Abstract. Keloids are proliferative growths of dermal collagen, usually resulting from excessive tissue response during wound healing. There is evidence that keratinocytes may promote keloidogenesis via epithelial-mesenchymal interactions. Metallothioneins (MTs) are known to be involved in the fundamental cellular processes of growth and apoptosis. In this study, we evaluated the expression of MT isoforms in normal and keloid keratinocytes. The expression patterns of ten functional MT isoforms were assessed using real-time RT-PCR in primary cultures of normal and keloid keratinocytes. The MT-2A isoform was the most abundant MT isoform in both normal and keloid keratinocytes while the MT-1B isoform was absent. There was a significant increase in the mRNA expression of four MT isoforms, viz. MT-1A, 1E, 1F and 2A in keloid keratinocytes as compared to normal keratinocytes. Up-regulation of MT-1A, 1E, 1F and 2A isoforms may play a part in the development of keloids by paracrine signaling.

Introduction

A keloid is an abnormality of the skin that results during the process of recovering from a skin injury. Keloid formation involves atypical proliferation and overproduction of extracellular matrix (1,2). Keloids occur when the scar matures and more collagen is produced than degraded, resulting in overgrowth of the skin and giving rise to an elevated and hyperaemic scar. As keloids extend beyond the injured site to invade the surrounding normal skin without spontaneous regression, they are considered as benign skin tumors.

MTs consist of a group of low molecular weight cysteine-rich proteins that have metal-binding properties (3). The MT protein was first identified as a cadmium-binding protein in equine kidneys and subsequently purified and characterized by Kagi and Vallee (4) and Kagi et al (5). MTs have selective binding to metals such as zinc, copper and cadmium (6) and are involved in the detoxification of heavy metals such as cadmium and mercury. MTs are known to be associated with protection against DNA damage and oxidative stress (7). MTs have been implicated in the fundamental processes of cell proliferation, differentiation and apoptosis (8). There are >10 functional MT isoforms in humans, which encode four main groups of MT proteins viz. MT-1, MT-2, MT-3 and MT-4 proteins (9-11). The MT-1 protein comprises many subtypes encoded by a set of MT-1 genes (12).

There is cumulative evidence that keratinocytes may promote keloidogenesis via epithelial-mesenchymal interactions. In this study, we compared the mRNA expression of the functional MT-1A, 1B, 1E, 1F, 1G, 1H, 1X, 2A, 3 and 4 isoforms in primary cultures of keloid keratinocytes (KK) with normal keratinocytes (NK).

Materials and methods

**Tissues.** Fresh tissue specimens of earlobe keloid scars were used in this project. No patient had previously received treatment for the keloids and excised specimens were confirmed histopathologically. Control tissue and cells were derived from discarded normal adult skin samples removed during plastic surgery procedures. The collection of pathological and normal tissue samples were approved by the Institutional Ethics Board and with informed patient consent.

**Primary keratinocyte cultures.** Primary cultures of keratinocytes derived from keloids and normal tissues were seeded at a density of 4x10^5 cells and maintained for 2 days in EpiLife medium (Cascade Biologics, OR) until 80% confluent. The medium was then changed to EpiLife basal medium for 48 h before subjecting to total RNA extraction.

**Transmission electron microscopy.** A fresh keloid and normal skin specimens were fixed in 3% glutaraldehyde and 2% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4) overnight, as previously described (13). Briefly, osmification was carried out with 1% osmium tetroxide for 1 h at room temperature. Samples were dehydrated in an ascending series of ethanol and embedded in araldite. Ultrathin sections were then cut and mounted on formvar-coated copper grids.
Sections were double stained with uranyl acetate and lead citrate before examination with a Philips CM 120 BioTwin electron microscope.

Real-time RT-PCR. Total RNA was isolated from the normal and keloid keratinocytes by TRIzol extraction. The concentration and the quality of the RNA extracted were examined by spectrophotometric absorbance readings at 260 and 280 nm. Five micrograms of total RNA isolates were transcribed into cDNA using SuperScript III 1st Strand Synthesis System (Invitrogen) with random hexamer primers following the manufacturer’s protocol. The cDNA products were kept at -20°C for real-time RT-PCR analysis. Each cDNA sample equivalent to 25 ng of total RNA was then used in each real-time RT-PCR reaction. The primers specific for the individual MT isoforms were adapted from Mididoddi et al (11). Real-time RT-PCR conditions were optimized by adjusting the annealing temperature. PCR was performed using a LightCycler (Roche Diagnostics) in a total reaction mixture of 10 μl containing 1X QuantiTect SYBR Green Master Mix (Qiagen), 0.5 μM of each primer and 25 ng of cDNA. After initial denaturation at 95°C for 15 min, 45 cycles were performed at 94°C for 15 sec, 60°C for 25 sec, and 72°C for 18 sec. Melting curve analysis was carried out at 65°C for 15 sec to verify the specificity of the amplification reaction. Triplicate reactions for each specimen were performed. Relative quantification was calculated using the ΔΔCT and 2^-ΔΔCT method (14), where ΔCT refers to the difference between the CT values of the target gene and the housekeeping gene, β-actin. The CT value is considered as the fractional cycle number at which the emitted fluorescence of the sample

Figure 1. Electron micrograph of keloid keratinocytes (A) showing the distinct presence of keratofilaments at a higher magnification (B). Bar, 5 μm (A); bar, 1 μm (B).

Figure 2. Electron micrographs of keloid fibroblasts (A-C) and normal skin fibroblast (D). Bar, 1 μm.
passes a fixed threshold above the baseline. For each MT isoform, NK cells were selected as the reference. ΔΔCT refers to the difference of the ΔCT between the KK and NK. Lower ΔCT values and ΔΔCT values reflect a relatively higher amount of MT transcript.

Statistical analysis. An unpaired two-tailed t-test was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software).

Results

Keloid morphology. The keloid tissue has an epithelial and mesenchymal compartment. Ultrastructural examination showed that keratinocytes in the keloid tissue were characterized by bundles of keratin tonofilaments (Fig. 1). In the mesenchymal compartment, there was proliferation of spindle-shaped fibroblasts with profuse deposition of collagen bundles (Fig. 2A) as opposed to the fibroblast seen with loose supporting collagenous tissue in normal skin (Fig. 2D). In this aberrant healing process, dense collagen fibers were deposited haphazardly and often as nodules in the dermis (Fig. 2A and B). At higher magnification (Fig. 2C), the presence of prominent endoplasmic reticulum signifies the protein secretory function of the fibroblasts.

MT-isoform expression. The expression of the ten functional MT isoforms in normal and keloid keratinocytes as evaluated by real-time RT-PCR are shown in Table I. Both NK and KK cells expressed the MT-1A, 1E, 1F, 1G, 1H, 1X and 2A isoforms. The MT-2A isoform was the most abundant MT isoform in both types of keratinocytes while the MT-1B mRNA transcript was absent. As the MT-3 and MT-4 isoforms showed either very low or no expression in NK and KK cells, they were considered as not computable. Expression of the the MT-1G and 1H isoforms were found to be low in both NK and KK. There was a significant difference in the expression of MT-1A, 1E, 1F and 2A isoforms in keloid keratinocytes as compared with normal keratinocytes (Table I). As shown in Fig. 3, the relative change in MT-1A was approximately 14.8-fold, MT-1E 7.9-fold, MT-1F 3.0-fold and MT-2A 7.4-fold. The specificity of the MT-1A (219 bp), MT-1E (284 bp), MT-1F (232 bp) and MT-2A (259 bp) PCR products is shown in Fig. 4.

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<th>Gene of interest</th>
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<th>Gene ΔC_T value</th>
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β-actin values for NK and KK are 16.75±0.86 and 17.73±1.69 respectively. Values shown are mean readings ± SD. ^P-value <0.05. NC, not computable.
Discussion

Wound healing is a complex process that involves the interaction of the epidermis and dermis. Keloidogenesis occurs when there is an abnormality in the proliferation and apoptosis of the fibroblasts in the dermal layer. However, this is not an isolated dermal disease as many studies have shown that epidermal-mesenchymal interactions play an important role in the formation of keloids (15-18).

There has been compelling evidence that autocrine, paracrine, and endocrine epithelial-mesenchymal interactions play a major role in normal skin homeostasis, growth, and differentiation (19,20). Keratinocytes are found to promote proliferation and inhibit apoptosis of the underlying fibroblasts (21). Lim et al has also reported that keratinocytes of keloid tissue has profound effects on the production and organization of collagen by fibroblasts in the dermis (17). These findings strongly indicate that the overlying keratinocytes play an important role in promoting the growth and apoptosis of the underlying fibroblasts. Moreover, many signaling proteins such as kinases, Bel-2 and TGF-ß were found to be expressed more in keloid fibroblasts co-cultured with keloid keratinocytes as compared to normal keratinocytes (18,22).

We examined MT expression in normal and keloid keratinocytes. MT is known to be expressed in the basal keratinocytes of normal skin and overexpressed in infiltrating basal cell carcinomas (23). In the present study, we observed for the first time that MT-1A, 1E, 1F, 1X and 2A isoforms were significantly expressed in KK as compared to NK cells. Thus far, the functional isoforms of MT have been reported to be involved in different functions. The MT-2A isoform is associated with cell proliferation (24), whereas both MT-1A and 2A isoforms are reported to protect against apoptosis and oxidative stress (25). It has also been posited that MT-1F and MT-2A isoforms may be involved in histological differentiation (24,26). However, all MTs have characteristic cys-x-cys, cys-x-y-cys, and cys-cys sequences (where x and y are non-cysteine amino acids) which are believed to bind a total of seven bivalent metal ions in two separate clusters (27).

The MT/thionein (MT/T) system is known to regulate and control the concentration and availability of zinc (28). The MT/T system has the capability to sequester zinc and at the same time release zinc when required (29). Bound zinc is released possibly as a result of oxidation and reduction of cysteine sulfur ligands present in MT (30). The role of zinc in modulating cell signaling, second messenger metabolism, stimulation of protein kinase phosphorylation and inhibition of protein phosphatase activity is well established (31). Moreover, zinc is a structural element of a huge number of transcription factors which contain zinc finger domains (32). The association of zinc with a multitude of biological functions and its related implications in health and disease, has led to this metal being touted as ‘the calcium of the twenty-first century’ (33).

As zinc promotes cell proliferation and differentiation as well as inhibits apoptosis (31), release of zinc by keloid keratinocytes could potentially serve as extracellular signals for the underlying fibroblasts to proliferate and produce collagen indiscriminately. Also, a lack of apoptotic mechanisms in keloids may play a pivotal role as alteration of oncogenes associated with apoptosis has also been previously reported (22). Conversely, sequestration of zinc in keratinocytes may reduce the availability of zinc to the underlying fibroblasts and low zinc levels inhibit collagenases, favoring collagen deposition (34,35).

In conclusion, modulation of MT-1A, 1E, 1F and 2A isoforms observed in this study may play a regulatory role in keloid formation by paracrine signaling. We postulate that up-regulation of MT in keratinocytes could induce the development of keloids via zinc signaling cascades. Further studies using keloid keratinocytes co-cultured with normal or keloid fibroblasts would be necessary to elucidate the role of these MT isoforms in keloidogenesis.

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References