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Analysis of Endothelial Progenitor Cells Recruitment to Tumor Angiogenesis

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The recruitment of endothelial progenitor cells (EPC) to sites of tumor angiogenesis has been described as a multistep process involving mobilization of precursor cells, adhesion, transendothelial migration, invasion into the surrounding tissue, formation of cell clusters in proximity of vascular sprouts and eventually incorporation into functional blood vessels. However the real contribution of EPC to the all process remains still controversial. In the present study the recruitment of EPC to tumor angiogenic sites, has been investigated at a cellular and a molecular level, using two different sources of EPC: human umbilical cord blood (UCB) and mouse embryonic EPC (e-EPC).

To define a population with enhanced tumor targeting capacity the hierarchy of human UCB-derived EPC has been evaluated. CD34+ mononuclear cells (MNC) were isolated from UCB. When cultured under endothelial condition these cells gave rise to a population identified as endothelial colony forming cells (ECFC). Human umbilical vein endothelial cells (HUVEC) served as mature endothelial control. In vitro, the expansion capacity as well as endothelial and stem cell marker expression were analyzed. To address the multistep nature of homing, adhesion, migration and transmigration assays were established. In vivo, the trafficking of CD34+ MNC, ECFC as well as HUVEC was analyzed using intravital microscopy. Cells were injected into mice bearing C6 glioma xenografts in the skin-fold chamber. Their recruitment in terms of adhesion, extravasation and formation of cell clusters was analysed. In *in vitro* functional assays, used to mimick the multistep nature of the homing process, ECFC showed an enhanced specific tumor tropism compared to HUVEC. In vivo ECFC showed a significant enhanced activity in initial interaction, adhesion, extravasation and cluster formation compared to CD34+MNC and to HUVEC. This is highly interesting as the phenotype, immune phenotype and expression of endothelial markers of ECFC strongly resembles mature HUVEC. Solely the expansion potential distinguishes ECFC from mature endothelial cells. The present study supports the notion that ECFC have significant higher potential for tumor targeting than non-cultivated CD34+MNC and mature endothelial cells. Accordingly, ECFC might be considered for future cell-based therapies to carry an anti-tumor gene to sites of tumor neo vascularization.

The molecular mechanisms that mediate EPC recruitment to tumor tissue remain to be largely understood. The Junctional Adhesion Molecule-C (JAM-C) is an adhesion molecule mainly expressed on endothelial and epithelial cells, localized at cell-cell contacts. It has been recently shown a role of JAM-C in leukocyte trans-endothelial migration and also inhibition of angiogenesis and tumor growth by using an antibody against JAM-C. Using the well characterized mouse e-EPC, the expression of the JAM-C protein was analyzed and its potential role during e-EPC recruitment in tumor angiogenesis was investigated, *in vitro* and *in vivo*. JAM-C expression analysis on e-EPC was studied on the m-RNA level and on the protein level. A Lewis Lung Carcinoma (LLC) syngenic tumor model was established by the implantation of LLC1 tumor cells in the skin-fold chamber, on C57/BL6 mice. Immunofluorescence analysis of JAM-C on tumor microvessels was performed. In vivo C57/BL6 mice bearing LLC1 tumour on the skin-fold chamber were systemically pre-injected with a monoclonal antibody anti JAM-C (H33), or with an IgG antibody or with PBS, used as controls. Fluorescently label e-EPC were integrated into animals and their recruitment in terms of interaction with the tumour microvessels, adhesion, extravasation, cluster formation and eventually incorporation was analysed by intravital multi-fluorescence microscopy. *In vitro*, blocking of JAM-C protein was assessed using different approaches (mAb H33, recombinant JAM-C and JAM-C siRNA). Adhesion, trans-endothelial migration and angiogenesis assays were performed to mimick the *in vivo* study. RT-PCR showed that JAM-C is expressed by e-EPC. Immunofluorescence demonstrated JAM-C protein expression and localiztion at the tight junctions. JAM-C was also found expressed on the LLC1 tumor endothelium. *In vivo* studies of e-EPC recruitment to tumor angiogenic sites showed that JAM-C is not involved in e-

EPC adhesion and extravasation. These results were confirmed by the *in vitro* adhesion and trans-endothelial migration. Two days after cell integration there was no differences among the three groups of animals (H33, IgG, PBS) in terms of number of recruited cells, but interestingly, mice treated with anti-JAM-C blocking antibody showed significantly fewer elongated, clustered cells compared to the PBS and the IgG treated control mice. In order to mimick the *in vivo* results, an *in vitro* angiogenesis assay was performed. Since e-EPC alone fail to form cord-like structures on matrigel™, e-EPC derived upon stimulation with c-AMP/retinoic acid (e-EPDC) were used. HUVEC were used as positive control. Blockade of JAM-C by mAb anti JAM-C (H33) and JAM-C recombinat protein showed a significant reduction on the formation of cord-like structures in e-EPDC as well as in HUVEC. Angiogenesis assay was also performed when JAM-C protein was knocked down by siRNA and significant differences were found between JAM-C siRNA and control siRNA or untransfeted cells, in e-EPDC as well as in HUVEC. The present study shows that JAM-C is expressed on EPC and that plays a specific role during EPC recruitment in tumour angiogenesis. In particular this study provides evidences that JAM-C is required by progenitor cells and by mature endothelial cells for building up a vascular network.

In conclusion the present study show evidences that both human UCB-derived EPC and mouse embryonic-EPC, *in vitro* as well as *in vivo* specifically home to sites of tumour angiogenesis and functionally take part to the process of tumour neovascularization. Moreover the data here presented provide novel contribution on the general understanding of EPC with respect to their role in tumour angiogenesis.