European Review for Medical and Pharmacological Sciences 2012; 16: 630-638

Interleukin-8 induces the endothelial cell migration through the Rac1/RhoA-p38MAPK pathway

Y. LAI[1], X.H. LIU[1], Y. ZENG[1], Y. ZHANG[2], Y. SHEN[1], Y.F. LIU[2]

[1]Institute of Biomedical Engineering, West China School of Preclinical and Forensic Medicine, Sichuan University, Chengdu (China)
[2]Laboratory of Biomedical Ultrasonics, West China Second University Hospital, Sichuan University, Chengdu (China)

Abstract. – *Background and Objectives:* **Endothelial cell migration is essential for tumor angiogenesis, and Interleukin-8 (IL-8) has been shown to play an important role in tumor growth, angiogenesis, and metastasis. The objective of this study was to investigate the molecular mechanism of IL-8 induced endothelial cell migration *in vitro*.**

Material and Methods: **Fluorescence microscope was used to study the distribution of cytoskeleton. The expression of Rac1 and RhoA protein was detected by western blotting. After endothelial cells were transfected by lipofectamine 2000 reagent, the Transwell chamber motility assay was applied to observe the migration of endothelial cells induced by IL-8. The active p38MAPK (mitogen-activated protein kinase) was evaluated by the p38MAPK activation assay.**

Results: **We demonstrated that IL-8 activated cell migration can be impaired by p38MAPK inhibitor, suggesting the participation of p38MAPK in the cell migration. Our results indicated that p38MAPK signaling is required for membrane ruffles, lamellipodia extensions, and actin stress fibers formation induced by IL-8. Furthermore, p38MAPK inhibitor led to increased Rac1 and RhoA expression in IL-8 treated EA.hy926 cells. In addition, IL-8 induced p38MAPK activation was suppressed by dominant-negative mutant for Rac1 and RhoA.**

Conclusions: **Our study demonstrates that IL-8-Rac1/RhoA-p38MAPK signaling pathway plays a vital role in the IL-8-induced endothelial cell migration, and it provides new insight into the molecular mechanisms by which IL-8 contributes to tumor angiogenesis and metastasis.**

Key Words:

Cell migration, IL-8, p38MAPK.

Introduction

As an inflammatory and chemotaxis factor, interleukin-8 (IL-8) belongs to the CXC family of proteins and it is readily purified from human monocyte culture supernatant stimulated with lipopolysaccharide (LPS) or phytohemagglutinin (PHA). It is discovered by Yoshimura[1] in 1987 and it is mainly secreted by monocytes and endothelial cells. IL-8 activates multiple intracellular signaling pathways downstream of two cell-surface receptors, CXCR1 and CXCR2. Increased expression of IL-8 and/or its receptors have been characterized in cancer cells, endothelial cells, infiltrating neutrophils, and tumor-associated macrophages, suggesting that IL-8 may function as a regulatory factor in the tumor microenvironment. Tumor-derived IL-8 has the capacity to exert profound effects on the tumor microenvironment. For example, IL-8 secretion can activate endothelial cells in the tumor vasculature to promote angiogenesis. The multiple effects of IL-8 on different cell types present within the tumor microenvironment suggest that targeting of IL-8 signaling may have important implications for sensitizing tumors resistant to chemotherapy and biologic agent.

The small G protein Rac1 is one of the main regulatory factors involved in the reassembly of the actin cytoskeleton, which plays an important role in coordinating cell migration. It has been found that external stimulus could be transducted through matrix-integrin-Rho GTPases signal pathway, thus suggesting that Rac1 may be one of the critical molecules involved in IL-8-induced endothelial cell migration. Mitogen-activated protein kinases (MAPKs), including Jun N-terminus kinase (JNK), p38 and Erk, play crucial roles in cell migration[2]. The activity of p38 is stimulated by many growth factors, cytokines, and chemotactic substances. P38MAPK is involved in growth factor and cytokine induced cell migration. Activation of p38MAPK is medi-

Corresponding Author: Liu Xiao-Heng, MD; e-mail: liuxiaohg@scu.edu.cn and liuxiaog@sina.com

ated by Rac1/cdc42 GTP binding proteins[3-6]. PKC isoforms are expressed to varying degrees in all mammalian cells and they have been linked to the change of cytoskeleton in many different cell types. A general impression of the literature is that particularly PKCα has the capacity to alter the cytoskeleton in a promigratory manner and to promote migration and that PKC δ is involved in the RhoA pathway[7,8].

Our previous study demonstrated that IL-8 could induce endothelial cell migration[9]. In addition, IL-8 induced the expression of Rac1, which then modulated cell migration[10]. However, whether p38MAPK and PKC involved in IL-8-induced endothelial cell migration or not remains largely unexplored. Therefore, the present study focused on the link between Rac1/RhoA and p38MAPK downstream of IL-8 in mediating endothelial cell migration. The results demonstrated that IL-8-Rac1/RhoA-p38MAPK signaling pathway plays a vital role in the IL-8-induced endothelial cell migration,

Materials and Methods

Cell Culture

The EA.hy926 cell line (Blood Research Institute, Jiangsu Province, China) is a hybridoma between human umbilical vein endothelial cells (HUVECs) and the epithelial lung tumor cell line A549[11]. This cell line retains most features of HUVECs, including the expression of endothelial adhesion molecules and human factor VIII-related Ag. EA.hy926 cells were grown to confluence in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS) (PromoCell, Heidelberg, Germany).

Antibodies and Reagents

RhoA monoclonal antibody and peroxidase conjugated goat anti-mouse secondary mAb were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rac1 monoclonal mAb was purchased from Cytoskeleton (Cytoskeleton, Denver, CO, USA). IL-8 was purchased from PeproTech (PeproTech, Rocky Hill, NJ, USA). BODIPY FL phallacidin was obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). DAPI was purchased from Sigma (Sigma, St. Louis, MO, USA). Staurosporine (st,a PKC inhibitor), sb203580 (sb, a p38MAPK inhibitor) were obtained from Calbiochem (Calbiochem, San Diego, CA, USA).

Cell Migration Assay

EA.hy 926 cells were incubated in serum-free medium for 12 hours prior to treatment with inhibitors. Transwell (Corning, NY, USA) filters were equilibrated in serum containing 1640 medium for 2 h. 1640 medium with 10% FBS was added into the lower chamber of the migration filters. Cells were plated in a volume of 100 ml serum-free DMEM per Transwell filter with a density of 1×10^6. Cells were pretreated with sb203580 (30 nm) or staurosporine (100 μm) for 30 min at 37°C before stimulated with 100 ng/ml IL-8 about 6h. Cells were allowed to migrate in 5% CO_2 at 37°C for 6 h and were subsequently fixed by immersion of the filters in methanol at room temperature for 15 min. Filters were washed with deionized water and stained in 0.2% crystal violet in a 20% methanol/water solution for 10 min. Cells were removed from the upper surface of the membrane with a cotton swab. Cells that had migrated to the underside of the membrane were counted at 200× magnification from five random fields on each membrane.

Western Blot Analysis

EA.hy 926 cells were incubated in serum-free medium for 12 hours prior to treatment with inhibitors. Cells were pretreated with sb203580 (30 nm) or staurosporine (100 μm) for 30 min at 37°C before stimulated with 100 ng/ml IL-8 at six time point (0 min, 5 min, 10 min, 30 min, 1 h, 6 h). Cells were lysed in buffer containing 20 mM Tris, pH 7.4, 137 mM NaCl, 1% NP-40, 10% glycerol, 20 mM NaF, 1 mM Na orthovanadate, 1 mM PMSF, 2 mg/ml aprotinin, and 2 mg/ml leupeptin. Protein concentrations in cell lysates were determined by the Bradford method. For Western blotting, cell lysates protein samples concentrations were determined using the Bio-Rad system (Bio-rad Hercules, CA, USA). Proteins were mixed with Laemelli sample buffer, boiled at 100°C for 5 min, and centrifuged at 12,000 rpm for 5 min. Pelleted proteins were then separated by 15% SDS-acrylamide electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Membranes were blocked overnight in PBS with 5% non-fat ried milk and 0.1% Tween-20. They were incubated with the relevant primary and secondary antibodies in blocking buffer for 1 h at room temperature, following three 10 min washes in PBS plus 0.1% Tween-20 after incubation. Blots were developed using the Super-Signal detection system (Pierce, Rockford,

IL, USA) and exposed to Hyperfilm (Amersham, Arlington Heights, IL, USA). Antibodies were diluted as follows: 1:200 for anti-RhoA monoclonal mAb, 1:500 for anti-Rac monoclonal mAb, and 1:4000 for peroxidase conjugated goat anti-mouse secondary mAb.

Immunofluorescent Staining

For immunofluorescence staining assays, cells were plated on collagen-coated coverslips for 24 hours before the experiment. Cells were pretreated with sb203580 (30 nm) or staurosporine (100 μm) for 30 min at 37°C before stimulated with 100 ng/ml IL-8 for various times (5 min, 10 min, 30 min, 1 h). Then, cells were rapidly fixed and permeabilized by immersion in 3.7% paraformaldehyde, 0.1% glutaraldehyde, and 0.15% mg/ml saponin in fix buffer at 37°C for 1 h. The fixed cells were then incubated with BODIPY FL phallacidin (Invitrogen, Carlsbad, CA, USA) and DAPI for 30 min and then mounted in 50% glycerol-PBS containing 6 mg/ml of N-propyl gallate. All fluorescent images were obtained using a 40 × 1.4 NA Olympics objective optics and a cooled CCD camera. All digital images were imported into NIS-Elements AR image software.

Plasmids

The expression plasmids dominant negative form of Rac1 (pcDNA3-EGFP-Rac1-T17N), containing the cDNA encoding the small GTPase Rac1, was purchased from Addgene (Boston, MA, USA)[12]. Plasmid dominant negative form of RhoA (RhoA188A) was kindly provided by Professor Chen Yong-Chang (Jiangsu University, China).

Transient Transfection

EA.hy926 cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and OptiMEM medium (Gibco BRL, Grand Island, NY, USA) in the absence of antibiotics according to the manufacturer's instructions. After transient transfection, the mRNA expression of Rac1 and RhoA was detected by RT-PCR. The transfection level was measured by a fluorescence microscope. Transfected cells were passaged at 56-60 h and used after 72 h.

Evaluation of p38MAPK Activity

EA.Hy926 cells were infected with T17NRac1 or RhoA188A, and then pretreated with sb203580 (30 nm) or staurosporine (100 μm) for 30 min at 37°C before stimulated with 100 ng/ml IL-8 for 6h. For measurement of p38MAPK activity, the phosphorylated p38MAPK in the lysate was immunoprecipitated and its activity was evaluated by its ability to phosphorylate the activating transcription factor-2 (ATF-2) fusion protein according to the manufacturer's instruction for p38MAPK assay kit from Cell Signaling Technology (Beverly, MA, USA).

Statistical Analysis

Data were presented as mean ± SD from at least 10 images obtained from three individual experiments, unless otherwise indicated. Statistical analysis was performed by one-way ANOVA test using SPSS 11.5 software package (Chicago, IL, USA). Differences in means were considered significant if $p < 0.05$.

Results

p38MAPK is Required in the Cell Migration Induced By IL-8

The Figure 1A showed the cell migration number change under different condition: inhibitor or inhibitor+IL-8. IL-8 increased the cell migration number respect to the control group ($p < 0.01$). The cell migration stimulated by IL-8 group in the cells pretreated of sb203580 was increased respect to the cells in sb203580 alone group ($p < 0.05$), and it had difference with cell migration number stimulated by IL-8 ($p < 0.01$), which means sb203580 can inhibit the cell migration induce by IL-8. Though staurosporine obviously increased the cell migration stimulated by IL-8, it had no difference respect to IL-8 group. Staurosporine might no involve in the cell migration induce by IL-8. Interestingly, the cell migration number of sb+IL-8 group had decreased respect to the st+IL-8 group. In EA.hy926 cells, IL-8 vividly induced migration, but sb203580 suppressed cell migration regardless of the presence of IL-8.

The Figure 1B showed the cell shape change when cells stimulated by IL-8 in present of these inhibitors (phase contrast images were taken at 200 × magnification). The cell form of IL-8 and sb+IL-8 group look similar, but both have differences respect to st+IL-8 group. Especially, the cell form in st+IL-8 group was abnormally: cell size decreased, cytoplasm and nucleus lessen, and the mechanism of this cell shape abnormal is unclear.

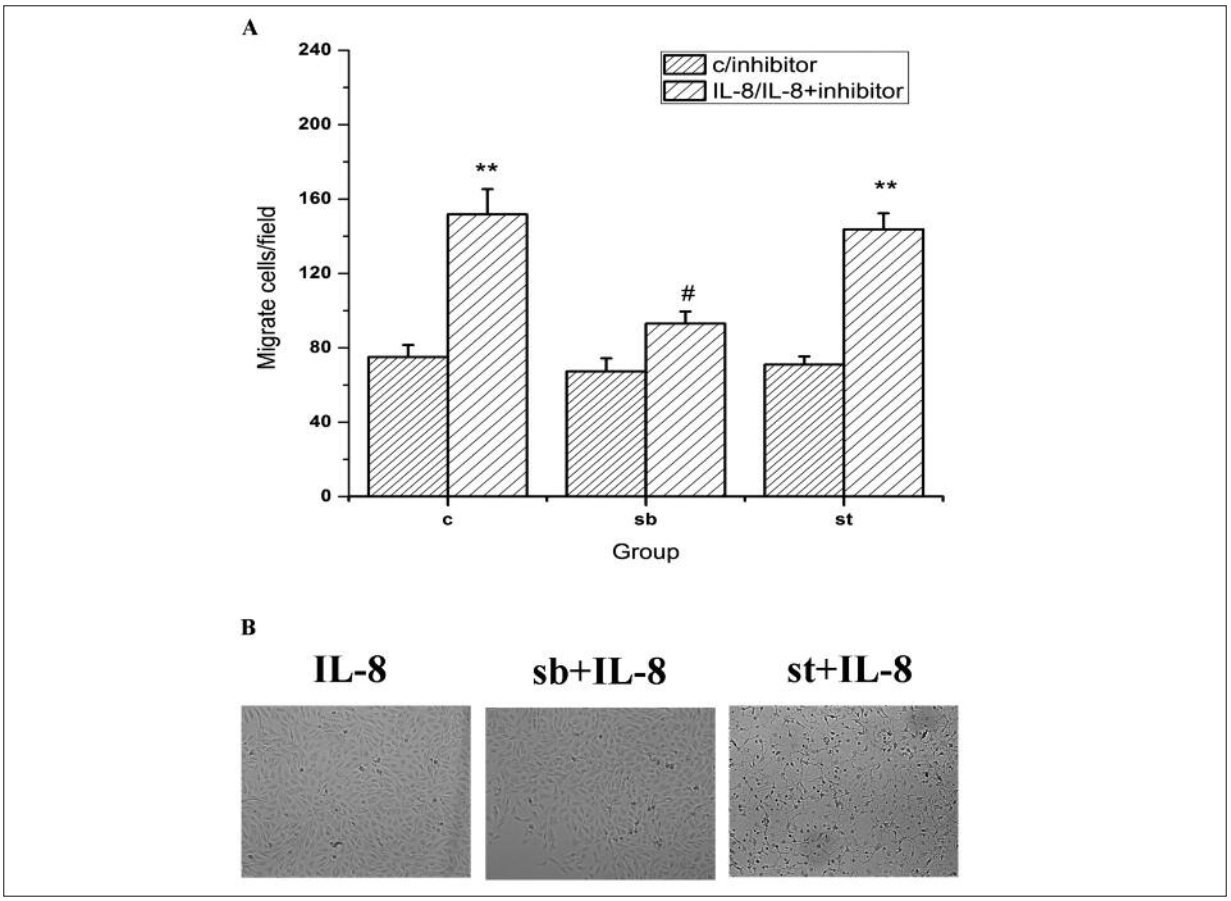

Figure 1. ***A,*** Effect of different inhibitors on IL-8-induced migration in EA.hy 926 cells. The cells were pretreated with sb203580, staurosporine for 30 min at 37°C before stimulation with 100 ng/ml IL-8. The migration was allowed to proceed as described in Materials and methods. $^{\#}p < 0.05$, the cell migration number of sb+IL-8 group VS that in IL-8 group; $^{**}p < 0.01$, the cell migration number of IL-8 group VS that in control group; $^{**}p < 0.01$, the cell migration number of st+IL-8 group VS that in st group. ***B,*** The cell morphology change in pretreatment cells when cells stimulated by IL-8. EA.hy 926 cells were pretreated for 30 min with sb203580 or staurosporine, and then stimulated by IL-8 (100 ng/ml). Phase contrast images were taken at 200 × magnification.

The Influence of Inhibitor on the Expressions of RhoA and Rac1 Proteins

In Figure 2A, the expression of Rac1 in DMSO group had no difference with control group (c group). After stimulated by IL-8 6 hour, the expression of Rac1 was increased ($p < 0.05$). Both of Rac1 expression in sb c (only addition sb203580), st c (only addition of staurosporine) group were increased respect to the control group. And, the Rac1 of sb+IL-8 (the cells pretreatment with sb203580 then stimulated by IL-8) group was increased respect to the IL-8 group ($p < 0.01$). As show in Figure 2C, IL-8 can induce the Rac1 level increase double of basis level (control group). But, in the cells pretreatment by staurosporine, the Rac1 had no change than before. Interestingly, the expression of Rac1 in sb+IL-8 was increased than these of sb c group. So, IL-8 increased the Rac1 expression in the cells pretreatd with sb203580.

In Figure 2B, DMSO slightly decreased the RhoA expression respect to the control group (c group), but it showed no difference with it. In cells stimulated by IL-8 for 6 h, the expression of RhoA increased respect to the control group ($p < 0.05$). The RhoA of sb+IL-8 group was increased respect to the IL-8 group ($p < 0.01$). Moreover, the RhoA expression of sb c group was increased almost double of basis level, which hints sb203580 increase the RhoA. Interestingly, in these cell which pretreatment by inhibitor (sb203580 or staurosporine) stimulated by IL-8, the RhoA also increased respect to the cells only addition inhibitor (as show in Figure 2D). When the cells pretreatment by staurosporine stimulated by IL-8, the expression of RhoA was in-

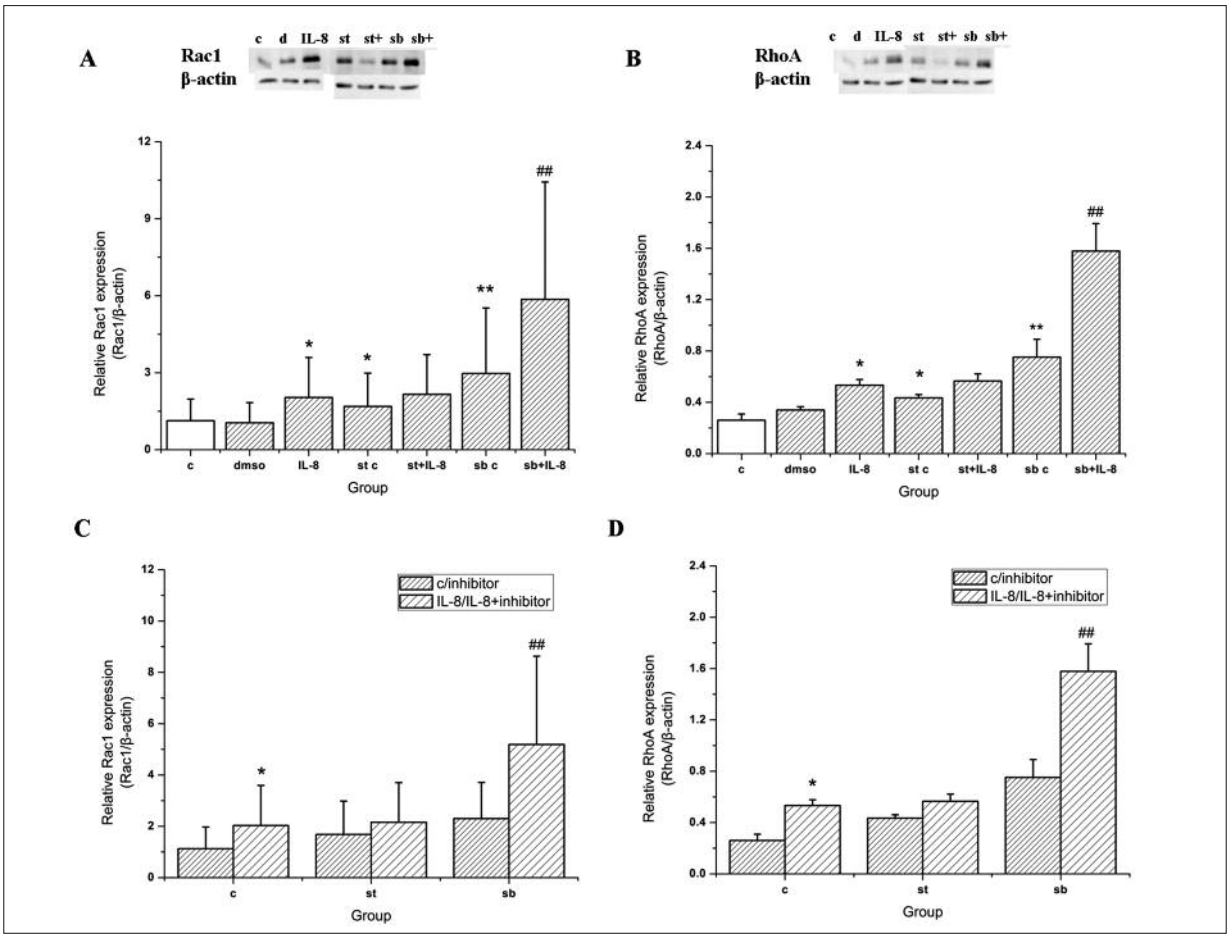

Figure 2. The Rac1 and RhoA expression of pretreatment cells when cell stimulated by IL-8. ***A,*** EA.hy 926 cells were pretreated with sb203580 or staurosporine for 30 min at 37°C before stimulation with 100 ng/ml IL-8. Cell lysates were prepared as described in Materials and methods. The protein bands were stained with antibodies recognizing Rac1. *$p < 0.05$, The Rac1 of st c and IL-8 group VS that in c group; **$p < 0.01$, The Rac1 of sb c group VS that of c group; $^{\#\#}p < 0.01$, The Rac1 of sb+IL-8 group VS that of IL-8 group. ***B,*** The protein bands were stained with antibodies recognizing RhoA. *$p < 0.05$, The RhoA of st c and IL-8 group VS that of c group; **$p < 0.01$, the RhoA of sb cgroup VS that of c group; $^{\#\#}p < 0.01$, the RhoA of sb+IL-8 group VS that of IL-8 group. ***C,*** The Rac1 expression between c/c+inhibitor or/ and IL-8/IL-8+inhibitor. *$p < 0.05$, the Rac1 of IL-8 group VS control group; $^{\#\#}p < 0.01$, the Rac1 of sb+IL-8 group VS that of sb group. ***D,*** The RhoA expression between c/c+inhibitor or/ and IL-8/IL-8+inhibitor. *$p < 0.05$, the RhoA of IL-8+inhibitor group VS control group; $^{\#\#}p < 0.01$, the RhoA of sb+IL-8 group VS that of sb group.

creased slightly respect to the st c group, and this rate of rise was be inferior to that between control and IL-8 group. But, in sb+IL-8 group, RhoA was increase than before, especially in sb+IL-8 group. Base on these results, sb203580 can also increase the expression of Rac1 and RhoA in cells.

The Influence of Inhibitors on the Cell Cytoskeleton

As show in the Figure 3, the cell morphology of st+IL-8 group was changed than other group in 5 min. Compare to st+IL-8 group, the cell edge in sb+IL-8 group was elong, and cell extend very wide from 10 min to 1 h. The cell morphology of st+IL-8 group unlike that of sb+IL-8 group: cell smaller respect to the sb+IL-8 group, and the fluorescence strength of F-actin was increased. All these inhibitors induce the cell shape change, and stress fiber cannot appear in observed time.

The Expression of RhoA and Rac1 proteins Change Induce by Inhibitor and IL-8

As shown in Figure 4A, the expression of Rac1 changes within different condition. In sb group, the exprsssion of Rac1 was decreased at 30 min than these of other time point ($p < 0.01$). The Rac1 of st group at 10 min and 30 min were

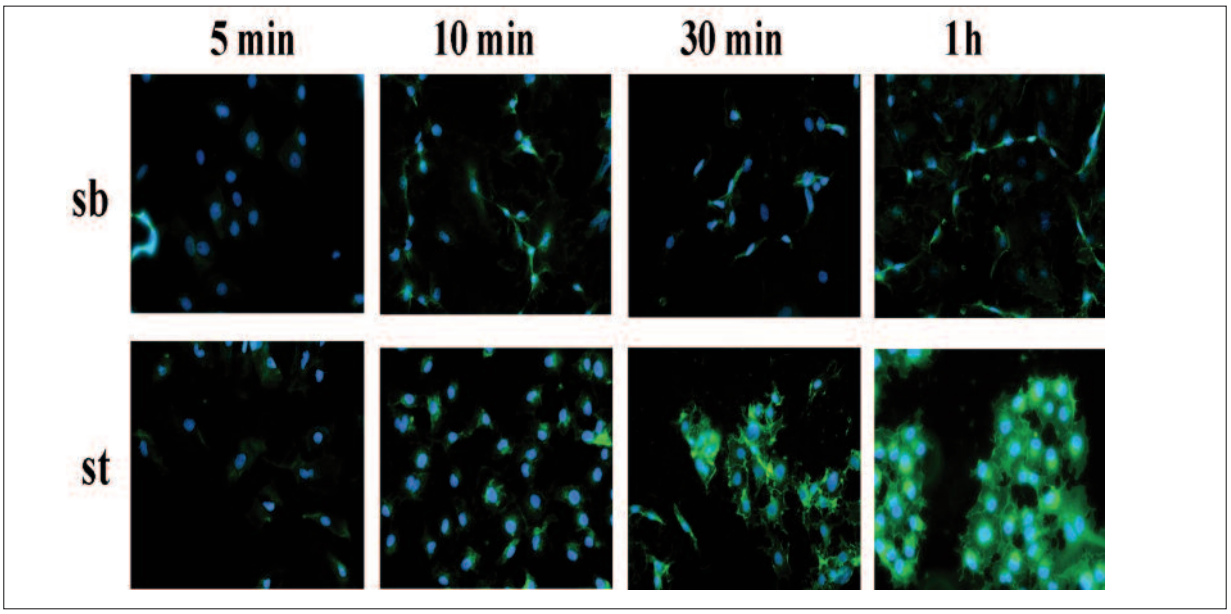

Figure 3. The influence of inhibitors on the cell cytoskeleton. EA.hy926 cells grown on glass coverslips.The cells were pretreated with sb203580 or staurosporine for 30 min at 37°C before stimulation with 100 ng/ml IL-8. Cells were fixed and stained with BODIPY FL phallacidin. Actin and DAPI staining were recorded from the same cells. Form the 10min, the cell morphology of sb+IL-8 group have been change: the cell edge was elong, and cell become wide and large. The cell morphology of st+IL-8 group unlike that of sb+IL-8 group: cell small than that of sb+IL-8 group, and the fluorescence strength of F-actin was increased.

different respect to 5 min, and the expression of Rac1 decreased again about stimulated by IL-8 1h. Figure 4C show that the Rac1 expression change in six time points: 0 min, 5 min, 10 min, 30 min, 1 h and 6 h. In 0 min group, the Rac1 in sb+IL-8 ($p < 0.01$) and st+IL-8 ($p < 0.05$) increased respect to the control group. Then, the expression of Rac1 in sb+IL-8 and st+IL-8 were decreased respect to IL-8 in next 10 minutes ($p < 0.05$). At the 30 min group, the Rac1 in IL-8 had no differences respect to st+IL-8 and sb+IL-8. In 1h group, the expression of Rac1 protein of IL-8 was different respect to sb+IL-8 ($p < 0.05$). It worth noting that the Rac1 of sb+IL-8 was increased than these of st+IL-8 and IL-8 group at 6 h ($p < 0.001$). Base on these results, the Rac1 in IL-8 was different respect to sb+IL-8 in all time point. The expression of Rac1 in st+IL-8 was different respect to IL-8 at 5 min and 10 min. st and sb in some degree have influence on the Rac1 expression change which induced by IL-8.

The expression of RhoA of 30 min and 1h had difference respect to 5 min and 10 min in sb group (Figure 4B, $p < 0.01$). The expression of RhoA in 30 min has no difference respect to 10 min, but was increased respect to 5 min in st group ($p < 0.05$). Compare to the RhoA expression of other time point in st group, that of 1 h was decreased ($p < 0.001$). The Figure 4D show that RhoA expression change under different condition: sb+IL-8 group, st+IL-8 group, IL-8 group at six time points. In 0 min group, the RhoA of sb and st group were increased respect to the control group ($p < 0.05$). The RhoA expression in IL-8 group of 5 min time point have difference respect to the other group ($p < 0.01$). In 10 min time point, the RhoA of IL-8 group had no difference respect to st+IL-8, but it was increased respect to the sb+IL-8 ($p < 0.05$). The expression of RhoA in IL-8 group of 30 min time point have difference with other group ($p < 0.001$). This expression diversity was enhanced in 1h time point. Interesting, the RhoA of sb+IL-8 was increased than these of st+IL-8 and IL-8 group at 6 h ($p < 0.001$).

IL-8 Actives p38MAPK in Rac1 and RhoA Depend Manner.

The IL-8-induced p38MAPK activation was increased respect to the basal level, and was attenuated by pretreatment with sb203580 (Figure 5A). The p38MAPK activation by IL-8 was suppressed by dominant-negative mutant for Rac1 (Figure 5A), suggesting that Rac1 could mediate p38MAPK activation. Moreover, the p38MAPK activation by IL-8 was suppressed by dominant-negative mutant for RhoA (Figure 5B), suggesting that RhoA could mediate p38MAPK activa-

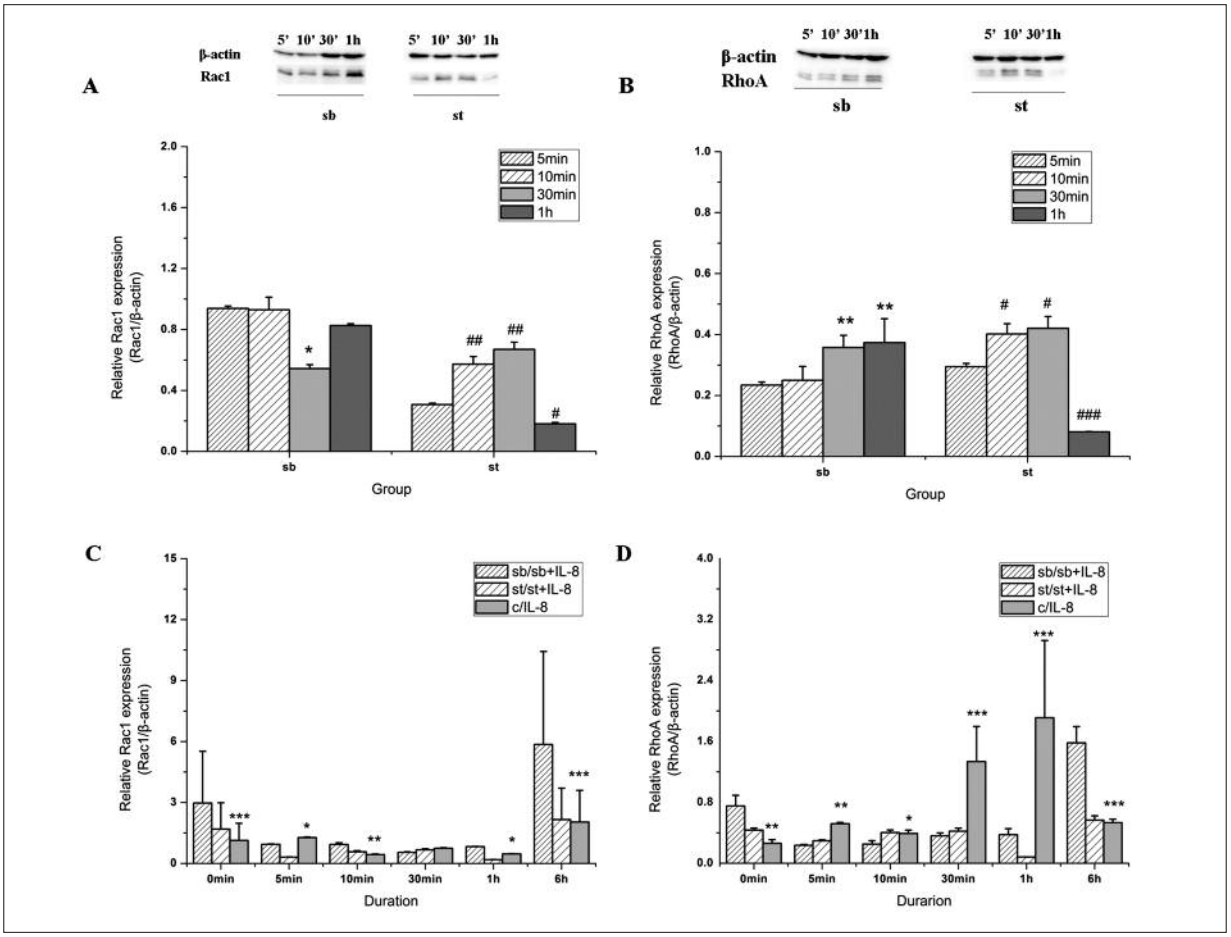

Figure 4. The exprsssions of RhoA and Rac1 proteins change induce by inhibitor and IL-8. ***A,*** Total Rac1 immunoblot analysis of whole-cell extracts from the pretreated cells(cells were pretreated with sb203580 or staurosporine for 30 min at 37°C) stimulated by IL-8 (100 ng/ml). *$p < 0.05$, the Rac1 of sb+IL-8 30 min group VS the Rac1 of sb+IL-8 5 min group; ##$p < 0.01$, the Rac1 of st +IL-8 10 min and 30 min group VS that of st+IL-8 5 min group; #$p < 0.05$, the Rac1 of st+IL-8 1h group VS that of st+IL-8 5 min group. ***B,*** Immunoblot detection of RhoA from cells treated with 100 ng/ml IL-8 in the pretreated with sb202190 or staurosporine. **$p < 0.01$, the RhoA of sb+IL-8 30 min and 1h group VS that of sb+IL-8 5 min group; #$p < 0.05$, the RhoA of st+IL-8 10 min and 30 min group VS that of st+IL-8 5 min group; ##$p < 0.01$, the RhoA of st+IL-8 1h group VS that of st+IL-8 5 min group. ***C,*** The expression of Rac1 in inhibitor/inhibitor+IL-8 in duration. ***$p < 0.001$, the Rac1 of sb and st group VS that of control group; *$p < 0.05$, the Rac1 of IL-8 VS these of sb+IL-8 and st+IL-8 group at 5min; **$p < 0.01$, The Rac1 of IL-8 VS these of st+IL-8 and sb+IL-8 group at 10 min; *$p < 0.05$, the Rac1 of IL-8 VS these of sb+IL-8 and st+IL-8 group at 1h; ***$p < 0.001$, the Rac1 of sb group VS that of IL-8 group at 6h. ***D,*** The expression of RhoA in inhibitor/inhibitor+IL-8 in duration. **$p < 0.01$, the RhoA of sb group VS that of control group; **$p < 0.01$, the RhoA of IL-8 VS these of sb+IL-8 and st+IL-8 group at 5 min; *$p < 0.05$, The RhoA of IL-8 VS that of sb+IL-8 group at 10 min; ***$p < 0.001$, the RhoA of IL-8 VS these of sb+IL-8 and st+IL-8 group at 1h; ***$p < 0.001$, the Rac1 of sb group VS that of IL-8 group at 6h.

tion. These results suggest that Rac1 and RhoA, both are genes in upstream of p38MAPK activation stimulated by IL-8.

Discussion

Cell migration is usually initiated in response to extracellular cues, including diffusible factors, signals on neighboring cells, and/or signals from the extracellular matrix. In our previous study[10], IL-8 could induce endothelial cell migration after 6h of IL-8 addition. IL-8 treatment not only induced the membrane ruffles and lamellipodia extensions, but also increased the actin stress fibers (unpublish data, s2).

The results of Transwell assay show that sb203580 decreased the cell migration induce by IL-8, which mean p38MAPK were involve on the process of IL-8 induce cell migration. In the present of staurosporine, IL-8 slightly decreased the cell migration than only addition of IL-8, but

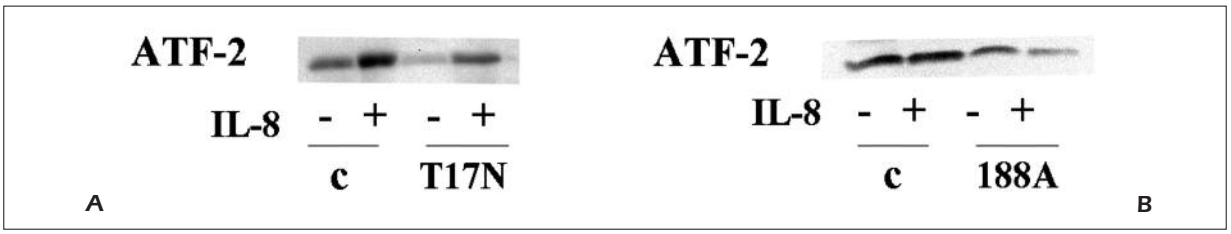

Figure 5. IL-8 actives p38MAPK in Rac1-dependent and RhoA-depend manner. ***A,*** EA.Hy926 cells were infected with T17NRac1, and then stimulated with IL-8 (100 ng/ml). Cell lysates were subjected to measurement of p38MAPK activity. ***B,*** EA.Hy926 cells were treated like Figura A. Cells were infected with plasmid containing RhoA188A, and then stimulated with 100 ng/ml IL-8. Cell lysates were subjected to measurement of p38MAPK activity.

had no difference with it. It suggested that PKC involves in the cell migration, but it might not take part in the cell migration induced by IL-8. Within IL-8 addition, staurosporine and sb203580 induce cell form change. Interestingly, the cell form change induced by staurosporine was not like as sb203580. The cell morphology induced by sb203580 look like same with that of the cells stimulated by IL-8 alone. This results hint PKC and p38MAPK might play different role in the cell migration stimulated by IL-8.

Western blotting indicated that both of staurosporine and sb203580 increased the expression of Rac1 and RhoA respect to the bascical line. And, staurosporine and sb203580 also upregulate the expression of RhoA and Rac1 when cells stimualed by IL-8. But, there were no differences respect to proteins expression stimulated by IL-8 in present or absence of staurosporine, which mean PKC might not take part in these proteins expression change induced by IL-8 at 6 h. Especially, the influence of sb203580 on these two protein expression was different respect to the staurosporine influence, which indicates that the p38MAPK was involved in the expression change of RhoA and Rac1 induced by IL-8.

The remodeling of actin cytoskeleton includes filopodia, lamellipodia, and stress fibers. All the three structures are essential to drive the several steps of actin-based endothelial cell motility. In our previous study, IL-8 treatment not only induced the membrane ruffles and lamellipodia extensions, but also increased the actin stress fibers (unpublish data, S2). The membrane ruffles appear when the cells stimulated by IL-8 5 min, and the formation of lamellipodia extensions start at 10 min. The actin stress fibers were induced by IL-8 about 1 h. In our study, all these inhibitors prevent the formation of membrane ruffle and stress fiber, because these had no appear in presence of inhibitors in all observed time. And, the expression of F-actin was enhanced in the cells pretreatment with staurosporine. So, PKC might influence the F-action expression, but have no effect on the cytoskeleton induced by RhoGTPase. SB203580 induce a plenty of parapodium emerge around cell periphery, which indicated p38MAPK might play a role in the formation of stress fiber-the process next to the parapodium formation.

Rho GTPases coordinately regulate multiple aspects of cell migration, affecting the different components of the cytoskeleton as well as cell-substrate adhesion and possibly matrix remodelling[13]. In our previous study, IL-8 induced a rapid rearrangement of the actin cytoskeleton in EA.Hy926 cells, generating extensions resembling membrane ruffling and stress fibers. This effect required parallel activation of the small GTPases Rac1 and RhoA. So, we detected the expression of Rac1 and RhoA when cells stimulated by IL-8 in present of staurosporine or sb203580 in these cytoskeleton change time (after the cells stimulated by IL-8 5 min, 10 min, 30 min, 1 h). The Rac1 of cells treated with IL-8 (100 ng/ml) in the presence of sb202190 and staurosporine were increased than these of cells stimualed by IL-8 alone except for two time point: 5 min and 30 min. This Rac1 expression decreased might be the reason of membrane ruffles, lamellipodia extensions disassembly. While the RhoA of sb203580 and staurosporine were obviously decreased than these of IL-8 group at 30 min and 1 h, and this might impair the cell stress fiber formation. These data suggest that p38MAPK contributes to the reorganization of the actin cytoskeleton induced by IL-8.

Hippenstiel et al[14] demonstrated at least 2 parallel LPS-induced pathways in endothelial cells that lead to NF-kB-dependent IL-8 expression: one via p38 kinase and one via Rho pro-

teins and tyrosine kinases. And selective activation of p38 kinase signaling pathway was sufficient and as effective as LPS stimulation to induce IL-8 production in endothelial cells. In our study, sb203580 decreased the IL-8-induced p38MAPK activation, and dominant-negative mutant for Rac1 and RhoA decreased the p38MAPK activation by IL-8. In Zhang's study[6], a dominant negative Pak1 suppresses both interleukin-1- and Rac/Cdc42-induced p38 activity. Rac and Cdc42 appear to regulate a protein kinase cascade initiated at the level of Pak and leading to activation of p38 and JNK. So, the signaling pathway induced by IL-8 might be: IL-8-Rac1/RhoA-Pak1-p38MAPK, and it still need further investigation.

In this study, we firstly confirmed the critical role of p38MAPK in IL-8-induced cell migration by demonstrating that sb203580 could obviously inhibit the cell migration induced by IL-8. The changes of cytoskeleton showed that pretreatment by sb203580 would reduce the membrane ruffling and stress fibers significantly. These observations suggested that p38MAPK signaling is required for these cytoskeletal changes. Furthermore, by western blot we found that SB203580 led to increased expression of Rac1 and RhoA protein in cells treated by IL-8. In addition, treatment of cells with sb203580 abolished the IL-8-induced p38MAPK activation, and the p38MAPK activation by IL-8 was suppressed by dominant-negative mutant for Rac1 and RhoA. Collectively, these data suggest that p38MAPK functions as a downstream activator of Rac1 and RhoA.

In summary, our study demonstrated p38MAPK involve in the cell migration induced by IL-8. Both Rac1 and RhoA might as an upstream of p38MAPK. The cell migration induced IL-8 via Rac1/RhoA-p38 kinase pathway. Further analysis of this signaling cascade will provides new insight into the molecular mechanisms by which IL-8 contributes to tumor angiogenesis and metastasis.

Acknowledgements

This study was supported in part by grant from National Natural Science Foundation of China (No. 30570450, 10772127, 10972148), Sichuan Youth Science & Technology Foundation (No. 06ZQ026-009), and Program for New Century Excellent Talents in University of China (No. NCET-06-0791).

References

1) Yoshimura T, Matsushima K, Oppenheim JJ, Leonard EJ. Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)-stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). J Immunol 1987; 139: 788-793.
2) Huang C, Jacobson K, Schaller MD. MAP kinases and cell migration. J Cell Sci 2004; 117: 4619-4628.
3) Coghlan MP, Chou MM, Carpenter CL. Atypical protein kinases Clambda and-zeta associate with the GTP-binding protein Cdc42 and mediate stress fiber loss. Mol Cell Biol 2000; 20: 2880-2889.
4) Tibbles LA, Ing Y, Kiefer F, Chan J, Iscove N, Woodgett J, Lassam N. MLK-3 activates the SAPK/JNK and p38/RK pathways via SEK1 and MKK3/6. EMBO J 1996; 15: 7026-7035.
5) Uddin S, Lekmine F, Sharma N, Majchrzak B, Mayer I, Young PR, Bokoch GM, Fish EN, Platanias LC. The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. J Biol Chem 2000; 275: 27634-27640.
6) Zhang S, Han J, Sells M, Chernoff J, Knaus U, Ulevitch R, Bokoch G. Rho family GTPases regulate p38 mitogen-activated protein kinase through the downstream mediator Pak1. J Biol Chem 1995; 270: 23934-23936.
7) Massoumi R, Larsson C, Sjölander A. Leukotriene D(4) induces stress-fibre formation in intestinal epithelial cells via activation of RhoA and PKCdelta. J Cell Sci 2002; 115: 3509-3515.
8) Larsson C. Protein kinase C and the regulation of the actin cytoskeleton. Cell Signal 2006; 18: 276-284.
9) Zou M, Liu X, Li Y, Lai Y. Experimental study on the migration of vascular endothelial cells stimulated by IL-8. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2006; 23: 1013-1016.
10) Lai Y, Liu X, Wu J, Zeng Y, Yue A, He X. Rac1 mediates the migration of endothelial cell induced by IL-8. Sheng Wu Yi Xue Gong Cheng Xue Za Zhi 2008; 25: 357-362.
11) Edgell C, McDonald C, Graham J. Permanent cell line expressing human factor VIII-related antigen established by hybridization. Proc Natl Acad Sci USA 1983; 80: 3734-3737.
12) Subauste MC, Von Herrath M, Benard V, Chamberlain CE, Chuang TH, Chu K, Bokoch GM, Hahn KM. Rho family proteins modulate rapid apoptosis induced by cytotoxic T lymphocytes and Fas. J Biol Chem 2000; 275: 9725-9733.
13) Ridley AJ. Rho GTPases and cell migration. J Cell Sci 2001; 114: 2713-2722.
14) Hippenstiel S, Soeth S, Kellas B, Fuhrmann O, Seybold J, Krull M, Eichel-Streiber C, Goebeler M, Ludwig S, Suttorp N. Rho proteins and the p38-MAPK pathway are important mediators for LPS-induced interleukin-8 expression in human endothelial cells. Blood 2000; 95: 3044.