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Keratinocyte Differentiation Inversely Regulates the Expression of Involucrin and Transforming Growth Factor β1

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Abstract Extensive skin loss from a variety of conditions such as severe thermal injury is associated with significant functional morbidity and mortality. In recent years, the healing quality has been improved for patients who suffer burns due in part to the usage of skin replacement mainly prepared from multi-layered sheets of cultured keratinocytes. Although it is known that keratinocytes are a rich source of wound healing promoting factors such as transforming growth factor-β1 (TGF-β1), it is not clear whether differentiated keratinocytes in a multi-layer form release this multi-functional growth factor and has any functional influence on dermal fibroblasts. This study examined the hypothesis that keratinocytes in mono- and multi-layer forms express different levels of TGF-β1. To address this hypothesis, keratinocytes were grown in serum free medium (KSFM) supplemented with bovine pituitary extract (50 μg/ml) and EGF (5 μg/ml). When cells reached confluency, conditioned medium was removed and replaced with 50% KSFM with no additives and 50% DMEM without serum and cells were allowed to form multi-layers and differentiate. The conditioned medium was then collected every 48 h up to 24 days and the level of TGF-β1 and the efficacy of a keratinocyte released fibroblast mitogenic factor were evaluated by ELISA and ^{3}H-thymidine incorporation, respectively. Northern analysis was also employed to evaluate the expression of TGF-β1, involucrin, TIMP-1, and 18 S ribosomal RNA in keratinocytes at different times of the onset of differentiation. The microscopic morphology of keratinocytes at different times of induction of cell differentiation showed detachments (nodules) of many regions of keratinocyte sheet from culture substratum within 1–2 weeks. The numbers and sizes of these nodules were increased as the process of keratinocyte differentiation proceed. The results of TGF-β1 evaluation revealed that mono-layers of cultured keratinocytes which were round, attached, and proliferating in KSFM + BPE and EGF containing medium released a significantly higher level of TGF-β1 (196±58 pg /ml) relative to those grown in multi-layer forms (28±7.8 pg/ml). A longitudinal experiment was then conducted and the results showed that cells on the onset of differentiation released even greater level of TGF-β1 (388±53 pg/ml) relative to those grown in KSFM + BPE and EGF. This finding was consistent with the expression of TGF-β1 mRNA evaluated in keratinocytes grown in test medium for various duration. In general, the level of TGF-β1 protein and mRNA gradually reduced to its lowest level within 12 days of growing cells in our test medium. When aliquots of the collected keratinocyte conditioned medium were added to dermal fibroblasts, the level of ^{3}H-thymidine incorporation increased only in those cells receiving aliquots of conditioned medium containing high levels of TGF-β1. When involucrin was used as a differentiation marker for keratinocytes at different time points, the highest level of involucrin mRNA expression was found at the later stage of cell differentiation. In conclusion, high involucrin expressing differentiated keratinocytes seem to be quiescent in releasing both TGF-β1 and a fibroblast mitogenic factor. J. Cell. Biochem. 83: 239–248, 2001.

Key words: keratinocyte; differentiation; involucrin; transforming growth factor-β1; skin; burn; fibroblasts; wound healing

Restoration of an epidermal barrier is an essential requirement for massive acute burns and for treatment of giant congenital nevi [Carter et al., 1987; Gallico et al., 1989], junctional epidermal bullosa [Kumagai et al., 1988], and in post-burn scar revision [Fisher, 1984]. In vitro cultivation of keratinocytes, with the support of a feeder layer of lethally

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irradiated 3T3-cells, was initially introduced by Rheinwald and Green [1975]. O'Connor et al. [1981] were the first to use human autologous cultured keratinocytes as a burn wound coverage. These investigators were later able to grow keratinocytes to confluency that were suitable for grafting [Hefton et al., 1983; Gallico et al., 1984]. The advantage of using autologous cultured keratinocytes is that a small uninjured biopsy specimen from the patient can be expanded 1,000-fold in 3 weeks providing sufficient epidermal sheets to cover a large body surface area and avoiding large donor site wounds [Hefton et al., 1983].

Several lines of evidence indicate that keratinocytes express many growth factors, cytokines and their receptors. Expression of epidermal growth factor (EGF), basic fibroblast growth factor (FGF-2), keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), transforming growth factor-α (TGF-α), interleukins (ILs), heparin-binding growth factors (HBGF), and amphiregulin have been demonstrated in keratinocytes. Cytokines such as IL-1, -4, -6, and -8 are believed to be synthesized by activated keratinocytes and function as potent growth factors [reviewed by Mckenzie and Sauder, 1990]. Proteins of the EGF family are key regulators of epidermal development and growth [Gniadecki, 1998]. Activation of the EGF receptor (EGFR) in human epidermal keratinocytes, mediates autonomous replication of keratinocytes and inhibits differentiation and apoptosis [Piepkorn et al., 1998]. It has been demonstrated that the EGFR which is an important contributor to cell proliferation and migration is transiently upregulated in wounded skin. The EGFR in the normal epidermis is important for autocrine growth in renewing tissue, promotion of cell survival, regulation of cell migration during epidermal morphogenesis, wound healing, and suppression of terminal differentiation [Hudson and McCawley, 1998]. It has also been demonstrated [Bechtel et al., 1998] that keratinocytes are able to express both plasminogen activator (PA) and its major inhibitor (PAI-2) during re-epithelialization of epidermal defects. Further, it has been shown that in an organotypic co-culture system, PAI-2 was strongly expressed in early cultures prior to formation of a differentiated epidermis-like structure [Bechtel et al., 1998]. Other studies have shown that PAI-1 mRNA expression appeared to be growth state-regulated and was associated with specific keratinocyte subpopulations undergoing migration and proliferation, which is a condition equivalent to wound regeneration [Staiano-Coico et al., 1996].

Dermal–epidermal interaction has been the subject of several recent studies. It has been demonstrated that when a cultured keratinocyte sheet is used as a temporary wound coverage, it promotes wound healing and increases wound epithelialization [Bolviar-Flores et al., 1990]. This was further confirmed by showing an acceleration of healing by 8 days in a keratinocyte lysate-treated group relative to a control group [Somers et al., 1996]. Further studies revealed that lysates of cultured keratinocytes contain mitogenic activity for keratinocytes, endothelial cells, and fibroblasts. Treatment with lysates of keratinocyte cultures had a 2-fold stimulation on epithelialization [Duinslaeger et al., 1996]. Proliferation of dermal fibroblasts and matrix modulation in response to keratinocyte-conditioned medium (KCM) has also been studied. The results revealed that KCM significantly increases fibroblast replication and decreases collagen synthesis. As keratinocytes and fibroblasts are located in two different skin layer, soluble cytokines and growth factors considered to be the main mediators in dermal–epidermal interaction. There is, therefore, a long list of cytokines and growth factors which are expressed by keratinocytes in culture [Mckenzie and Sauder, 1990].

Transforming growth factor-β1 (TGF-β1) is a multi-functional cytokine that influences many physiological and pathological processes. TGF-β1 functions as a chemoattractant for dermal fibroblasts and monocytes/macrophages, influences fibroblast cell proliferation and matrix deposition. These diverse biological effects on a variety of cell types established the fact that TGF-β1 is a key cytokine in the process of wound healing [Border and Noble, 1994]. On the other hand, TGF-β1 has been implicated in the pathogenesis of fibrosis in several organs including the skin. Thus, the expression of TGF-β1 in deficient or excessive amounts at the wound site determines the ultimate quality of tissue repair, ranging from non-healing to fibrosis. For these reasons, TGF-β1 has been considered as a double-edged sword with both therapeutic and pathological potential [Border and Noble, 1995]. In fact, fibrosis of injured tissues, including the dermis, is not a unique pathologic process but

may be caused by the same biologic events which are involved in the normal process of tissue repair [Wakefield et al., 1987]. Considering the fact that keratinocytes are a major source of cytokines and growth factors including TGF-β1 in culture, there would be a need to examine how proliferating keratinocytes in cultures are different from those in multilayered forms which are used as a wound coverage. This study is, therefore, conducted to examine the hypothesis that proliferating and differentiated keratinocytes express different levels of TGF-β1. Our findings revealed that involucrin expressing differentiated keratinocytes are quiescent in releasing both TGF-β1 and an unknown fibroblast mitogenic factor(s).

MATERIALS AND METHODS

Cell Cultures

The procedure of Rheinwald and Green [1975] was used for cultivation of human foreskin keratinocytes using serum-free keratinocyte medium (GIBCO) supplemented with bovine pituitary extract (50 μg/ml) and EGF (5 μg/ml). Primary cultured keratinocytes at passages 3–5 were used. Keratinocytes were then grown in our test medium consists of 50% DMEM and KSFM each without any additives. Keratinocyte conditioned medium was then collected every 48 h up to Day 22 and evaluated for TGF-β1 and fibroblast mitogenic factor(s). In another set of experiments keratinocytes which were kept in test medium for 0, 2, 5, 14, 18, and 22 days were harvested, total RNA was extracted and evaluated by Northern analysis. To establish fibroblast cultures, either fetal foreskin or normal skin punch biopsies obtained from patients undergoing elective reconstructive surgery were established in DMEM supplemented with 10% FBS as previously described [Ghahary et al., 1994]. Strains of dermal fibroblasts at passages 3–7 were used in this study.

Enzyme-Linked Immunosorbent Assay (ELISA) for TGF-β1

To determine the amounts of TGF-β1 produced by keratinocytes grown in either KSFM or test medium at different time intervals, a sandwich ELISA for TGF-β1 was used [Danielpour, 1993]. Briefly, 96-well plates were coated with 100 μl/well of monoclonal antibody to human TGF-β (Genzyme, Cambridge, MA) at a concentration of 1 μg/ml in PBS. The plates were incubated for 2 h at room temperature (RT) followed by 16 h at 4°C. After washing twice with PBS-Tween-20 (PBS-T), the plates were blocked with 1% bovine serum albumin (BSA, crystallized, Sigma, St. Louis, MO) for 60 min at RT and washed three times with PBS-T. One milliter of conditioned medium from each culture sample was acidified with 24 μl of 5N HCl for 15 min at RT and neutralized with 40 μl of 1 M HEPES/5 N NaOH (5/2). One hundred microlitres of the acidified/neutralized samples was added to each well of the plates, which were then incubated at RT for 60 min. After washing, the plates were incubated with 100 μl/well of chicken anti-human TGF-β1 (R&D Systems) at a concentration of 2.5 μg/ml for 60 min at RT with shaking. After washing five times with PBS-T, the plates were incubated with alkaline phosphatase-conjugated rabbit anti-chicken IgG (Sigma) at RT for 60 min followed by five washings with PBS-T. After adding substrate (O-nitrophenyl phosphate, 1 mg/ml, Sigma), the plates were incubated at RT for 60 min and the optical density was read using a THERMOmax (Molecular Devices, Menlo Park, CA) microplate reader at a wave length of 405 nm. Serial dilutions (0, 125, 250, 625, 1,250, to 2,500 pg/ml) of recombinant human TGF-β1 (Genzyme) were used to prepare a standard curve.

Extraction of Cellular RNA and Northern Analysis

To demonstrate whether TGF-β1 mRNA expression is consistent with the level of TGF-β1 detected in keratinocyte conditioned medium, keratinocytes grown in either KSFM or in test medium for 0, 2, 5, 14, 18, and 22 days were harvested, washed in cold PBS, and lysed in 6 ml of guanidinium thiocyanate (GITC) as previously described [Ghahary et al., 1994]. Total RNA was extracted by the GITC/CsCl procedure of Chirgwin et al. [1979], separated by electrophoresis and blotted onto nitrocellulose filters. Filters were then baked under vacuum for 2 h at 80°C and pre-hybridized in a solution containing 50% formamide, 0.3 M NaCl, 20 mM Tris HCl (pH 8.0), 1 mM EDTA, 1× Denhardt's solution (1× = 0.02% bovine serum albumin, Ficoll and polyvinylpyrrolidone), 0.005% salmon sperm DNA, and 0.005% poly (A) for 2–4 h at 45°C. Hybridization was performed in the same solution at 45°C for 16–20 h using cDNA probes for either human TGF-β1, tissue

inhibitor metalloproteinase-1 (TIMP-1) or 18 S ribosomal RNA. The probes were labeled with ^{32}P-α-dCTP (DuPont Canada, Streetsville, Mississauga, Ontario, Canada) by nick-translation. Filters were initially washed at room temperature with 2× SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate) and 0.1% SDS for 30 min, then for 20 min at 65°C in 0.1× SSC and 0.1% SDS solution. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at −70°C in the presence of an enhancing screen. The human TGF-β1 cDNA was a gift from Dr. G.I. Bell (Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biology and Medicine, University of Chicago, IL). The cDNA for human involucrin was a gift from Dr. Howard Green (Howard Medical School, Department of Cell Biology, Boston, MA). cDNAs for TIMP-1 and 18 S ribosomal RNA were obtained from the American Type Culture Collection (Rockville, MD).

Fibroblast Cell Proliferation Assay

To examine whether mono- (proliferating) and multi-layered (differentiating) forms of keratinocytes release any factor which might be mitogenic for either dermal fibroblasts or keratinocytes into their conditioned medium, ^{3}H-thymidine incorporation, as an index for cell proliferation, was used. Briefly, subconfluent cultures of either fibroblasts or keratinocytes were trypsinized, washed in assay medium (DMEM, 0.2% FBS, 10 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid and 1% penicillin/streptomycin antibiotic), seeded at 5×10^5/0.5 ml/well in 24-well plates, and incubated for 3 h at 37°C in an atmosphere of 5% CO_2. Cells were then received 50% DMEM and 50% keratinocyte conditioned medium which have been collected every 48 h up to 24 days from keratinocytes grown in test medium. Twenty-two hours later 0.25 mCi of ^{3}H-labeled thymidine was added to each well for 2 h and incubation continued. Cells were then fixed, washed, and the radioactivity was measured in the cell lysate collected from each well.

Statistical Analysis

The difference in fibroblast cell proliferation and TGF-β1 protein production between treated and control cells was determined and compared. The statistical significance was evaluated using Student's t-test. P values of < 0.05 were considered significant.

RESULTS

The morphology of keratinocytes grown in KSFM (Fig. 1, panel A) and test medium (50/50 KSFM + DMEM) (Fig. 1, panel B) for 48 h showed that cells in both culture conditions are in mono-layered form in which the borders of the cells are defined. While cells grown in test medium for a week showed some areas of special structures, called nodules (Fig. 1, panel C). The numbers and sizes of these nodules were increased with time (Fig. 1, panel D and E). The cell density in nodules was very high compared to surrounding area. Cells in these nodules were overlapped and that indicates that keratinocytes are formed in multi-layers in these areas. The regions of the keratinocyte sheets in which nodules were present found to be detached from the substratum. As shown in Figure 1, panel E, the culture conditioned medium was trapped underneath of these bubble type structures. To examine whether there is a correlation between keratinocyte cell morphology and expression of TGF-β1, the level of protein and mRNA for this muti-functional cytokine was evaluated at different time intervals prior and post differentiation. The profile of TGF-β1 production by keratinocytes at different time points of the differentiation onset is shown in Figure 2. The quantity of TGF-β1 in conditioned medium derived from cells grown in KSFM for 48 h was 196±58 pg/ml. While replacing this medium with our test medium for the same period of time resulted in almost 2-fold increase (388±53 vs. 196±58 pg/ml) in production of TGF-β1. However, when conditioned medium was collected every 2 days up to Day 24, a significant reduction in the level of TGF-β1 was found at the later time points. The lowest level of TGF-β1 was found in conditioned medium collected from keratinocytes grown in test medium on Day 18. To demonstrate whether the pattern of TGF-β1 production by keratinocytes is consistent with TGF-β1 mRNA, cells which were grown in test medium for 0, 2, 4, 5, 14, 18, and 22 days, were harvested and evaluated for expression of TGF-β1 and 18 S ribosomal RNA. The results showed that cells grown in either KSFM or test medium for only 2 days express a high level (1.2±0.04 and 0.99±0.2, respectively) of TGF-β1 mRNA and this expression declined to 50% (0.66± 0.05) of that expressed in KSFM within 5 days and remained low (0.36± 0.2) up to Day 22 examined.

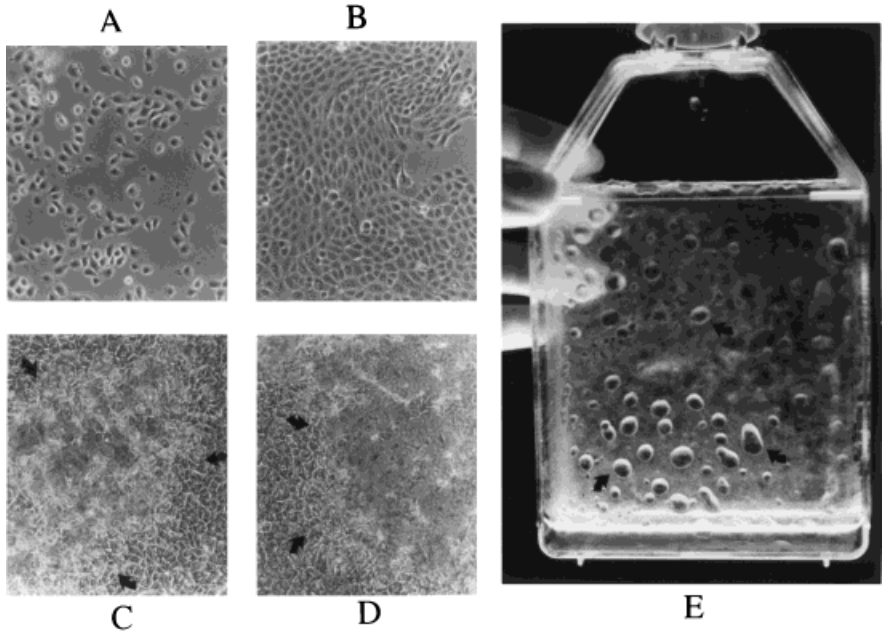

Fig. 1. Morphology of cultured keratinocytes in test medium at different durations. Keratinocytes were cultured in either KSFM alone for 48 h **(panel A)** or test medium containing 50% DMEM and 50% KSFM for 48 h **(panel B)**, 1 week **(panel C)**, 2 weeks **(panel D)**, and 3 weeks **(panel E)**. Arrows confine the microscopic regions of the keratinocytes (nodules) in which cells form multi-layers and become detached from substratum. Panel E shows the macroscopic appearance of keratinocyte sheet in which many nodules (arrows) filled with conditioned medium are seen after 3 weeks of being cultured in test medium.

This alteration was not due to variation in total RNA loading, as the pattern of 18 S ribosomal RNA showed no marked variation in RNA loading from one sample to another (Fig. 3). The specificity of the profile of TGF-β1 mRNA expression was confirmed by re-probing the same blot with tissue inhibitor metalloproteinase-1 (TIMP-1). As shown in Figure 4,

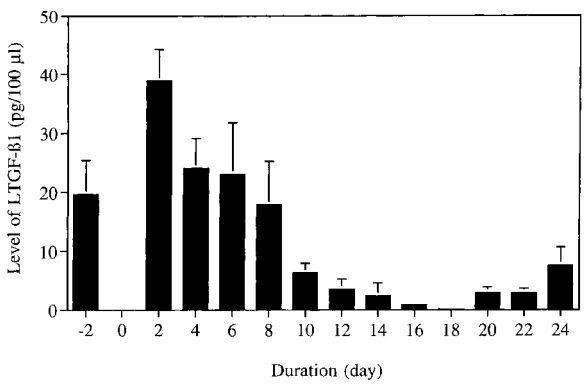

Fig. 2. Levels of TGF-β1 protein by keratinocytes reduced as the process of differenciation proceed. When primary cultured keratinocytes reached 90% confluency, keratinocyte serum free conditioned medium was exchanged with test medium containing 50% DMEM and 50% KSFM with no additive. Conditioned medium was then collected every 48 h thereafter up to Day 24 and tested for TGF-β1 protein by ELISA as described in the Materials and Methods. The level of TGF-β1 expressed as pg/100 µl of conditioned medium derived from keratinocytes grown in KSFM for 48 h (−2 bar) was also evaluated as a control. Data represent mean±SEM of five separate experiments conducted in triplicates.

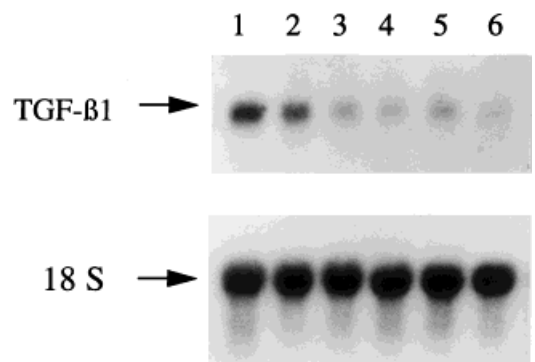

Fig. 3. Differentiation of keratinocytes is associated with reduction of TGF-β1 mRNA expression. Primary keratinocytes were cultured in either KSFM for 48 h (**lane 1**) or test medium containing 50% DMEM and 50% KSFM for 48 h (**lane 2**), Day 5, 14, 18, and 22 (**lanes 3–6**, respectively). Total RNA was then extracted individually and subjected to Northern analysis. The blot was initially hybridized with TGF-β1 cDNA and subsequently with cDNA specific for 18 S ribosomal RNA used as a control for RNA loading. The autoradiogram represents three different blots prepared from three separate experiments.

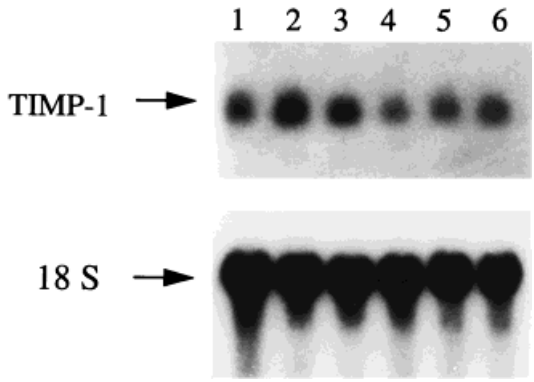

Fig. 4. Tissue metalloproteinase inhbitor-1 is constitutively expressed by keratinocytes. Primary keratinocytes were cultured in either KSFM for 48 h (**lane 1**) or test medium containing 50% DMEM and 50% KSFM for 48 h (**lane 2**), Day 5, 14, 18, and 22 (**lanes 3–6**, respectively). Total RNA was then extracted individually and subjected to Northern analysis. The blot was initially hybridized with TIMP-1 cDNA and subsequently with cDNA specific for 18 S ribosomal RNA. The autoradiogram represents three different blots obtained from three separate experiments.

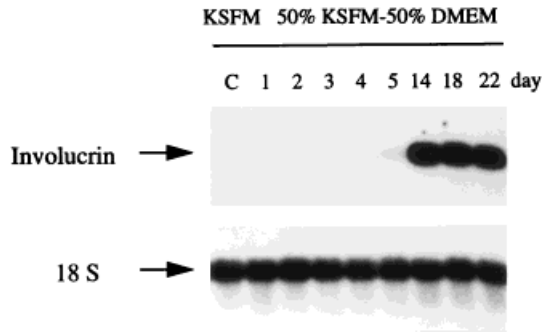

Fig. 5. Time dependent expression of involucrin in keratinocytes. Primary keratinocytes were cultured in either KSFM for 24 h (**lane 1**) or test medium containing 50% DMEM and 50% KSFM for 24 h (**lane 2**), Day 2, 3, 4, 5, 14, 18, and 24 (**lanes 3–9**, respectively). Total RNA was then extracted individually and subjected to Northern analysis. The blot was initially hybridized with involucrin cDNA as a marker for cell differentiation and subsequently with cDNA specific for 18 S ribosomal RNA. The autoradiogram represents two different blots obtained from three separate experiments.

keratinocytes surprisingly express TIMP-1 mRNA constitutively. The expression of TIMP-1 transcript in keratinocytes grown in KSFM was lower relative to that of cells grown in test medium. The pattern of 18 ribosomal RNA shown in Figure 4, indicates that this alteration is not due to the RNA loading. However, the level of this transcript was also reduced in keratinocytes at their later stage of differentiation but not to the same extend as that seen for TGF-β1.

Involucrin is one of the well established keratinocyte differentiation markers which is normally expressed by irreversible differentiated keratinocytes [Watt, 1987]. We therefore, evaluated the expression of involucrin mRNA in keratinocytes at different time points as a differentiation marker. To demonstrate whether the pattern of TGF-β1 production and mRNA by keratinocytes correlates with involucrin expression, total RNA from the same samples which were used to evaluate the expression of TGF-β1 mRNA, was used to re-hybridize with a cDNA for involucrin. The results of Northern analysis shown in Figure 5 indicates that involucrin is absent in keratinocyte harvested at Days 0, 2, and 4 and its expression become faintly apparent on Day 5 and reached its maximum levels on Day 14, 18, and 22 examined. These findings indicate that the onset of differentiation for keratinocytes grown in our test medium might even be earlier than Day 5 in which the level of involucrin become detectable by Northern analysis. This result may also indicate a correlation between expression of involucrin, keratinocyte differentiation, and suppression of TGF-β1 expression.

The release of fibroblast mitogenic factor from keratinocytes has been reported before [Carter et al., 1987]. However, the presence and quantity of any mitogenic factor which may release during keratinocyte differentiation has not be examined and compared. The conditioned medium of cells grown in either KSFM or in test medium for various time intervals was, therefore, collected and tested for any mitogenic factor for both dermal keratinocytes and fibroblasts (Fig. 6). The results showed that proliferating keratinocytes grown in KSFM and cells grown in test medium for less than a week produce an unknown factor which was mitogenic for dermal fibroblasts. The efficacy of this factor released from keratinocyte on Day 2 and 4 was greater in stimulating fibroblast cell proliferation than DMEM plus either 2 or 10% FBS. The level of this keratinocyte releasable fibroblast mitogenic factor was reduced to its lowest level on Day 8 and gradually increased and reached to another maximal level on Day 14. The second wave of reduction began on Day 16 and reached to its lowest level on Day 24. In general, cells which were grown in the test medium and were at their later stage of differentiation were less active in expressing such a

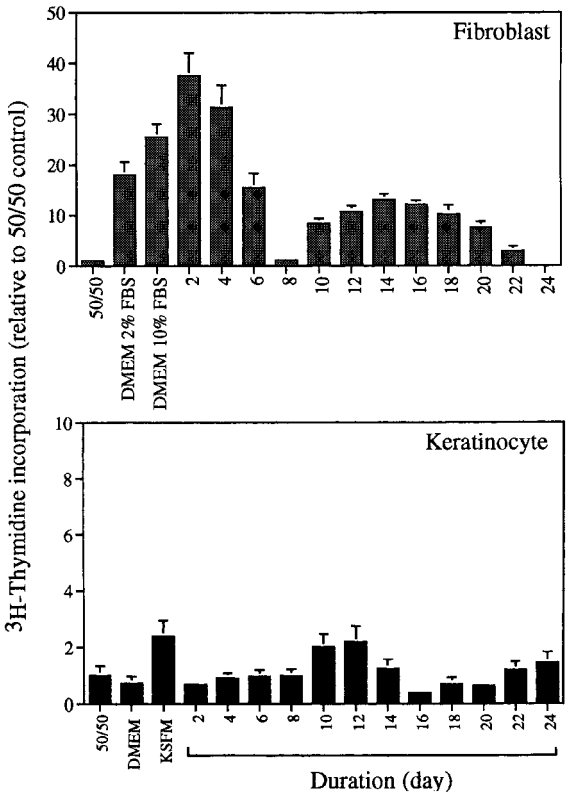

Fig. 6. Keratinocytes in mono-and multi-layered form release different levels of a fibroblast mitogenic factor. Primary cultured keratinocytes were grown in KSFM and when they reached 90% confluency, medium was exchanged with test medium containing 50% DMEM and 50% KSFM with no additive. Conditioned medium was then collected every 48 h thereafter up to Day 24 and tested for either a fibroblast **(upper panel)** or keratinocyte **(lower panel)** mitogenic factor(s) using ^{3}H-thymidine incorporation as an index for cell proliferation as described in the Materials and Method Section. Data represent mean±SEM of five separate experiments conducted in triplicates.

factor. When the same samples were tested on proliferation of keratinocytes, only samples collected on Day 10 and 12 showed some keratinocyte proliferating effects relative to cells received unconditioned test medium. These findings indicate that cells at different stages of differentiation may produce different levels and types of either fibroblast or keratinocyte mitogenic factors.

DISCUSSION

The present study was conducted to examine the hypothesis that keratinocytes in mono- and multi-layer forms express different levels of TGF-β1 as well as a mitogenic factor for fibroblasts. The rationale for this study is that cultured epithelial autografts (CEA) can successfully be used for wound coverage in patients with large thermal injuries, giant congenital nevi [Green et al., 1979; Gallico et al., 1989], junctional epidermal bullosa [Carter et al., 1987], and in post-burn scar revision [Kumagai et al., 1988]. As keratinocytes express high level of TGF-β1 in culture, it would be of particular interest to know whether differentiated keratinocytes in a multi-layer form also produce the same amount of TGF-β1 and any fibroblast mitogenic factor(s). In our study, we selected TGF-β1 because the multi-functional characteristics of TGF-β1 makes it a unique cytokine for possible treatment of non-healing wounds or to accelerate the normal process of healing in severe thermal injury. This is based on the fact that this cytokine modulates the synthesis and deposition of various extracellular matrix (ECM) proteins such as fibronectin, collagenase, type I and type III collagen by dermal fibroblasts [Goldstein et al., 1989; Roberts and Sporn, 1990; Hill et al., 1992]. This cytokine also enhances the production of protease inhibitors such as tissue metalloproteinase inhibitor-1 (TIMP-1) [Edwards et al., 1987; Overall et al., 1989], glycosaminoglycans [Ignotz and Massague, 1986; Sporn et al., 1987] and neovascularization [Allen et al., 1993].

In this study, the level of TGF-β1 was significantly greater in cells which were grown in KSFM, a well established culture condition in which keratinocytes are attached to substratum and have capacity to proliferate. Similarly, when cells were grown in test medium, for several days at which keratinocytes were still attached to the culture substratum, the level of TGF-β1 production was still very high. However, the concentration of TGF-β1 in keratinocyte conditioned medium reduced to its lowest level within one week post-culturing the cells in test medium. The expression of TGF-β1 mRNA in keratinocytes at early time points which was evaluated by Northern analysis was consistent with the level of TGF-β1 production. The specificity of the pattern of TGF-β1 expression was confirmed by probing the same blots with TIMP-1 cDNA showing a constitutive expression of TIMP-1 mRNA at different time points. These findings reveal that the expression of mRNA for TGF-β1 and to a lesser extent TIMP-1 is markedly influenced by the status of keratinocyte differentiation.

The release of a fibroblasts mitogenic factor from cultured keratinocytes is not surprising as

several studies indicated that keratinocyte-derived factors are likely to be a mixture of several factors with some overlapping properties. It is also likely that some of these biological activities are related to the previously identified cytokines or growth factors such as IL-1α [Sauder et al., 1990; Guolet et al., 1996]. However, there may also be a fibroblast mitogenic factor released from keratinocytes which has not been characterized before. In a keratinocyte/fibroblast co-culture system, Guolet et al. [1996] reported a cellular cooperation between human keratinocytes and dermal fibroblasts such as an increase in DNA synthesis by keratinocytes through secretion of some soluble factors in the culture medium. A protein with an apparent size of 35–40 kDa was also identified by ^{35}S-methionine labeling in conditioned medium from keratinocyte/fibroblasts co-culture system which has not been further characterized [Guolet et al., 1996]. Eisinger et al. [1988] also found that epidermal cells release a factor with a molecular weight of ~1,000 kDa that stimulates their own proliferation and that its biological effect is different from that of EGF. In our study, the results of ^{3}H-thymidine incorporation experiments demonstrate that conditioned medium with higher concentration of TGF-β1 were able to stimulate fibroblasts to proliferate. The overlapping profile of TGF-β1 production by keratinocytes and proliferation of fibroblasts in response to keratinocyte conditioned medium suggest a direct correlation between expression of TGF-β1 and fibroblast cell proliferation. The novelty of our finding is that: (1) the release of both TGF-β1 and this fibroblast mitogenic factor is regulated by the status of keratinocyte differentiation and (2) as low concentration of TGF-β1 is mitogenic for fibroblasts (unpublished observations), this overlapping profiles suggest that this fibroblast mitogenic factor might in fact be keratinocyte released TGF-β1 rather than a new factor. As reported by Thannickal et al. [1998], it is also possible that keratinocyte released TGF-β1 increases fibroblast cell proliferation through induction of fibroblast growth factor receptors. However, this was not the case for keratinocytes because no correlation in pattern of TGF-β1 production and keratinocyte cell proliferation was found. This finding indicates that mono-, but not multi-layer form of keratinocytes release a mitogenic factor for dermal fibroblasts. Interestingly, neither TGF-β1 nor this fibroblast mitogenic factor was strongly expressed by differentiated keratinocytes at the later time points. Instead, keratinocytes expressed a very high level of involucrin mRNA which has been used as a differentiation marker. These findings indicate that expression of TGF-β1 and a differentiation factor such as involucrin is inversely regulated in keratinocytes. Although, the status of keratinocyte morphology may control the expression of TGF-β1 production, cell detachment at the later time points might also be another factor in regulation of TGF-β1 expression. This suggestion is based on our previous findings that cytoskeletal alteration induced changes in fibroblast morphology was associated with an elevation of TGF-β1 mRNA expression [Varedi et al., 1995]. As shown in Figure 1, keratinocyte cell death was not the reason for detachment of regions of keratinocyte sheet in which formation of nodules was observed. This is because keratinocytes began to express involucrin mRNA from Day 5, a time at which keratinocytes in test medium are forming keratinocyte nodules which become apparent within 7 days of culture. The numbers and sizes of these nodules were increased with time. A direct correlation between cell differentiation and detachment of regions of keratinocytes from substratum in our system is not surprising. This is because there is a clear indication in the literature [Boyce and Ham, 1983] that higher concentration of Ca^{++} (1.0 mM) induces keratinocyte differentiation. Since 50% of our test medium is DMEM which contains a higher Ca^{++} concentration (4.99 mM) than that of KSFM (0.09 mM), keratinocytes gradually become differentiated and begin to express several differentiation factors including involucrin in a sequential manner [Watt, 1987]. Similar to the in vivo condition, differentiated keratinocytes tend to migrate upward in the keratinocyte sheets. In our system, movement of differentiated keratinocyte to the upper layer of the keratinocyte sheet seem to be the reason for detachment of keratinocyte sheets from the substratum.

In summary, data presented here clearly demonstrate that the expression of TGF-β1 is governed by the status of keratinocyte differentiation. The overlapping profile of the release of TGF-β1 and an unknown fibroblast mitogenic factor at different time points of the onset of cell differentiation suggest that this unknown mitogenic factor may in fact be keratinocyte

released TGF-β1. Further experiments such as the use of anti-TGF-β1 neutralizing antibody is currently carried out to confirm this point.

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