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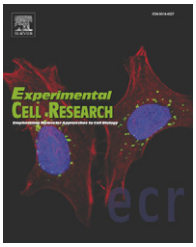

Research Article

Expression of RAC2 in endothelial cells is required for the postnatal neovascular response

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ABSTRACT

Herein, we describe an obligate role for the hematopoietic specific GTPase, RAC2 in endothelial integrin signaling and the postnatal neovascularization response *in vivo*. Using a *Rac2* knockout mouse model, we discovered that despite the presence of both RAC1 and RAC2 protein in endothelial cells, RAC2 is obligately required for the postnatal neovascular response and αvβ3/α4β1/α5β1 integrin-directed migration on vitronectin, H296 and CH271, fibronectin fragments, respectively. The molecular basis for RAC2 specificity was explored. A genetic analysis of Syk −/+ or Syk−/+;Rac2 −/+ mice revealed that SYK kinase is required for the integrin induced activation of RAC2. The analysis of endothelial cells from Rac2−/+ versus Syk−/+;Rac2−/+ mice provided genetic evidence that SYK-RAC2 signaling axis regulates integrin (αvβ3, α4β1 and α5β1) dependent migration. Our results provide evidence that a specific region of the nonreceptor protein tyrosine kinase, SYK, the B linker region containing Y342 and Y346 is required for SYK's regulation of RAC2 and integrin dependent migration. Moreover, the capacity of mice to vascularize the ischemic hindlimb following femoral artery ligation or matrigel plugs was markedly reduced in mice homozygous deficient for the *Rac2* gene. These findings identify a novel signaling axis for the induction and potential modulation of postnatal angiogenesis.

Introduction

The signal transduction events within endothelial cells (EC) which encode the complex cascade of events that are required for angiogenesis are just now coming to light [1]. Angiogenesis was originally defined as the process by which new blood vessels are formed from pre-existing vascular structures. The process is important for certain neovascular events which result in the reestablishment of a vascular blood supply following ischemia and in tumor-induced angiogenesis [2]. More recent evidence suggests that certain types of neovascularization responses are dependent upon bone marrow derived endothelial cells and/or circulating endothelial precursor cells (CEP or EPC) or hemangiocytes populations, which can be detected resident within mature blood vessels as a source for angiogenic responsiveness [3,4]. More recently, Zengin et al. observed that a vascular wall resident endothelial progenitor population of cells is important in postnatal angiogenesis [5]. Regardless of the source of EC, it is likely that a

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Abbreviations: BMEC, bone marrow derived endothelial cells; EC, endothelial cells; MEF, mouse embryo fibroblasts; VN, vitronectin; FN, fibronectin; LDPI, Laser Doppler Perfusion Imaging

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better understanding of the fundamental signaling events responsible for endothelial signal relay will prove useful in the identification of new molecular targets for vascular therapeutics [3]. Remodeling of extracellular matrix and alterations in integrin-mediated adhesion/migration within endothelial cells are required for neovascularization/angiogenesis *in vivo* [6]. The small guanosine triphosphatases (GTPases) of the Rho family have been shown to participate in these important processes in a number of cell types including endothelial cells [7,8].

The RAC family of small G proteins is composed of three isoforms, RAC1, RAC2 and RAC3 [9]. The RAC1 and RAC3 are ubiquitously expressed whereas RAC2 is selectively expressed in hematopoietic cells [10]. Experiments performed in the Rac2 knockout mouse model have established a prominent role for RAC2 in hematopoietic cells including neutrophil, macrophage and mast cell defects [11–13]. Since hematopoietic cells and endothelial cells are both derived postnatally from the same bone marrow compartment and share the expression of certain common signaling proteins, we hypothesized that hematopoietic specific small GTPase, RAC2 may play a role in endothelial cells and hence may be important signaling pathways in the control of angiogenesis.

To test this hypothesis, we utilized a Rac2 knockout mouse model to examine the role of Rac2 loss in endothelial cell function (s) and angiogenesis. Herein, we demonstrate RAC2 expression in endothelial cells and provide direct evidence that this small G protein is required for integrin ($\alpha v\beta 3$, $\alpha 4\beta 1$ and $\alpha 5\beta 1$) directed migration of endothelial cells and the angiogenic response *in vivo*. Results from analysis of mouse genetic models (Syk−/+, Syk−/+; Rac2−/+) provide evidence that SYK kinase is required for the activation of RAC2 and endothelial cell migration via the $\alpha v\beta 3$ integrin. Moreover, using a reductionistic approach in COS7 cell (transfection with B linker region SYK mutations at Y317F, Y342F, Y346F and catalytically dead SYK, K396R) generate additional evidence that SYK can selectively mediate the activation of RAC2 downstream of $\alpha 4\beta 1$ integrin engagement and SYK can augment RAC2-dependent migration via this integrin. Moreover, we have identified a subregion of SYK, the B linker sequence, required for the activation of RAC2 and cell migration. These combined observations suggest that SYK-RAC2 signaling axis and specifically the B linker region of SYK are new molecular targets for the regulation of neovascular/angiogenic response of endothelial cells *in vivo*. Considering the importance of postnatal neovascularization, the identification of a novel molecular pathway, SYK-RAC2 signaling axis in endothelial cells that controls post embryonic angiogenesis may result in the development of innovative therapeutic strategies (e.g. RAC2 or SYK inhibitors).

Materials and methods

Animals, antibodies and reagents

Rac2 knockout and Syk haploinsufficient (−/+) mice and normal littermates in C57BL/6J genetic background have been described [11,14,15]. We adhere to Emory University Animal Care and Use Committee approved protocols and NIH guidelines. The FVN/N-TgN (Tie2-GFP) transgenic mice were obtained from commercial stocks at the Jackson Laboratory (Bar Harbor, ME). Human brain endothelial cells (HBEC) were obtained as described [16]. The HUVEC, J774 and COS7 cells were purchased from ATCC (Rockville, MD). RAC2 Ab was a gift from Drs. G. Bokoch and U. Knaus (The Scripps Research Institute, La Jolla, CA). PAK-1 PBD (RAC/CDC42 assay reagent), agarose for pull down of the activated RAC1 and RAC2 and monoclonal RAC1 antibody were from Upstate Biotechnology (Lake Placid, NY). Recombinant human fibronectin peptides, H296 and CH271 were obtained from Collaborative Biomedical (Bedford, MA) or were a gift from Takara Shuzo (Otsu, Japan). Vitronectin was purified as described [17]. GM-CSF was bought from Peprotech (Rocky Hill, NJ). Dil acetylated low-density lipoprotein (Ac-LDL) and vWF-conjugated FITC rat anti-mouse mAbs were procured from Molecular probes (Eugene, OR). All other chemicals were purchased from Sigma (St. Louis, MO).

Isolation of bone marrow derived endothelial cells (BMEC)

Bone marrow derived endothelial cells (BMEC) were generated by flushing the marrow cavity with culture media (DMEM, Life Technologies, Rockville, MD) followed by a separation on histopaque199 (Sigma, St. Louis, MO). Cells were collected, washed and resuspended in DMEM supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin and plated on 10 cm dish and cultured for 24 h at 37 °C (5% CO_2). The next day, media containing nonadherent cells was collected, spun at 1200 rpm and plated on 60 mm dish. After 48 h culture in 5% CO_2 incubator, nonadherent cells were collected, spun at 1200 rpm, re-suspended in complete media containing 400 ng/ml GM-CSF and cultured for 5–7 days in 5% CO_2/5% O_2 incubator at 37 °C.

Immunolabeling and Dil-labeled acetylated-LDL uptake assay

For staining, cells grown on tissue culture dishes were washed with PBS, fixed with 4% paraformaldehyde at 4 °C for 30 min and washed three times with PBS. The cells were then immersed in PBS containing 3% milk, 0.025% Triton X 100 (PMT solution) for 30 min to block nonspecific protein interactions and permeabilize the cell membranes. The cells were stained with anti vWF mouse antibody and FITC rat anti-mouse Abs in PMT solution and incubated at 4 °C for overnight. Macrophages and appropriate isotype antibodies (IgG2a) were used as negative controls. For uptake of Dil-labeled ac-LDL, cells were incubated with 10 μg/ml Dil-labeled ac-LDL (Biomedical Technologies, Stoughton, MA) for 4 h at 37 °C. After incubation, cells were washed three-times with PBS, fixed with 4% paraformaldehyde for 30 min and visualized with a fluorescent microscope (Nikon). We utilized the Tie2-EGFP transgenic mouse model where the Tie2 promoter elements drive the endothelial cell specific expression of green fluorescent protein [18] to visualize and isolate EGFP positive endothelial cells from different organs e.g. heart, lung, kidney and brain *in vivo*. Cell suspensions from different tissues were prepared as described [19] and subjected to FACS sorting to isolate a highly purified population of EGFP positive cells for further analysis.

Immunoblot analysis of RAC2 expression

Lysates of bone marrow derived endothelial cells (BMEC), macrophages (BMMθ), mouse macrophage cell line (J774 A.1), human brain endothelial cell line (HBEC), human umbilical vein endothelial cells (HUVEC), EGFP sorted cardiac endothelial cells from Tie2 transgenic mice and mouse embryo fibroblast (MEF), bone marrow derived macrophages from Rac2 deficient mice (both for negative