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Mesangial cell-derived transforming growth factor-β1 reduces macrophage adhesiveness with consequent deactivation

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Mesangial cell-derived transforming growth factor-β1 reduces macrophage adhesiveness with consequent deactivation. Adhesion of macrophages is a crucial event that determines the number and function of macrophages at inflammatory sites. The aim of this study was to elucidate the role of mesangial cells in the regulation of macrophage adhesiveness. J774.2 macrophages were suspended in serial dilutions of mesangial cell conditioned medium (MC medium) and seeded on plastic tissue culture plates. MC medium did not affect the initial adhesion of macrophages but induced subsequent detachment in a concentration-dependent manner. A similar effect was observed when macrophages were plated on plastic coated with laminin, collagen type IV or Matrigel. The reduced adhesiveness was reversible, and cell viability was unaffected by MC medium, indicating that the effect is not due to cytotoxicity. Conditioned media from fibroblastic, epithelial and endothelial cell lines did not induce macrophage detachment. To identify the active component in MC medium, we examined the involvement of transforming growth factor-β1 (TGF-β1) in the process. Mesangial cells constitutively expressed TGF-β1 mRNA, and MC medium contained the active form of TGF-β1. Exogenously added TGF-β1 induced macrophage detachment in a dose-dependent manner, and an anti-TGF-β1 neutralizing antibody partially abolished the activity of MC medium, indicating the involvement of TGF-β1 as an active component. Compared to adherent cells, detached macrophages showed reduced mitogenic activity and blunted induction of IL-1β and IL-6 in response to lipopolysaccharide. These data demonstrate that TGF-β1 is a mesangial cell-derived factor that impairs adhesiveness of macrophages and confers blunted responses to a specific stimulus. These findings suggest one potential mechanism for macrophage clearance from inflamed glomeruli.

Accumulation of mononuclear cells in the glomerulus is a characteristic feature of glomerulonephritis [1]. The major population of infiltrating cells is a monocyte/macrophage lineage [2]. Depletion of these cells by irradiation, antibodies or pharmacological agents prevents glomerular hypercellularity and matrix expansion and reduces the degree of proteinuria in several models of glomerulonephritis [3–6]. This line of evidence suggests a crucial role of infiltrating macrophages in glomerular injury. Activated macrophages are capable of producing a variety of inflammatory mediators including cytokines, proteinases and reactive oxygen radicals [7], and may thereby contribute to glomerular damage [8–10]. Using glomerular cells in culture, several studies have reported the role of macrophages in glomerular cell activation. For example, conditioned media from activated macrophages induce mesangial cell mitogenesis and matrix production [11, 12]. In contrast, little attention has been paid to the control of macrophage activity by intrinsic glomerular cells. It has been shown that activated mesangial cells have the ability to produce monocyte chemoattractant protein-1 (MCP-1) and colony stimulating factors [13–15]. This implies that glomerular cells could participate in the recruitment and accumulation of macrophages in pathological situations. It is still not known how glomerular cells modulate certain functions of macrophages, particularly their adhesive properties, and what the consequence of altered adhesiveness may be on their function.

Adhesiveness of macrophages is an important factor which determines retention of macrophages at inflammatory sites. Adhesion also serves as a priming stimulus for functional alteration in monocyte/macrophages, such as differentiation, migration, phagocytosis, oxidative burst and gene expression [16–21]. These findings indicate that adhesion of macrophages within the glomerulus is a crucial event that regulates the activity of local macrophages. In this study, we aimed to elucidate how resident glomerular cells, especially mesangial cells, affect adherence of macrophages to substrata. To investigate whether altered adherence is associated with functional change, the differences in mitogenesis and cytokine production between adherent and non-adherent cells were examined. We demonstrate that mesangial cell-derived factors induce detachment of macrophages from substrata leading to their subsequent deactivation. We have identified transforming growth factor-β1 (TGF-β1) as one of the mesangial cell-derived molecules involved in this process.

Methods

Cells

Rat mesangial cells were cultured from isolated glomeruli of an adult male Sprague-Dawley rat as described before [22]. A mesangial cell clone SM43 was established by a limiting dilutional method and identified as being of mesangial cell phenotype as described elsewhere [23]. A macrophage cell line J774.2 and an endothelial cell line ECV304 were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and a macrophage cell line NR8383, a fibroblastic cell line NRK49F and epithelial cell lines LLCPK1 and CCL64 from American Type Culture Collection (Rockville, MD, USA). These cells were maintained in Dulbecco's MEM/F-12 (DME-F12; GIBCO BRL, Gaithersburg, MD, USA) supplemented with 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 0.25 μg/ml of amphotericin B,

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and 10% fetal calf serum (FCS). DME-F12 containing 1% FCS (1% FCS/DME-F12) was generally used as the basal assay medium.

Conditioned media

To prepare conditioned media, confluent cultures of each cell line grown in the presence of 10% FCS in 100 mm culture plates (Greiner, Gloucestershire, UK) were washed twice and incubated in 4 ml of 1% FCS/DME-F12 at 37°C for 48 hours. The conditioned media were then passed through a 0.2 μm filter (Nalgene, Rochester, NY, USA) and stored at −80°C until use. The culture medium conditioned by SM43 was used as the standard mesangial cell conditioned medium (MC medium). DME-F12 containing 1% FCS was also incubated in the absence of cells for 48 hours, filtered, and used as control medium for assays. In a certain experiment, conditioned medium prepared from serum-depleted, inactivated mesangial cells was utilized. For this purpose, confluent SM43 cells were precultured in 0.5% FCS/DME-F12 for 72 hours and then incubated in 4 ml of 1% FCS/DME-F12 for 48 hours.

Assessment of adhesiveness

Murine J774.2 macrophages (5×10^4 cells/well) were suspended in 2 to 10 times diluted MC medium or control medium and plated in 24-well tissue culture plates (Becton Dickinson, Lincoln Park, NJ, USA). MC medium diluted at 1:1 with 1% FCS/DME-F12 was generally used for studies. The number of floating cells was determined at 30 minutes and 24 hours after seeding. Viability of the cells was assessed by trypan blue exclusion. Reversibility of macrophage adhesiveness was examined by replating detached cells in fresh control medium. To establish that the effect is not specific to J774.2 cells, the same study was conducted with NR8383 rat macrophages. All the experiments were performed in the presence of 1% FCS.

To investigate the effect of MC medium on the adhesion of macrophages onto extracellular matrices (ECM), tissue culture plates were coated with laminin, collagen type IV or basement membrane Matrigel™, following the protocols provided by the manufacturer. These matrix materials were purchased from Collaborative Biomedical Products/Becton Dickinson Labware (Bedford, MA, USA). The effect of MC medium was tested as described above.

To examine the effect of TGF-β1 on macrophage adhesiveness, human TGF-β1 (0 to 10 ng/ml; Genzyme, Cambridge, MA, USA) was added to cultures of macrophages. To investigate the involvement of TGF-β1 as an active component in MC medium, conditioned medium was prepared in the absence of FCS. The conditioned medium was pre-incubated with or without 25 μg/ml of an anti-TGF-β1 neutralizing antibody (Promega, Southampton, UK) for 10 minutes at room temperature, diluted at 1:9 with 1% FCS/DME-F12, and retested. This concentration of the anti-TGF-β1 antibody is sufficient to abolish completely the suppressive effect of MC medium on macrophage cytokine synthesis, as reported elsewhere [24].

Heat-inactivated conditioned medium was utilized to examine involvement of thermolabile factors as active entities in MC medium. MC medium or control medium diluted at 1:9 was heated at 80°C for 10 minutes, and the efficacy was retested. Generally, heat treatment inactivates many peptide factors with the exception of certain molecules including TGF-β1 [24].

Assessment of mitogenic activity

J774.2 macrophages suspended in MC medium diluted at 1:1 were seeded in 24-well plates at a density of 2.5×10^4 cells/well. After incubation for 48 hours, the cells were pulsed with 1 μCi of [^{3}H]-thymidine per well for three hours. Adherent and non-adherent macrophages were then harvested separately, and the cell numbers were counted using a hemocytometer. Incorporation of radioactivity was measured by liquid scintillation counting, and values expressed as [^{3}H]-thymidine incorporation per cell.

Northern blot analysis of cytokine expression

J774.2 macrophages were cultured for 24 hours in tissue culture plates in the presence of MC medium (1:1 dilution) or control medium and stimulated by lipopolysaccharide (LPS; 1 μg/ml; Sigma Immunochemicals, St. Louis, MO, USA) for additional 24 hours. Adherent and non-adherent cells were separately harvested from identical culture plates, and Northern blot analysis was performed on expression of IL-1β and IL-6, as described before [25]. In brief, total RNA was extracted by a single-step method [26], and RNA samples (10 μg/lane) were electrophoresed on 1.2% agarose gels and transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). For hybridization, mouse IL-1β cDNA [27] and mouse IL-6 cDNA [28] were labeled with ^{32}P-dCTP using a random priming method [29]. The membranes were hybridized with the probes overnight at 65°C, washed at 50°C and exposed to Kodak XAR films at −80°C. Densitometric analysis was performed using a computerized program, NIH Image. Values were normalized relative to the level of β-actin mRNA expression which was used as the loading control.

Expression of TGF-β1 in mesangial cells

To identify the active component in MC medium, we focused on TGF-β1 since (i) TGF-β is one of the most potent suppressors of macrophage function [30], (ii) cultured mesangial cells have the ability to produce active TGF-β1 [24, 31], and (iii) TGF-β1 may inhibit expression of adhesion receptors in macrophages [32, 33]. Expression of TGF-β1 mRNA in mesangial cells was evaluated by Northern blot analysis. This was performed using a porcine TGF-β1 cDNA probe [34].

Western blot analysis of active TGF-β1 was performed as described previously [35]. Two milliliters of control media or conditioned media containing 0.5% FCS was precipitated with the same volume of 10% trichloroacetic acid at 4°C. After centrifugation, each pellet was dissolved in 10 μl of 0.5 N NaOH, electrophoresed in a 10% polyacrylamide gel, and transferred electrophoretically onto a nitrocellulose membrane (Schleicher and Schuell). Analysis was performed using an anti-human TGF-β1 antibody (1:500 dilution; Promega), a Vectastain ABC Kit (Vector Laboratories, Peterborough, UK) and a peroxidase substrate kit AEC (Vector Laboratories) following the protocols provided by the manufacturers.

The growth inhibition assay of the mink lung epithelial cell line, CCL64 [36], was used to determine the biological activity of TGF-β in MC medium [35]. In brief, CCL64 cells were seeded in 24-well plates at a density of 5×10^3 cells/well. After incubation for 24 hours in the presence of 10% FCS, cells were further incubated in 1% FCS/DME-F12 for 24 hours and then exposed to serially diluted MC medium. After 48 hours, [^{3}H]-thymidine (1

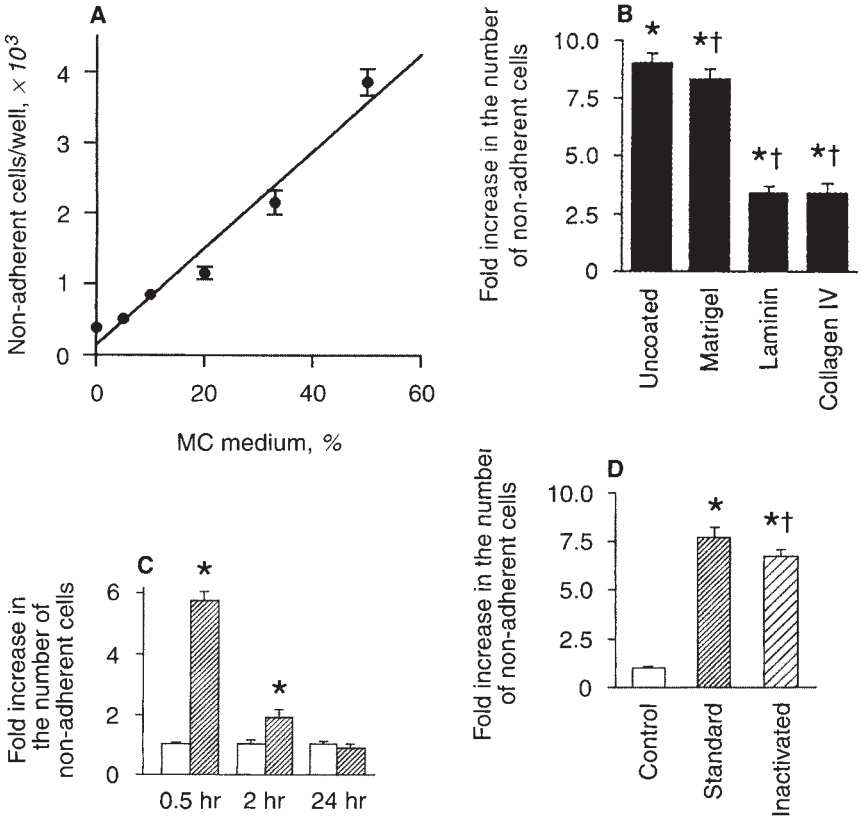

Fig. 1. *Effect of mesangial cell conditioned medium (MC medium) on macrophage adhesiveness.* Confluent rat mesangial cell line SM43 cultured in a 100 mm culture plate was incubated in 4 ml of 1% FCS/DME-F12 at 37°C for 48 hours. The conditioned medium was passed through a 0.2 μm filter and used as MC medium. DME-F12 containing 1% FCS was also incubated in the absence of cells for 48 hours, filtered, and used as control medium for assays. **A.** Dose-dependent effect of MC medium. J774.2 macrophages were suspended in 2 to 10 times diluted MC medium or control medium and plated in 24-well tissue culture plates (5×10^4 cells/well). After 24 hours, the number of detached cells was counted. Assays were performed in quadruplicate. Data are presented as means ± SE. **B.** Effect of MC medium on macrophage adhesiveness to different matrix constituents. Tissue culture plates were coated with basement membrane Matrigel, laminin, or collagen type IV, and the effect of MC medium (1:1 dilution) was tested. Statistically significant differences ($P < 0.05$) compared to the effect of control medium on each substrate (*) or to the effect of MC medium on uncoated plastic (†) are shown. **C.** Reversibility of impaired adhesiveness of macrophages. Following the treatment with MC medium for 24 hours, detached macrophages were suspended in fresh control medium and replated into 24-well plates (▨). As a control experiment, macrophages were pretreated with control medium for 24 hours, suspended in fresh control medium and replated (□). After 0.5, 2, and 24 hours, the number of floating cells was counted, and fold increases against the values of control were calculated. Asterisks indicate statistically significant differences ($P < 0.05$) compared to controls. **D.** Effect of MC medium derived from serum-depleted mesangial cells. Confluent SM43 cells were precultured in 0.5% FCS/DME-F12 for 72 hours and incubated in 4 ml of 1% FCS/DME-F12 for 48 hours. The prepared conditioned medium from inactivated mesangial cells (inactivated) was diluted at 1:1 and added to macrophages. Statistically significant differences ($P < 0.05$) compared to the effect of control medium (control) (*) or standard MC medium (standard) (†) are shown.

μCi/well) was added, incubated for three hours, and incorporation of radioactivity was measured by liquid scintillation counting. As a standard, activated human TGF-β1 (0 to 5 ng/ml; Genzyme) was used.

Statistical analysis

Assays were performed in quadruplicate. Data are expressed as means ± SE. Statistical analyses were performed using the non-parametric Mann-Whitney test. P values less then 0.05 were considered to be significantly different.

Results

Effect of MC medium on macrophage adhesiveness

Murine J774.2 macrophages were suspended in serial dilutions of MC medium and plated on plastic substrata. The number of floating cells was determined at 30 minutes and 24 hours after seeding. After 30 minutes, over 99% of macrophages adhered, and MC medium did not affect this initial adhesion (not shown). However, within 24 hours, MC medium significantly induced detachment of macrophages in a concentration-dependent manner (Fig. 1A). The number of detached cells in MC medium diluted at 1:1 was approximately 10 times (7 to 13 times) higher than that in control medium. To establish that the effect was not specific to J774.2 cells, the same study was conducted with NR8383 rat macrophages. MC medium affected adhesion of the rat macrophages in a similar fashion; a 1:1 diluted conditioned medium generated a 19 ± 1 fold (mean ± SE) increase in the number of detached NR8383 cells (data not shown). Subsequent studies were therefore confined to J774.2 cells.

A similar inhibitory effect of MC medium on adhesiveness was observed in macrophages seeded on Matrigel, laminin, or collagen type IV (Fig. 1B). Interestingly, MC medium was more effective in

macrophages seeded on uncoated plastic than those on ECM-coated plates. All further experiments were, therefore, conducted using uncoated plastic as described below. Throughout the studies, more than 98% of detached macrophages were viable as determined by trypan blue exclusion.

To investigate whether the reduced adhesiveness was reversible, detached macrophages pretreated with MC medium were suspended in control medium and replated into 24-well plates. As shown in Figure 1C, re-seeded macrophages exhibited significant impairment of initial adhesion at 30 minutes, but the majority of the cells regained adhesiveness within 24 hours. No significant difference in the number of non-adherent cells was detected at 24 hours compared to MC medium-untreated macrophages.

We tested whether the activity of MC medium is affected by activation states of mesangial cells. As demonstrated in Figure 1D, the conditioned medium from serum-depleted "inactive" cells also substantially reduced adhesiveness of macrophages. Compared to the standard MC medium, however, the medium from inactivated cells showed modest but significant reduction in this activity [87 ± 2% versus standard MC medium (100%), mean ± SE, $P < 0.05$].

Effect of conditioned media from non-mesangial cell lines

Conditioned media derived from LLCPK1 epithelial cells, NRK49F fibroblasts, and ECV304 endothelial cells were tested for the effect on macrophage adhesiveness. J774.2 macrophages were suspended in 1:1 diluted conditioned media and seeded in 24-well culture plates. After 24 hours, the number of floating cells was evaluated. In contrast to MC medium, none of the conditioned media from non-mesangial cell lines induced macrophage detachment (Fig. 2).

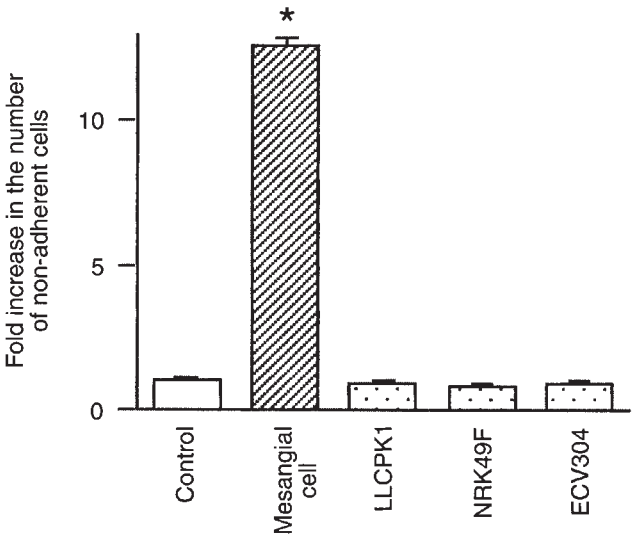

Fig. 2. *Effect of media conditioned by non-mesangial cell lines on macrophage adhesiveness.* A fibroblastic cell line NRK49F, an epithelial cell line LLCPK1, and an endothelial cell line ECV304 were cultured in 100 mm culture plates. The confluent cultures were incubated in 4 ml of 1% FCS/DME-F12 at 37°C for 48 hours, and passed through a 0.2 μm filter. Adhesion assays were performed using 1:1 diluted conditioned media. Fold increases in the number of non-adherent cells compared to the value of control medium (control) are demonstrated. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference ($P < 0.05$) compared to control. Assays were performed in quadruplicate.

Expression of TGF-β1 by mesangial cells

Expression of TGF-β1 mRNA in SM43 mesangial cells was examined by Northern blot analysis. Mesangial cells constitutively expressed a 2.5 kb transcript, consistent with the predicted size of TGF-β1 mRNA, as shown in Figure 3A. Immunoblot analysis on MC medium detected a 25 kDa band corresponding to the active form of TGF-β1 (Fig. 3B). By using the growth inhibition assay of the mink lung epithelial cell line CCL64, we confirmed that MC medium contained biologically active TGF-β (Fig. 3C). The estimated concentration of active TGF-β1 was 5 ng/ml. In contrast, conditioned medium derived from NRK49F fibroblasts, LLCPK1 epithelial cells or ECV304 endothelial cells did not exert any inhibitory effect on the mitogenesis of CCL64 cells (data not shown).

Identification of TGF-β1 as an active component of MC medium involved in macrophage detachment

To examine the effect of TGF-β1 on macrophage adhesiveness, several concentrations of TGF-β1 were added to cultures of macrophages. As shown in Figure 4A, TGF-β1 induced macrophage detachment in a dose-dependent manner. The effect was detected at concentrations more than 50 pg/ml and reached to maximum at 5 ng/ml. The number of detached cells induced by 5 ng/ml TGF-β1 was 10 times higher than that of control.

To elucidate the involvement of TGF-β1 as an active entity of MC medium, diluted conditioned medium was pretreated with an anti-TGF-β1 neutralizing antibody, and the efficacy was retested. Compared to untreated medium, the effect of MC medium treated with the antibody was partially but significantly (35 ± 4%, mean ± SE, $P < 0.05$) abrogated (Fig. 4B). These data suggested that TGF-β1 is an active component involved in the regulation of macrophage adhesiveness by MC medium.

To examine involvement of other soluble factors as active entities in MC medium, diluted MC medium was heated at 80°C, and the efficacy was retested. Although the active form of TGF-β1 is known to be heat-stable, the heat treatment significantly reduced the total activity of MC medium [67 ± 4% versus unheated MC medium (100%); Fig. 4C]. It is noteworthy that the heat-inactivated MC medium still retained the substantial ability to induce macrophage detachment compared to control medium.

Deactivation of macrophages following reduced adhesiveness

We examined whether the reduced adhesiveness of macrophages treated with MC medium is associated with altered mitogenic activity. Macrophages were pretreated with MC medium, and incorporation of [^{3}H]-thymidine was examined. Adherent and non-adherent macrophages were harvested separately, and incorporation of radioactivity per cell was evaluated. Compared to attached cells, detached macrophages showed a significant reduction in mitogenic activity (Fig. 5A). The mean value in non-adherent cells was 0.038 ± 0.013 cpm/cell versus 0.265 ± 0.021 cpm/cell in adherent cells ($P < 0.05$).

J774.2 macrophages stimulated by LPS express proinflammatory cytokines IL-1β and IL-6 [24]. Using these molecules as indicators of activation, we examined whether altered adhesiveness affects the response of macrophages to a specific stimulus. Macrophages were pretreated with diluted MC medium or control medium and stimulated by LPS. Adherent and non-adherent

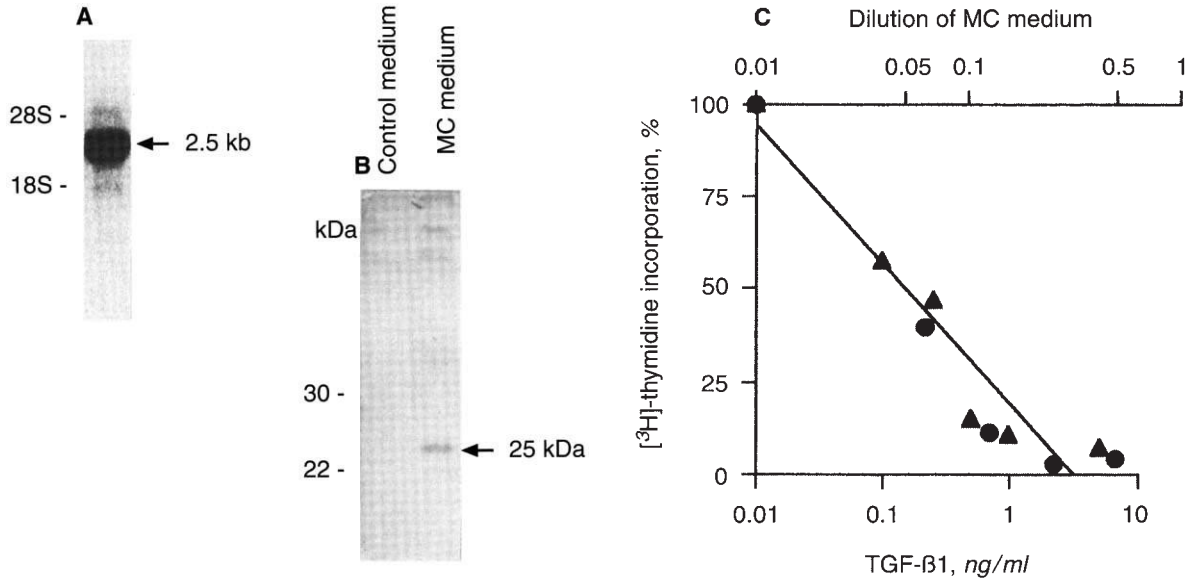

Fig. 3. *Expression of transforming growth factor-β1 (TGF-β1) in mesangial cells under basal culture conditions.* **A.** Northern blot analysis. Total RNA was extracted from confluent SM43 mesangial cells, and Northern blot analysis was performed on the expression of TGF-β1. The position of 28S and 18S ribosomal RNAs is shown on the left. **B.** Immunoblot analysis of TGF-β1 in MC medium. Control medium and MC medium were precipitated with 10% trichloroacetic acid and electrophoresed on a 10% polyacrylamide gel. Immunoblot analysis was performed using an anti-TGF-β1 antibody which recognizes the active form of TGF-β1. The color reaction was developed using an immunoperoxidase method. The position of molecular-weight markers (30 and 22 kDa) is shown on the left. **C.** Bioassay of TGF-β activity. TGF-β bioactivity was evaluated by a growth inhibition assay using the indicator cell line, CCL64. Cells were exposed to 2 to 16 times diluted mesangial cell conditioned media (●), pulsed with [^{3}H]-thymidine, and incorporation of radioactivity was measured by liquid scintillation counting. As a standard, human TGF-β1 (0 to 5 ng/ml; ▲) was used.

cells were separately harvested from identical culture plates, and Northern blot analysis was performed. As demonstrated in Figure 5B, treatment with MC medium markedly suppressed the expression of IL-1β and IL-6 in adherent macrophages, as reported previously [24]. Under the same culture conditions, detached macrophages exposed to MC medium exhibited lower levels of cytokine expression than adherent cells. The normalized values of mRNA level in non-adherent cells were 36% (IL-1β) and 37% (IL-6) compared to adherent cells (100%).

Discussion

Adhesion is a crucial event for macrophages to remain and function at inflammatory sites. In various forms of glomerulonephritis, accumulation of macrophages is one of the most typical pathological features [1], whereas little is understood about the mechanisms involved in macrophage adhesiveness within the glomerular microenvironment. In this report, we describe a novel mechanism whereby glomerular mesangial cells regulate adhesiveness of macrophages. We address the fact that mesangial cells, but not other cells tested, release factors which induce macrophage detachment from substrata. This activity is not restricted to a particular macrophage cell line, since the same effect of MC medium was observed with another macrophage line NR8383.

There are several possible mechanisms involved in the negative effect of MC medium on macrophage adhesiveness. Certain factors secreted from mesangial cells could reduce adhesiveness via cytotoxicity. However, our data showed that the decreased adhesiveness was reversible and that cell viability was unaffected by MC medium. Mesangial cell-derived factors might directly interfere with interactions between macrophages and substrata, but the fact that the initial adhesion of macrophages was not affected by the MC medium, excluded this possibility. Mesangial cells may modulate macrophage adhesiveness by suppressing expression of certain adhesion receptors. Indeed, our data could imply the requirement of new protein synthesis for the adhesive ability of macrophages, since (i) macrophages pretreated with MC medium showed reduced initial adhesion, and (ii) this impaired adhesiveness gradually recovered to a normal level following the withdrawal of MC medium.

Macrophages express a variety of adhesion receptors for cellular and matrix ligands, including integrins and scavenger receptors. Monocytes/macrophages utilize integrins for adhesion to ECM within the microenvironment [37, 38]. Macrophages also use scavenger receptors and complement receptor 3 (CR3; β2 integrins) to adhere to various biological and artificial substrata including tissue culture plastic [32, 39, 40]. It has been reported that TGF-β1 down-regulates expression of scavenger receptors and CR3 in macrophages [33, 40]. Together with our present finding that TGF-β1 produced by mesangial cells is a negative regulator for macrophage adhesiveness, inhibited expression of these adhesion receptors by TGF-β1 might explain the suppressive effect of MC medium on macrophage adhesiveness.

Using an anti-TGF-β1 neutralizing antibody, we found that the contribution of TGF-β1 was partial, since an excess of anti-TGF-β1 antibody inhibited only 35% of the activity of MC medium. Furthermore, total activity of MC medium was decreased by heat treatment, suggesting the involvement of other thermolabile molecules. IL-10 or tumor necrosis factor-α

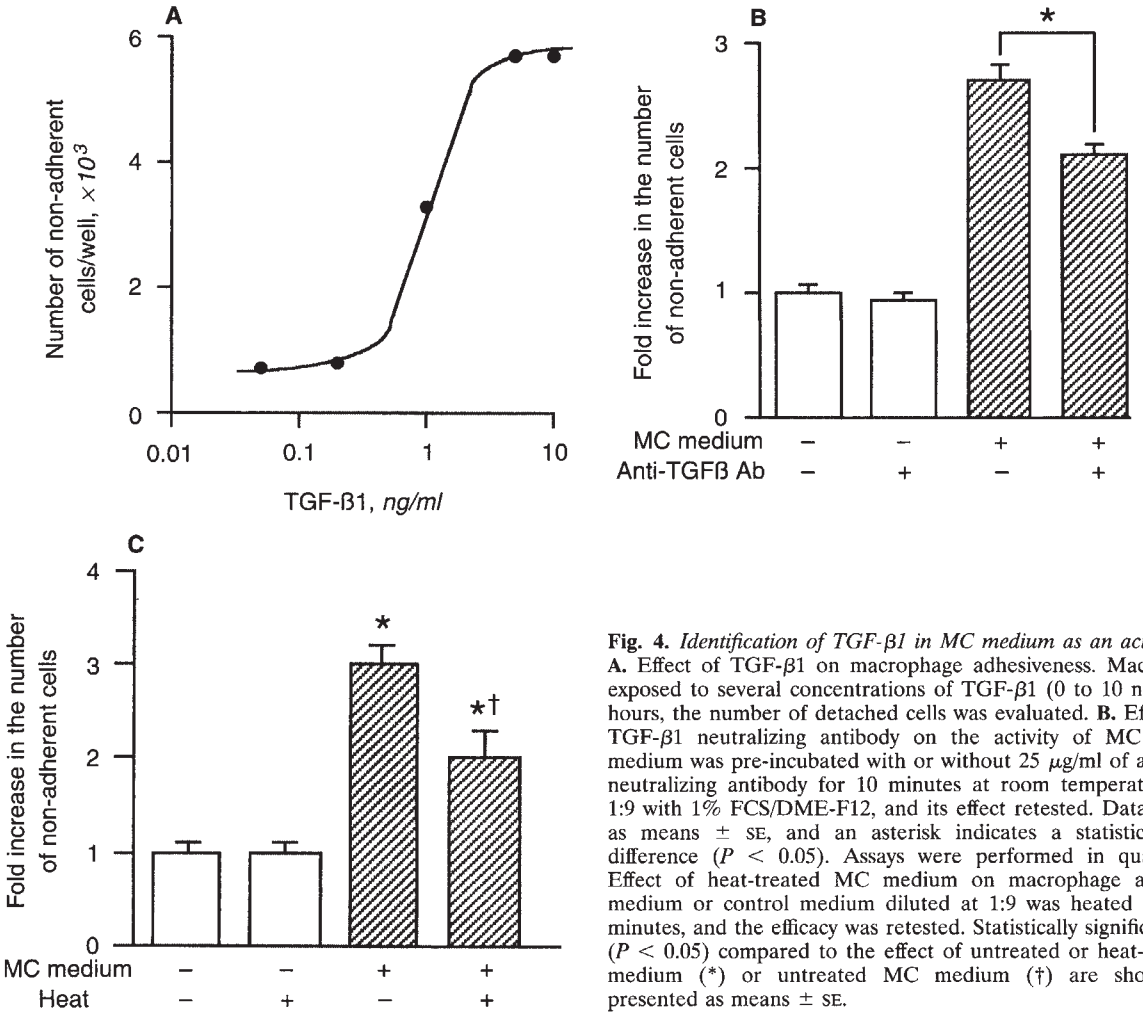

Fig. 4. *Identification of TGF-β1 in MC medium as an active component.* **A.** Effect of TGF-β1 on macrophage adhesiveness. Macrophages were exposed to several concentrations of TGF-β1 (0 to 10 ng/ml). After 24 hours, the number of detached cells was evaluated. **B.** Effect of an anti-TGF-β1 neutralizing antibody on the activity of MC medium. MC medium was pre-incubated with or without 25 μg/ml of an anti-TGF-β1 neutralizing antibody for 10 minutes at room temperature, diluted at 1:9 with 1% FCS/DME-F12, and its effect retested. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference ($P < 0.05$). Assays were performed in quadruplicate. **C.** Effect of heat-treated MC medium on macrophage adherence. MC medium or control medium diluted at 1:9 was heated at 80°C for 10 minutes, and the efficacy was retested. Statistically significant differences ($P < 0.05$) compared to the effect of untreated or heat-treated control medium (*) or untreated MC medium (†) are shown. Data are presented as means ± SE.

(TNF-α) could be candidates in this regard [41, 42], but expression of these cytokines is not normally detected in SM43 mesangial cells [24]. Further investigation will be required to elucidate the unidentified components in MC medium.

In this study, we utilized plastic plates coated with different ECM components which are found in the normal glomerulus. MC medium significantly reduced adhesiveness of macrophages seeded on laminin, collagen type IV or basement membrane Matrigel as well as on plain plastic. Interestingly, MC medium was most effective on macrophages adhered to plastic, rather than those on ECM-coated plates. This difference may be explained by the enhancement of macrophage adhesiveness by cell-ECM interaction since expression of integrins may be up-regulated by TGF-β1 [43].

Adherence is a priming trigger for a variety of monocyte/macrophage functions. For example, adhesion promotes differentiation of monocytes to tissue macrophages and induces migration, phagocytosis, respiratory oxidative burst, and expression of certain cytokines and proto-oncogenes [16–21]. These findings indicate that adhesiveness of macrophages within the glomerulus may play an important role in the regulation of cell function. We, therefore, examined the relationship between macrophage adhesiveness and its activity following the exposure to MC medium. Compared to adherent cells, non-adherent macrophages showed reduced mitogenic activity and blunted expression of cytokines in response to LPS. These findings indicate that mesangial cell-derived factors, including TGF-β1, induce detachment of macrophages followed by their blunted responses to a specific stimulus.

In the acute, reversible model of anti-Thy1 nephritis in the rat, a transient accumulation of monocytes/macrophages is observed within 24 hours following injection of an anti-Thy1 antibody [44]. The increased number of local macrophages is sustained up to day 7 and declines thereafter. At day 14, the majority of the inflammatory macrophages disappear from the glomerulus [44], conceivably by trafficking to draining lymph nodes [45]. In this nephritis model, up-regulation of glomerular TGF-β1 is detected from day 4 and continues at least until day 14 [46]. Interestingly, the anti-Thy1 antibody re-injected at day 14 does not induce macrophage accumulation [44]. These data suggest that up-regulation of TGF-β1 is closely correlated not with accumulation but with reduced macrophage retention in the glomerulus. In the recovery from glomerulonephritis, reduced adhesiveness of macrophages would be required. Our data demonstrated that serum-activated mesangial cells exhibited higher levels of anti-adhesion activity than serum-depleted, inactivated mesangial cells. The negative effect of mesangial cell-derived factors including TGF-β1 on macrophage adhesiveness may play a role in the clearance of macrophages from the nephritic glomeruli and, in part, participate in the recovery of the glomerulus from acute inflammation.

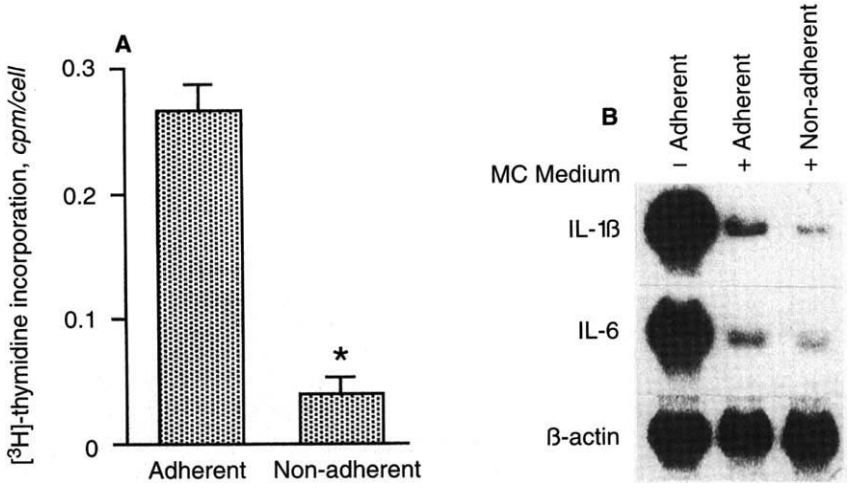

Fig. 5. *Activity of detached macrophages following the treatment with MC medium.* **A.** Mitogenic activity. Macrophages suspended in MC medium (1:1 dilution) were seeded in 24-well plates at a density of 2.5×10^4 cells/well. After incubation for 48 hours, the cells were pulsed with 1 μCi of [^{3}H]-thymidine per well for 3 hours. Adherent and non-adherent macrophages were then harvested separately, and the cell numbers were determined. Incorporation of radioactivity was measured by liquid scintillation counting, and values expressed as [^{3}H]-thymidine incorporation per cell. Data are presented as means ± SE, and an asterisk indicates a statistically significant difference ($P < 0.05$). Assays were performed in quadruplicate. **B.** Cytokine expression. Macrophages were cultured for 24 hours in the presence or absence of MC medium (1:1 dilution) and stimulated by lipopolysaccharide (LPS; 1 μg/ml) for an additional 24 hours. Adherent and non-adherent cells were separately harvested from identical culture plates, and Northern blot analysis was performed for evaluation of IL-1β and IL-6 mRNAs. As a loading control, expression of β-actin mRNA is shown.

The pathophysiological roles of TGF-β in the glomerulus are complicated. Constitutive expression of TGF-β1 may be detected in the normal rat glomerulus [46–48]. This expression is up-regulated in several glomerular diseases, especially in glomerulonephritis, and may facilitate matrix accumulation leading to glomerulosclerosis [49]. However, there is an additional line of evidence that suggests an anti-inflammatory property of TGF-β. TGF-β1 is known to inhibit proliferation of glomerular cells [50–53], suppress their responses to the proinflammatory cytokine IL-1β [35], and could participate in re-differentiation of activated glomerular cells [54]. We recently reported that mesangial cell-derived TGF-β1 suppresses production of proinflammatory cytokines by activated, adherent macrophages [24]. Furthermore, TGF-β1 is known to have suppressive effects on several other functions of macrophages; that is, this molecule strongly inhibits macrophage production of potentially injurious inflammatory mediators such as reactive oxygen radicals and nitric oxide at picomolar concentrations [30, 55]. Indeed, Largen and coworkers recently reported that MC medium inhibited nitric oxide production by rat peritoneal macrophages [56]. These findings, together with our current data, reinforce the anti-inflammatory potential of TGF-β1 in early glomerular injury. Further investigation will be required to elucidate fully the beneficial properties of TGF-β, so as to determine the time point at which its anti-inflammatory properties spill over into prosclerotic properties in glomerulonephritis.

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