主論文の要旨

Important Role of Concomitant Lymphangiogenesis for Reparative Angiogenesis in Hindlimb Ischemia

（虚血肢における修復性血管新生にはリンパ管新生が重要な役割を果たす）

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【Introduction】

Critical limb ischemia (CLI) represents a severe stage of peripheral artery disease (PAD) with pain at rest and/or ischemic ulcers or gangrene. Therefore, it is imperative to establish therapeutic strategies to prevent a progression to CLI in these patients.

Augmentation of angiogenesis by administration of pro-angiogenic cytokines or by transplantation of stem/progenitor cells is an additional therapeutic option for severe ischemic diseases. More recently, we have shown that implantation of autologous adipose-derived regenerative cells (ADRCs) promoted angiogenesis and healing of ischemic ulcers in similar patients. Lymphatic vessels may function as the clearance system for extracellular fluid, inflammatory cytokines and cells. Thus, this system may be necessary and important for angiogenic recovery after severe ischemic insult. However, the role of lymphangiogenesis in a reparative angiogenesis in ischemic tissue is largely unknown.

Accordingly, we investigated the potential role of lymphangiogenesis in reparative angiogenesis using a well-established mouse model of hindlimb ischemia. We further examined the role of concomitant lymphangiogenesis during ADRC-mediated therapeutic angiogenesis

【Methods and Results】

First, we investigated the changes in the capillary lymphatic density and VEGF-C expression in HLI in the setting of blood perfusion recovery at post-operative days (POD) 0, 14, and 28. Immuno-staining was performed to detect vascular endothelial cells [CD31-positive cells (red)] and lymphatic endothelial cells [LYVE-1-positive cells (green)] in HLI (Figure 1A). Our analysis revealed that angiogenesis emerged after POD 3, whereas lymphangiogenesis emerged after POD 5. A remarkable change in terms of those the morphology and the size of lymphatic vessels could not be detected in ischemic adductor muscles. Both angiogenesis and endogenous lymphangiogenesis were induced in a time-dependent manner after ischemic injury in HLI (Figure 1B and C). Further, the expression of VEGF-C was upregulated from POD 3 to 7 and its peak was observed at POD 5 in response to ischemic injury in the skeletal muscles of our model (Figure 2D).

To determine the impact of functional lymphatic transport capacity loss in HLI, we created an excessive and prolonged tissue edema model mimicking lymphatic drainage deficiency in the local tissue of HLI (Figure 1D and E) and investigated its effect on the blood perfusion recovery. Forced edema significantly inhibited angiogenesis and blood perfusion recovery (Figure 1H and I). The amputation-free survival rate was low in the artificial edema model in HLI (Figure 1F and G).

We investigated VEGF-C expression in ADRCs under a hypoxic condition both *in vitro* and in an ischemic hind limb tissue after ADRCs implantation to determine whether ADRCs have the potential ability to promote lymphangiogenesis in a paracrine manner.

qPCR demonstrated that hypoxic stimuli significantly increased VEGF-C expression in ADRCs compared with normoxia (Figure 2C) and that the VEGF-C expression in the ADRC-treated ischemic muscles was upregulated compared with than that in the PBS group (Figure 2D). ADRC implantation in HLI could promote blood perfusion recovery (Figure 2A and B) with augmentation of angiogenesis (Figure 2E and F).

The immunofluorescence analysis demonstrated that the number of macrophages was significantly upregulated in the response to the ischemic injury in the damaged tissue (Figure 3A). Conversely, the accumulation of macrophages was improved by augmentation of lymphangiogenesis with ADRC implantation at POD 14 (Figure 3A and B). qPCR showed that the expressions of the inflammatory factors, such as TNF-a, IL1-β, IL6, and TGF-β, were upregulated in an ischemic tissue at POD14 after injury. However, these inflammatory reactions were attenuated in the ADRC group (Figure 3C). Figure 3D demonstrates that the expression of vasohibin, angiostatin, and endostatin in HLI was upregulated in the PBS group. Conversely, these responses were partially canceled in the ADRC group compared with those in the PBS group (Figure 3D).

For further confirmation of the contribution of lymphangiogenesis on angiogenesis in HLI, we used MAZ51, which is a VEGFR-3 kinase inhibitor, to block lymphangiogenesis. Inhibition of lymphangiogenesis by MAZ51 enhanced inflammatory cell infiltration, gene expression of TNFα, IL-1β, IL-6, TGFβ, angiostatin, vasohibin and endostatin, and tissue edema (Figure5). Thereafter, we found that inhibition of lymphangiogenesis blocked endogenous ischemia-induced angiogenesis and suppressed the ADRC-induced angiogenesis in HL (Figure4).

【Discussion】

Lymphatic vessels play a pivotal role in the lymphatic fluid transport, lipid absorption, and immune cells drainage from the interstitial space to venous circulation. Although lymphatic vessels are known to exist in the skeletal muscles[29] in the same manner as blood vessels, limited knowledge is available regarding their kinetics and role in HLI. Herein, we first demonstrated that endogenous lymphangiogenesis was induced during lymphatic rarefaction via ischemic injury, and VEGF-C expression was upregulated in a series of adaptive lymphangiogenesis accompanied with endogenous angiogenesis in the ischemic limb. Moreover, inhibition of VEGFR3 signaling by MAZ51, confirmatively suppressed lymphangiogenesis in HLI. Taken together, our data indicate that postnatal lymphangiogenesis in HLI was induced at least in part via the VEGF-C-VEGFR3 signaling pathway in the ischemic hind limb, regardless of whether angiogenesis and lymphangiogenesis were independent of each other.

Our present study also found that the expression of some anti-angiogenic factors such as angiostatin, vasohibin, and endostatin, apparently increased in response to the

accumulation of inflammatory cytokines after HLI induction; these reactions deteriorated by inhibition of lymphangiogenesis but were ameliorated by the augmentation of lymphangiogenesis. More importantly, the concept of elimination of prolonged severe inflammation and edema modulating lymphatic revascularization could help in the formation of a new mechanistic approach for HLI.

【Conclusions】

lymphangiogenesis contributes to ischemia-induced angiogenesis by improving the surrounding resistance factors in HLI. Our findings could help in the formulation of a new mechanistic concept for therapeutic angiogenesis and might provide relevant information regarding additional novel therapeutic targets for CLI.