The Protein C-Thrombomodulin Mechanism:
Regulating Multiple Biological Systems
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Reasons to think about vascular disease
- Pulmonary emboli: 100,000 deaths/yr
- Coronary artery disease: 1 million deaths/yr
- Strokes: 0.5 million deaths/yr
- Infections/inflammation: 15 million deaths/yr
- Cancer-related: 14 million deaths/yr
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The inert vessel wall...

Questions
- Mechanisms to protect the vessel wall
- Links between coagulation and inflammation
- Regulation - spatially and temporally
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Outline

- The protein C-thrombomodulin mechanism
- Links between coagulation, inflammation, innate immunity and cell proliferation
- Physiologic relevance
- Research directions

Coagulation pathway

Vessel injury or endotoxin → TF → VIIa → IX → Xa → IXa → VIIIa → Fibrinogen → Fibrin

Discovery of protein C (PC)

by
Johan Stenflo, 1975

Protein C (PC) → Thrombin → Activated protein C (APC) → FV/Va → FVIII/VIIIa → AT III
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Protein C (PC)
- Vitamin K-dependent
- Synthesized mostly in liver
- 461 amino acid residue precursor
- Plasma level 3-5 mg/ml
- Apparent molecular weight 62 kDa
- 3 structural domains

Structure of PC
- 9 Gla residues
- Calcium-dependent interactions with cell membrane surfaces and with EPCR and TM
- EGF1 with site for factor Va and VIIIa
- Calcium-dependent activation of PC by thrombin-TM

Extrahepatic sites of PC synthesis
- Renal tubular epithelial cells
- Prostate
- Testis
- Bronchial epithelial cells
- Neurons in brain
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Major players: TM, PF4, EPCR, thrombin, PC

Thrombomodulin (TM) and protein C activation

APC promotes fibrinolysis
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Rapid "onkoff" nature of PC activation

PC activation

Cytosol

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Physiologic importance of PC (1)

- PC deficiency
  - Thrombotic tendency with 50% levels
  - >100 mutations - type I most frequent
  - Type II mutations - less frequent
- Heterozygous PC deficiency
  - DVT, PE most common
  - Occasionally unusual locations of thrombosis
  - Variable expression of thrombotic phenotype
  - With AT deficiency - increased severity

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Physiologic importance of PC (2)

- Homozygous PC deficiency
  - Neonatal - life-threatening diffuse thrombosis
    - Venous and arterial
    - "Purpura fulminans"
- Acquired PC deficiency
  - Warfarin-induced skin necrosis
    - More frequent in patients with PC or PS deficiency
  - Liver disease, sepsis, DIC
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Resistance to APC: factor V<sub>Leiden</sub>

Factor V<sub>Leiden</sub>
- Allelic frequency 2-15%
- Geographic variability
- Thrombotic risk
  - 5-10 fold increase in heterozygous deficiency
  - 50-100 fold increase in homozygous patients
- Transgenic mouse models

Benefits of factor V<sub>Leiden</sub>
- Less bleeding in peripartum period
- Less bleeding in patients with hemophilia
- Decreased sepsis-related mortality
  - Due to higher levels of APC?
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The PC-TM system and inflammation

- Inflammatory response in E. coli-induced sepsis
  - Protective: PC, APC, PS
  - Exacerbates: anti-PC Ab, C4bBP

The PC-TM system and inflammation

- PC +/- mice
  - Increase sensitivity to sepsis
    - Short survival, elevated cytokines, hypotensive
- Administration of APC in rodent models
  - Suppresses TNF and iNOS
  - Prevents neutrophil infiltration
  - Prevents hypotension

Anti-inflammatory properties of APC

- Inhibits PMN activation
- Decreases PMN elastase and ROS release
- Blocks PMN interactions with selectins
- Decreases tissue factor expression
- Prevents cytokine (TNF) release from ECs and monocytes
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**Anti-inflammator properties of APC-EPCR**

- Increased EPCR
- Increased Bcl2
- Decreased A20, IAP
- Decreased p53

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**Vasculoprotective properties of APC-EPCR**

- Increased Vascular integrity
- Increased permeability

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**Therapeutic importance of APC**

- Decreased mortality in patients with severe sepsis
  - 6.1% reduction in 28-day all cause mortality (PROWESS)
  - More rapid resolution of hemostatic, cardiovascular and respiratory failure
  - Adverse effect - non-significant increased bleeding
  - Mechanisms - probably multiple
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APC versus PC

- Is PC an effective treatment for sepsis?
  - Controversial
  - Unresolved
  - Anecdotal success in meningococcemia and purpura fulminans
  - Needs further study

Thrombomodulin and the thrombin-activatable fibrinolysis inhibitor (TAFI)

Role of C-type lectinlike domains

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Deletion of the lectin-like domain of thrombomodulin

Increased sensitivity of TM^{LeD/LeD} mice to LPS

Higher serum cytokines in TM^{LeD/LeD} mice after low dose LPS
Increased PMN (arrows) accumulation in TM<sup>LeD/LeD</sup> lungs

Increased myocardial infarcts after ischemia-reperfusion (I/R)

Increased PMN adhesion to TM<sup>LeD/LeD</sup> ECs
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**Upregulated ICAM, VCAM and pERK<sub>1/2</sub> endothelial cells of TM<sub>LeD/LeD</sub> mice (Western immunoblots)**

<table>
<thead>
<tr>
<th>Mice</th>
<th>WT</th>
<th>LeD</th>
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<tbody>
<tr>
<td>LPS</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

- ICAM-1
- VCAM-1
- pERK<sub>1/2</sub>
- ERK<sub>1/2</sub>

**Soluble thrombomodulin (sTM)**
- sTM in plasma and urine
- From proteolytic degradation of membrane TM
- Comprised of several fragments (EGF 1-6, lectin domain)
- Normal plasma levels 3-50 ng/ml
- Increased with vascular damage
- Inverse correlation of plasma levels with new onset coronary heart disease (controversial)

**TM<sub>lec155</sub> suppresses leukocyte adhesion to endothelial cells**

<table>
<thead>
<tr>
<th>Human TM&lt;sub&gt;lec155&lt;/sub&gt; (mg/ml)</th>
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<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>10</td>
</tr>
<tr>
<td>40</td>
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</table>

# of PMNs adhering to endothelial cells/cm<sup>2</sup>
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TM_{lec155} suppresses TNF-induced activation of ERK_{1/2} in HUVECs

Co-ordinate action of TM and APC-EPCR

HMGB1
High mobility group box 1 = amphoterin
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N-terminal domain of TM: a natural inhibitor of HMGB1

- Critical cofactor for thrombin-mediated generation of APC
- TM sequesters thrombin, i.e., shifts its substrate specificity
  - Prevents thrombin from exerting pro-coagulant and pro-inflammatory functions (e.g., inducing iNOS, cytokine release, leukocyte chemotaxis, complement activation)
- Critical cofactor for generation of TAFIa
- Anti-inflammatory effects of N-terminal lectin-like domain
  - Via HMGB1 and other partner proteins

Summary of role of TM in inflammation

Role of TM in cancer

- Many tumors express TM
- Inverse correlation between TM and metastasis/malignancy
- Lectin-like domain has tumor suppressor properties
### Regulation of TM

<table>
<thead>
<tr>
<th>Up-regulating factors</th>
<th>Down-regulating factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thrombin</td>
<td>Hypoxia</td>
</tr>
<tr>
<td>VEGF</td>
<td>Oxidized LDL</td>
</tr>
<tr>
<td>Histamine, retinoic acid</td>
<td>TGF-beta</td>
</tr>
<tr>
<td>Heat shock</td>
<td>TNF-α, IL-1β</td>
</tr>
<tr>
<td>Statins</td>
<td>Endotoxin</td>
</tr>
<tr>
<td>TNF-α, IL-1β (in macrophages)</td>
<td>Oxidation of Met388</td>
</tr>
</tbody>
</table>

### Regulation of EPCR

- Downregulated by LPS, IL-1β, TNFα
- Soluble form (sEPCR) by MMP cleavage
  - Increased in sepsis
  - Complexes with proteinase-3 (PR3) and interferes with neutrophil-endothelial cell interactions
  - APC-sEPCR may traffic to PMN nucleus

### TM and proteinase activated receptors

- Thrombin
- PAR1
- Gi
- pERK1/2, NFκB
TM mutations and disease

- Transgenic TM<sup>pro/pro</sup> mice - hypercoagulable
- Human TM gene mutations
  - A455V and -1208-1209TTdelTT variants
    - Increase risk for coronary heart disease
  - Ala25Thr substitution - risk for ischemic heart disease

PC-TM system in development

- TM<sup>-/-</sup> mice - embryonic lethal E7.5-8.5
- EPCR<sup>-/-</sup> mice - embryonic lethal E10.0
- TM and EPCR expressed by giant trophoblasts
- Low TF rescues TM<sup>-/-</sup> and EPCR<sup>-/-</sup> embryos
- Endothelial TM critical for survival

Based on Isermann, et al., Nature Medicine, 2003, 331-337
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Pulling it together...

Local response to injury

Protection of adjacent tissue

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Future directions

Therapeutics of PC/APC

- Sepsis
- Thrombo-embolic disease
- Ischemia-reperfusion injury
- Stroke
- Lung inflammation
- "Safer" forms of APC

Therapeutics of EPCR

- Soluble EPCR??
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Therapeutics of thrombomodulin

- EGF1-6 for DIC
- Lectin-like domain of TM for sepsis
- Other

Diagnostic insights

- Inflammation/infection
- Coagulation
- Innate immunity
- Neoplasia

Potential impact

- New insights into mechanisms underlying disorders of coagulation, inflammation, angiogenesis, neoplasia, and immunity
- Novel therapeutic approaches

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...We need to do more research...