

Ultraviolet B irradiation increases endothelin-1 and endothelin receptor expression in cultured human keratinocytes

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Abstract The effect of ultraviolet B (UVB) irradiation on endothelin-1 (ET-1) and ET receptor expression was examined using cultured normal human keratinocytes. Keratinocytes secreted ET-1 in the medium at a level of 2.1 pg/day/10⁵ cells. UVB irradiation up to 10 mJ/cm² increased ET-1 secretion 3-fold, and potentiated expression of mRNA for ET-1. Both ET_A and ET_B receptor mRNAs were detected in keratinocytes, and their expression was up-regulated by 5 mJ/cm² UVB irradiation.

Key words: Endothelin-1; Endothelin receptor; Ultraviolet B; Keratinocyte

1. Introduction

The endothelins (ET) are a family of highly conserved 21-amino acid peptides originally isolated from vascular endothelial cells on the basis of their potent vasoconstrictor activity [1]. ET consists of three isopeptides, termed ET-1, ET-2 and ET-3 (ETs), while their receptors are classified, both pharmacologically and molecularly, into ET_A and ET_B subtypes [2–5]. The ET_A receptor has different affinities for the three ET isoforms in the order of ET-1>ET-2>ET-3. The ET_B receptor binds ET-1, -2 and -3 with similar affinity. Intensive studies have increased the knowledge on processing, pharmacological effects, receptors, signal transduction and antagonists of ETs [for review see 6–11]. As a mitogen, ET-1 has been reported to stimulate DNA synthesis in Swiss 3T3 fibroblasts [12–14], Rat-1 fibroblasts [15], rat mesangial cells [16] and glial cells [17].

Recently, ET-1 secreted from keratinocytes has been shown to act as a paracrine growth factor for melanocytes [18–20], while we demonstrated that ET-1 also works as an autocrine growth factor for keratinocytes [21]. In our aforementioned study, keratinocytes treated with tumor necrosis factor- α (TNF- α) or interleukin-1 α (IL-1 α) was found to have increased levels of secreted ET-1. Since TNF- α and IL-1 α are known to be two major cytokines released by ultraviolet B (UVB) irradiation [22], we speculated that ET-1 is involved as a mediator during UVB-induced inflammation. In this study, we examined the effect of UVB irradiation on the ET-1 and ET receptor expression of cultured keratinocytes.

2. Materials and methods

2.1. Cell culture and UVB irradiation

Normal human keratinocytes from infant foreskins were cultured in serum-free medium [23,24]. Keratinocytes were grown in keratinocyte growth medium (KGM, Kurabo Co., Osaka, Japan). KGM contained epidermal growth factor (10 ng/ml), insulin (5 μ g/ml), hydrocortisone (0.5 μ g/ml), ethanolamine (0.1 mM), phosphoethanolamine (0.1 mM), CaCl₂ (0.15 mM), and brain pituitary extract (0.4%, v/v), in modified MCDB 153 medium. All experiments were carried out using third- or fourth-passage keratinocytes grown in KGM. After KGM was replaced with phosphate-buffered saline (PBS), the cells were exposed to UVB (FL20SE30 lamps, Toshiba, Japan), followed by incubation with keratinocyte basal medium (KBM, Kurabo Co.) which contained only ethanolamine and phosphoethanolamine in modified MCDB 153 medium.

2.2. Radioimmunoassay (RIA)

ET-1-like immunoreactivity in the conditioned medium was determined using an ET-1, -2 (high sensitivity) [¹²⁵I] assay system (RPA 545, Amersham International, Buckinghamshire, UK) according to the manufacturer's instructions. Rabbit anti-ET antibody used in this assay kit cross-reacts with ET-1, -2 and big ET-1, but not ET-3. Keratinocytes in a 35-mm size dish were exposed to UVB and then incubated in KBM. After 48 h incubation, the conditioned medium was collected and the number of cells in the plate counted after tripan blue staining. ET-1-like immunoreactivity was expressed as pg/day/plate.

2.3. Northern blot analysis

Keratinocytes, prior to subconfluency, in 100-mm size dishes were exposed to 5 mJ/cm² UVB and further cultured in KBM for 12 h. Total RNA was extracted by the guanidinium thiocyanate/phenol/chloroform method [25]. Ten μ g of total RNA was separated by formaldehyde/1.1% agarose gel electrophoresis and transferred to a Hybond-N membrane (Amersham). The cDNA for human ET_B receptor [26] was labeled to a specific activity of 8 \times 10⁸ c.p.m./ μ g with [α -³²P]dCTP by the random priming method, and used as a probe. Hybridization and washing were performed as described [26], followed by autoradiography at -80°C for 5 days.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was performed using GeneAmp RNA PCR Kit (Perkin-Elmer Co., Norwalk, CT) according to the manufacturer's instructions. cDNA was generated using 1 μ g of total RNA as a template in the presence of cloned murine Moloney leukemia virus reverse transcriptase and random hexamers. ET-1 primers were designed as 5'-TGC TCC TGC TCC CTG GTT GAT AAA GAG TGT GTC-3' (sense) and 5'-GGT CAC ATA ACG CTC TCT GGA GGG CTT-3' (antisense) [27]. The primers were predicted to amplify a 462 base pair DNA fragment (nucleotides 157 to 618) [2]. ET_A receptor primers were designed as 5'-AGC TTC CTG GTT GAT ACC CAT CAA-3' (sense) and 5'-T CAA CAT CTC ACA AGT CAT GAG-3' (antisense) [28]. The primers were predicted to amplify a 714 base pair DNA fragment (nucleotides 136 to 850) [29]. PCR was performed for various set number of cycles (at 94°C for 1 min, 60°C for 1 min, 72°C for 2 min) in the presence of AmpliTaq DNA polymerase. Aliquots of each of the amplification products were applied to a 6% polyacrylamide gel for electrophoresis, and DNA was visualized by ethidium bromide stain-

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Abbreviations: ET, endothelin; UVB, ultraviolet B.

ing. PCR products were subjected to direct sequencing by the dideoxy chain termination method [30].

3. Results

3.1. Effect of UVB irradiation on ET-1 secretion

Fig. 1 shows the ET-1-like immunoreactivity secreted from keratinocytes during the 48 h incubation period after exposure to UVB irradiation. Non-treated keratinocytes secreted ET-1 at a level of 9.4 pg/day/plate (2.1 pg/day/ 10^5 cells), and UVB-irradiated keratinocytes increased ET-1 secretion dose-dependently. An approximate 3-fold increase of ET-1 secretion was achieved by 10 mJ/cm² UVB, however an excessively high dose of UVB irradiation reduced the ET-1 secretion (Fig. 1). Morphological observations (Fig. 2) and trypan blue staining of the cells prior to collection of the media (legend of Fig. 1) confirmed that 10 mJ/cm² or a higher dose of UVB irradiation damaged the keratinocytes. Accordingly, the remaining experiments were carried out at 5 mJ/cm² UVB.

3.2. Effect of UVB irradiation on ET-1 and ET receptor mRNA expression

Ten μ g of total RNA, which had been extracted from the keratinocytes 12 h after 5 mJ/cm² UVB irradiation, was separated by agarose gel electrophoresis (Fig. 3A). The transferred RNA was hybridized with a probe for ET_B receptor (Fig. 3B). When compared with non-treated cells, UVB irradiation significantly increased the ET_B receptor expression in keratinocytes. Since ET-1 and ET_A mRNAs were not detected by Northern blot analysis at the condition tested (10 μ g of total RNA), RT-PCR (Fig. 3C and D) was performed using 1 μ g of total RNA as a template from each sample shown in Fig. 3A. The PCR products were found to coincide to human ET-1 and ET_A receptor, respectively, by direct sequencing [2,29]. Fig. 3C clearly indicates that UVB irradiation increased the ET-1

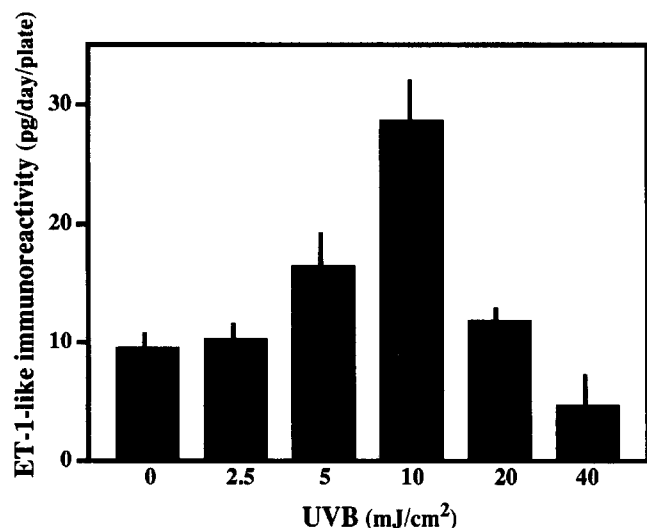


Fig. 1. Effect of UVB irradiation on ET-1 secretion of keratinocytes. The ET-1-like immunoreactivity secreted from keratinocytes during the 48 h incubation period after exposure to UVB irradiation was measured by a radioimmunoassay. ET-1-like immunoreactivity was expressed by pg/day/plate. The experiment was repeated four times with similar results being achieved each time. $n = 3$, mean \pm S.D. Cell numbers in each plate were 4.5×10^5 cells (0 mJ/cm²), 4.3×10^5 cells (2.5 mJ/cm²), 3.6×10^5 cells (5 mJ/cm²), 0.9×10^5 cells (10 mJ/cm²), 0.2×10^5 cells (20 mJ/cm²), 0.03×10^5 cells (40 mJ/cm²).

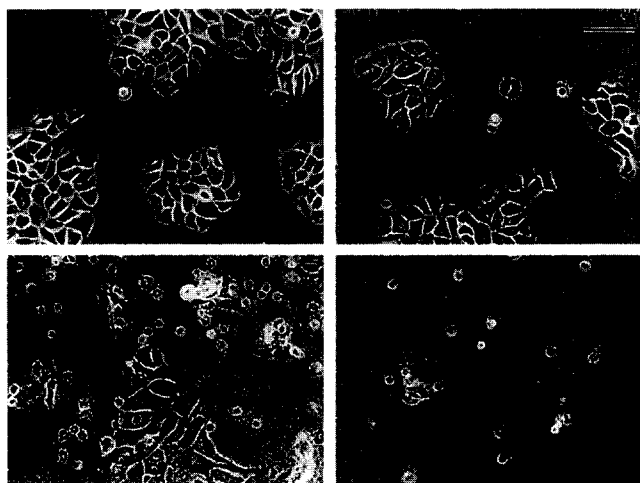


Fig. 2. Morphological observation of the UVB-irradiated cells. (A) 0 mJ/cm², (B) 5 mJ/cm², (C) 10 mJ/cm², (D) 20 mJ/cm². Bar: 100 μ m.

mRNA expression. Meanwhile, Fig. 3D demonstrates that ET_A mRNA is expressed in non-treated keratinocytes (slightly positive at 25 cycles), and that UVB irradiation enhances the mRNA expression.

4. Discussion

Ultraviolet absorption by skin leads to molecular and cellular damage with consequent impairment of tissue function. Keratinocytes comprise the majority of epidermal cells and protect skin from ultraviolet damage with the assistance of melanocytes and Langerhans cells. Cutaneous inflammation caused by UVB irradiation is mediated by various cytokines, among which TNF- α and IL-1 α have been thought to be the two major cytokines [22]. The stimulatory effect of TNF- α and IL-1 α on ET-1 expression has been shown in cells of epithelial origin [19,21,31,32]. In this study, we clearly demonstrated that UVB irradiation increases mRNA and secreted protein levels for ET-1 in keratinocytes. This result suggests that a portion of the effect induced by UVB irradiation is exerted through keratinocyte-secreted IL-1 α and TNF- α . At a low dose of UVB irradiation (5 mJ/cm²), an increased amount of ET-1 may contribute to the re-growth of melanocytes [18–20] and keratinocytes [21]. However, at a high dose of UVB irradiation, ET-1 secretion from keratinocytes was found to be reduced and keratinocytes destroyed.

We were able to detect mRNAs for ET_A and ET_B receptors in keratinocytes for the first time. This result is compatible with our previous data showing that iodinated ET-1 competed with unlabeled ET-1 and -2 with similar affinities, but with a lower affinity with unlabeled ET-3 [21]. UVB irradiation up-regulated the expression of both ET_A and ET_B receptors in keratinocytes. The increase in receptor expression as well as in ET-1 expression confirms that ET-1 is involved as a mediator during the inflammation process caused by UVB irradiation. These findings are supported by in vivo data that immunoreactive ET-1 and ET-1-binding sites in mice skin are increased by UVB irradiation (manuscript in preparation).

ETs have short-term actions such as vasoconstriction and long-term actions such as cell growth-stimulatory activity [6–

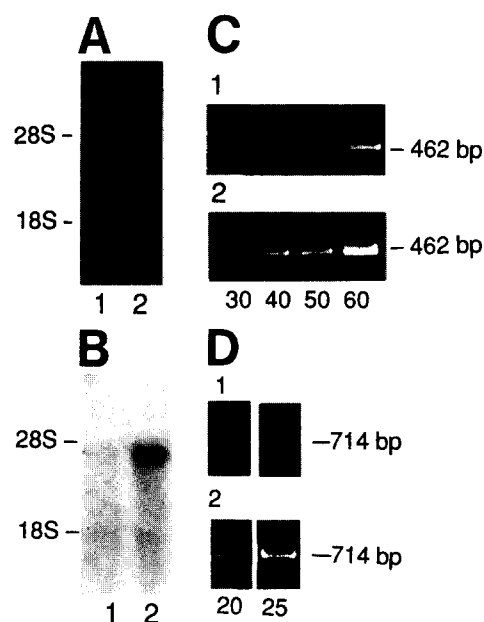


Fig. 3. The effect of UVB irradiation on ET-1 and ET receptor mRNA expression in keratinocytes. Total RNA was extracted from the keratinocytes 12 h after 5 mJ/cm² UVB irradiation. (A) ethidium bromide staining of the gel (10 µg of total RNA in each lane), (B) Northern blot analysis for ET_B receptor from the gel shown in A, (C) RT-PCR for ET-1 (1 µg of total RNA as a template from each sample shown in A), (D) RT-PCR for ET_A receptor (1 µg of total RNA as a template from each sample shown in A). The numbers under the bands represent PCR cycles. 1, 0 mJ/cm² UVB; 2, 5 mJ/cm² UVB.

11]. Since keratinocytes expressed ET_A receptor, we expected a short-term action of ET-1. In preliminary experiments, we observed that ET-1 stimulated the contraction of collagen gel embedded with fibroblasts and/or keratinocytes. However, we failed to demonstrate such an activity by UVB irradiation in this model, thereby suggesting that UVB irradiation has various effects on keratinocytes other than the enhancement of ET-1 secretion. We therefore speculate that UVB-induced ET-1 from keratinocytes may work as a paracrine factor for melanocyte growth and melanization [19], and as an autocrine growth factor for keratinocytes [21].

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