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Monocytes Induce Proximal Tubular Epithelial–Mesenchymal Transition Through NF-kappa B Dependent Upregulation of ICAM-1

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ABSTRACT

Inflammatory cell infiltration plays a key role in the pathogenesis of tubulointerstitial damage in chronic renal diseases. In addition to secreting the profibrotic cytokines, monocytes themselves have been demonstrated to be directly associated with renal fibrogenesis. However, how infiltrating monocytes interact with resident cells and the underlying mechanisms remain elusive. In this study we investigated the effects of monocytes on phenotypic changes of human proximal tubular HK-2 cells. The typical epithelial cell morphology of HK-2 cells disappeared after co-culture with monocytes, accompanied by decreased E-cadherin expression, and increased α-SMA and fibronectin expression, suggesting that HK-2 cells undergo epithelial–mesenchymal transition (EMT). Further analysis revealed that the effects were dependent on direct contact of the two types of cells as conditioned medium had no effects. Interestingly, administration of CD18 antibody directly inhibited this process. Furthermore, by microarray and RT-PCR we found that NF-kB signaling may play a role in this process and blockade of this signaling pathway in HK-2 cells could inhibit ICAM-1 expression and EMT phenotypes. Taken together, these findings suggest that monocytes infiltration could directly induce EMT of HK-2 cells via upregulation ICAM-1 through NF-kB signaling pathway. J. Cell. Biochem. 112: 1585–1592, 2011.

KEY WORDS: MONOCYTE; PROXIMAL TUBULAR CELLS; EMT; NF-κB; ICAM-1

Renal tubulointerstitial fibrosis (TIF), characterized by the accumulation of extracellular matrix (ECM), is considered as the hallmark of progressive renal disease. Recently, increasing evidence suggested that epithelial–mesenchymal transition (EMT), by which epithelial cells lose their epithelial specific markers, undergo cytoskeletal remodeling, and gain a mesenchymal phenotype, plays a prominent role in TIF [Rastaldi et al., 2002; Liu, 2004; Li, 2007; Vitalone et al., 2008; Acloque et al., 2009]. Infiltration of inflammatory cells, particularly monocyte/macrophage, has also been implicated in the pathogenesis of TIF [Lavaud et al., 1996; Ko et al., 2008; Wang et al., 2008]. EMT develops upon inflammation during wound healing and tissue regeneration but then stops once inflammation is attenuated. Nevertheless, in the setting of organ fibrosis, EMT continues to respond to ongoing inflammation, eventually leading to organ destruction [Kalluri and Weinberg, 2009]. Once present at interstitial sites, monocyte interacts with resident cells and ECM to generate a proinflammatory microenvironment that amplifies tissue injury via monocyte/macrophage recruitment [Vielhauer et al., 2010].

Intercellular adhesion molecule-1 (ICAM-1) is the principal adhesion molecule expressed on resident interstitial cells and recognized by monocytes. As an inducible transmembrane receptor, ICAM-1 forms the counter-receptor for the monocyte β2-integrins (CD18). ICAM-1 appears to be functionally the most important adhesion molecule in monocytes accumulation in many renal diseases. Our previous study demonstrated that binding of monocytes to proximal tubular cells (PTCs) through ICAM-1 stimulated the synthesis of TGF-β1, which is involved in TIF [Zhang et al., 2005]. However, the association between infiltration of monocytes and tubular EMT, two key events to the development of TIF, has not been directly addressed so far.

Therefore, in the present study, we established a monocytes tubular cells co-culture model and investigated whether monocytes could induce tubular cells EMT. Our results suggest that monocytes

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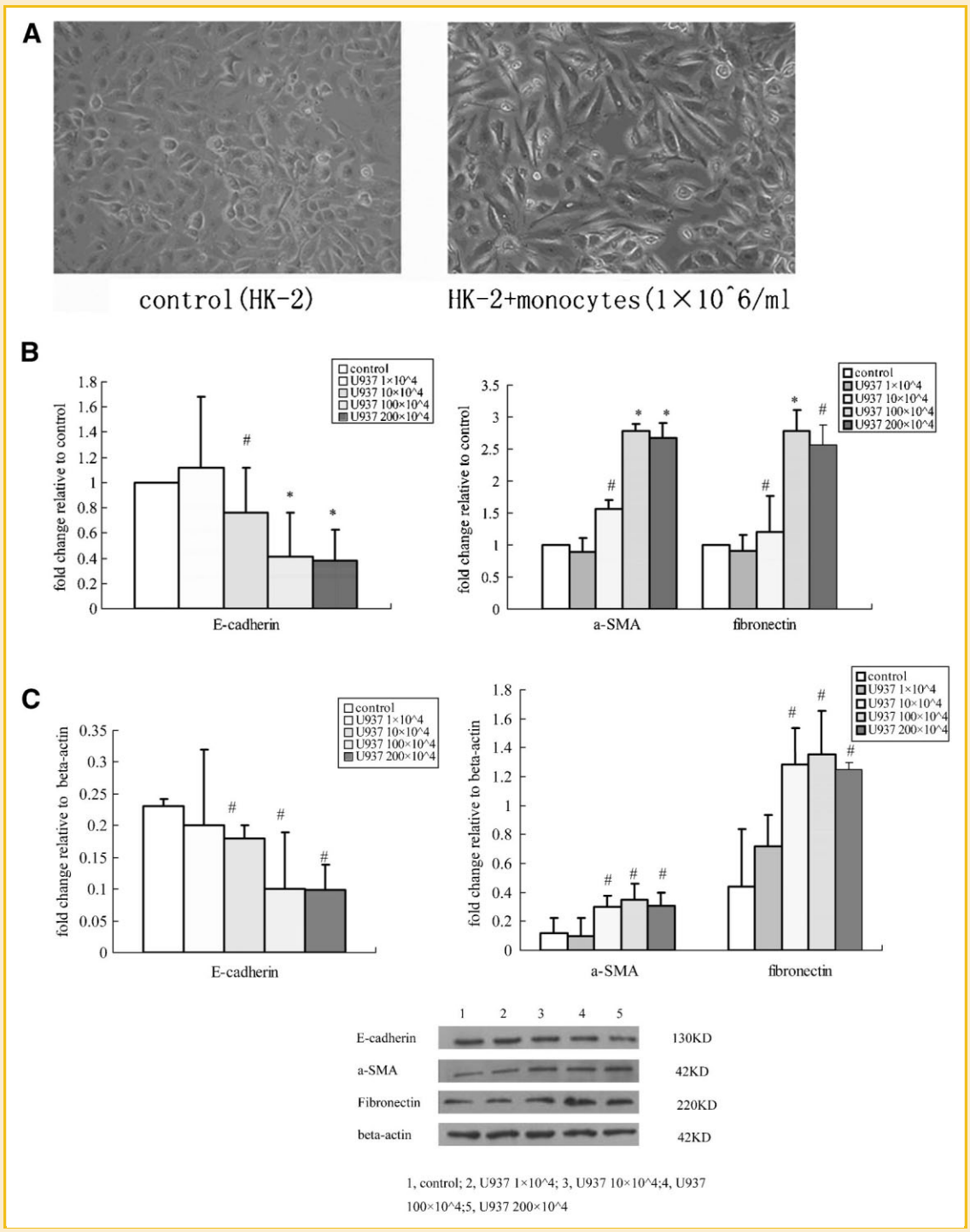

Fig. 1. HK-2 cells undergo EMT upon co-culture with monocytes. A: Phase contrast microscopy of phenotypic change of HK-2 cells. The normal phenotypes of HK-2 cells were disappeared after co-culture with monocytes (1×10^6/ml) for 24 h, HK-2 cells developed a series of phenotypic changes including elongation, branching, and lose of a cobblestone appearance under phase contrast microscopy (10×10). B: Real-time PCR of the total E-cadherin, α-SMA, and fibronectin mRNA prepared from HK-2 cells co-cultured with monocytes showed that E-cadherin mRNA level in HK-2 cells was significantly downregulated when co-cultured with monocytes ($\#P < 0.05$, $^*P < 0.01$ vs. control). α-SMA and fibronectin mRNA levels in HK-2 cells were significantly upregulated when co-cultured with monocytes in a dose-dependent manner ($\#P < 0.05$, $^*P < 0.01$ vs. control), GAPDH was used as mRNA loading control. C: Western blot of total E-cadherin, α-SMA, and fibronectin protein prepared from HK-2 cells showed that E-cadherin protein level in HK-2 cells was significantly downregulated when co-cultured with monocytes ($\#P < 0.05$ vs. control). α-SMA and fibronectin protein levels in HK-2 cells were significantly upregulated when co-cultured with monocytes in a dose-dependent manner ($\#P < 0.05$ vs. control),β-actin was used as a protein loading control.

infiltration could directly induce EMT of HK-2 cells via upregulation ICAM-1 through NF-kB signaling pathway.

MATERIALS AND METHODS

CELL CULTURE

HK-2 cells, human proximal tubular epithelial cells immortalized by transduction with human papilloma virus 16 E6/E7 genes, were cultured in DMEM/Ham's F12 (Gibco, UK) supplemented with 10% heat-inactivated fetal calf serum (FCS, Sijiqing, China). U937 cells, originally derived from a human histocytic lymphoma, were grown in suspension culture in RPMI medium supplemented with 5% FCS, L-glutamine and penicillin/streptomycin. For co-culture of HK-2 and U937 cells, the co-culture experiments were performed in six-well plates. U937 cells were washed once with serum-free RPMI 1640 medium and different number of U937 cells was added to the HK-2 cell monolayer in 1 ml serum-free DMEM medium and the cells were co-cultured.

PREPARATION OF CONDITIONED MEDIUM

Conditioned medium was prepared either from U937 cells cultured alone (cultured medium, CM1) or from U937 cells exposed to HK-2 cell monolayer for 24 h (CM2).

REAL-TIME PCR

Total RNA was extracted from HK-2 cells by RNAiso plus reagent and cDNA was then synthesized by using a reverse transcription (RT) system kit (Takara, Japan). RT-PCR was performed on ABI PRISM 7300 real-time PCR System (Applied Biosystems). The primers used for real-time PCR were as follows: α-SMA: 5′-GACAATGGCTCTGGGCTCTGTAA-3′ 5′-ATGCCATGTTCTATCGGGTACTTCA-3′; Fibronectin: 5′-GAGCTGCACATGTCTTGGGAAC-3′ 5′-GGAGCAAATGGCACCGAGATA-3′; E-cadherin: 5′-GGATTGCAAATTCCTGCCATTC-3′ 5′-AACGTTGTCCCGGGTGTCAAG-3′; GAPDH: 5′-GCACCGTCAAGGCTGAGAAC-3′ 5′-ATGGTGGTGAAGACGCCAGT-3′.

WESTERN BLOT ANALYSIS

Total proteins were extracted from HK-2 cells, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and electrotransferred onto PVDF membranes. The membranes were blocked with 5% nonfat milk in Tris-buffered saline with 0.5% Tween 20 (TBST) overnight at 4°C, incubated with primary antibodies of mouse anti-human E-cadherin (Cell signaling), rabbit anti-human α-SMA (Abcam, UK), mouse anti-human fibronectin, rabbit anti-human NF-kB(p65) (Cell signaling), or mouse anti-human β-actin (Santa Cruz), for 1 h at room temperature, followed by the incubation with HRP-conjugated secondary antibodies of goat anti-rabbit or goat anti-mouse for 1 h. Finally, the signals were detected using ECL advanced system (GE Healthcare, UK).

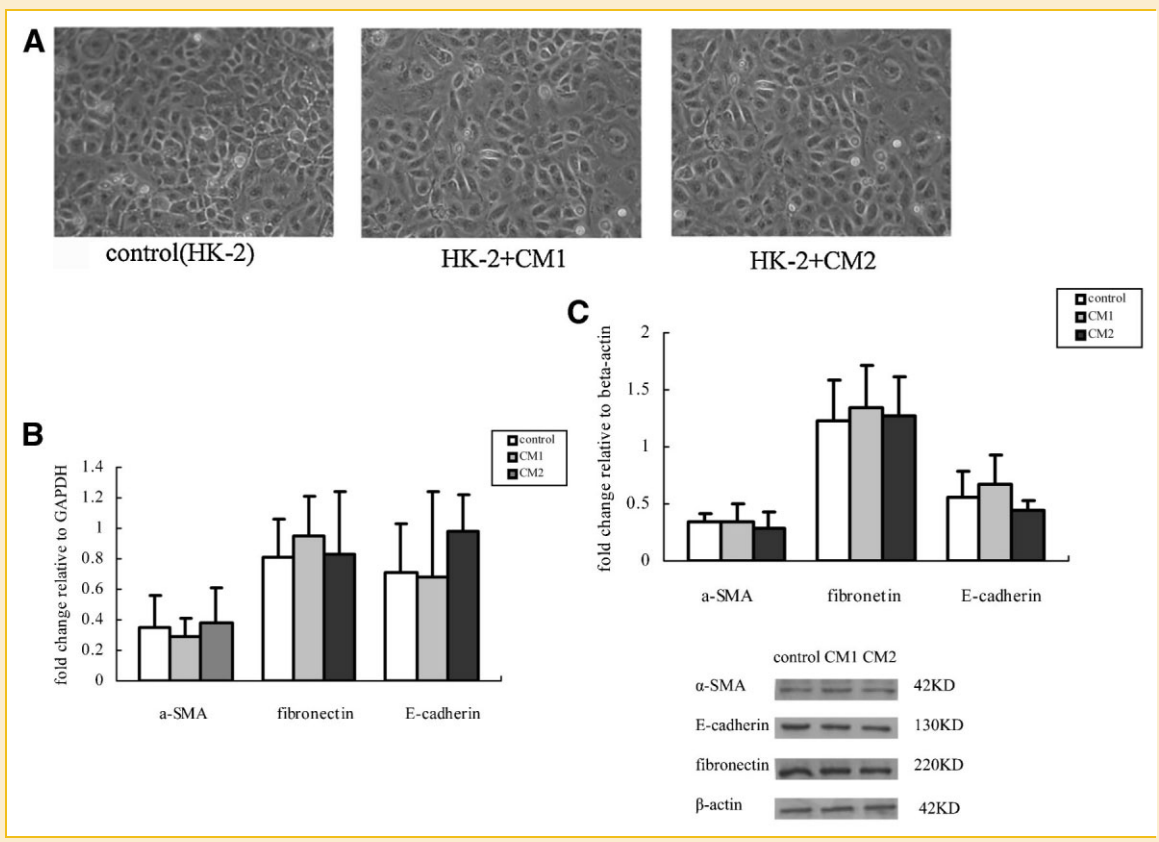

Fig. 2. HK-2 cells fail to undergo EMT upon incubation with conditioned medium from monocytes. A: Phase contrast microscopy of phenotypic change of HK-2 cells treated with conditioned medium showed that no difference was observed among phenotypes of HK-2 cells untreated, treated with CM1 for 24 h, or treated with CM2 for 24 h (10 × 10). B: Results of real-time PCR analysis showed that there were no significant changes in the expression of E-cadherin, α-SMA, or fibronectin at mRNA level, GAPDH was used as a mRNA loading control. C: Western blots analysis of the protein level was the same with mRNA level. β-actin was used as a protein loading control.

STATISTICAL ANALYSIS

All the data were expressed as mean ± SD and analyzed by one-way ANOVA using SPSS13.0 statistical software. Values of $P < 0.05$ were considered to be statistically significant.

RESULTS

HK-2 CELLS UNDERGO EMT UPON CO-CULTURE WITH MONOCYTES

Upon co-culture with monocytes (1×10^6/ml) for 24 h, HK-2 cells developed a series of phenotypic changes including elongation, branching, and losing cobblestone like feature under phase contrast microscopy while the cells that were not co-cultured with monocytes failed to demonstrate these changes (Fig. 1A), suggesting that the cells may undergo EMT. Thus we went on to examine the expression of E-cadherin, α-SMA, and fibronectin in these cells since the downregulation of E-cadherin and upregulation of α-SMA and fibronectin are hallmarks of EMT. By real-time PCR analysis we indeed found that the mRNA level of E-cadherin in HK-2 cells was decreased gradually upon co-culture with increasing amount of U937 monocytes and the mRNA levels of α-SMA and fibronectin

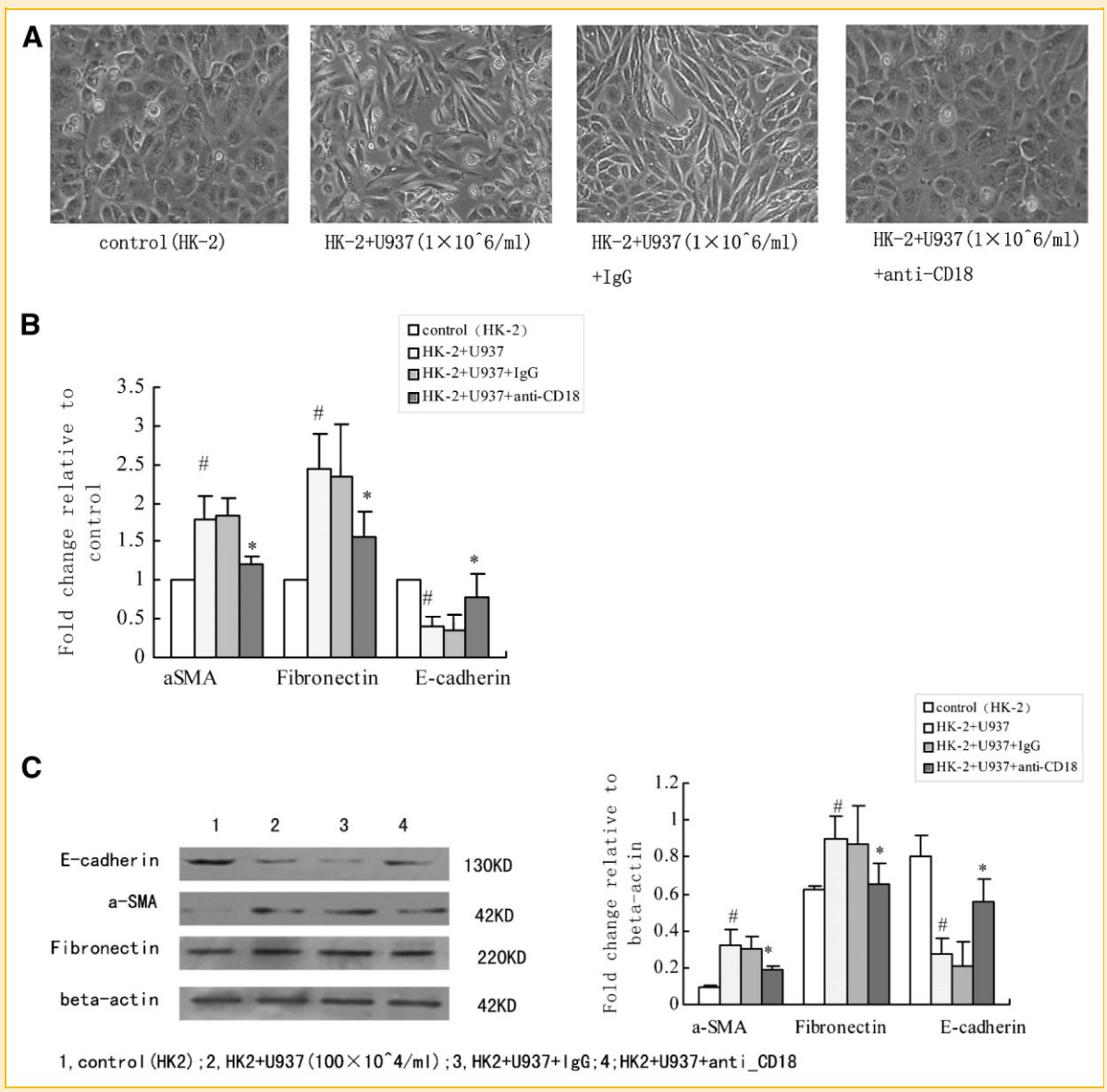

Fig. 3. Blockage of ICAM-1–CD18 interaction inhibits monocyte induced HK-2 cells transition. A: Phase contrast microscopy of phenotypic change of HK-2 cells, treated with monocytes, and blocked by anti-CD18. The normal phenotypes of HK-2 cells disappeared after co-culture with U937 monocytes (1×10^6/ml) for 24 h, HK-2 cells became elongation, branching, and losing a cobblestone feature. Anti-CD18 antibody reversed the phenotype changes of HK-2 cells. IgG was used as a control (10×10). B: Real-time PCR of the total E-cadherin, α-SMA, and fibronectin mRNA prepared from HK-2 cells showed that anti-CD18 reversed the downregulation of E-cadherin, and the upregulation of α-SMA and fibronectin induced by monocytes (#$P < 0.05$ vs. control, *$P < 0.05$ vs. monocytes group). C: Western blots of total E-cadherin, α-SMA, and fibronectin protein prepared from HK-2 cells were the same with mRNA levels (#$P < 0.05$ vs. control, *$P < 0.05$ vs. monocytes group).

were significantly higher in HK-2 cells co-cultured with U937 monocytes than in HK-2 cells cultured alone (Fig. 1B; $\#P < 0.05$, $^{*}P < 0.01$ vs. control). Furthermore, by Western blot we found that the protein level of E-cadherin in HK-2 cells was similarly decreased gradually upon co-culture with increasing amount of U937 monocytes and protein levels of α-SMA and fibronectin were significantly higher in HK-2 cells co-cultured with U937 monocytes than in HK-2 cells cultured alone ($\#P < 0.05$ vs. control; Fig. 1C). Taken together, these data strongly suggest that HK-2 cells undergo EMT upon co-culture with monocytes.

HK-2 CELLS FAIL TO UNDERGO EMT UPON INCUBATION WITH CONDITIONED MEDIUM

One obvious possibility is that EMT of HK-2 cells is induced by cytokines secreted by the monocytes alone or soluble factors produced after the two types of cells come into direct contact. To explore this possibility we prepared two kinds of conditioned medium, i.e., from U937 cells cultured alone (CM1) or from U937 cells exposed to HK-2 cell monolayer for 24 h (CM2), then we treated HK-2 cells with these conditioned medium. Interestingly, we found that neither CM1 nor CM2 induced the phenotype changes of HK-2 cells (Fig. 2A). Next we examined the effects of conditioned medium on the expression of E-cadherin, α-SMA, and fibronectin in HK-2 cells. Real-time PCR and Western blot analysis revealed that there was no significant changes in the expression of E-cadherin, α-SMA, or fibronectin in HK-2 cells at both mRNA (Fig. 2B) and protein levels (Fig. 2C). Altogether, these results suggest that cytokines or soluble factors released from monocytes cultured alone or co-cultured with HK-2 cells are unlikely to be responsible for EMT of HK-2 cells.

BLOCKAGE OF ICAM-1–CD18 INTERACTION INHIBITS MONOCYTES INDUCED HK-2 CELLS TRANSITION

The next possibility is that the direct cell–cell contact accounts for EMT of HK-2 cells. ICAM-1 is the principal adhesion molecule expressed on resident interstitial cells and recognized by monocytes. Therefore, we employed anti-CD18 to block ICAM-1–CD18 mediated cell–cell contact between monocytes and HK-2 cells. Notably, anti-CD18 antibody blocked U937 monocytes induced phenotypic changes of HK-2 cells (Fig. 3A). Furthermore, anti-CD18 reversed the downregulation of E-cadherin, and the upregulation of α-SMA and fibronectin induced by monocytes at both mRNA (Fig. 3B) and protein levels (Fig. 3C; $\#P < 0.05$ vs. control, $^{*}P < 0.05$ vs. monocytes group).

MONOCYTES ACTIVATED NF-kB SIGNALING IN HK-2 CELLS

To gain further insight into the mechanisms involved in monocytes induced EMT of HK-2 cells, we performed microarray experiments and found that molecules involved in NF-kB signaling such as CCL20, IL2, IL8, LTA, and PECAM1 were upregulated significantly in HK-2 cells co-cultured with U937 monocytes (Fig. 4A; $\#P < 0.05$, $^{*}P < 0.01$ vs. control). By Western blot analysis, very low level of NF-kB (p65) was detected in HK-2 cells that had not been activated with monocytes (lane 1), while NF-kB level was increased upon co-cultured with increasing amount of monocytes (lanes 2–4; Fig. 4B; $\#P < 0.05$ vs. control). These data suggested that NF-kB signaling might be involved in the monocytes induced EMT of HK-2 cells.

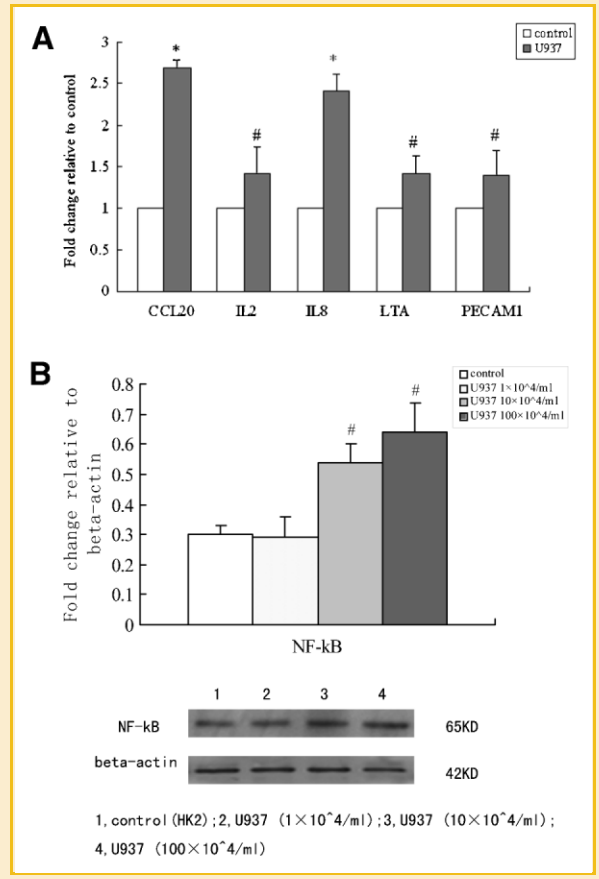

Fig. 4. Monocytes activated NF-kB signaling in HK-2 cells. A: Microarray experiments showed that the expression of CCL20, IL2, IL8, LTA, and PECAM1 were upregulated in HK-2 cells co-cultured with U937 monocytes ($\#P < 0.05$, $^{*}P < 0.01$ vs. control). B: Western blot analysis showing low level of NF-kB (p65) in HK-2 cells cultured alone (lane 1) and higher level of NF-kB in HK-2 cells co-cultured with increasing amount of monocytes (lanes 2–4; $\#P < 0.05$ vs. control).

BLOCKING NF-kB SIGNALING INHIBITS EMT OF HK-2 CELLS VIA THE DOWNREGULATION OF ICAM-I

Since ICAM-1 is a crucial molecule that mediates the direct contact between monocytes and HK-2 cells as shown above, first we examined the potential involvement of NF-kB signaling in the regulation of ICAM-1 expression. By using NF-kB inhibitor PDTC we demonstrated that it inhibited U937 monocyte-induced upregulation of ICAM-1 in HK-2 cells at both protein (Fig. 5A) and mRNA levels (Fig. 5B; $\#P < 0.05$ vs. control, $^{*}P < 0.05$ vs. monocytes group). Next we examined the effects of PDTC on EMT of HK-2 cells and found that the phenotype changes of HK-2 cells induced by direct contact with monocytes was prevented by PDTC (Fig. 5C). Consistent with this observation, we further demonstrated that PDTC promoted the expression of E-cadherin and inhibited the expression of α-SMA and fibronectin in HK-2 cells co-cultured with U937 monocytes at both mRNA (Fig. 5D) and protein levels

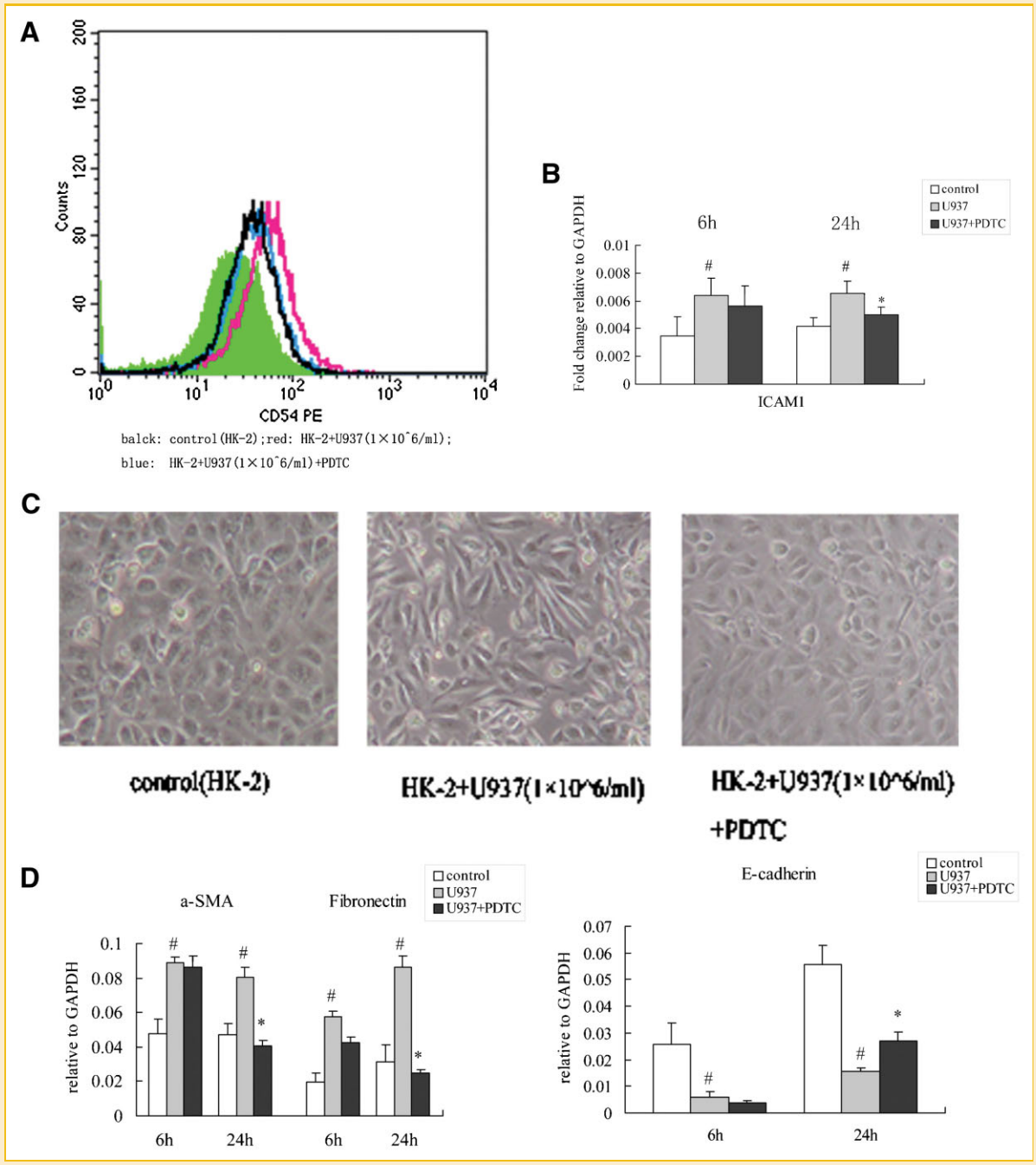

Fig. 5. Blocking NF-kB signaling inhibits EMT of HK-2 cells via the downregulation of ICAM-1. A: Results from flow cytometry showed that ICAM-1 protein level in HK-2 cells was decreased after PDTC treatment compared with untreated cells. B: Real-time PCR of the total ICAM-1 mRNA level in HK-2 cells showed that the ICAM-1 mRNA was decreased after PDTC treatment compared with untreated cells (#$P < 0.05$ vs. control, *$P < 0.05$ vs. monocyes group). C: Phase contrast microscopy showed that PDTC reversed the phenotype changes of HK-2 cells induced by monocytes to normal (10 × 10). D: Results of real-time PCR analysis showed E-cadherin mRNA in HK-2 cells was significantly upregulated, whereas, those of α-SMA and fibronectin were significantly downregulated after PDTC treatment compared with untreated cells (#$P < 0.05$ vs. control, *$P < 0.05$ vs. monocyes group). E: Western blots analysis showed that E-cadherin protein in HK-2 cells was significantly upregulated, whereas, those of α-SMA and fibronectin were significantly downregulated after PDTC treatment (#$P < 0.05$ vs. control, *$P < 0.05$ vs. monocyes group). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

(Fig. 5E; #$P < 0.05$ vs. control, *$P < 0.05$ vs. monocytes group). Taken together, these results suggest that direct contact between monocytes and HK-2 cells leads to the activation of NF-kB signaling in HK-2 cells, which in turn upregulates ICAM-1 expression and induces EMT of HK-2 cells.

DISCUSSION

TIF is a frequent consequence of chronic renal disease irrespective of its etiology and represents the major lesion of end-stage renal diseases. TIF is characterized by tubular atrophy/dilation, interstitial

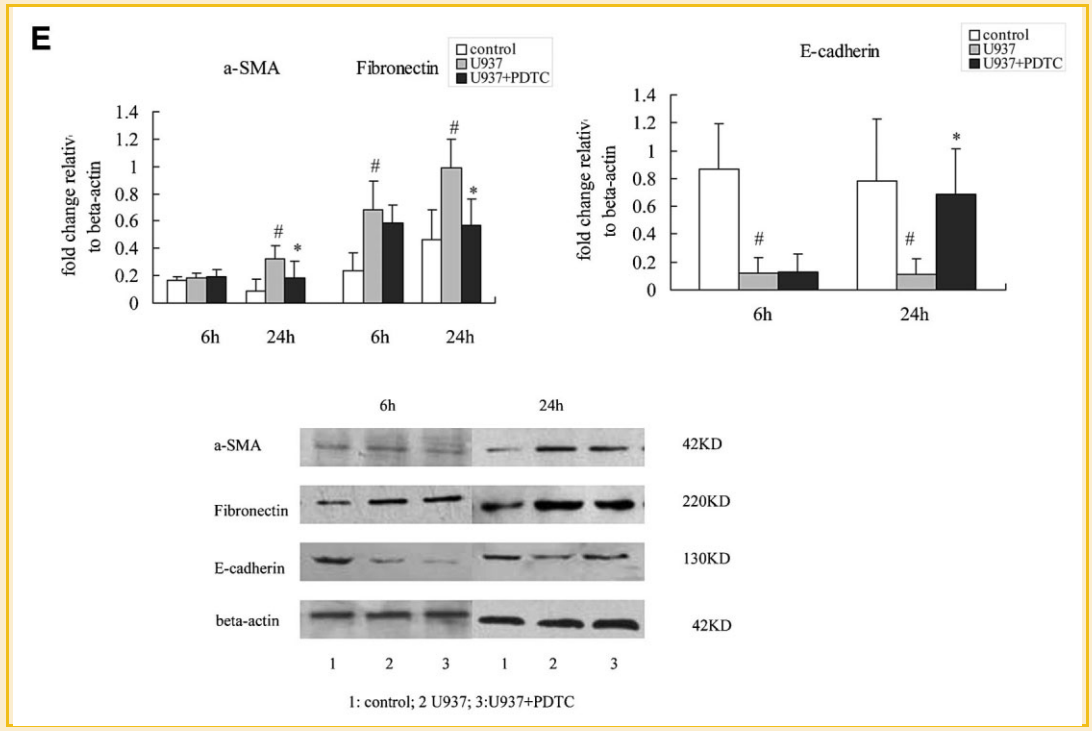

Fig. 5. (*Continued*)

monocyte infiltration, accumulation of fibroblasts, and increased ECM deposition [Zeisberg et al., 2001]. During the progression of TIF, the synthesis of ECM is increased while its degradation is reduced, consequently leading to the accumulation of ECM in the interstitial space. Although many different cell types are involved, myofibroblasts are considered as the main initiator of fibrosis in the kidney [Iwano et al., 2002; Strutz and Zeisberg, 2006]. More recent evidence suggested that myofibroblasts could be transdifferentiated from the tubular epithelial cells via EMT or endothelial cells via EndMT [Zeisberg et al., 2007]. Rastaldi et al. [2002] reported that the number of cells with EMT markers was correlated with the degree of interstitial damage and renal dysfunction, indicating the significant importance of the phenotypic change of resident renal cells in the progression of chronic renal disease.

Infiltration of inflammatory cells, particularly monocyte/macrophage, becomes as a known feature of tubulointerstitial damage in many chronic renal diseases. Complete inhibition of renal monocyte/macrophage accumulation prevented the upregulation of the macrophage-associated proinflammatory mediators, tumor necrosis factor (TNF)-alpha, and matrix metalloproteinase-12, and significantly reduced tubular apoptosis [Ma et al., 2009]. Nevertheless how infiltrating inflammatory cells interact with resident cells and subsequently lead to TIF remains a largely unexplored field. In the present study, we demonstrated that co-culture of monocyte and HK-2 cell induced the phenotypic change of HK-2 cells characterized by cell elongation, branching, and losing cobblestone like feature. Notably, co-culture induced the downregulation of E-cadherin and upregulation of α-SMA and fibronectin in HK-2 cells, suggesting that monocytes promote the phenotypic change of PTCs via EMT.

To unravel whether the effect is induced by cytokines or other soluble factors secreted by monocytes or direct contact between monocytes and HK-2 cells, different conditioned medium were prepared and used to treat HK-2 cell, the results suggested that the conditioned medium had no significant effects on HK-2 cells. Instead we found that direct contact of monocytes with HK-2 cells may play a key role in EMT. This is consistent with our previous study that monocyte induced TGF-β1 expression in HK-2 cells via direct contact of the two types of cells rather than conditioned medium [Zhang et al., 2004].

ICAM-1 are constitutively expressed at low level on the surface of a wide variety of cells including fibroblasts, leukocytes, keratinocytes, endothelial cells, hepatocytes, smooth muscle, and epithelial cells [Rothlein et al., 1986]. ICAM-1 has five Ig-like domains, which function in mediating cell–cell and cell–matrix interactions. Interaction between CD18 on the surface of monocytes and its ligand ICAM-1 on the surface of resident cells initiates the activation of resident cells, such as epithelial cells. In present study, the direct contact of monocytes and HK-2 cells seems to be mediated via CD18–ICAM-1 interaction, since anti-CD18 could inhibit the phenotypic change of HK-2 cells. This is in agreement with previous findings that direct contact of leucocytes and human renal fibroblasts expressing ICAM-1 led to further upregulation of ICAM-1 expression [Blaber et al., 2003]. These data suggest an alternative mechanism of inflammatory amplification, which may be characteristic of a persistent leukocytic involvement in areas of

chronic inflammation rather than in cytokine-induced acute inflammation. Although ICAM-1 is predominantly expressed on the apical site of PTCs, numerous studies have demonstrated its expression on their baso-lateral site in renal disease, consistent with the finding of polarized interactions between interstitial inflammatory cells and PTCs in in vivo study [Daniel et al., 2001; Le Hir and Besse-Eschmann, 2003].

In order to gain further insight into the mechanisms involved in monocytes induced EMT, we performed microarray, RT-PCR and WB experiments and revealed the engagement of NF-kB signal in this process. The transcription factor NF-kB regulates the expression of many inflammatory genes and adhesion molecules [Blackwell and Christman, 1997], when it is freed from an inhibitory action of IkB [IM, 1997; Liu, 2006]. In our study we found that co-culture activates NF-kB signaling and induces ICAM-1 expression in HK-2 cells, thus promoting the contact between monocytes and HK-2 cells and finally leading to EMT of HK-2 cells.

In summary, we have identified a novel mechanism that regulates monocytes dependent PTCs activation. Specifically, we demonstrate that via ICAM-1–CD18 mediated direct cell–cell contact monocytes promote the activation of NF-kB signaling in PTCs, which in turn induces the transition of PTCs to myofibroblasts, a process characterized by the downregulation of E-cadherin and upregulation of α-SMA and fibronectin.

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