CD34^+VEGFR-3^+ progenitor cells have a potential to differentiate towards lymphatic endothelial cells

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Introduction

Lymphatic vessels

- no basement membrane
- no pericytes
- functions: draining excess fluid from extracellular space, absorbing & transportation of lipids, transporting leukocytes & APC, removal of cell debris, dust particles & microorganisms, tumour metastasis

Lymphangiogenesis

- occurs in wound healing, embryonic development, tissue regeneration, inflammation, tumour metastasis

newly formed lymphatic vessels sprout from pre-existing vessels by proliferation, migration and tube formation of endothelial cells

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Introduction

Endothelial progenitor cells

- are circulating cells that promote neovascularization at sites of ischemia, injury, hypoxia or tumour formation
- majority resides in bone marrow
- number of EPCs in human cord blood is much higher than in peripheral blood
- were already detected at growing lymphatic vessels in the cornea of mouse & transplanted human kidney

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De Biase C (2013). Effects of physical activity on endothelial progenitor cells
Introduction

CD34 haematopoetic cells, EPCs
CD133 haematopoetic stem/progenitor cell marker
VEGF-C
VEGFR-3 receptor of VEGF-C/-D, expressing specifically on lymphatic endothelial
Prox-1 a lymphatic specific marker expressed in nuclei
5’Nase marker for lymphatics
LYVE-1 marker or lymphatics, may play a role in lymphatic hyaluronan transport
CD44 is a receptor for hyaluronic acid

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Van Der Auwera (2006). First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours.
Methods

Harvesting of mononuclear cells

40-60 ml human umbilical cord blood
mononuclear cell isolation by density-gradient centrifugation (percoll)
examination of cells under confocal laser microscope

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Methods

Isolation & identification of EPCs

detection of CD34\(^+\)VEGFR-3\(^+\) and CD133\(^+\)VEGFR-3\(^+\) cells by fluorescence-activated cell sorting

examination of CD133 expression on CD34\(^+\)VEGFR-3\(^+\) cells

evaluation of uptake of acetylated low-density lipoprotein (Dil-Ac-LDL) and Ulex Europaeus agglutinin-1 (UEA-1)
Methods

Induction of cell differentiation

seeding of CD34+VEGFR-3+ cells on dishes (pre-coated with fibronectin)
incubation in medium enriched with VEGF-C for at least 14 days
observation of morphological changes at day 1, 7, 10, 14 & 21

for following experiments only cells which were incubated in VEGF-C enriched medium for 14 days were used
Methods

Immunocytochemistry to evaluate if VEGF-C induced EPCs differentiated towards lymphatic endothelial cells, they were seeded on coverslips (pre-coated with polylysine) and stained for

5’Nase
LYVE-1
Prox-1
CD44
Methods

Quantitative real-time PCR

- extraction of total RNA from CD34⁺VEGFR-3⁺ cells treated with VEGF-C at different time-points
- determination of VEGFR-3 mRNA level
- control: CD34⁺VEGFR-3⁺ cells without VEGF-C treatment
RNA interference to evaluate the effect of VEGF-C/VEGFR-3 pathway on lymphangiogenesis.

3 different siRNAs targeting human VEGFR-3 and 1 irrelevant siRNA (control) were designed.

Transfection of cells with Lipofectamine 2000 → incubation in VEGF-C enriched medium → assessment of VEGFR-3 mRNA levels by RT-PCR.
Methods

Proliferation assay

cells were seeded on fibronectin pre-coated dishes
after 24 h incubation cells were counted with a haemocytometer
assays were repeated 5 times
Methods

Transmigrating assay

VEGF-C was placed in the lower chamber

after 12 h incubation, cells in the lower chamber were counted

assays were repeated 3 times
Methods

Wounding assay

- Cells in dishes were scraped on 4 different sides.
- Cells were counted in successive 100 μm section of 300 μm wide.
- Maximal distance of cell migration from the wounded edge was measured.
- Assays were repeated 3 times.

5 groups:
- Vehicle
- bFGF
- VEGF
- VEGF-C
- VEGF-C + VEGFR-3 siRNA
Methods

Tube formation in 3D collagen gel & Matrigel

- collagen gel: collagen type I from rat tail, dissolved in acetic acid
- DMEM
- NaHCO$_3$

Cells were seeded on dishes (pre-coated with collagen), when cells grew to monolayer they were treated and afterwards a layer of collagen was made.

Tube formation & area + length of tubes were assessed using TEM

LYVE-1 staining for identifying as lymphatic capillary like structures

Assays were repeated 3 times

4 groups
- vehicle
- VEGF-C
- VEGF-C + VEGFR-3 siRNA
- VEGF-C + irrelevant siRNA

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Results

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Results

Table 1: Effects of bFGF, VEGF and VEGF-C on migration of the EPC-derived cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cell numbers</th>
<th>Maximal distance (µm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14 ± 3</td>
<td>239 ± 36</td>
</tr>
<tr>
<td>bFGF</td>
<td>36 ± 8*</td>
<td>398 ± 28*</td>
</tr>
<tr>
<td>VEGF</td>
<td>30 ± 6*</td>
<td>386 ± 42*</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>48 ± 10*</td>
<td>578 ± 48*</td>
</tr>
<tr>
<td>si-VEGFR-3 +VEGF-C</td>
<td>16 ± 5*</td>
<td>242 ± 39*</td>
</tr>
</tbody>
</table>

The values are mean ± SD.
*P < 0.01 versus control group, †P < 0.01 versus bFGF and VEGF groups, ‡P < 0.01 versus VEGF-C group. n = 16.
Results

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Conclusion

VEGFR-3⁺CD34⁺ cells were found & isolated in human cord blood

VEGF-C induction lead to differentiation into LECs
proliferation of LECs
migration of LECs

LECs could form tubes which connected and formed a network in 3D collagen gel

VEGF-C/VEGFR-3 signalling pathway may be crucial in lymphangiogenesis