Inhibitory Effects of Retinoids on Vascular Endothelial Growth Factor Production by Cultured Human Skin Keratinocytes

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Key Words
Vascular endothelial growth factor - Retinaldehyde

Abstract
Background: Vascular endothelial growth factor (VEGF), a potent angiogenic factor and vasodilator, is strongly expressed by epidermal keratinocytes in many angiogenesis-dependent skin disorders. Retinoids may modulate VEGF in skin and this may be related to an effect on rosacea. Aim: To investigate the effect of retinaldehyde on VEGF production by human keratinocytes. Methods: The effects of different concentrations of retinoids (all-trans-retinal and all-trans-retinoic acid) on VEGF production by cultured human skin keratinocytes in both cell extracts and supernatants were determined. Expression of VEGF was analyzed by enzyme-linked immunosorbent assay (ELISA) and RT-PCR. Results: The amount of cell-associated and secreted VEGF strongly decreased with retinoid concentration (e.g., 48, 69% inhibition at 0.1 μM all-trans-retinal and -retinoic acid, respectively, in the supernatants). In parallel, approximately 25% inhibition of VEGF mRNA expression was obtained in the presence of 0.01 μM all-trans-retinal. Conclusion: The decrease in VEGF expression by keratinocytes on contact with retinoids may prevent skin neoangiogenesis in certain skin diseases.

Introduction
Keratinocytes are the most abundant cells in the epidermis. They synthesize various active biological mediators in response to different extracellular signals present in their environment, especially angiogenic growth factors which play an important role in stimulating and maintaining skin vascularization. They therefore constitute a good cellular model to determine the in vitro ability of different molecules to modulate angiogenic factor production. Vascular endothelial growth factor (VEGF) is regarded as the most important positive regulator of angiogenesis and vascular permeability [for reviews, see 1–3].

Previous studies showed that cultured normal human keratinocytes synthesize this cytokine [4]. VEGF is strongly expressed in many disorders of the skin [5–7] and seems to be an interesting candidate to determine the effects of retinoids on skin vascularization. Various studies showed that retinoids inhibit angiogenesis in several experimental systems. Retinoids inhibit vessel ingrowth into the chorioallantoic membrane of the chick [8, 9]. Retinoids also abrogate tumor-associated angiogenesis in vivo [10]. In the present study, we used ELISA and RT-PCR techniques to assess VEGF protein synthesis and gene expression in normal human keratinocytes cultured with retinaldehyde or retinoic acid.
Materials and Methods

**Immunoassay**

Early-passage (P2) normal human skin keratinocytes were seeded into 6-well plates at a density of 1 × 10⁶ cells/ml DMEM/well. The amount of VEGF in centrifuged supernatants and in the cell extracts was evaluated using ELISA kits (R & D systems). This type of assay was used to evaluate the effect of retinoids (Sigma) on VEGF secretion; keratinocytes were incubated for 24 h with various concentrations of retinaldehyde or retinoic acid (0.1, 1, 3 μM) in serum-free medium (Gibco). Simultaneous control incubations of keratinocytes without retinoids were performed. This assay was done using three keratinocyte lines. All assays were carried out in triplicate for each agent.

All-trans-retinoic acid and all-trans-retinal stock solutions were prepared in dimethylsulfoxide (1 μM) and stored protected from light at −20°C. Working dilutions were prepared with serum-free medium (Gibco-BRL).

An aliquot of the suspension was set aside to measure the protein concentration. Cell proteins were measured according to a Biorad protein assay (Biorad) protocol using bovine serum albumin (Sigma) as a standard. Final results are expressed as the amount of VEGF related to total protein.

**RT-PCR Analysis**

Total RNA (1 μg) from cultured skin keratinocytes was reverse transcribed at 37°C for 60 min into cDNA using the Access RT-PCR System (Promega) in a 50-μl volume.

Polymerase reactions were performed using oligonucleotides complementary to the 5' and 3' ends of the coding sequence of VEGF (CTGCTCTTGGGTTCACTGC and CACCGCCTTGGCCTTGGTGCACTAT). Amplification was performed for 30 cycles (94°C for 40 s, 57°C for 1 min; 72°C for 1.5 min) in a DNA thermal cycler heat block (Perkin-Elmer, Gene Amp PCR system 2400). The amplified PCR products were separated by 3% agarose electrophoresis.

Results

**Expression of VEGF Protein Is Inhibited in Keratinocytes after Exposure to Retinaldehyde and Retinoic Acid**

The total content in VEGF was inhibited in a dose-dependent manner by retinaldehyde as well as retinoic acid which induced a higher inhibition (table 1). In cell extracts, as compared with untreated cells, retinaldehyde induced a decrease in VEGF protein content only at the highest concentration of 3 μM (table 1); retinoic acid induced a significant decrease at 1 μM. Decreases in the levels of VEGF protein were similarly observed in cell supernatants where the inhibition upon retinaldehyde treatment was higher at the lowest concentrations; a similar effect was observed upon retinoic acid treatment, although the inhibition was higher than that induced by retinaldehyde.

**Table 1. Effects of retinoids on VEGF protein production in cell extracts and supernatants of cultured normal human keratinocytes**

<table>
<thead>
<tr>
<th>Supematants</th>
<th>Cell extracts</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg/μg proteins</td>
<td>pg/μg proteins</td>
<td>pg/μg proteins</td>
</tr>
<tr>
<td>x100</td>
<td>SD</td>
<td>x100</td>
</tr>
<tr>
<td>Control</td>
<td>17.86</td>
<td>2.98</td>
</tr>
<tr>
<td>Retinal</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>9.32</td>
<td>2.02</td>
</tr>
<tr>
<td>1 μM</td>
<td>11.23</td>
<td>1.56</td>
</tr>
<tr>
<td>3 μM</td>
<td>11.31</td>
<td>1.6</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 μM</td>
<td>5.37</td>
<td>0.99</td>
</tr>
<tr>
<td>1 μM</td>
<td>10.68</td>
<td>2.89</td>
</tr>
<tr>
<td>3 μM</td>
<td>10.18</td>
<td>1.21</td>
</tr>
</tbody>
</table>

VEGF concentrations in the supernatants were determined by ELISA. Keratinocytes were incubated for 24 h in the presence of different concentrations of retinaldehyde or retinoic acid. VEGF production by keratinocytes related to the total cell protein content is expressed as picograms per microgram protein. Values are the mean of 3 assays ± SEM.

*p < 0.05: significant difference compared to the control using the Dunnett test.

**VEGF mRNA Expression Is Decreased in Keratinocytes after Exposure to Retinaldehyde and Retinoic Acid**

VEGF mRNA was detected in cultured keratinocytes by RT-PCR (fig. 1). A 25% inhibition of VEGF mRNA was observed after treatment of cells with retinaldehyde 0.01 μM; no significant modification of the VEGF mRNA was observed after treatment with retinoic acid at 0.01 μM.

Discussion

In this study we confirm and extend our observations upon the inhibition of VEGF expression [11] by all-trans-
retinaldehyde and all-trans-retinoic acid in primary cultured human normal keratinocytes. Such an inhibition was subsequently confirmed by Weninger et al. [12] who observed that all-trans- and 13-cis-retinoic acid as well as all-trans-retinol inhibited VEGF in normal human keratinocyte supernatants.

Low concentrations of retinaldehyde induced a greater decrease in VEGF in the supernatant than high concentrations, whereas the reverse was seen for the cell-associated VEGF. A similar effect was seen with retinoic acid. This indicates a dose-dependent effect of the two retinoids on the distribution/secretion of VEGF in cultured keratinocytes. As a whole the VEGF protein was inhibited by 33% by 3 μM retinaldehyde, and up to 67% by similar dose of retinoic acid.

The low concentration 0.01 μM of retinaldehyde inhibited VEGF gene expression by 25% after 24 h of incubation which is consistent with the decrease in secreted protein. No inhibition of VEGF mRNA was observed with 0.01 μM retinoic acid at 24 h incubation; this is probably due to a difference in the time course regulation of VEGF between retinoic acid, and retinaldehyde; the latter, comparatively to retinoic acid would act in a delayed manner.

The inhibition of VEGF production by the retinoids tested in the present study points to an effect of retinaldehyde in skin vascularization. The level of VEGF is an important parameter in maintaining balanced skin angiogenesis.

The decrease in VEGF production by keratinocyte cells may prevent skin necroangiogenesis and inflammation developing in rosacea or other angiogenesis-dependent diseases of the skin in which a dense network of microcapillaries is produced and inflammatory cells are present.

The inhibition of VEGF, which is a potent chemotactic agent for inflammatory cells and endothelial cells [13, 14], provides direct evidence that retinaldehyde may contribute to the reduction of inflammation sites by regulating inflammatory mediator synthesis in these diseases. This molecule should play a role in the prevention of uncontrolled microvascularization in skin diseases, but its potential clinical use in such a context needs further investigation in vivo.

References