Manipulating pro-angiogenic effect of Circulating Angiogenic Cell cultured Collagen Matrices through Integrins

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Introduction:

Circulating angiogenic cells (CAC) have the ability to promote blood vessel formation and restore blood flow to ischemic tissue by directly incorporating into new vessels and by secreting pro-angiogenic cytokines. While the therapeutic potential for these cells has been demonstrated in both animal models and clinical trials, cellular retention and engraftment remain significant hurdles. As a strategy to help overcome these challenges, we have developed a type I collagen-based matrix to deliver CACs. The collagen matrix has been shown to enhance cell retention and improve perfusion of ischemic tissue. Furthermore, CAC survival, migration, angiogenesis potential, adhesion and pro-angiogenic cytokine secretion are all improved in CACs cultured on collagen matrix compared to traditional fibronectin. How the matrix enhances CAC function and therapeutic potential remains unknown but likely involve integrin proteins. Integrins are a large family of proteins (18 α and 8 β) that form 24 αβ heterodimeric transmembrane glycoproteins, which regulate a number of cellular processes including survival, proliferation, differentiation and migration. This study aimed to investigate the involvement of integrins in promoting the function of CACs when cultured on matrix. We hypothesize that this will enable us to improve the therapeutic potential of CAC-matrix treatment.

Materials and Methods:

The expression of several collagen-binding (α1, α2, α10, α11 and β1) and pro-angiogenic integrin genes (α5, αV, β3 and β5) were evaluated in human CACs by qRT-PCR following a 4-day culture on fibronectin or collagen matrix. Increased mRNA expression of α5 in collagen matrix cultured CACs (cmCACs) translated to increased α5 protein expression, assessed by western blot. Using a specific blocking antibody (Abcam) against α5, the role of this protein was examined in cmCACs by characterizing cell phenotype using flow cytometry, as well as their adhesion, migration and angiogenic potential. The essential role of α5 in the therapeutic effect of cmCACs was confirmed *in vivo* using a hindlimb ligation CD-1 immunodeficient mouse model. Animals were injected 20 minutes after ligation with PBS (control), cmCACs, or cmCACs with blocked α5. Blood flow was monitored by laser Doppler, and arteriole density was evaluated in hindlimb muscle tissue sections 2 weeks post-ligation by immunocytochemistry. To further investigate the mechanism of α5 pro-angiogenic effects, cmCACs were stimulated with angiopoietins for 2h prior to functional analysis such as adhesion, migration and proliferation.

Results:

Compared to fibronectin, the mRNA and protein expression of integrin α5 was increased in CACs after 4 days of culture on collagen matrix. The functional importance of this adhesion protein was demonstrated by blocking α5 for two hours prior to functional assays. A significant

reduction was observed in the ability of α5-blocked cmCACs to adhere to collagen ($p<0.014$), migrate in response to VEGF ($p<0.031$), and incorporate into tubule-like structures formed during an angiogenesis assay ($p<0.039$). *In vivo*, the perfusion of ischemic hindlimbs was decreased in mice treated with α5-blocked cmCACs compared to the control cmCACs-treated group at day 7 ($p=0.0148$) and day 14 ($p=0.025$) post-ligation. The number of SMA^+ blood vessels per field-of-view was also lower in animals treated with α5-blocked cmCACs ($p=0.022$), compared to the cmCACs control group. These results demonstrate the essential role of α5 in the angiogenic therapeutic effect of cmCACs. To then further stimulate these cells *in vitro*, angiogenic factors known to interact with integrin α5 – angiopoietin-1 and -2 – were added to the culture media. Angiopoietin-1- stimulated cmCACs had a 1.57-fold increase in migration ($p=0.025$) and a 1.78-fold increase in cell incorporation into tube-like vessels during angiogenesis assay compared to control cmCACs ($p=0.042$).

Discussion

Collagen matrix culture enhances the angiogenic potential of CACs, at least in part, through the regulation of specific integrins. While α5 is not a collagen binding protein, it is still regulated by attachment of CACs to a collagen matrix, which was seen through mRNA and protein expression. Blocking the activity of α5 in CACs after four days on collagen matrix reversed the improvement in their function and pro-angiogenic effect, as it was shown *in vitro* and *in vivo*. This study also presents a possible new approach to increase of therapeutic effect of cmCACs by the use of angiopoetin-1. It provides insight into novel mechanisms for improving the expansion and function of therapeutic CACs.