Circulating Endothelial Cells and Their Clinical Significance

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The author states that he has no conflict of interest.
The walls of blood vessels are formed by vascular endothelial cells. These cells rarely divide, doing so only about once every 3 years on average. However, when the situation requires it, angiogenesis can stimulate them to divide.
Why count endothelial cells?

- **Marker for vascular injury**
  - Shed from damaged vasculature
  - Increased levels in disease associated with vascular involvement: cancer, myocardial infarction, vasculitis…

- **Amount of CECs might correlate with disease severity**
- **Increase or decrease in CECs will serve as marker for efficacy of angiogenesis inhibiting therapy (e.g. Bevacizumab, Sunitinib)**
- **Baseline CECs might serve as prognostic factor for various disease**
What are the mechanisms of endothelial cell detachment?

- Mechanical injury
- Protease-or Cytokine-mediated detachment
- Defective adhesive properties of endothelial cells
- Activation of apoptotic programs/loss of survival tone
- Imbalance of pro/anti-angiogenic systems

=> Heterogeneous population of cells according to activation / viability / apoptosis status
Issues in CEC enumeration

- **Widely different methods in use:**
  - immunomagnetic bead enrichment, immunocytochemistry, flow cytometry

- **Extremely rare events**
  - CTCs and CECs are present as rare events against a background of high numbers of host cells
  - Typically 0-20 CEC/mL or 0-20 CEC/5*10^6 WBC in healthy donors

- **Uncommon morphology (usability of FSC/SCC ?)**

  - **Most current methods of quantifying these rare cells in patients are inconsistent and lacking in sensitivity**

  - **Overlapping phenotype CEC and Platelets**
Rare event analysis by FCM: Critical factors

- At what frequency do the target events occur in the sample population?
  - Poisson statistics apply
  - S.D. = \( \sqrt{\text{target events counted}} \)

- \( r = (100/\text{CV})^2 \), where \( r \) = the number of events meeting the required criterion.
  Can be used to determine the number of events to be acquired that will provide a given precision.
Calculation of the total number of events required for a given precision

<table>
<thead>
<tr>
<th>For a CV of (%)</th>
<th>1.0</th>
<th>5.0</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td># of positive events to be recorded</td>
<td>10000</td>
<td>400</td>
<td>100</td>
<td>25</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>When occurring at a frequency of (%)</th>
<th>1:n</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>0.1</td>
<td>1000</td>
</tr>
<tr>
<td>0.01</td>
<td>10,000</td>
</tr>
<tr>
<td>0.001</td>
<td>100,000</td>
</tr>
<tr>
<td>0.0001</td>
<td>1,000,000</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Total # of events which must be collected</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>20</td>
</tr>
<tr>
<td>0.1</td>
</tr>
<tr>
<td>0.01</td>
</tr>
<tr>
<td>0.001</td>
</tr>
<tr>
<td>0.0001</td>
</tr>
</tbody>
</table>

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### How rare is rare?

<table>
<thead>
<tr>
<th>Cell Number</th>
<th>Cell Type</th>
<th>Cel Frequency (Leukocytes)</th>
<th>Assay Volume for a CV &lt; 20% *</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.000.000.000 / mL</td>
<td>Erythrocytes</td>
<td>1 in $10^{-2}$</td>
<td>0.000025 µL</td>
</tr>
<tr>
<td>100.000.000 / mL</td>
<td>Platelets reticulocytes</td>
<td>1 in $10^{-1}$</td>
<td>0.00025 µL</td>
</tr>
<tr>
<td>10.000.000 / mL</td>
<td>Neutrophiles</td>
<td>1 in $10^{0}$</td>
<td>0.025 µL</td>
</tr>
<tr>
<td>1.000.000 / mL</td>
<td>Lymphocytes Monocytes</td>
<td>1 in $10^{1}$</td>
<td>0.25 µL</td>
</tr>
<tr>
<td>100.000 / mL</td>
<td>Eosinophiles Basophiles</td>
<td>1 in $10^{2}$</td>
<td>2.5 µL</td>
</tr>
<tr>
<td>10.000 / mL</td>
<td>Hematopoetic Progenitors (CD34+)</td>
<td>1 in $10^{3}$</td>
<td>2,5 µL</td>
</tr>
<tr>
<td>1.000 / mL</td>
<td>Nucleated Erythrocytes</td>
<td>1 in $10^{4}$</td>
<td>25 µL</td>
</tr>
<tr>
<td>100 / mL</td>
<td>CD34+CD38- Cells</td>
<td>1 in $10^{5}$</td>
<td>250 µL</td>
</tr>
<tr>
<td>10 / mL</td>
<td></td>
<td>1 in $10^{6}$</td>
<td>2,5 mL</td>
</tr>
<tr>
<td>1 / mL</td>
<td><strong>Endothelial Cells</strong></td>
<td>1 in $10^{7}$</td>
<td>25 mL</td>
</tr>
<tr>
<td>0,1 / ml</td>
<td><strong>Tumor Cells</strong></td>
<td>1 in $10^{8}$</td>
<td>250 mL</td>
</tr>
</tbody>
</table>
Technical considerations

- Proportion of target population vs sample volume
- Enrichment; when?
- Enrichment techniques
  - positive: size, immunomagnetic
  - negative: immunomagnetic, lysing, density (ficoll)
- Use a multi-parameter approach
  - negative selection marker(s)
  - positive selection markers; including a DNA stain
  - controls; use internal controls, isotype controls are never fully representative for your experimental mAb
CEC analysis
(adapted from Leon Terstappen)

**Orthogonal Light scatter**

**100μl Blood**

**Gate on Nucleated Cells**

<table>
<thead>
<tr>
<th>Definition</th>
<th>CD31+, CD45-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definition 1</td>
<td>8.4 x 10^5 /100μl</td>
</tr>
<tr>
<td>Definition 2</td>
<td>CD31+, CD146+ CD45- 1.9 x 10^3 /100μl</td>
</tr>
<tr>
<td>Definition 3</td>
<td>CD31+, CD45-, DNA+ 9.7 x 10^3 /100μl</td>
</tr>
<tr>
<td>Definition 4</td>
<td>CD31+, CD146+, CD45-, DNA+ 2 /100μl</td>
</tr>
</tbody>
</table>
Own work – CEC FCM assay

- Fast: capable of batch analysis
- Cheap: compared to CellTracks assay
- Robust: low inter- and intratest CV
- Multi-parameter: assure high sensitivity and specificity
- Customizable: additional markers of activation, malignancy
- Validated!: extensive testing of endothelial origin
Design steps

- Marker selection for available fluorescence parameters
- Enrichment technique
  - Bulk lysis (4 mL peripheral blood)
- Necessity of blocking agent
  - IgG (human, murine)
  - Specific FcRγ blocking (CD32)
- Obtaining absolute numbers (Cells/ml)
Confirmation of selected markers on isolated CEC from vein wall dissection using collagenase digestion (1)
Confirmation of selected markers on isolated CEC from vein wall dissection using collagenase digestion (2)
Regeneration of EC from pre-existing EC: Angiogenesis

Potente et al. Cell sept 2011
Preferred combination

- **Positive selection**
  - CD34, CD146, *(CD105)*
  - DRAQ5

- **Negative selection**
  - CD45

- **Final panel:**
  - CD34-FITC, *CD105-PE*, CD45-PerCP, CD146-APC, DRAQ5
Lyse Stain Wash Procedure

- 4 mL of peripheral blood
- Bulk lysis using 45 mL of NH$_4$Cl lysing buffer, 15 min at RT
- Spin down 10’, 1000g
- Stain pellet in 250 µL of mAb cocktail
- Incubate 15 min at RT in darkness
- Wash with 50 mL PBS and Spin down 10’, 1000g
- and resuspend in 1 ml PBS (0.5 mL + 0.5 mL Rinse)
- Acquisition on FACSCanto Flow cytometer, store nucleated CD34++ cells
CEC – flow cytometer analysis
First 10,000 cells
CEC – flow cytometer analysis
First 100,000 cells

1024
768
512
256
0
0 256 512 768 1024
FSC-H

1024
768
512
256
0
0 256 512 768 1024
CD45 PerCP-A

1024
768
512
256
0
0 256 512 768 1024
DRAQ5 APC-Cy7-A

1024
768
512
256
0
0 256 512 768 1024
CD34 FITC-A

Gate 1

Gate 2

Gate 3

Gate 3

Gate 3
CEC – flow cytometer analysis
All CD34+ cells in 4 ml blood (>50 Million cells)

Cancer patient

Treshold on CD34+ cells, nucleated cells
CEC—flow cytometer analysis and sort

Patient

Healthy Donor

Morphology and vWF on FACS sorted CEC
CEC phenotype

[Four scatter plots showing the expression levels of CD146 APC-A and CD146 PE-A, CD141 PE-A, CD133 PE-A, and CD144 PE-A.]
Validation

- Sorted Cells have CEC morphology

- Sorted cells have CEC specific gen profile
  
  \( \text{Expression of } vWF, \ MCAM, \ CDH5 \ - \ Negative \ for \ CD45, \ BST1 \)

- Same phenotype as cells isolated cells form fresh vein vessel wall and tissues
  
  CD34++, CD146+, CD45-, CD31++, CD105+, CD144+, CD309(+)
Prevalence of CECs using CEC Count assay

Donor / Patient Type

HD
Metastatic cancer
Prior to SCT

CECs / 4mL

P=0.0017
P=0.0007
Conclusions

- The measurement of CEC is a non-invasive and specific way to measure endothelial injury/turn over

- CEC levels correlated with established markers of endothelial dysfunction

- CD31+, CD45- phenotype not sufficient

- Requires measurement of DNA content

- CEC frequency requires pre-enrichment and/or large blood volume
A new approach for rapid and reliable enumeration of circulating endothelial cells in patients

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Prostate cancer

- Strijbos MH et al., Eur J Cancer. 2010: Circulating endothelial cells, tissue factor, endothelin-1 and overall survival in prostate cancer patients treated with docetaxel.
### Study – CEC kinetics in SCT

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range)</td>
<td></td>
<td>51 (19-66)</td>
</tr>
<tr>
<td>Sex female (%)</td>
<td></td>
<td>48 (43)</td>
</tr>
<tr>
<td>Diagnosis (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALL</td>
<td></td>
<td>13 (12)</td>
</tr>
<tr>
<td>AML</td>
<td></td>
<td>50 (45)</td>
</tr>
<tr>
<td>CLL</td>
<td></td>
<td>7 (6)</td>
</tr>
<tr>
<td>CML</td>
<td></td>
<td>6 (5)</td>
</tr>
<tr>
<td>MDS</td>
<td></td>
<td>9 (8)</td>
</tr>
<tr>
<td>MM</td>
<td></td>
<td>3 (3)</td>
</tr>
<tr>
<td>NHL</td>
<td></td>
<td>10 (9)</td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td>14 (12)</td>
</tr>
<tr>
<td>Graft source (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sib</td>
<td></td>
<td>37 (33)</td>
</tr>
<tr>
<td>MUD</td>
<td></td>
<td>56 (50)</td>
</tr>
<tr>
<td>dUCBT</td>
<td></td>
<td>19 (17)</td>
</tr>
<tr>
<td>Conditioning type (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAB</td>
<td></td>
<td>25 (22)</td>
</tr>
<tr>
<td>RIC</td>
<td></td>
<td>87 (78)</td>
</tr>
<tr>
<td>Conditioning regimen (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flu+Cyclo+TBI</td>
<td></td>
<td>27 (24)</td>
</tr>
<tr>
<td>Flu+TBI</td>
<td></td>
<td>59 (53)</td>
</tr>
<tr>
<td>Cyclo+TBI</td>
<td></td>
<td>21 (19)</td>
</tr>
<tr>
<td>Busu+Cyclo</td>
<td></td>
<td>2 (2)</td>
</tr>
<tr>
<td>TBI only</td>
<td></td>
<td>3 (2)</td>
</tr>
</tbody>
</table>
Influence of condition regimens on CEC kinetics in SCT

Conditioning intensity and time of measurement

RIC

pre 3 months 6 months 12 months 24 months

MAB

pre 3 months 6 months 12 months 24 months

P = 0.002

P = 0.000

P = 0.000
CEC numbers in patients with and without GVHD
No Presence of donor specific CECs

Pre-SCT

Post-SCT
Circulating endothelial cell enumeration demonstrates prolonged endothelial damage in recipients of myeloablative allogeneic stem cell transplantation.

<table>
<thead>
<tr>
<th>Month of CEC count</th>
<th>Variable</th>
<th>Beta</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre</td>
<td>Low HCT comorbidity index (0)</td>
<td>-0.19</td>
<td>0.038</td>
</tr>
<tr>
<td></td>
<td>CMV reactivation in month 1-3</td>
<td>-0.22</td>
<td>0.016</td>
</tr>
<tr>
<td>3</td>
<td>aGVHD grade 2-4</td>
<td>-0.26</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>MAB conditioning</td>
<td>0.67</td>
<td>0.000</td>
</tr>
<tr>
<td>6</td>
<td>cGVHD present</td>
<td>-0.24</td>
<td>0.008</td>
</tr>
<tr>
<td></td>
<td>MAB conditioning</td>
<td>0.63</td>
<td>0.000</td>
</tr>
<tr>
<td>12</td>
<td>cGVHD present</td>
<td>-0.32</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>MAB conditioning</td>
<td>0.42</td>
<td>0.001</td>
</tr>
</tbody>
</table>
CEC in Oncology

- CEC in cancer patients, composite of both:
  - Normal CEC
    (shed from endothelial wall due to vascular damage from therapy)
  - Tumor derived CEC
    (shed from tumor lesions)

- Important to discriminate between normal and tumor derived CEC in monitoring studies
Normal vs. tumor vasculature

**Normal vessels**
- quiescent EC
  - Regular hierarchy
  - Controlled permeability
  - Stable blood flow

**Tumor vessels**
- activated EC
  - Irregular
  - Increased branching
  - Dilated
  - Vascular leak
  - Disturbed blood flow
Candidate “tumor endothelial markers”

Surface markers (gene and/or protein expression); results of literature search

- Absence of CD54*
- CD106
- CD109
- CD137
- CD276
- CXCR7

* Present on normal EC and all immune cells
Study of tumor endothelial markers (TEM) on homogenized tissue: method

- Isolation of EC from normal and tumor tissue
- Dissociation of tissues with collagenase into single-cell suspensions using the gentleMACS™ dissociator (Miltenyi Biotec)
- Stain Cells (6-color):
  - CD34 FITC, TEM-PE, CD45 PerCP
  - CD146 APC, DRAQ5 (nucleus dye), DAPI (viability)
- Analyze EC; CD34+, CD45-, CD146+, DRAQ5+, DAPI-
- Compare TEM expression on normal vs. tumor-derived EC
Example of EC in normal tissue

Endothelial cells, blue frame; pericytes, red frame

CD29+
CD31+
CD54+
CD105+
CD144+
CD146+

CD29+
CD31−
CD54−
CD105(+)  
CD144−
CD146++
CD276 is overexpressed in tumor EC

Normal Tissue

Tumor Tissue

Endothelial cells, blue frame/dots; pericytes, red frame/dots
CD105, CD146 and CD276 are over-expressed in tumor endothelial cells (metastatic colon cancer; n=16)
CD276 expression, a novel tumor associated marker on CEC

Healthy donor  Patient 1  Patient 2

CEC are depicted in violet
CD276 expression on CEC in healthy donors and cancer patients
Prognostic value of circulating endothelial cells in patients with recurrent glioblastoma Treated with bevacizumab
Future perspectives

- **(Circulating) endothelial cells in cancer, an emerging area:**
  - possibly new insights into tumor biology
  - tool to evaluate tumor growth and angiogenesis
  - tool to monitor anti-angiogenic drug activity

**Ongoing clinical studies:**
- CEC levels during systemic treatment (SU11248 – ifosfamide)
- CEC levels in Glioblastoma patients treated with bevacizumab (EORTC 26101)
- TEM expression intensity in advanced colorectal cancer (ORCHESTRA study)

**Translational study (on going):**
- Interaction between EC and CTC for formation of metastases;
  Identification of antigens and their ligands on both cell types
The Rotterdam CEC team

- Nick Beije
- John Foekens
- Jan Gratama
- Jaco Kraan
- Wendy Onstenk
- Anieta Sieuwerts
- Stefan Sleijfer
- Michiel Strijbos
- Mai Van