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Rapid communication

Proteomic characterization of human early pro-angiogenic cells

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ABSTRACT

Early pro-angiogenic cells (EPCs) have been shown to be involved in neovascularization, angiogenesis and re-endothelialization and cathepsin L inhibition blunted their pro-angiogenic effect. In the present study, we have analysed and mapped the proteome and secretome of human EPCs, utilizing a combination of difference in-gel electrophoresis (DIGE) and shotgun proteomics. A population of 206 protein spots were analysed, with 171 being identified in the cellular proteome of EPCs. 82 proteins were identified in their conditioned medium, including the alternative macrophage markers C–C motif chemokine 18 (CCL18) and the hemoglobin scavenger receptor CD163 as well as platelet factor 4 (CXCL4) and platelet basic protein (CXCL7) with "platelet alpha granule" being returned as the top category according to the Gene Ontology Annotation. Apart from cathepsin L, the cathepsin L inhibitor also attenuated the release of a wide range of other cathepsins and lysosomal proteins such as legumain, but stimulated the secretion of members of the S100 protein family. The data presented here are the most comprehensive characterization of protein expression and secretion in human EPCs to date and highlight the potential importance of cysteine proteases in the processing of platelet factors for their pro-angiogenic potential. This article is part of a special issue entitled, "Cardiovascular Stem Cells Revisited".

1. Introduction

The concept of progenitor cells is attracting considerable interest in cardiovascular research and early pro-angiogenic cells (EPCs) have obtained particular attention. EPCs, previously referred to as endothelial progenitor cells, were first described in 1997 by Ashara et al. [1] who demonstrated that these cells were derived from CD34+-enriched mononuclear cells in peripheral blood, and had the ability to participate in vasculogenesis in the animal model of hindlimb ischaemia. EPCs are supposed to represent a subset of circulating bone marrow cells among peripheral blood mononuclear cells (PBMNC), which have the capacity to differentiate into endothelial cells *in vivo*. Numerous publications have shown that EPCs are involved in neovascularization, angiogenesis and re-endothelialization, with cathepsin L playing a crucial role [2]. However, the nomenclature and the phenotype of EPCs are subject to ongoing controversy and there are still no specific markers, which unambiguously identify these cells [3,4]. By now, the inconsistent therapeutic effects of cell therapy have been attributed to the different isolation procedures [5].

Using proteomics, we have recently analysed the protein composition of microparticles originating from EPC cultures. Our data revealed that conventional methods for isolating PBMNC using density barrier centrifugation lead to a contamination with platelets [6]. Platelets disintegrate into platelet microparticles, which can transfer "endothelial" characteristics, such as CD31, von Willebrand factor (vWF) and UEA-1 staining, to the PBMNC population and influence their angiogenic properties [6]. While platelets may promote an angiogenic monocyte phenotype [7,8], these findings highlight the need for a more comprehensive analysis of EPCs. So far, we have reported a transcriptomic dataset of EPCs [2,9] and proteomic datasets of Hill colony-forming units [10] and smooth muscle progenitors [11]. In these proteomic studies, EPCs served as a reference for comparison with other putative progenitor cell populations. A comprehensive proteomic dataset of early outgrowth EPCs, however, has not been published so far. The aim of this study is to

Abbreviations: EPC, early pro-angiogenic cells; DIGE, difference in-gel electrophoresis; FCS, foetal calf serum; HUVEC, human umbilical vein endothelial cells; PBMNC, peripheral blood-derived mononuclear cells; qPCR, real-time PCR.

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characterize the proteome and secretome of EPCs using a combination of difference in-gel electrophoresis (DIGE) and shotgun proteomics for cellular and secreted proteins, respectively and to assess the effects of cathepsin L inhibitors on their secretory potential.

2. Materials and methods

PBMNC were isolated by density gradient centrifugation with Ficoll from peripheral blood of healthy human volunteers and cultivated on fibronectin in the presence of VEGF as previously described [7–9]. EPCs were incubated for 3 h in serum-free medium with the cathepsin L inhibitor (Z-FF-FMK, 10 μM) or high glucose (25 mM), then washed with PBS, and incubated with serum-free medium for 24 h without further stimulation. Proteomics analysis were performed as previously described [6,10,11]. A detailed protocol is provided online.

3. Results and discussion

3.1. Comparison of EPCs and HUVECs

Flow cytometry analysis demonstrated the presence of the VEGF-R2 (KDR) and the functionally important SDF-1 receptor CXCR4 in both EPCs and HUVECs (Fig. 1(A)), but in agreement with previous reports [3,4] their proteome was very different (Fig. 1(B)). To analyse the proteins predominantly expressed by EPCs, 206 spots were excised and of these 171 were identified by LC–MS/MS (Supplemental Fig. 1), leaving 35 spots (16.9%) unidentified. The majority of proteins were enzymes (40%), followed by structural proteins (22%), chaperones (13%) and signalling proteins (11%). All identifications are listed in Supplemental Table I. Among the identified proteins, which were abundant in EPCs compared to HUVECs, were several anti-oxidative enzymes such as mitochondrial superoxide dismutase and hemoxygenase-1, confirming our previous finding of a high expression of

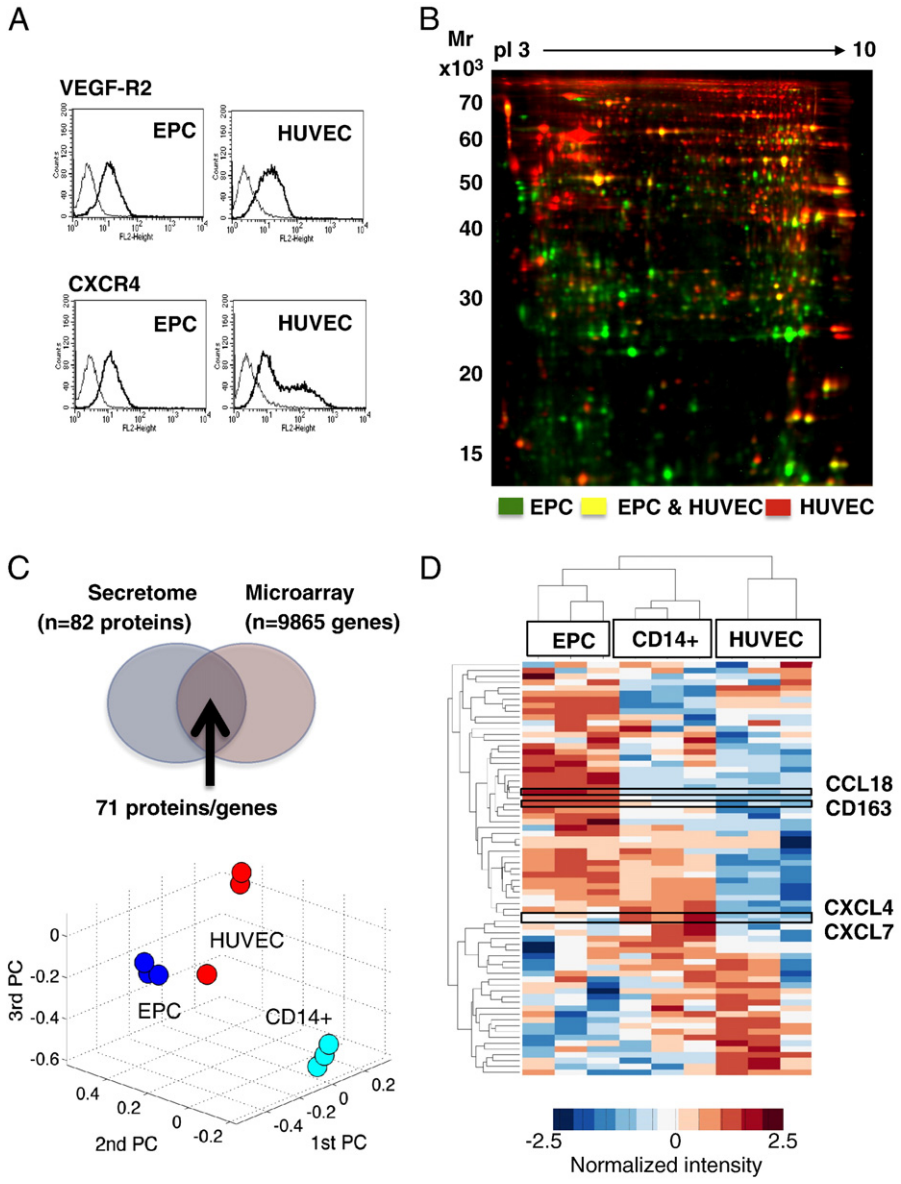

Fig. 1. Proteomics comparison of EPCs and HUVECs. (A) FACS analysis of markers in EPCs and HUVECs. Cells were incubated with either isotype controls (thin line) or antibodies to VEGF-R2 and CXCR4. (B) The cellular proteome of EPCs and HUVEC was compared using DIGE (green colour indicates EPCs and red colour indicates HUVECs). Note that the cellular proteome of EPCs does not resemble the one of a mature endothelial phenotype. (C) Venn diagram and PCA plot of 71 proteins identified in the conditioned medium of EPCs that were also observed at the probe level in the microarray dataset. The mRNA expression levels of the 71 proteins are sufficient to distinguish EPCs, HUVECs, and CD14+ monocytes. (D) Unsupervised hierarchical clustering of 71 secreted proteins/genes. The blue colour indicates low expression, and the red colour indicates high expression at the mRNA level. Transcripts for CXCL4 and CXCL7 are abundant in freshly isolated CD14+ monocytes while alternative macrophage markers CCL18 and CD163 are expressed in EPCs.

anti-oxidative enzymes leading to the resistance of EPCs towards apoptosis [12], and members of the cathepsin family. Notably, cathepsin L inhibition has been shown to block the pro-angiogenic activity of EPCs [2].

3.2. The secretome of EPCs

To complement the analysis of the cellular proteome, the conditioned media of 4 independent EPC preparations were investigated using shotgun proteomics. This analysis returned 82 human protein features (Supplemental Table II), including CXCL4, CXCL7, fibronectin, thrombospondin 1 and fibrinogen. Thus, the classification according to the Gene Ontology Annotation returned "extracellular space" and "platelet alpha granule" as the top categories for the secretome of EPCs (Supplemental Table III). The presence of platelet alpha granules was confirmed by electron microscopy (Supplemental Fig. 2). 71 of the 82 identified protein features in the conditioned medium could be mapped to our previously published microarray dataset [2]. The gene expression profile of these 71 secreted proteins was sufficient to separate peripheral blood-derived CD14+ monocytes, HUVECs and EPCs in principal component analysis (PCA, Fig. 1(C)). Supplemental Fig. 3 lists the genes/proteins according to their statistical significance. CXCL4 and CXCL7 transcripts were more abundant in freshly isolated CD14+ monocytes than cultured EPCs (Fig. 1(D)). Yet, CXCL4 and CXCL7 were identified in the conditioned medium of EPCs expressing the alternative macrophage markers CCL18 and CD163.

3.3. Effect of a cathepsin L inhibitor

Since platelets are rich sources of angiogenic growth factors, variations in platelet contamination may complicate the interpretation of the EPC culture assays [6,8]. Thus, DIGE was employed to assess the effect of cathepsin L inhibitors on the secretome of EPCs (Fig. 2(A)). The analysis of 99 differentially expressed protein spots (Supplemental Fig. 4) by LC–MS/MS resulted in the identification of 81 non-redundant proteins (Supplemental Table IV). All peptide identifications are

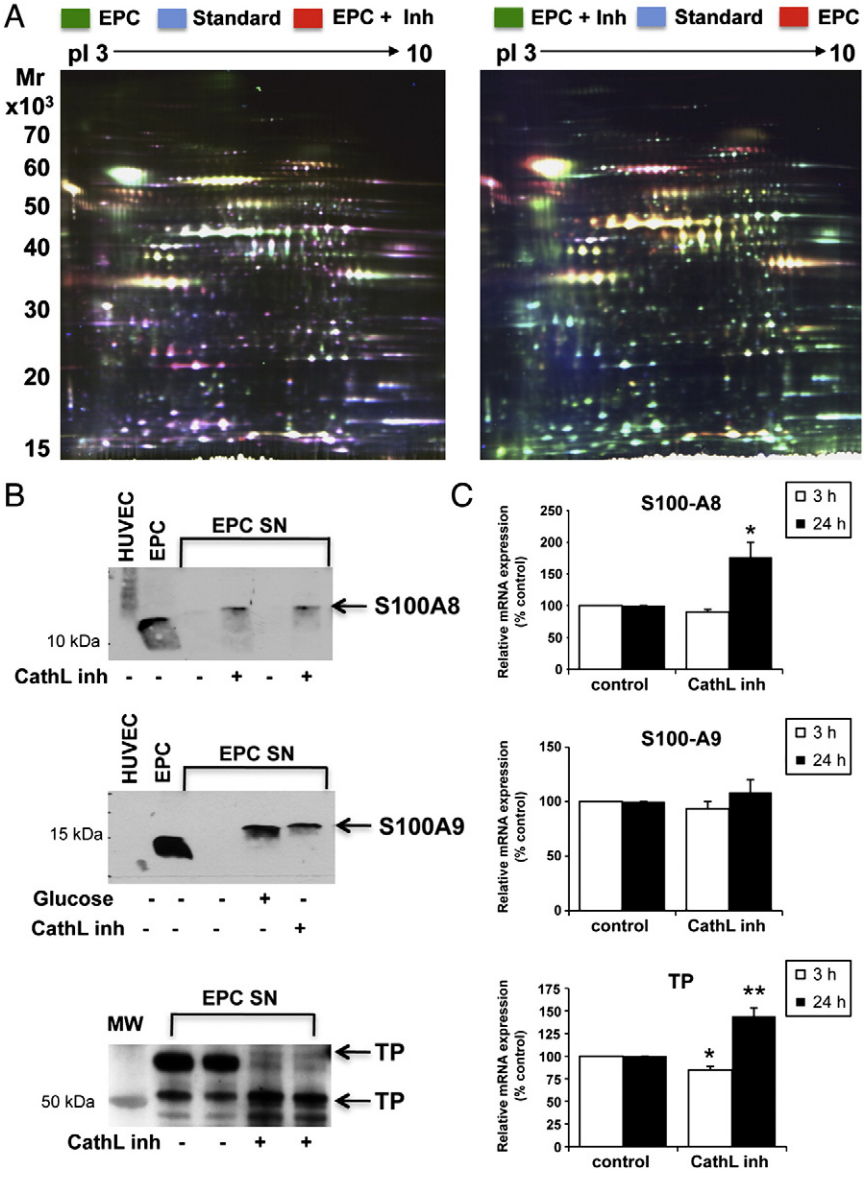

Fig. 2. The effect of a cathepsin L inhibitor on the secretome of EPCs. (A) DIGE comparison of conditioned medium from EPCs treated with a cathepsin L inhibitor (+ Inh). Results are reproduced with different biological replicates using reverse-labeling. (B) Proteins in the supernatant (SN) of control EPCs and EPCs treated with a cathepsin L inhibitor were immunoblotted and probed with antibodies for S100 A8, S100 A9 and thymidine phosphorylase. Cellular lysates of HUVECs and EPCs served as additional controls. MW denotes molecular weight marker. Note that thymidine phosphorylase is also present in platelets and that two distinct bands are visible on the immunoblot (TP). (C) Concordant regulation at the mRNA and the protein level was observed for S100 A8, but not for S100 A9 and thymidine phosphorylase, suggesting that the cathepsin L inhibitor alters the release of these proteins. * denotes $p<0.05$; ** $p<0.01$.

provided in Supplemental Table V. The cathepsin L inhibitor effected the secretion of a wide range of other cathepsins, lysosomal proteins (beta-hexosaminidase beta chain, ganglioside GM2 activator, and legumain), and thymidine phosphorylase. Thymidine phosphorylase, also known as platelet-derived endothelial growth factor, is an intracellular enzyme that produces an angiogenic metabolite and has been shown to contribute to the angiogenic activity of EPCs [10]. In contrast, members of the S100 protein family (A8, A9, and A11) were increased. The changes for S100 A8, S100 A9 and thymidine phosphorylase were subsequently confirmed by immunoblotting (Fig. 2(B)), but there was no concordant regulation for S100 A9 and thymidine phosphorylase at the mRNA level (Fig. 2(C)). Expression changes for legumain, leptin, S100 A11, enolase, Rantes and IL-8 are shown in Supplemental Fig. 5.

3.4. Platelet contamination of EPCs

Originally, EPCs were thought to be a subpopulation of PBMNC that have the potential to differentiate into mature endothelial cells. In some of the common culture assays, however, the cell type consistent with current definitions of an EPC phenotype may have arisen from an uptake of platelet antigens by mononuclear cells. This was highlighted by our previous proteomic analysis of microparticles from EPCs [6]. In the present study, we analyse the cellular proteome and the secretome of EPCs. This analysis resulted in the identification of several platelet factors: CXCL7 is a key angiogenic chemokine that binds to CXCR2. Blockade of CXCR2 significantly reduced EPC adhesion on platelet-coated endothelial matrix [13]. CXCL4 is a platelet-derived chemokine that promotes macrophage differentiation from monocytes and negatively regulates CD163 expression. The expression of alternative macrophage markers CD163 and CCL18 increased in early outgrowth EPCs compared to CD14+ monocytes. Similarly, classical macrophages do not express legumain.

3.5. Beyond cathepsin L inhibition

Our analysis highlights that the cathepsin L inhibitor induces a complex cellular response encompassing a wide range of seemingly unrelated proteins. While some of the factors, such as thymidine phosphorylase, have previously been reported to contribute to the angiogenic potential of EPCs [10], others, such as the protein S100 family (A8, A9, A11) have not been implicated so far. The S100 A9 protein (referred to as calgranulin B and migration inhibitory factor-related protein 14) controls leukocyte migration and infiltration at sites of wounding. It plays a role as a pro-inflammatory mediator in acute and chronic inflammation, in particular it is known to up-regulate IL-8. Similarly, the release of S100 A9 in the presence of the cathepsin L inhibitor was associated with increased expression of IL-8 by EPCs (Supplemental Fig. 5, 879.7 ± 35.2 pg/ml in the conditioned medium of control EPCs vs $10{,}010.1 \pm 8025.4$ pg/ml ml in the conditioned medium of EPCs treated with the cathepsin L inhibitor). Supplementing the culture medium with high glucose (25 mM), which reduces the secretion of cathepsin L and the invasion of EPCs [14], also promoted the release of the S100 A9 protein (Fig. 2(B)). However, since the cathepsin L inhibitor had a broad effect on other members of the cathepsin family and lysosomal enzymes, it cannot be excluded that this inhibitor may have influenced the release or processing of platelet factors, i.e. proteolytic processing of CXCL7 is known to be accomplished by neutrophil-derived cathepsin G and is inhibited by interaction of CXCL7 with CXCL4 [15], adding another layer of complexity to the study of angiogenic effects in EPC cultures.

4. Conclusions

Complex read-outs such as angiogenesis depend on the net effect of all the proteins present in the conditioned medium. Here we present a map of the cellular proteome and secretome of EPCs. We demonstrate that the proteome of EPCs is largely different from those of mature endothelial cells, that the conditioned medium of EPC cultures is rich in platelet proteins, and we identify novel targets of the cathepsin L inhibitor, which has previously been shown to block the angiogenic activity of EPCs [2].

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.yjmcc.2010.11.022.

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