Lecture 4

Regulation of VEGF expression
**VEGF**
vascular endothelial growth factor

**Induced at pathological conditions**

Hypoxia

Inflammatory cytokines & growth factors
- IL-1β
- TNFα
- IL-6
- MCP-1
- GM-CSF
- NO
- HO-1

**Required for developmental angiogenesis**

*Ferrara & Alitalo, Nature Med. 2000*
Regulation of VEGF expression

1. Enhancement of VEGF mRNA expression
   - activation of VEGF promoter
   - increased stability of VEGF mRNA

2. Enhanced translation through internal ribosome entry site (IRES)

**Hypoxia – one of the strongest inducers of VEGF expression**

*Half-life of endogenous VEGF mRNA is about 65 min
  stability increases ~ 3 times in hypoxia*

*HuR – a member of Elav-like family of binding proteins; binds to distal AU-rich region in the VEGF 3’UTR. Stabilizes VEGF mRNA*
Structure of VEGF human promoter
Is hypoxia do only one activator of HIF-1?

\[ \text{L-Arg + O}_2 \xrightarrow{\text{NOSI, NOSII, NOSIII cofactors}} \text{NO}^\cdot + \text{L-Cit} \]
Nitric oxide induces VEGF synthesis

L-Arg + O₂ \[\rightarrow\] NOS II (iNOS) \[\rightarrow\] NO + L-Cit

NOS III (eNOS) \[\rightarrow\] IL-1β \[\rightarrow\] VEGF

Dulak et al., ATVB, 2000; Atherosclerosis 2001; JACC 2001; Jozkowicz et al., Cardiovasc Res 2001
Effect of IL-1β on VEGF mRNA expression in rat vascular smooth muscle cells

Li et al., JBC 1995
IL-1β induces also the expression of inducible nitric oxide synthase.

Dulak et al., ATVB 2000: 20: 656-666
Expression of VEGF is enhanced by cytokine stimulation

Dulak et al., ATVB 2000: 20: 656-666
NO induces VEGF synthesis

L-Arg + O₂ \[\rightarrow\] NOS II \[\uparrow\] NO\cdot + L-Cit

IL-1β \[\rightarrow\] VEGF

\[
\begin{align*}
\text{NO} & \quad \text{NO}_2^- (\mu\text{M}) \\
\text{Control} & \quad \text{IL-1β} & \quad \text{IL-1β} \\
& & \quad +\text{L-NAME}
\end{align*}
\]

\[
\begin{align*}
\text{VEGF} \quad \text{pg/ml} \\
\text{Control} & \quad \text{IL-1β} & \quad \text{IL-1β} \\
& & \quad +\text{L-NAME}
\end{align*}
\]

Dulak et al., ATVB, 2000
Role of NO in regulation of VEGF synthesis in pathological conditions

1. Induction of iNOS expression in tumors and other inflammatory diseases

2. Inhibition of NO production in cardiovascular diseases
Role of nitric oxide in tumors
Nitric oxide in cardiovascular system

Nitric Oxide - The Pluripotent Molecule

Leukocytes

Platelets

L-Arginine → NOS → NO

Endothelium

VSMC

cGMP

Vasodilation

anti-aggregatory

anti-adhesive

anti-inflammatory

anti-proliferative

anti-migratory
Modulation of NO and VEGF synthesis by glucose

**NOS II**
- 5,5 mM
- 25 mM

**VEGF**
- 5,5 mM
- 25 mM

*Dulak et al., Life Sci, 2004*
Mechanisms of induction of VEGF by NO
Interleukin-1β induces VEGF through NO

VEGF synthesis is enhanced by NO
- generated by NOS II after cytokine stimulation
- derived from NOS II or NOS III after gene transfer
- released from NO-donors

Dulak et al., ATVB, 2000; Atherosclerosis 2001; JACC 2001; Jozkowicz et al., Cardiovasc Res 2001
Possible mechanisms of NO-induced HIF-1α stabilization

1. NO
   - PI3/Akt
   - p42/44
   - phosphorylation

2. Hydroxylation of P402 and P564 by prolyl-4-hydroxylase

3. Hydroxylation of Asn803 by asparaginyl hydroxylase

4. Degradation by E3/VHL ubiquitin ligase complex

- Nitrosylation of E3 ligase

- Inhibition of p300/CBP binding

Dulak & Józkowicz, Antioxid Redox Signal, 2003
Regulation of VEGF synthesis by cytokines

- IL-1β
  - p42/44 kinase
  - PI3K/Akt
  - NF-κB
  - NOS II
  - NO
  - VEGF

- AP-2/Sp-1
  - HIF-1

PI3K/Akt → HIF-1
Modulation of VEGF synthesis by gene transfer of NOS
Enhancement of VEGF synthesis by gene transfer of nitric oxide synthases

- Nitrite (μM)
- VEGF (pg/ml)

- Control
- β-gal
- Transient cNOS
- Stable transfection with cNOS
- +NAME +arginine

* indicates significant difference from control
$ indicates significant difference from β-gal
# indicates significant difference from transient cNOS

bovine eNOS or human iNOS cDNA

Overexpression of eNOS enhances angiogenesis in hind limb ischemia

Namba K et al., Circulation 2003: 108:2250-2257
Regulation of VEGF production by reactive oxygen species
Hypoxia inducible factor – a crucial mediator of hypoxia- and NO-induced VEGF expression
Other potential modulators of HIF-1 activity

\[ \text{L-Arg} + \text{O}_2 \xrightarrow{\text{NOSI, NOSII, NOSIII}} \text{NO}^\cdot + \text{L-Cit} \]

\[ \text{O}_2 \xrightarrow{\text{NAD(P)H oxidase}} \text{O}_2^\cdot \xrightarrow{\text{SOD1, SOD2, SOD3}} \text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{H}_2\text{O} \]
Hydrogen peroxide augments VEGF synthesis in 3T3 fibroblasts and vascular smooth muscle cells

Grzenkowicz-Wydra et al., Mol Cell Biochem, 2004
Stable overexpression of *SOD1* augments VEGF synthesis in 3T3 fibroblasts

**VEGF mRNA expression**

**VEGF promoter activity**

**VEGF protein synthesis**

*Grzenkowicz-Wydra et al., Mol Cell Biochem, 2004*
Overexpression of SOD1 enhances VEGF synthesis through HIF-1 transcription factors

Grzenkowicz-Wydra et al., Mol Cell Biochem, 2004
Enhancement of VEGF expression through VEGF promoter activation

Hypoxia, NO, ROS

IL-6, OSM

Cytokines, growth factors

IL-6, OSM

translation
Other factors involved in HIF-1 activation of hypoxia-responsive element
Regulation of VEGF expression
Role of heme oxygenase-1 in angiogenesis
Heme oxygenase activity

HO-1 – a stress-inducible gene
HO-2 – a constitutive gene
HO-3 – function not well known
How HO-1 influences VEGF synthesis?

SnPPIX

heme → HO-1/HO-2 → biliverdin → CO

iron
Expression of VEGF enhanced by different stimuli parallels HO-1 expression

**NO**

A7R5
vascular smooth muscle cells

<table>
<thead>
<tr>
<th>Control</th>
<th>SNAP</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td></td>
</tr>
<tr>
<td>HO-1</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td></td>
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</tbody>
</table>

**15d-PGJ₂**

HMEC-1
endothelial cells

<table>
<thead>
<tr>
<th>control</th>
<th>15d-PGJ₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>3 μM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

β-actin
Overexpression of HO-1 enhances VEGF synthesis

- in vascular smooth muscle cells and microvascular endothelial cells (Dulak et al., Antioxid Redox Signal 2002; 4: 229-240
   Józkowicz et al., Antioxid Redox Signal 2002: 4: 557-585)

![Image of gel electrophoresis showing HO-1, VEGF, and GAPDH bands with % of control graph]
Induction of VEGF synthesis by factors stimulating HO-1 and inhibition by SnPPIX, an inhibitor of HO activity.
Basal VEGF synthesis is lower in aortic endothelial cells from HO-1 knocked-out mice

Cisowski et al., BBRC 2005
Possible mediators of HO-1 induced VEGF synthesis

Heme → HO-1 → CO → Hb

eg. PGJ₂ → iron → ferritin

ODQ

Hb

soluble guanylate cyclase → cGMP

Biliverdin → Bilirubin
Inhibition of HO-1 activity, scavenging of CO and inhibition of guanylate cyclase attenuates PGJ₂-induced VEGF synthesis in HMEC-1

**CO**

**VEGF**

*Józkowicz et al. Antioxid Redox Signal, 5:155-162, 2003*
CO (1%) enhances VEGF synthesis in vascular smooth muscle cells

Dulak et al., Antioxid Redox Signal 2002;4:229-240
Iron inhibits VEGF synthesis

The chart shows the effect of iron ions on VEGF synthesis. Iron ions (Fe) at different concentrations and forms (Fe^{3+} and Fe^{2+}) were compared to control conditions.

- **Control** (purple bars) shows baseline VEGF levels.
- **Fe^{3+}** at 10 µm (yellow bar with *) and 100 µm (yellow bar with *).
- **Fe^{2+}** at 10 µm (yellow bar with *) and 100 µm (yellow bar with *).

The graph indicates a decrease in VEGF levels with the addition of iron ions, suggesting an inhibitory effect on VEGF synthesis.
Iron, HIF-1 activation and VEGF synthesis

Iron inhibits HRE activation
Deferoxamine (iron chelator) enhances HRE activation

Dulak et al. in: Heme Oxygenase in Biology and Medicine, 2002
Mechanisms of heme-induced VEGF expression in human keratinocytes
Physiological angiogenesis in adults is restricted

placenta

uterus

Hair growth

Wound healing

Blood vessels
Heme is released during skin injury and induces HO-1 expression

Frank, Wagener et al, Blood, 2003

Hanselmann et al., 2001
Angiogenesis and wound healing

- VEGF and other angiogenic mediators are produced after injury

- angiogenesis is required for a proper wound healing

- wound healing is impaired by diabetes

- no studies so far addressed the role of HO-1 in regulation of the expression of angiogenic mediators in keratinocytes and wound healing angiogenesis
Hemin induces HO-1 expression and activity in HaCaT keratinocytes

**HO-1 expression**

Control | Hemin 10 μM | Hemin 30 μM
---|---|---

**HO-1 protein-Western**

Marker | Control | Hemin 10 μM

**HO-1 protein-ELISA**

<table>
<thead>
<tr>
<th>Condition</th>
<th>HO-1 protein [ng/mg protein]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>200</td>
</tr>
<tr>
<td>Hemin 30 μM</td>
<td>1200</td>
</tr>
</tbody>
</table>

**HO-1 activity**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bilirubin [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
</tr>
<tr>
<td>Hemin 30 μM</td>
<td>800</td>
</tr>
<tr>
<td>Hemin + SnPPIX 10 μM</td>
<td>200</td>
</tr>
</tbody>
</table>

High glucose impairs VEGF synthesis, hemin restores it

Control
Hemin 10 μM
Hemin 30 μM

5.5 mM glucose

Control
Hemin 30 μM

SnPPIX 30 μM
CuPPIX 30 μM
Hemin 30 μM

25 mM glucose

siRNA against HO-1 attenuates VEGF production in HaCaT keratinocytes

**HO-1 expression**

**HO-1 protein-Western**

**HO activity**

**VEGF ELISA**

*Jazwa et al. Free Radical Biol Med., 2006*
Hypoxia induces HO-1 and VEGF in HaCaT keratinocytes. 

Hemin additionally up-regulates hypoxia-induced VEGF production

Conditioned media from cells overexpressing HO-1 and producing more VEGF stimulate angiogenesis

![Graph showing BrdU incorporation](image)

- **BrdU incorporation [% of control]**
  - Conditioned media from cells overexpressing HO-1 and producing more VEGF stimulate angiogenesis
  - Comparison with control and various treatments:
    - DMEM medium
    - VEGF 10 ng/ml
    - Control
    - Hemin 30 μM
    - SnPPIX 30 μM
    - CuPPIX 30 μM

**HUVEC proliferation**

- **Control**
- **Hemin 30 μM**
- **Hemin + SnPPIX 30 μM**
- **Hypoxia**

*Jazwa et al. Free Radical Biol Med., 2006*
Which product(s) of HO-1 mediates enhancement of VEGF synthesis in HaCaT keratinocytes?
Inhibition of HO-1 activity, scavenging of CO and inhibition of guanylate cyclase attenuates PGJ$_2$-induced VEGF synthesis in HMEC-1

CO does not enhance VEGF synthesis in HaCaT

Loboda et al., in preparation
Biliverdin dose- and time-dependently enhances VEGF gene expression

Control 10 μM 30 μM

Control 3 h 6 h 12 h 24 h

VEGF (% of control)

30 μM biliverdin

dose-dependent

time-dependent

Loboda et al., in preparation
Biliverdin activates proximal fragments of VEGF gene promoter

Loboda et al., in preparation
Activation of Sp-1/Sp-3 transcription factors and p42/p44 MAPK is responsible for VEGF protein accumulation after biliverdin treatment

Loboda et al., in preparation
HO-1 product induce VEGF expression in various cell types

1. Endothelial cells - CO
2. Vascular smooth muscle cells - CO
3. Keratinocytes, some tumor cells - biliverdin
Effect of HO-1 on angiogenesis in vivo
Facilitated angiogenesis induced by heme oxygenase-1 gene transfer in a rat model of hindlimb ischemia

Capillary density

VEGF

Suzuki M et al., Biochem Biophys Res Commun 2003
Aggravation of diseases by HO-1-dependent angiogenesis
HO-1 increases B16(F10) melanoma cells proliferation

*Was et al., submitted*

**BrdU incorporation**

**Overexpression**

![Bar chart showing cell proliferation (% of control) for wild type and HO-1 overexpression](chart1)

**Pharmacological activation**

![Bar chart showing cell proliferation (% of control) for various hemin concentrations](chart2)

• RT-PCR

WT  transfected with pcDNAHO-1

![RT-PCR gel](gel1)

HO-1  GAPDH

control  hemin

![Western blot for HO-1](blot1)
HO-1 augments angiogenic properties of melanoma cells

Stimulation of HUVEC with media harvested from wild type or HO-1 overexpressed B16(F10)

Stimulation of HUVEC with media harvested from wild type B16(F10) treated with hemin

Endothelial cell proliferation (% of control)

- Unstimulated HUVEC spheroids
- Media from unstimulated WT
- Media from WT stimulated with hemin
- Media from HO-1 overexpressed B16(F10)
HO-1 overexpression increases tumor vascularization

*CD31 staining*

**Wild type**

**HO-1 overexpression**

**Vessel density in tumors**

![Image of CD31 staining showing vessel density comparison between wild type and HO-1 overexpression tumors.](image)

**Legend:**

- WT: Wild type
- HO-1 overexpression

**Graph:**

- Y-axis: Vessel number (0-70)
- X-axis: WT vs. HO-1 overexpression

**Statistics:**

- N=8-10 tumors/group

*Was et al., submitted*
partially (?) in a VEGF-dependent way

VEGF in serum of mice injected with wild type and HO-1 overexpressed B16(F10)

- No cancer
- WT
- HO-1 overexpression

VEGF expression in wild type and HO-1 overexpressed tumors

- WT
- HO-1 overexpressed

*N=10

* p<0.05 vs no cancer

# p<0.01 vs no cancer

Was et al., submitted
NO and HO-1 (CO) as down-stream mediators of VEGF-induced angiogenesis
VEGF-induced signaling in endothelial cells

Zachary, Cardiovasc Res 2001
NO works upstream and downstream of VEGF

- eNOS/iNOS induction or over-expression
- VEGF
- VEGFR-2
- NO
- SMC
- keratinocytes
- endothelial cells
- EC migration/morphogenesis/survival

*Dulak & Józkowicz, Card Res 2002*
Does HO-1 work upstream and downstream of VEGF?
VEGF induces HO-1 expression in endothelial cells

*HUVEC*

<table>
<thead>
<tr>
<th>Control</th>
<th>Hemin</th>
<th>VEGF</th>
</tr>
</thead>
</table>

| HO-1     | EF2   |

*HUVEC*

<table>
<thead>
<tr>
<th>Control</th>
<th>VEGF 24 h</th>
<th>VEGF 48 h</th>
</tr>
</thead>
</table>

| HO-1     | actin    |

*Dulak et al., Antioxid Redox Signal, in press*

*Bussolati et al., Blood 2004:103: 761-766*
Inhibition of HO-1 blocks angiogenic activity of endothelial cells

Overexpression of HO-1 enhances angiogenic activity of endothelial cells.

- VEGF
- VEGF + HO-1 transfection
- VEGF + β-gal transfection
- VEGF + CORM, 1 μM

The cumulative length of sprouts (% of control value) is shown in the graph.

* Statistically significant difference.
HO-1 deficiency impairs production of VEGF and decreases proliferation of endothelial cells

**VEGF synthesis**

**VEGF-induced proliferation**

Cisowski et al., BBRC 2005: 326: 670-676

Dulak et al., in: Heme Oxygenase in Biology and Medicine, Nova Press, 2005
HO-1 works upstream and downstream of VEGF

- VEGF
- eNOS overexpression
- iNOS induction
- NO
- HO-1
- SMC keratinocytes

EC

- VEGFR-1/2
- EC migration/morphogenesis/survival
- CO?
- NO
- eNOS
- HO-1
Heme Oxygenase

The Elegant Orchestration of its Products in Medicine

Leo E. Otterbein
Brian S. Zuckerbraun
Editors

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