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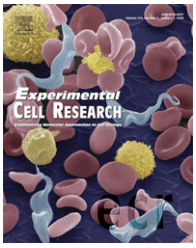

Research Article

MARCKS is a downstream effector in platelet-derived growth factor-induced cell motility in activated human hepatic stellate cells

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ABSTRACT

Myristoylated alanine-rich protein kinase c substrate (MARCKS) has been suggested to be implicated in cell adhesion, secretion, motility and mitogenesis through regulation of the actin cytoskeletal structure. In the present study, a possible link between MARCKS and the platelet-derived growth factor (PDGF) signaling pathway was investigated in activated human hepatic stellate cells (hHSC), critical regulators of hepatic fibrogenesis.

PDGF-BB stimulation resulted in a bi-directional movement of MARCKS that coincided with the phosphorylation of MARCKS and the activation of both PKCε and PKCα. Biochemical inhibition of PKC kinase activity and small interfering RNA (siRNA) against PKCε demonstrated that PKCε is indispensable for PDGF-BB-induced MARCKS phosphorylation and cell migration. Immunoprecipitation studies revealed an association between MARCKS and the PDGFβ-receptor, while the PDGFβ-receptor and PKCα associated with focal adhesion kinase (FAK). Transient transfection with MARCKS DNA plasmid remarkably reduced PDGF-BB stimulated cell motility. In contrast, siRNA against MARCKS increased cell migration in RNAi treated cells in comparison to the scrambled control cells.

In conclusion, the present study indicates that MARCKS play a major key role in PDGF-BB-induced chemotaxis in activated hHSC.

Introduction

Several studies have investigated the intracellular molecular events responsible for the mitogenic and motogenic action of platelet-derived growth factor (PDGF) on activated human or rodent hepatic stellate cells (HSC), a cell type responsible for hepatic fibrogenesis following chronic liver damage [1,2]. Of all three PDGF dimeric isoforms tested it was PDGF-BB, through its binding with PDGF-β receptor, that showed to be the strongest mitogen and chemoattractant for hHSC. This is in agreement with the predominant expression of PDGF receptor-β subunits compared with PDGF-α subunits in hHSC [3–7]. Hence, in part due to the low level of PDGF-α receptor, PDGF-AA is unable to stimulate HSC chemotaxis despite its ability to

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promote mitogenesis and PI3-K activation [8]. Therefore, the PDGF-α and PDGF-β receptor signal partially through different pathways, and the pathways leading to cell proliferation are different from those that lead to chemotaxis. Overall, our previously published studies have provided a considerable advance in the knowledge of the intracellular signaling pathways activated by PDGF-BB and in the cross-talk between PDGF-receptor and the molecular effectors residing in the focal adhesion complex [9,10]. However, only very scarce information is available concerning the molecular pathways connecting these events and the PDGF-induced re-organization of the actin cytoskeleton necessary for the finalization of PDGF biologic effect, particularly cell migration.

Current experimental evidence suggests that myristoylated alanine-rich protein kinase c substrate (MARCKS) is involved in cellular processes such as cell adhesion, cell spreading, membrane trafficking and cell motility through regulation of the actin cytoskeletal structure. Several binding partners are already described as well as the sub-cellular localization of MARCKS. However, MARCKS' fine molecular mechanisms have not been elucidated [11–13]. MARCKS is known as a conservative protein that is present in all cell types investigated and is associated with membranes by the cooperative interaction of two membrane-binding domains: the myristoyl moiety inserts into the hydrophobic lipid bilayer, and the basic effector domain (ED) interacts electrostatically with the inner side of the plasma membrane. The effector domain (ED) regulates the local availability of phospholipids and is responsible for the interactions with other proteins such as calcium-calmodulin (Ca^{2+}-CM) and filamentous actin (F-actin) [11,14]. The bundling and cross-linking of actin filaments by MARCKS is known to function in a direct way through the presence of two actin binding sites in the effector domain [15–17]. This dynamic interaction contributes to the formation of a strong cortical actin cytoskeleton at the plasma membrane and to membrane stability in a non-stimulated state [18,19]. In contrast, upon growth factor stimulation PKC becomes activated and phosphorylates the serine residues within the effector domain of MARCKS, thereby disrupting the electrostatic interaction and releasing the protein from the membrane. As a consequence, once MARCKS is phosphorylated, the protein loses its binding capacity to F-actin [11]. After dephosphorylation of MARCKS the protein returns to the plasma membrane and restores its F-actin binding activity [11,20]. Therefore, it has been postulated that the cycling of MARCKS between cytoplasm and plasma membrane via its phosphorylation and dephosphorylation status affects the dynamics of the membrane–actin interactions that stabilize a rigid F-actin cytoskeleton in non-stimulated cells and the re-organization of the actin cytoskeleton upon appropriate stimulation [14–17]. However, no information is available concerning the involvement of the MARCKS system in PDGF-BB-induced chemotaxis, and, in general, in cell motility in primary non-transformed human cells, such as human hepatic stellate cells.

Therefore, in reason of these relevant features of MARCKS and its role in the regulation of cell plasticity, this study was designed in order to elucidate whether or not this system could represent a suitable molecular link between PDGF-BB signaling and the cytoskeletal modifications necessary for PDGF-induced cell migration in human hepatic stellate cells, key players in liver fibrogenesis.

Methods

Reagents and antibodies

Alpha-smooth muscle actin antibody (α-SMA, mouse clone1A4), β-actin (AC-15) and all reagents used in this study are from Sigma Aldrich (Saint Louis, MI, USA), or otherwise mentioned. PDGF-BB was purchased from Peprotech (Rockhill, NJ, USA). PKC inhibitors GÖ6976/GÖ6850/GÖ6893 and rottlerin were obtained from Calbiochem (CA, USA). Primary antibodies against MARCKS (N-19, M20), P-MARCKS (Ser159/163), P-PKCε (Ser729), nPKCε (C-15), cPKCα (H-7), FAK (C-20) were purchased from Santa Cruz Biotechnology (CA, USA) as well as their secondary antibodies, respectively HRP-conjugated goat or rabbit IgG. Antibodies against PDGFβ-receptor (06-498) and P85-PI3K (clone UB93-3) were purchased from Upstate Biotechnology (Lake Placid, NY, USA). Fluorescent secondary antibody Alexa Fluor 488 rabbit anti-goat IgG as well as the nuclear staining TO-PRO-3 were purchased from Molecular Probes (Leiden, The Netherlands). All antibodies were used according to the manufacturer's manual.

Isolation and culture of human hepatic stellate cells (hHSC)

Cells were isolated from wedge sections of normal human liver unsuitable for transplantation by a collagenase/pronase digestion and centrifugation on UF Powder gradients (Larex Inc, M, USA) and were cultured under standard conditions as extensively described elsewhere [21,22]. Experiments described in this study were performed on cells between passage 3 and 7. At these stages of culture, cells showed functional and ultrastructural features of fully activated hHSC-"interface" myofibroblasts [23].

Immunofluorescence staining

After 48 h of serum deprivation, cells were treated with 10 ng/ml of PDGF-BB for 1 h. Afterwards, an immunofluorescence staining was employed against P-MARCKS and nuclei were stained with TO-PRO-3 (0.12 μM) as described elsewhere [10]. To detect intracellular P-MARCKS images were taken using a LSM 510 META confocal laser microscope (Carl. Zeiss Inc. Thornwood NY, USA) with an objective lens 40×.

Protein analysis and western blot analysis

Total protein extraction

Cells were stimulated with 10 ng/ml of PDGF-BB for 5, 15, 30 min and up to 1 h. Preparation of the cell lysates, SDS-PAGE, Western blot analysis and co-immunoprecipitation assays were performed using standard protocols as previously described [22,24]. Briefly, cells were lysed in a RIPA buffer containing 20 mM/l Tris/HCl, pH 7.4, 150 mM/l NaCl, 5 mM/l EDTA, 1% Nonidet P-40, 1 mM/l Na_3VO_4, 1 mM/l PMSF, 1:100 protease inhibitor cocktail. Insoluble proteins were discarded by centrifugation at 10,000 rpm at 4 °C and total proteins were measured (Pierce, Rockford, IL, USA) and stored at −80 °C for further analysis. Other cell cultures were pre-treated for 15 min with different PKC inhibitors; GÖ6976, GÖ6850, GÖ6893 (5 μM final concentration) [25,26] and rottlerin (2 μM final concentration) [27] followed by