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Research paper

In vivo validation of signaling pathways regulating human monocyte chemotaxis

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Abstract

Identification of novel signal transduction pathways regulating monocyte chemotaxis can indicate unique targets for preventive therapies for treatment of chronic inflammatory diseases. To aid in this endeavor we report conditions for optimal transfection of primary human monocytes coupled with a new model system for assessing their chemotactic activity *in vivo*. This method can be used as a tool to identify the relevant signal transduction pathways regulating human monocyte chemotaxis to MCP-1 in the complex *in vivo* environment that were previously identified to regulate chemotaxis *in vitro*. MCP-1-dependent chemotaxis of monocytes is studied in an adoptive transfer model where human monocytes transfected with mutant cDNAs are transferred to mice followed by initiation of peritonitis. Harvesting peritoneal cells at 24 h diminishes the contribution of immunologic responses to the cross-species transfer. Validation of relevant regulatory molecules *in vivo* is critical for understanding the most relevant therapeutic targets for drug development.

Keywords: Monocytes; Transfection; Adoptive transfer; Chemotaxis; PKCβ

1. Introduction

The non-transfectable nature of primary human monocytes has made it difficult to study the signal transduction pathways that are important for regulating monocyte function. Since transfection of primary monocytes with cDNA was not available until recently, we have instead used antisense oligonucleotides as specific inhibitors of mRNA and protein expression to explore monocyte signaling (Li and Cathcart, 1994, 1997; Roy and Cathcart, 1998; Li et al., 1999, 2007; Bey and Cathcart, 2000, 2002; Carnevale and Cathcart, 2001, 2003; Zhao et al., 2002, 2005; Bey et al., 2004; Xu et al., 2004; Bhattacharjee et al., 2006). We report here the optimization of an efficient method for transfecting primary monocytes that has dramatically

Abbreviations: MCP-1, Monocyte chemoattractant protein 1; OPT, Opti-MEM I medium; MNM, Human monocyte nucleofector medium; BCS, Bovine calf serum.

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expanded the conventional approaches for studying potential signaling pathways involved in the pathogenic processes accompanying several inflammatory diseases including atherosclerosis.

Monocyte migration into the intima of an arterial wall is thought to be one of the regulatory steps in atherogenesis. Recruitment of monocytes from the peripheral blood is a multistep process in which locally produced chemokines are believed to play a crucial role. Monocyte chemoattractant protein 1 (MCP-1) is important in attracting monocytes to sites of inflammation and has been shown to be particularly important in atherosclerotic lesions (Wilcox et al., 1994; Boring et al., 1998; Chen and Dennis, 1998; Gu et al., 1998; Aiello et al., 1999; Ross, 1999). We have been studying the pathways that regulate monocyte chemotaxis to MCP-1 because of their pathologic significance and potential for offering new approaches for manipulating chronic inflammation.

Our studies to date have identified several unique signaling pathways that regulate monocyte chemotaxis *in vitro* (Carnevale and Cathcart, 2001, 2003) but it is important to validate the role of these pathways *in vivo* in relevant inflammatory responses. One kinase shown to regulate the *in vitro* chemotactic response of human monocytes to MCP-1 is PKCβ (Carnevale and Cathcart, 2003). To confirm the relevant *in vivo* role for PKCβ, we optimized transfection conditions in primary human monocytes and then transfected them with wild type and dominant negative PKCβ cDNA. We then examined the *in vivo* relevance of PKCβ for regulating monocyte chemotaxis using a novel model of inflammation incorporating adoptive transfer of human monocytes into mice. This model is based on the fact that migration of monocytes to the peritoneum in thioglycolate-induced peritonitis is dependent on MCP-1 (Lu et al., 1998). We therefore combined this adoptive transfer model and our optimal transfection protocols to evaluate the effect of a key regulatory molecule on MCP-1-induced chemotaxis of primary human monocytes *in vivo*.

2. Materials and methods

2.1. Reagents

Human MCP-1 from Pharmingen was diluted to 50 μg/ml with Dulbecco's PBS containing 1 mg/ml BSA as a stock solution and was used at 50 ng/ml to attract human monocytes. Anti-GFP antibody (monoclonal JL-8) was obtained from Clontech (Mountain View, CA). Antibody to PKCβII (anti-PKCβ) was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Human Dendritic Cell Nucleofector kit and Human Monocyte Nucleofector kit were both purchased from Amaxa Biosystems (Gaithersburg, MD). GFP-tagged PKCβ wild type (GFP-PKCβ-WT, pBK-CMV-GFP-PKCβII) and the dominant negative mutant (GFP-PKCβ-DN, pBK-CMV-GFP-PKCβII K371R) were kindly provided by Yusuf Hannun (Medical University of South Carolina, Charleston, SC). PKH26 red fluorescent cell linker kits were obtained from Sigma-Aldrich Co. (St. Louis, MO). Four to eight week old BALB/CJ female mice were from The Jackson lab, Bar Harbor, Maine. Isofluorane USP was from Abbott Laboratories, North Chicago, IL. Heparin was obtained from Amersham Life Sciences, Arlington Heights, NJ.

2.2. Isolation of human monocytes

Human peripheral blood monocytes (PBM) were isolated either by separation of mononuclear cells followed by adherence to BCS-coated flasks as described earlier (Cathcart et al., 1989) or by Ficoll-Hypaque sedimentation followed by countercurrent centrifugal elutriation (Wahl et al., 1984a,b). PBM purified by these two methods were consistently >95% $CD14^+$. These studies complied with all relevant federal guidelines and institutional policies regarding the use of human subjects. All data shown in this manuscript are from studies with elutriated monocytes.

2.3. Immunoblotting

PBM were either nucleofected with pmaxGFP or GFP-PKCβ-WT or GFP-PKCβ-DN using the method providing optimal transfection efficiency. Whole cell extracts were prepared by previously published protocols (Rosen et al., 1996; Roy et al., 2002). Lysates were resolved by 12% SDS-PAGE, transferred to a PVDF membrane, blocked with 5% BSA in PBS with 0.1% Tween 20 and subjected to immunoblotting with monoclonal anti-GFP antibody (diluted 1:1000 in 3% BSA in PBS with 0.1% Tween 20) overnight. The hybridization signal was detected using SuperSignal West Pico Chemiluminescent substrate (Pierce, Rockford, IL). The immunoblots were then stripped and reprobed with PKCβ antibody according to our previously published protocol.

2.4. Transfection of primary human monocytes

Monocytes (5×10^6 in 2 ml 10% BCS/DMEM) were kept in polypropylene tubes in a 37 °C incubator with