Decreased EGFR mRNA expression in response to antipsoriatic drug dithranol \textit{in vitro}

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Dithranol is enormously effective in the treatment of psoriasis; however its molecular mode of action should be further elucidated. Since epidermal growth factor receptor (EGFR) is involved in the pathogenesis of psoriasis, the objective of this study was to investigate the transcriptional effect of dithranol on EGFR gene expression in the HaCaT keratinocyte cell line, which is commonly employed as a model system to study psoriasis including experiments examining the effects of therapeutic drugs and cellular regulators on keratinocytes. Cultured HaCaT cells were treated with 0.1-0.5 µg/ml dithranol for 30 min. After 4 h, total cellular RNA isolated from HaCaT cells was reverse transcribed to cDNA which was subjected to polymerase chain reaction (PCR) with specific primer pair for EGFR. We found that dithranol treatment down-regulated the EGFR mRNA of HaCaT cells in a concentration-dependent manner. Our result was further substantiated using a quantitative real-time PCR approach. Taken together, the dithranol-induced down-regulation of the EGFR in cultured human keratinocytes might help to disclose the molecular therapeutic action of the drug.

Key words: Psoriasis, dithranol, EGFR, gene expression, human keratinocytes.

INTRODUCTION

Psoriasis, a chronic inflammatory skin disease affecting approximately 2-4% of the population worldwide, is not curable although many treatments are available to reduce the bothersome symptoms and appearance of the disease. Dithranol (1, 8-dihydroxy-9-anthrone) also referred to as anthralin is one of the critical topical antipsoriatic agents (Pavithran, 2001). It is a vastly effective com-
not studied. The first two studies concerning dithranol mentioned here also have certain weak points. Gottlieb et al. (1992) utilized such cellular model that might not be most suitable for psoriasis, and they did not investigate the transcriptional effect of dithranol on EGFR gene expression. Although the inhibition of binding between EGFR and ligands was discovered, suggesting the functional effect of dithranol on altering components of the EGFR pathway in cultured keratinocytes, insights into the EGFR gene expression have not been elucidated. Like the study of Gottlieb et al. (1992), Kemény et al. (1993) revealed their findings based solely on the ligand binding assays, and they employed the squamous cell carcinoma cell line (SCL-II) of human origin, which was also not the frequently analyzed cellular model for psoriasis.

To date, the EGFR and its ligands have been principally recognized to be one of the most powerful and complex signaling networks, especially in diverse effects on skin biology and pathology (Schneider et al., 2008). Herein, the objective of this study was to investigate the transcriptional effect of dithranol on EGFR gene expression in the HaCaT keratinocyte cell line. HaCaT cells were established as the first permanent epithelial cell line from adult human skin, and has been evident to exhibit normal differentiation, thus providing a promising tool for studying regulation of keratinization in human cells (Boukamp et al., 1988). The HaCaT cell line has been the most widely used cellular model to study hyperproliferative skin diseases such as psoriasis (Ryle et al., 1989; Ockenfels et al., 1995; Lehmann, 1997; Paramio et al., 1998, 1999; Garach-Jehoshua et al., 1999; Farkas et al., 2001, 2003; Kim et al., 2007; Belsõ et al., 2008). Thus, HaCaT keratinocytes were chosen as proper candidates for studying the molecular effect of such therapeutic drug as dithranol on the EGFR gene expression.

### MATERIALS AND METHODS

#### Cell culture

The human keratinocyte cell line HaCaT (kindly provided by Dr. N.E. Fusenig, German Cancer Research Centre, Heidelberg, Germany) was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and cells were maintained at 37°C in a humidified atmosphere at 5% CO₂.

### Dithranol treatment

Dithranol treatment was performed following a procedure previously described by Farkas et al. (2001) with minor modifications. Briefly, cultured HaCaT cells at about 90% confluence in a 60 mm plate were washed with phosphate buffered saline (PBS) under sterile conditions and were incubated for 30 min at 37°C in a humidified atmosphere at 5% CO₂ with increasing concentrations of dithranol (0.1, 0.25 and 0.5 μg/ml) (Sigma, St. Louis, MO) in DMEM containing 0.5% FBS. Dithranol was always freshly dissolved in acetone and used immediately. Control cells were treated with acetone only or left untreated.

### Total RNA extraction and semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

After dithranol treatment, treated cells were washed with PBS and were incubated in DMEM containing 10% FBS for 4 h at 37°C in a humidified atmosphere at 5% CO₂ prior to total cellular RNA isolation using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The amount of RNA was determined by absorbance at 260 nm. Prior to RT-PCR reaction, about 1 μg of the total RNA was treated with 10⁵ U deoxyribonuclease I (DNase I) (Invitrogen, Carlsbad, CA) for 15 min at 25°C. First-strand cDNA was synthesized from about 100 ng of DNase I-treated RNA using ImProm-II™ Reverse Transcription System (Promega, Madison, WI) with oligo(dT)₁₇ primer following the manufacturer’s protocol. Reverse transcription was carried out at 50°C for 60 min. After cDNA synthesis, the desired DNA fragment was then amplified for 30-35 cycles using RealTag DNA Polymerase (Real Biotech Corp., Taipei, Taiwan) and specific primers listed in Table 1 for EGFR transcripts and β-actin transcripts as controls. Each cycle of PCR consisted of a 30 s denaturation at 94°C, a 30 s annealing at 52°C for EGFR and 58°C for β-actin and a 45 s extension at 72°C. The products of RT-PCR were resolved by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. The DNA bands corresponding to EGFR and β-actin transcripts were 518 base pairs (bp) and 656 bp, respectively.

### Quantitative real-time PCR

Real-time PCR was performed on a LightCycler instrument using the LightCycler FastStart DNA MasterPLUS SYBR Green I Kit according to the manufacturer’s instructions (Roche Applied Science, Mannheim, Germany). Together with the specific primers, the master mix was added to cDNA solutions, including water blanks as negative controls. The PCR was run for 10 min at 95°C and followed by 50 cycles of 10 s at 95°C, 10 s at 52°C and 35 s at 72°C. After amplification, melting curve analysis was performed to verify specificity. For data analysis, we used the LightCycler Data Analysis software 4.0. The expression of EGFR target gene was normalized to that of β-actin, and the data for each dithranol concentration treatment represented fold change in normalized.

### Table 1. Specific oligonucleotide primers used in RT-PCR.

<table>
<thead>
<tr>
<th>Name</th>
<th>Oligonucleotide sequence (5' → 3')</th>
<th>Product length (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGFR-sense</td>
<td>GCTACGATTGGCTGAAGTAC</td>
<td>518</td>
<td>Kubota et al. (2001)</td>
</tr>
<tr>
<td>EGFR-antisense</td>
<td>ATGGGTTGAGAGACTGGA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-actin-sense</td>
<td>ACGGTTCACCACACTGTGC</td>
<td>656</td>
<td>Fisker et al. (2004)</td>
</tr>
<tr>
<td>β-actin-antisense</td>
<td>CTAGAAGCATTTGCGGTGGACGATG</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
mRNA expression relative to that at 0 ug/ml of dithranol. The relative expression in fold change was arbitrarily set at 1. To compare expression results between each dithranol concentration treated, a relative quantification model was used (Pfaffl, 2001).

**Statistical analysis**

All band densities was analyzed by 1D-Multi Lane Densitometry program in an Alphalmager 2000 (Alpha Innotech Corp., San Leandro, CA). The EGFR density from each dithranol concentration was normalized to β-actin density. Each experiment was performed in triplicate, and data were reported as fold over 0 μg/ml dithranol concentration. The data represented the mean ± S.D. of three independent experiments and were analyzed by the Student’s t-test.

Differences at $P < 0.05$ were considered to be significant.

**RESULT AND DISCUSSION**

To study the effect of dithranol on EGFR mRNA expression, we performed the semi-quantitative RT-PCR approach. After 4 h of treatment, our result showed that normalized EGFR mRNA transcripts of HaCaT cells were decreased as dithranol concentrations used were increasing (Figure 1). As compared to 0 ug/ml dithranol concentration, the difference in EGFR mRNA transcripts was statistically significant ($P < 0.05$) when 0.25 and 0.5 ug/ml
Dithranol were used for treatment. The down-regulation effect of dithranol was concentration-dependent as analyzed using Pearson’s correlation (Figure 2). In addition, the quantitative measurement of EGFR transcripts was carried out using the real-time PCR method. We found that normalized EGFR mRNA transcripts, as dithranol concentrations increasing from 0.1, 0.25 and 0.5 ug/ml were down-regulated to 0.60, 0.70 and 0.48-fold over that of 0 ug/ml dithranol treatment, respectively.

Therefore, dithranol down-regulated the EGFR mRNA expression of HaCaT cells in a dose-dependent manner. However, inconsistent result was found between the two experimental approaches. The semi-quantitative RT-PCR technique showed significant reduction of normalized EGFR mRNA transcripts when HaCaT cells were treated with dithranol concentrations of 0.25 and 0.5 ug/ml, whereas we could observe that significant drop using the quantitative real-time PCR approach even in the presence of dithranol concentration as low as 0.1 ug/ml for treatment. This might be explained by the difference in sensitivity between the two techniques. Using the quantitative real-time PCR, normalized EGFR mRNA transcripts were found to be 0.60 and 0.70-fold over that of 0 ug/ml dithranol in response to treatment with dithranol concentrations of 0.1 and 0.25 ug/ml, respectively. However, these levels of normalized EGFR mRNA were comparable. In the present study, we utilized only one housekeeping gene, β-actin, for normalization since it has been often used for this regard. It should be noted using more than one reference genes is better for assuring data reliability.

Previous studies have shown that various cytokines and growth factors are over-expressed in psoriatic epidermis (Schon and Boehncke, 2005). Keratinocytes are the major component cells of the epidermis, and their growth is controlled by both negative and positive mediators (Hashimoto, 2000). Of these mediators, the most crucial mechanism for the proliferation of keratinocytes is the signal from the EGFR. The EGFR family consists of EGFR, TGF-α, heparin binding EGF-like growth factor (HB-EGF), amphiregulin, epiregulin, betacellulin, epigen, neuregulin (NRG)-1, NRG-2, NRG-3, and NRG-4, and the EGFR family consists of EGFR (also called ErbB1, ErbB2, ErbB3, and ErbB4 (Hashimoto, 2000). Previous reports have shown that TGF-α, amphiregulin and HB-EGF are over-expressed in psoriatic epidermis (Gottlieb
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