主論文の要旨

Therapeutic Angiogenesis by Autologous Adipose-Derived Regenerative Cells: Comparison With Bone Marrow Mononuclear Cells

（自己脂肪組織由来再生細胞による血管新生療法：骨髄単核球細胞との比較）

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Introduction

We have reported that therapeutic angiogenesis using autologous bone marrow-mononuclear cell (BM-MNC) transplantation augmented the angiogenesis in ischemic tissues and achieved a positive clinical outcome in patients with critical limb ischemia (CLI). However, long-term clinical outcome of BM-MNC-based study clarified that the survival and amputation-free rate were decreased in CLI patients suffered from arteriosclerosis obliterans. Therefore, we have kept searching a novel cell source in lieu of BM-MNC.

Stromal vascular fractions (SVFs) are mesenchymal progenitor cells isolated from the adipose tissue that possess the ability to stimulate the regeneration when introduced to the damaged tissues. To represent their regenerative abilities, this population is termed adipose-derived regenerative cells (ADRCs). Therefore, for the first time, we set a pre-clinical study to compare the therapeutic capacity of ADRC with BM-MNC using a rabbit model of hindlimb ischemia (HLI), and further examine the underlying mechanism of ADRC-related host macrophages reprograming capacity.

Methods

Animal Model: Male New Zealand White rabbits and C57BL/6J mice were used. Animals were anesthetized and the entire femoral arteries were ligated and removed. Four weeks after treatment, blood pressure, subcutaneous blood flow, collateral vessels formation and capillary density were measured respectively to analyze the therapeutic effects in each of the groups.

Isolation of ADRCs and BM-MNCs: Under general anesthesia, the adipose tissue was harvested from inguinal fat pads and minced and digested with collagenase. SVFs and mature adipocytes were separated by centrifugation. We have defined ADRCs as a subpopulation of fresh isolated SVFs. BM-MNCs were then isolated by centrifugation through a Histopaque density gradient procedure.

Flow Cytometric: Cells were washed in flow cytometric buffer, and incubated with antibody for 30 min and washed twice with buffer. Data was collected by FACS Canto flow cytometer.

Anti-IL-10 Neutralizing MAb Injection: Anti IL-10 neutralizing MAb or nonspecific rat IgG as a control were administered intraperitonealy into mice treated with or without ADRCs.

Results

ADRCs and BM-MNCs Augmented Angiogenesis: Four weeks after autologous ADRCs or BM-MNCs transplantation, either cell-therapy treatment augmented angiogenesis and collateral vessel formation (blood pressure recovery, blood perfusion, angiography and capillary density) in the ischemic hindlimb compared with saline (Fig. 1).

ADRC-PGE_2 Polarized Host Macrophages: ADRCs secreted more PGE_2 compared with BM-MNCs under normal condition and/or hypoxic condition. ADRC-CM slanted the polarity of macrophage to M2 phenotype in vitro and this specific effect was abolished by administration

of EP2/4 receptor antagonists. To assess the polarization of the infiltrated macrophages in the ischemic muscle, we performed flow cytometric analysis to identify the percentage of M2 macrophage in total. A greater percentage of CD206+ macrophage was observed in ADRC group than other two groups (Fig. 2).

Cytokines Expression: We confirmed that mRNA expression of angiogenic cytokines (VEGF and HGF) were unregulated in the macrophages cultured with ADRC-CM. A greater concentration of IL-10 was detected in the supernatant of macrophages cultured with ADRC-CM compared with other two groups. This effect was attenuated by the administration of EP2/4 receptor antagonists in ADRC-CM group rather than BM-MNC-CM group (Fig. 3).

ADRCs Create a Favorable Microenvironment: The gene expressions of TNF-α and IL-6 were suppressed in ischemic muscle 2 days after ADRCs treatment. Enhanced IL-10 expression was determined in the ischemic tissue in ADRCs group. The Bcl-xl/Bax protein expression ratio was profoundly increased in ADRCs group compared with other 2 groups (Fig. 4).

IL-10 Blockade Attenuated ADRC-Induced Neovascularization: An intraperitoneal injection of anti-IL-10 neutralizing MAb significantly suppressed blood flow recovery and collateral vessel formation in ADRC-treated group. MAbs caused muscular atrophy and increasing of TUNEL signal expressing cells in the ischemic thigh (Fig. 5).

Discussion

Several investigators demonstrated that various angiogenic cytokines secreted from ADRCs are important for regenerating damaged tissues. We previously found ADRC transplantation inhibited the invasion of immunocytes at the regenerative site, suggesting its anti-inflammatory manner. Increasing evidence suggests that macrophages have conflicting characteristics, such as pro-inflammatory and anti-inflammatory macrophages. Our data suggested ADRC therapy appears to promote angiogenesis through its ability of muting inflammation and polarizing $CD206^+$ macrophage via PGE_2-EP2/4 axis.

ADRCs released not only angiogenic growth factors, but also PGE_2 that stimulated the host M2 macrophages polarization via EP2/4 receptors. This notion is further supported by diminished angiogenic response and elevated apoptosis of vascular-lineage cells in mice treated anti-IL-10 MAb. Thus, these results suggest that the enhanced expression of IL-10 plays protective role in the process of tissue protection against ischemia-induced apoptosis.

Conclusion

In conclusion, autologous ADRC transplantation is capable to reach the similar therapeutic efficacy compared with BM-MNCs in a rabbit model of HLI; and more importantly, ADRCs transplantation creates a suitable microenvironment for tissue regeneration by polarizing IL-10 releasing M2 macrophages in local area. From a clinical point of view, therapeutic angiogenesis using autologous ADRC transplantation seems to be a useful strategy for patients with CLI.