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Research Article

PKCε is a negative regulator of PVAT-derived vessel formation

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ABSTRACT

Rationale: Vessel formation is a crucial event in tissue repair after injury. Thus, one assumption of innovative therapeutic approaches is the understanding of its molecular mechanisms. Notwithstanding our knowledge of the role of Protein Kinase C epsilon (PKCε) in cardio-protection and vascular restenosis, its role in vessel progenitor differentiation remains elusive.
Objective: Given the availability of PKCε pharmacological modulators already tested in clinical trials, the specific aim of this study is to unravel the role of PKCε in vessel progenitor differentiation, with implications in vascular pathology and vasculogenesis.
Methods and results: Mouse Peri-Vascular Adipose Tissue (PVAT) was used as source of mesenchymal vessel progenitors. VEGF-induced differentiation of PVAT cells down-regulates both PKCε and p-PAK1 protein expression levels. PKCε overexpression and activation: i) reduced the expression levels of SMA and PECAM in endothelial differentiation of PVAT cells; ii) completely abrogated tubules formation in collagen gel assays; iii) increased the expression of p-PAK1.
Conclusion: PKCε negatively interferes with vessel progenitor differentiation via interaction with PAK-1.

Introduction

Angiogenesis is the formation of new vessels by resident endothelial cells that proliferate and remodel. Differently, vasculogenesis is the "de novo" formation of vessels by stem cells that generate a vascular network [1]. Although vasculogenesis occurs primarily in the embryo, it has been found that adult bone marrow-derived endothelial progenitor cells can generate new vessels [2]. Both angiogenesis and vasculogenesis are involved in the pathophysiology of tumour growth and in tissue repair after injury, like myocardium infarct. Thus, understanding the molecular mechanisms of vessel formation is crucial to block disease development and favor tissue regeneration.

Vascular adventitia is currently seen as a dynamic compartment for trafficking in and out of the arterial vessels, mediating

Abbreviations: PKCε, Protein Kinase C epsilon; PVAT, Peri-Vascular Adipose Tissue; PAKs, p21-Activated Kinases

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communications between endothelial, smooth muscle cells and surrounding tissues [3]. Moreover, the adventitia contains the microvessels that nourish the media, lymphatics and autonomic nerve fibers for the tonic regulation of the vessel wall. Interestingly, there is solid evidence showing that the adventitia is a stem cell niche providing progenitors of both endothelial and mural cells, potentially able to respond to arterial injury [4,5]. The Perivascular Adipose Tissue (PVAT) is often associated with large arteries [3], it contains adipocytes and pre-adipocytes and shares common features with the *tunica adventitia* such as the presence of fibroblasts, macrophages and vascular progenitors. It localizes externally to the adventitia [7] and is responsible for the production of several vasoactive paracrine factors (reviewed in [8]). Moreover, together with adventitia, PVAT represents a source of mesenchymal progenitors that participate in microvascular network remodeling [6].

The Protein Kinase C (PKC) ε plays a pivotal role in cell proliferation, apoptosis and differentiation [9,10]. We have previously demonstrated that high levels of PKCε impair intestinal progenitor cell differentiation and rescue cells from TRAIL-induced apoptosis, preserving the pool of proliferating cells at the bottom of the intestinal crypt [11]. In human hematopoiesis, while PKCε is up-regulated during erythropoiesis, preventing erythroblasts apoptosis induced by TRAIL [12], it must be down-regulated during megakaryocytopoiesis for mature platelet production [13]. Persistent levels of PKCε mRNA in platelets are strongly associated with platelet reactivity and myocardial infarct [14]. Moreover, beside its role in the cardioprotection from hypoxia-reperfusion induced cell death [15,16], PKCε regulates the expression of the cardiac genes *nkx2.5* and *gata4* during differentiation of bone marrow-derived mesenchymal stem cells [17]. Interestingly, Deuse et al. [18] recently showed that PKCε is involved in the development of neointimal hyperplasia during vascular restenosis after balloon injury, regulating endothelial cell proliferation and extracellular fibrosis. This observation, together with the role of PKCε in cell commitment, prompted us to focus on its potential role in the expression of vascular differentiation markers by *in vitro* and *in vivo* experiments. As *in vitro* model of endothelial and smooth muscle differentiation we used PVAT-derived progenitors. Vascular differentiation potential of PVAT-derived cells has been described by Passmann et al. [5].

To investigate the downstream signaling of PKCε in PVAT cells vascular commitment, we focused on p21-Activated Kinases (PAKs) because PAK1, 2 and 4 are important factors in blood vessel development and structure [19]. Moreover, the activation of the Cdc42-PAK signaling cascade plays a key role in endothelial cell lumen and tube formation and requires the coordination of PKCε and Src family kinases [20], suggesting that PAK proteins could be downstream target of PKCε.

Materials and methods

Cell culture

Mice were anesthetized with isofluoran 5% and then sacrificed by cervical dislocation.

PVAT cells were isolated from adipose adventitial tissue between aortic arch and pulmonary artery (black circle in Fig. 1A). Tissue obtained from 5 adult CD1 mice was digested with elastin (0.75 mg/ml, Sigma, Milan Italy) and collagenase II (1 mg/ml, Sigma, Milan, Italy) (3 digestions of 30 min each). Stem Cell Antigen 1+ (Sca1+) positive cells were isolated using anti-Sca1 immuno-magnetic microbeads and the MACS cell separation system (Miltenyi, Italy).

PVAT cells were maintained in culture with D-MEM (Sigma, Italy) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 2 mM glutamine, 1% antibiotics and maintained in a humidified 5% CO_2 atmosphere at 37 °C. Cells cultured in low serum medium (2% horse serum) were differentiated to smooth muscle lineage by 10 ng/ml TGF-β1 (Peprotech, Tebu-bio, Italy), or to endothelial lineage by 10 ng/ml VEGF (Sigma, Milan Italy). After 72 h cell cultures were harvested and processed for western blot analysis or immunofluorescence. Osteogenic and adipogenic differentiation was induced with specific media from Stem Cell Technologies (Vancouver, Canada) following manufacturer's instructions for 20 days, then tested by Alizarin red and Oil red Oil staining, respectively [21]. For the *in vitro* vasculogenesis assay PVAT cells were embedded in collagen I matrix from rat tail (BD Biosciences, MA USA) and cultured for 3 days. The matrix (collagen gel) was prepared following manufacturer's instructions. Cells were resuspended in ice-cold collagen matrix (50,000/mL) and seeded in 96-well plates (modified from Ref. [22]). After 15 min at room temperature the plate was transferred at 37 °C for 30 min to promote gelation. Each experiment was performed in triplicate and at least nine images were caught by Leica ICC50 HD camera and processed with Image J software.

PKCε activity was pharmacologically modulated by the εV1-2 (CEAVSLKPT) and ψεRACK (CHDAPIGYD) peptides, conjugated to TAT_{47-57} (CYGRKKRRQRRR) by a cysteine disulfide bond [23]. Free

Fig. 1 – Peri-Vascular Adipose Tissue (PVAT) cells: anatomical site and differentiation potential. (A) Picture of the superior part of an adult mouse heart. Circle indicates the tissue that was isolated to obtain PVAT cells. AA, aortic arch; PA, pulmonary artery. Scale bar, 50 μm. (B) Phase contrast picture of the PVAT cell cultures grown in complete medium. Scale bar, 40 μM. (C) Alizarin Red Staining of the PVAT cell cultures differentiated with osteogenic medium. Arrow head indicates calcium deposit. Scale bar, 30 μM. (D) Oil Red Oil staining of the PVAT cell cultures differentiated with adipogenic medium. Arrow head indicates lipid droplets. Scale bar, 20 μM. (E) Flow cytometry analysis of the $Sca1^+$ population isolated from Perivascular Adipose Tissue (PVAT). Cells were stained with antibodies anti-CD45, -CD14, -CD105, and -CD90 labeled with phycoerythrin (PE) or with antibodies anti-CD34 and -CD44 labeled with Fluorescein Iso-thiocianate (FITC). Autofluorescence was evaluated by staining cells with isotype-matched control antibodies (Isotype cont.). Percentage of positive cells are reported in the panels. Data shown are representative of three replicates. (F) Western blot detection of PKCε, SMA, PECAM, p-PAK1, p-PAK2, p-PKCα/β II and p-PKCδ/θ proteins in untreated (C), in TGF-β1− (T) and VEGF-treated PVAT cell cultures (V) for 3 days. GAPDH has been used as loading control. (G) Densitometry measurements of western blots assays. The protein levels of PKCε, SMA, PECAM, p-PAK1, p-PAK2, p-PKCα/β II and p-PKCδ/θ were expressed as arbitrary units with respect to the control (indicated as C). Values had been normalized with the GAPDH of the same sample. Means ± SD of three independent experiments are reported. Statistical analysis was performed with One-Way Anova and Dunnett's Test for multiple comparisons (*$p<0.05$; **$p<0.005$).