged culture. Recent *in vitro* studies have shown that inhibition of Mϕ infiltration of the mesangium by maneuvers such as irradiation (24), use of anti-Mϕ serum (25) or induction of essential fatty acid deficiency (26) results in significant amelioration of the development of GS in animal models of glomerular injury. In addition to previous *in vitro* studies demonstrating modulation of MC proliferation and matrix synthesis by MϕSN, the present study provides additional support for a potential role for the Mϕ in the pathogenesis of GS perhaps by enhancing both MC migration as well as focal accumulations of MC and mesangial matrix.

We conclude that Mϕ may play a direct role in the modulation of mesangial expansion following renal injury via the effects of their secretory products on MC migration and ability to promote the formation of focal accumulation of cells and matrix as suggested by the present *in vitro* study, effects which may in part account for the apparent contributions of the Mϕ to the development of GS and atherosclerosis.

This work was supported by National Institutes of Health Grant R01 DA 06753 (Dr. Singhal) and by a research fellowship

grant from the National Kidney Foundation of NY/NJ (Dr. Mattana). Portions of this work were published in abstract form for the Annual Meeting of the American Federation for Clinical Research, 1993.

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