Dibutyryl Cyclic AMP Modulates Keratinocyte Migration Without Alteration of Integrin Expression

Toshiroh Iwasaki, John D. Chen, Janice P. Kim, Kimberly C. Wynn, and David T. Woodley
Department of Dermatology, Stanford University School of Medicine, Stanford, California, U.S.A.

Cyclic adenosine monophosphate (cAMP) has long been regarded as a second messenger and a regulator of human keratinocyte proliferation. It has been demonstrated that cAMP inhibits keratinocyte proliferation when used at high concentrations. Nevertheless, new recent reports have demonstrated that cAMP may stimulate or inhibit keratinocyte growth depending upon the concentration used. Studies to examine the influence of cAMP upon the migration of other cell types have been contradictory. To determine the direct effect of dibutyryl cAMP (DBcAMP) upon human keratinocyte migration, we used a quantitative locomotion assay using a wide range of DBcAMP concentrations. We found a bi-phasic effect of DBcAMP on keratinocyte migration across connective tissue matrices. Keratinocyte locomotion on the matrices was promoted at 10^{-5} M and 10^{-6} M of DBcAMP, but not at higher or lower concentrations. Time-course experiments demonstrated that the effect of DBcAMP on keratinocyte locomotion and proliferation occurred independently. Fluorescence-activated cell sorter analysis demonstrated that the effect of DBcAMP on the migration of human keratinocytes was independent from the modulation of integrin receptors. Although the cellular mechanisms by which DBcAMP promotes keratinocyte migration is unclear, the addition of DBcAMP or TPA to keratinocyte cultures enhanced the synthesis of a 92-kDa metalloproteinase in association with enhanced cellular migration. These observations suggest a possible link between metalloproteinase expression and cellular migration. *J Invest Dermatol 102:891–897, 1994*

The process of wound healing includes coagulation of blood products, formation of a fibrin clot, inflammation, re-epithelialization, angiogenesis, wound contraction, connective tissue repair, and remodeling [1,2]. Re-epithelialization involves two mechanisms. First, there is a rapid migration of keratinocytes over the wound bed. Second, epidermal cell proliferation occurs within 48–72 h after the epidermal cells migrate [2]. Therefore, both keratinocyte migration and cell division may play important roles in re-epithelialization. Keratinocyte migration is influenced by the connective tissue matrix to which the cells are apposed [3]. For example, matrices of type I collagen, type IV collagen, and fibronectin dramatically promote keratinocyte locomotion, whereas laminin, a large basement membrane zone glycoprotein, uniquely inhibits it [3–5].

Soluble compounds that directly affect keratinocyte locomotion are less well defined. Transforming growth factor-beta (TGF-β) has been shown to promote the epidermal outgrowth of split-thickness explant cultures [6]. However, this is a complex culture system containing fibroblasts, endothelial cells, and dendritic cells in addition to keratinocytes. In addition to the possible occurrence of cell-cell interactions, the explant outgrowth phenomenon is a composite of cellular division and motility.

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Reprint requests to: David T. Woodley, Department of Dermatology, Northwestern University, Northwestern University School of Medicine, 303 E. Chicago Avenue, Chicago, IL 60611.

Abbreviations: cAMP, cyclic AMP, adenosine 3′:5′-cyclic monophosphate; DBcAMP, dibutyryl cyclic AMP, N^6-2′-o-dibutyryl adenosine cyclic monophosphate; MI, migration index; TPA, 12-O-tetradecanoylphorbol-13-acetate.

High concentrations of intracellular cyclic adenosine monophosphate (cAMP) or the exogenous addition of dibutyryl cAMP (DBcAMP) to keratinocyte explant cultures have been reported to inhibit epidermal outgrowth [7,8]. Because these experiments used explant cultures [7] or *ex vivo* assays [8], it is difficult to evaluate the direct effect of these agents on keratinocytes. As noted above, these systems cannot discern the relative contributions made by cell motility and cell division.

Using a pure keratinocyte culture system, Green [9] reported 3×10^{-5} M DBcAMP enhances human keratinocyte proliferation. Falanga *et al* [10] also demonstrated that DBcAMP can stimulate the proliferative potential of normal human keratinocytes. Thus, DBcAMP has been shown to modulate human keratinocyte proliferation. Moreover, DBcAMP applied topically to human wounds stimulates wound healing [11]. However, whether the DBcAMP effect on wounds is due to an influence on cell division, cell motility or both is not known.

In this study, we evaluated the direct effect of DBcAMP on keratinocyte locomotion using a phagokinetic track assay in which cellular migration is quantitated by computerized image analysis [3]. In parallel, we examined the time sequence of the DBcAMP influence on the proliferative potential of human keratinocytes using the thymidine incorporation assay of O'Keefe and Chiu [12]. To determine the cellular mechanisms by which DBcAMP influences keratinocyte migration, we examined the expression of keratinocyte integrin receptors with or without the presence of DBcAMP using fluorescence-activated cell sorter (FACS) analysis. Petersen *et al* [13] demonstrated that 12-O-tetradecanoylphorbol-13-acetate (TPA) dramatically enhanced keratinocyte collagenase. Other studies [14–16] showed that keratinocyte-derived collagenases were enhanced when the cells were stimulated to migrate. Therefore, we evaluated the influences of both DBcAMP and TPA on keratinocyte migration and expression of metalloproteinases.

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MATERIALS AND METHODS

Cell Culture Human keratinocytes from neonatal foreskin were initiated into culture by the method of Rheinwald and Green [17]. Keratinocytes were harvested, plated, and subcultured in low calcium, serum-free KGM (keratinocyte growth medium, Clonetics, San Diego, CA) by the method of Boyce and Ham [18] as modified by Chiu and O'Keefe [19]. Cultures were passaged twice to remove any contaminating fibroblasts, as previously described [13].

Matrix Molecules and Materials DBcAMP, TPA, type I collagen, and pepsinized human placental type IV collagen were purchased from Sigma Chemicals (St. Louis, MO). Human plasma fibronectin was obtained from GIBCO Laboratories (Grand Island, NY). Rat monoclonal antibody to $\beta 1$ (A2B2), mouse monoclonal antibody to $\alpha 2$ (VM1), and mouse monoclonal antibody to $\alpha 6$ (G0H3) integrin subunits were generous gifts from Dr. Randall Kramer at the University of California at San Francisco. Mouse monoclonal antibodies to $\alpha 3$ (P1B5) and $\alpha 5$ (P1B6) integrin subunit were applied by Telios Inc. (San Diego, CA). Fluorescein isothiocyanate (FITC)–conjugated goat anti-mouse IgG and goat anti-rat IgG were purchased from Jackson Immuno Res (Bar Harbor, ME). These antibodies were used in FACS analysis experiments *(vide infra)*.

Migration Assays A modification of the phagokinetic track assay of Albrecht-Buehler [20] was established for human keratinocytes. Pure cell locomotion was quantitated by computer-assisted image analysis as previously described [3]. Briefly, coverslips were coated with colloidal gold salts and placed in 12-well dishes (Corning). The coverslips were then washed with 2 ml of Hanks' balanced salt solution (HBSS) supplemented with calcium and magnesium. Extracellular matrix molecules at various concentrations (0–90 μg/ml) were added in 0.8 ml of HBSS with calcium and magnesium and incubated for 2 h at 37°C. Sixty to seventy percent of radioactively labeled matrix molecules were bound to the coverslips after vigorous washing [3]. Four thousand keratinocytes were plated in each well (816 cells/cm^2) in serum-free KGM. Cultures were incubated for 16–24 or 48 h, washed, and fixed in 3% formaldehyde in phosphate-buffered saline (PBS).

A Jandel Video Analysis computer system was used for quantitative analysis of keratinocyte locomotion. Five random, non-overlapping fields from each dish were captured by the computer and the percentage of the total field area taken up by migration tracks was calculated for each field. The percentage of total field area taken up by migration tracks was defined as the migration index (MI). Each condition was assayed in duplicate.

The KGM group without added DBcAMP or TPA was treated as the control baseline group within each experimental set. The MIs for each DBcAMP or TPA concentration were determined. Each MI represents the mean track area divided by the field area from at least ten non-overlapping fields viewed by the video camera and analyzed by the computer. The percent difference between the MIs of those assays performed in the presence of DBcAMP or TPA and those assays without the addition of these factors (i.e., control baseline group) was calculated over a wide range of different concentrations.

Statistical Analysis Results were evaluated using the Student t test. MIs between the baseline control group (KGM group without DBcAMP or TPA) and each concentration of DBcAMP or TPA group were compared. The level of significance was defined as $p < 0.05$.

Thymidine Incorporation Assay For the evaluation of the effect of DBcAMP on keratinocyte proliferative potential, we used the thymidine incorporation assay for keratinocytes described by O'Keefe and Chiu [12]. All assays were performed in 24-well Corning trays with 4 μg/ml of type I collagen using 1 ml of KGM with or without the addition of DBcAMP. Keratinocytes were incubated in KGM with each concentration of DBcAMP for 24 or 48 h.

After incubation with DBcAMP, 2 μCi of ^{3}H-thymidine (ICN Laboratories, Irvine, CA) was added to each well and pulsed for 6 h. Keratinocytes were washed twice with cold PBS and then washed once with ice-cold 5% trichloroacetic acid. Keratinocytes were solubilized by 0.1% sodium dodecylsulfate (SDS). Solubilized radioactivity was counted in a scintillation counter with Safety Solve (Research Products International, Mount Prospect, IL). Each group of thymidine incorporation assay was composed of six wells.

FACS Analysis for Integrin Receptor Subunits Keratinocytes were incubated with or without DBcAMP for 16 h. Keratinocytes were then incubated with phosphate-buffered saline (PBS) containing 2 mM ethylene diaminetetraacetic acid for 30 min and 1×10^5 cells were resuspended in PBS with 1% fetal bovine serum per condition. Cells were incubated with primary antibodies to the integrin receptor subunits (1:100, final concentration) for 60 min at 4°C. Then, cells were incubated with FITC-conjugated secondary antibody (1:100) for 20 min at 4°C. The cells were fixed with 0.5% formaldehyde in PBS and FACS analysis was done using FACStar or FACScan (Becton Dickinson Immunocytometry Systems Inc., San Jose, CA) flow cytometer.

Zymogram Analysis of Type IV Collagenase/Gelatinase Zymogram analysis was performed according to the method described previously [16]. Briefly, conditioned media from cultured keratinocytes incubated with or without DBcAMP or TPA for 24 h was suspended in loading dye (0.25 M Tris-HCl, pH 6.8, 5% SDS, 0.1% bromophenol blue, 4% sucrose). Samples were not reduced or heated, and were loaded on a 10% SDS polyacrylamide gel containing 0.1% gelatin. Following electrophoresis, the gel was soaked in 2.5% Triton X-100 for 30 min at room temperature and subsequently incubated in 0.05 M Tris containing 5 mM calcium chloride at 37°C for 48 h. The gels were stained for 1 h with Coomassie brilliant blue and then destained.

Enzyme-Linked Immunosorbant Assay (ELISA) for Interstitial Collagenase Keratinocyte conditioned media with or without DBcAMP were directly assayed for collagenase activity using ELISA [21]. Samples were incubated with rabbit anti–type I collagenase antibody (a generous gift from Eugene A. Bauer, M.D., Department of Dermatology, Stanford University, Stanford, CA) at 4°C overnight, then incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (diluted 1:1000) at 37°C for 1 h. Optical densities were measured at 410 nm by an ELISA reader after color development.

RESULTS

Keratinocyte Migration Without Matrix Keratinocytes apposed to coverslips without extracellular matrices exhibited minimal locomotion. The cells made very small round halo-shaped tracks. The MIs of keratinocytes apposed to the coverslips without matrix components were 1.7 ± 0.6 (mean $\pm$ SEM). The presence of DBcAMP had no effect on the migration of keratinocytes apposed to coverslips without extracellular matrix molecules.

Keratinocyte Migration On Collagen Matrices In contrast, human keratinocytes demonstrated vigorous migration when apposed to a matrix of interstitial (type I) collagen. The migrating keratinocytes made oblong and long linear tracks in addition to some halo-shaped tracks. MIs of keratinocytes migrating on interstitial collagen were 16.4 ± 1.3 when 4 μg/ml of collagen was absorbed to the dishes. When 15 μg/ml of collagen was absorbed to the dishes, the migrating keratinocytes produced higher MIs (33.7 ± 2.7). When the keratinocytes were apposed to an interstitial collagen matrix, the presence of DBcAMP altered the level of migration depending upon the concentration used (Fig 1*A,B*).

On a "suboptimal" matrix of type I collagen (4 μg/ml), concentrations of DBcAMP between 10^{-6} M and 10^{-5} M significantly enhanced human keratinocyte locomotion over that of control keratinocytes by 46% ($p < 0.05$) and 53% ($p < 0.05$), respectively (Fig 1*A,B*). When the concentration was increased to 10^{-3} M, locomotion was actually reduced. However, this high concentration of DBcAMP is toxic to human keratinocyte cultures [10]. Very dilute concentrations (10^{-8} M and 10^{-7} M) had minimal influence on the migration of keratinocytes apposed to collagen.

When an "optimal" type I collagen matrix was provided (15 μg/ml), DBcAMP at 10^{-6} M also significantly enhanced the keratinocyte migration by 32% ($p < 0.05$) over the control assays without DBcAMP (Fig 2*B*). However, all other concentrations of DBcAMP did not alter the migration of human keratinocytes on an "optimal" type I collagen matrix to a statistically significant level.

Similar to an interstitial collagen matrix, human keratinocytes apposed to basement membrane (type IV) collagen demonstrated high levels of migration. On a "suboptimal" matrix of type IV collagen (4 μg/ml), the MIs were 7.5 ± 0.4. The migration was greater (MIs = 13.9 ± 1.8) on an "optimal" type IV collagen matrix (15 μg/ml).

When the keratinocytes were apposed to an "optimal" type IV collagen matrix, the presence of DBcAMP at concentrations of 10^{-6} M and 10^{-5} M significantly increased migration by 72% ($p < 0.05$) and 60% ($p < 0.05$), respectively, when compared to control assays without DBcAMP (Fig 2*A,B*). Other concentrations of DBcAMP did not alter keratinocyte migration on basement mem-

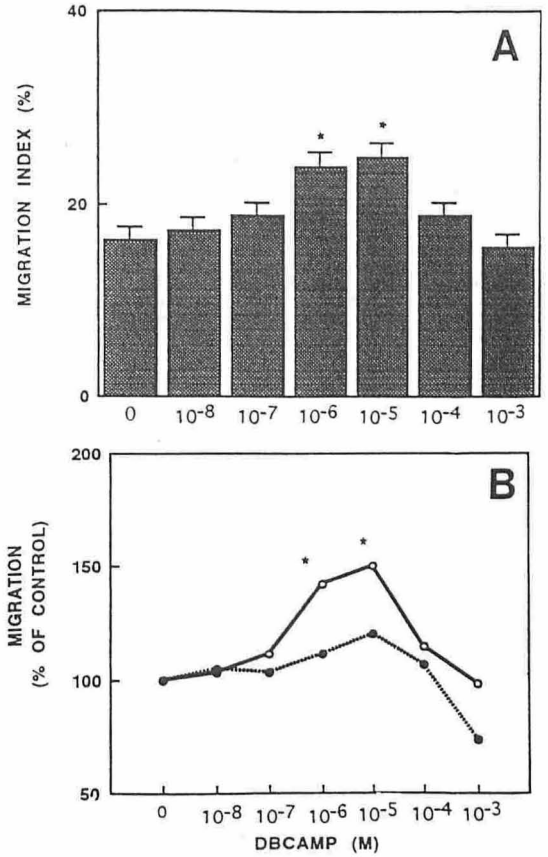

Figure 1. Effect of DBcAMP on human keratinocyte locomotion on "suboptimal" concentrations (4 μg/ml) of type I and type IV collagen. Cover slips were coated with colloidal gold salts and placed in 12-well dishes. Four micrograms per milliliter of type I or IV collagens were added and incubated for 2 h at 37°C. Human keratinocytes (4×10^3 cells/cm^2) were plated in each well in KGM cultured for 16 h and fixed in 3% formaldehyde in PBS. Keratinocyte migration tracks were measured by computerized image analysis (see *Materials and Methods*). Ten different fields were analyzed per condition. Results were evaluated with the Student t test. MIs on type I collagen in the presence of various concentrations of DBcAMP is shown in *A*. *B* shows the percent difference between the MIs of the experimental dishes containing DBcAMP and the control dishes on type I collagen *(open circles)* and type IV collagen *(closed circles)*. *Bar,* mean MIs from at least 10 non-overlapping fields. *Error bars,* SEM. * $p < 0.05$.

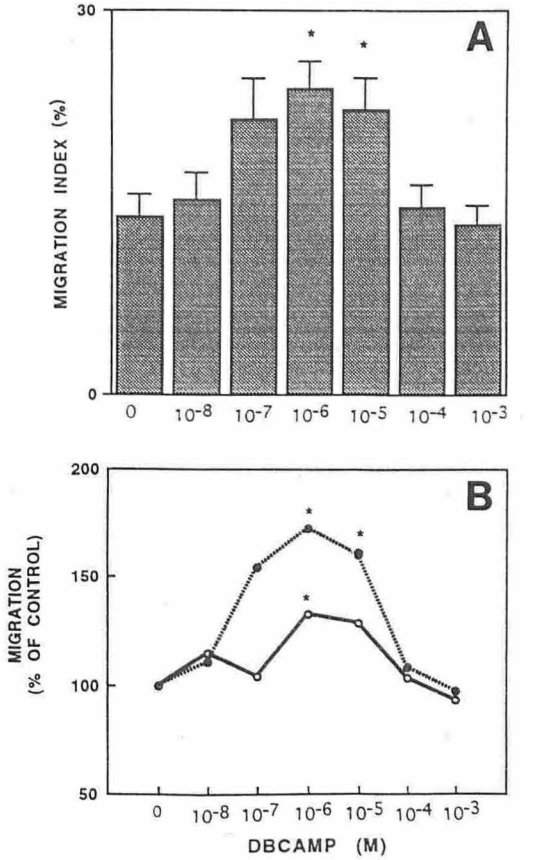

Figure 2. Effect of DBcAMP on human keratinocyte locomotion on "optimal" concentrations (15 μg/ml) of type I and type IV collagen. The experimental methods were identical to those in Fig 1. The results were evaluated with the Student t test. In *A*, the average MIs of cell migrating on type IV collagen with and without DBcAMP are demonstrated. In *B*, the percent difference between the MIs of the experimental dishes containing DBcAMP and the control dishes (those without DBcAMP) is shown for each situation. *Bar,* mean MIs from at least 10 non-overlapping fields. *Error bars,* SEM. * $p < 0.05$.

brane collagen significantly. Likewise, when a "suboptimal" matrix of type IV collagen (4 μg/ml) was used, DBcAMP did not significantly alter migration (Fig 1*B*).

The effect of TPA on keratinocyte migration was evaluated by the addition of 10 ng/ml of TPA to keratinocytes apposed to a matrix of type I collagen (4 μg/ml) for 16 h. This concentration of TPA has been demonstrated to increase the constitutive synthesis of collagenase by human keratinocytes in culture [13]. TPA also changes the cell morphology of human epidermal carcinoma cells and alters protein kinase C expression [22]. Keratinocyte migration on type I collagen was enhanced by 40% when the cells were exposed to 10 ng/ml of TPA (Fig 3).

Migration on Matrix Glycoproteins Laminin is a major noncollagenous glycoprotein in basement membranes [23]. Fibronectin is a major glycoprotein found in plasma and in a number of connective tissues including dermis [24]. Both human fibroblasts and keratinocytes synthesize fibronectin [25,26] and laminin [27]. It has been shown previously that a matrix of fibronectin induces human keratinocyte migration, whereas a laminin matrix inhibits it [3]. Preliminary experiments demonstrated that the fibronectin-induced migration is concentration dependent and that a plateau is reached when 60 μg/ml of fibronectin is absorbed to the coverslips. For the experiments here, we selected a suboptimal amount of fibronectin of 20 μg/ml, which provides MIs approximately 50% of the maximal level. As shown in Fig 4*A,B*, the only concentration of DBcAMP that enhanced keratinocyte migration on a fibronectin matrix was a concentration of 10^{-5} M. When an optimal fibronectin matrix (60 μg/ml) was used, DBcAMP did not significantly promote migration at any of the concentrations tested (data not shown).

Laminin inhibits keratinocyte locomotion, and the inhibition is maximal at 90 μg/ml [4]. At the concentrations tested, DBcAMP neither significantly increased or decreased keratinocyte migration on a laminin matrix (data not shown).

Time Course of Keratinocyte Migration Versus Proliferative Potential Induced by DBcAMP To determine when the effect of DBcAMP on keratinocyte migration occurred, time-course experiments were performed in which human keratinocytes were apposed to an optimal matrix of type I collagen and the levels of migration assessed with or without the presence of 10^{-5} M DBcAMP at 24 and 48 h. As shown in Fig 5, keratinocyte migration on type I collagen without the presence of DBcAMP is approximately the same at 24 and 48 h. Compared with the assays performed without DBcAMP, the presence of DBcAMP in the dishes enhances keratinocyte migration by 40% at 24 h ($p < 0.05$). Within this time frame, there is no DBcAMP effect on cell prolifer-

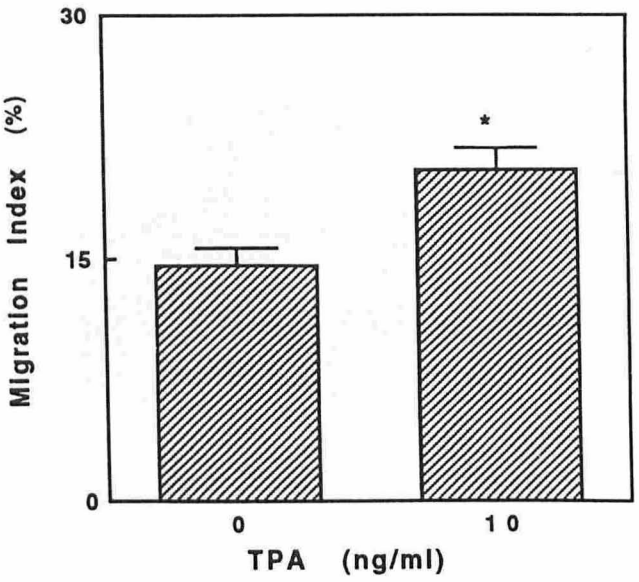

Figure 3. Effect of TPA on keratinocyte locomotion on type I collagen. Four micrograms per milliliter of type I collagen were added to the coverslips, and the wells were incubated for 2 h at 37°C. The methods were then identical to those in Fig 1, except that, rather than the addition of DBcAMP, keratinocytes were incubated for 16 h with or without 10 ng/ml of TPA. Results were evaluated with the Student t test. *Bar,* mean MIs from at least 10 non-overlapping fields. *Error bars,* SEM. * $p < 0.05$.

ation as judged by the thymidine incorporation assay *(vide infra)*. In the standard migration assay, the keratinocytes have essentially stopped migrating between 24 and 30 h. We observed little or no enhancement of keratinocyte migration with or without the presence of DBcAMP at 48 h. Therefore, DBcAMP provided no further enhancement in keratinocyte migration between the 24-h and 48-h period. It appears that most of the enhanced migration due to the presence of DBcAMP occurs within the first 24 h. However, during the period between 24 and 48 h when keratinocyte migration was not enhanced, this is the time period when DBcAMP induced an enhancement in the proliferative potential of the cells *(vide infra)*.

DBcAMP has been shown to increase the proliferative potential of human keratinocytes [10]. To examine when the effect of DBcAMP upon the proliferative potential of human keratinocytes ocurred, we performed the thymidine incorporation assay of O'Keefe and Chiu [12] on keratinocyte cultures at 24 and 48 h using the concentrations of DBcAMP indicated in Fig 6. As shown in Fig 6, at 24 h none of the concentrations of DBcAMP tested increased the proliferative potential of the cells. However, by 48 h, the presence of 10^{-5} M of DBcAMP significantly enhanced keratinocyte incorporation of thymidine above that of controls.

Integrin Receptor Expression Five different α subunits and two β subunits of integrin receptors are recognized in human skin [28]. The known ligand of the $\alpha 2$ subunit is collagen. The ligands for the $\alpha 3$ subunit include laminin, collagen, and epiligrin. The $\alpha 5$ subunit recognizes fibronectin. The $\alpha 6/\beta 4$ integrin complex is located within hemidesmosomes [29]. The $\beta 1$ and $\alpha 3$ integrin receptor subunits were highly expressed in keratinocytes with or without DBcAMP. The $\alpha 2$, $\alpha 5$, and $\alpha 6$ integrin receptor subunits were moderately expressed on normal human keratinocytes with or without DBcAMP. However, the presence of DBcAMP did not modulate the expression of any of the integrin receptors on keratinocytes (Fig 7).

Collagenase Expression Exogenous addition of collagenase has been shown to enhance the motility of some tumor cells [30]. Human keratinocytes constitutively produce and secrete type I collagenase and 72- and 92-kDa type IV collagenases [13–16]. The

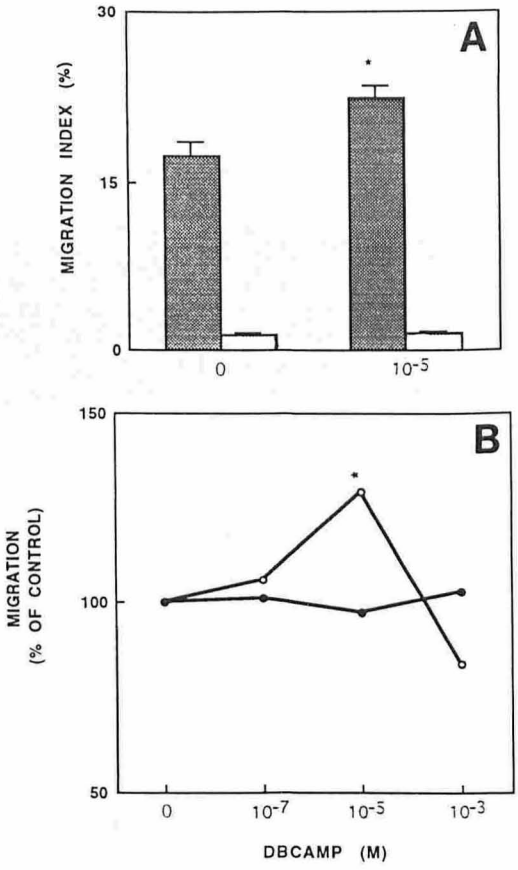

Figure 4. Effect of DBcAMP on keratinocyte locomotion on matrices of fibronectin (20 μg/ml) and bovine serum albumin (control). The methods were otherwise identical to those in Fig 1. Results were evaluated with the Student t test. The matrices to which the cells are apposed in *A* are fibronectin *(dark bars)* or albumin *(light bars)*. In *B*, the cells were apposed to matrices of fibronectin *(open circles)* or albumin *(closed circles)*. The percent difference of the MIs between experimental assays (containing DBcAMP) and control assays (without DBcAMP) are demonstrated for both matrices. *Bar,* mean of MIs from at least 10 non-overlapping fields. *Error bars,* SEM. * $p < 0.05$.

control and DBcAMP groups of keratinocyte cultures both synthesized and secreted 72- and 92-kDa type IV collagenase by zymography analysis (Fig 8*a*). There was no difference in the expression of 72-kDa type IV collagenase between control and DBcAMP groups. However, the presence of 10^{-4} and 10^{-6} M concentrations of DBcAMP promoted the expression of the 92-kDa type IV collagenase by the keratinocytes as compared to control. The addition of TPA to the cultures also enhanced the expression of both the 92- and 72-kDa type IV collagenase in a dose-dependent fashion (Fig 8*b*). The concentrations of type I collagenase in keratinocyte culture media range between 12.0 and 16.9 ng/ml, values which are 40 to 60 times lower than those of fibroblasts extracts [21]. The presence of DBcAMP in the cultures did not alter the secretion of type I collagenase as measured by ELISA (data not shown). These results suggest that the 92-kDa type IV collagenase is specifically influenced by the addition of DBcAMP.

DISCUSSION

cAMP acts as a second messenger of the cells [31], and the elevation of intracellular cAMP by the exogenous addition of DBcAMP to cells or tissues mediates various kinds of actions such as the dilatation of peripheral vessels, the enhancement of glycogenolysis, and the inhibition of platelet aggregation [32]. Most of the studies involving cAMP and keratinocytes have focused on cellular proliferation. There are no studies that have evaluated the direct effect of DBcAMP on human keratinocyte migration.

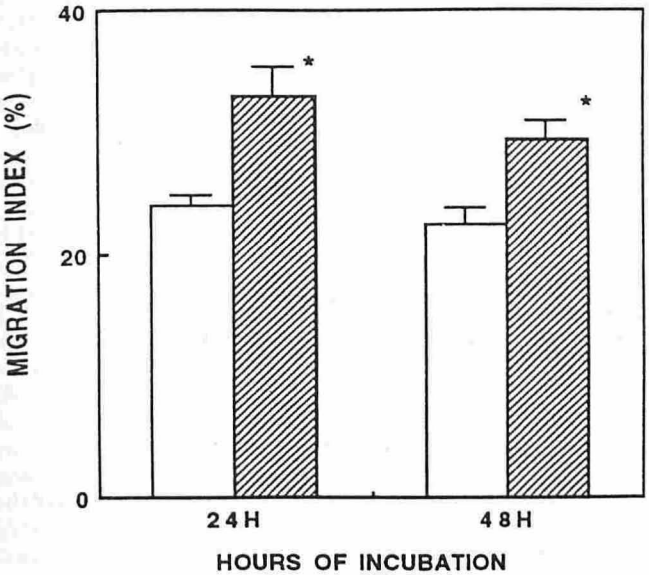

Figure 5. Human keratinocyte locomotion on type I collagen after 24 and 48 h. Migration assays performed at 24 and 48 h with 10^{-5} M of DBcAMP *(striped bars)* or without DBcAMP *(white bars)*. *Bar,* mean of MIs from at least 10 non-overlapping fields. *Error bars,* SEM. * $p < 0.05$ derived by a direct comparison of assays with and without DBcAMP at 24 and 48 h.

However, the effect of cAMP on cell locomotion has been studied in several other cell types using cAMP-elevating agents such as DBcAMP [33], cholera toxin [34], or β-receptor stimulants [35]. Most of these studies show an inhibition of cell locomotion by an elevation of intracellular cAMP. For example, cholera toxin and insulin inhibit the locomotion of 3T3 cells [34]; retinal pigment

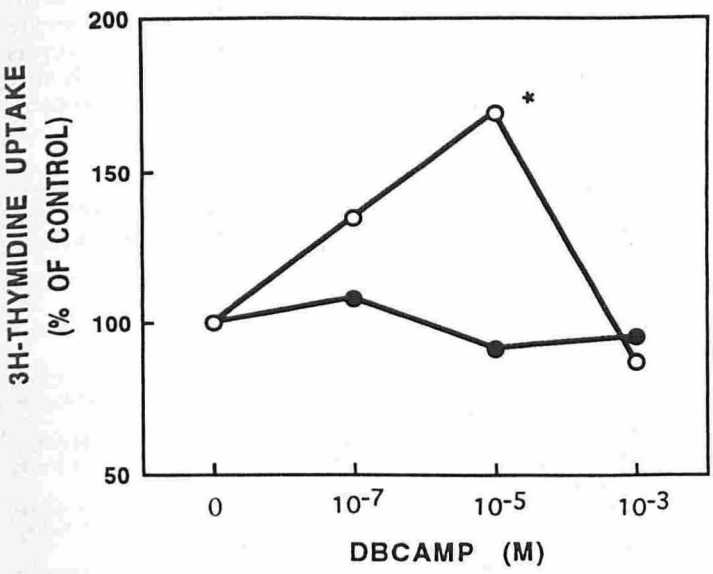

Figure 6. Effect of DBcAMP on ^{3}H-thymidine incorporation (proliferative potential) after 24 and 48 h of incubation. Keratinocytes were incubated on 4 μg/ml of type I collagen in KGM with each concentration of DBcAMP for 24 or 48 h. After the incubation with DBcAMP, 2 μCi of ^{3}H-thymidine was added to each well and pulsed for 6 h. Keratinocytes were washed with PBS and 5% trichloracetic acid. Cells were solubilized by 0.1% SDS. Solubilized radioactivity was counted in a scintillation counter. Each point on the graph represents six identical assays. The mean values of each experimental situation were compared with parallel control assays in which no DBcAMP was added. The percentage difference of thymidine incorporation between the experimental and control assays are demonstrated at 24 h *(closed circles)* and 48 h *(open circles)*.

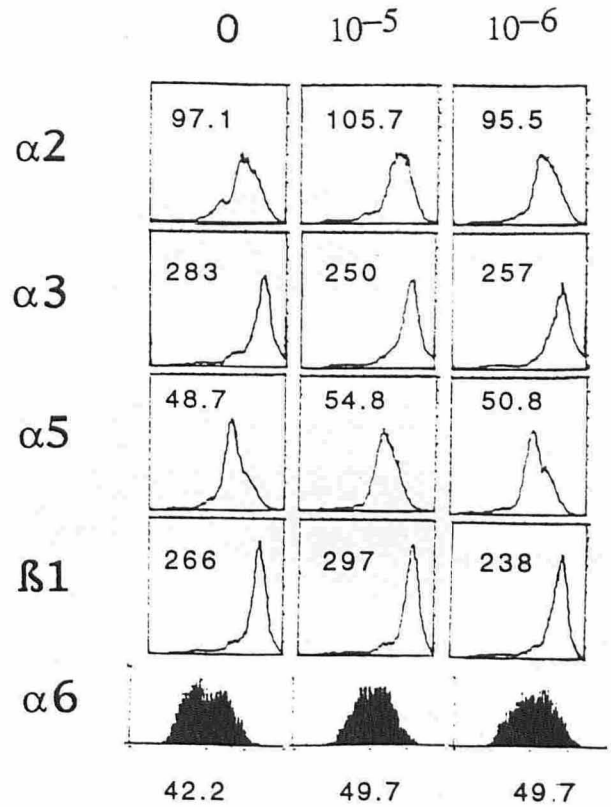

Figure 7. Effect of DBcAMP on the expression of integrin receptor subunit by FACS analysis. Keratinocytes were incubated with or without DBcAMP for 16 h. Cells were incubated with antibody to each integrin receptor subunit and then incubated with FITC-conjugated secondary antibody. Cells were analyzed by a fluorescence activated cell sorter. *Horizontal lines,* intensity of fluorescence; *vertical lines,* number of cells. The number enclosed within in each graph is the modal fluorescence value.

epithelial cell migration is inhibited by DBcAMP [33]. Similarly, spontaneous motility of rabbit peritoneal neutrophils is inhibited by a number of substances that elevate cAMP [36]. However, there have been few reports on the assessment of the effect of DBcAMP on keratinocyte locomotion. Using the wound closure of newt limbs, Donaldson *et al* [37] suggested that the effect of cAMP on epidermal cell migration depends on its concentration. Stenn and Depalma [2] support this notion and suggest that cAMP at specific concentrations may modify the movement of keratinocytes.

Our experiments show that DBcAMP at appropriate concentrations can significantly enhance the migration of keratinocytes on connective tissue matrices of fibronectin, interstitial collagen, and basement membrane collagen. The enhancement is biphasic because high and low concentrations of DBcAMP have either no effect or a slight suppressive effect upon keratinocyte locomotion. High concentrations of DBcAMP (10^{-3}M) are toxic to human keratinocytes [10]. Falanga *et al* [10] also reported that DBcAMP stimulated the proliferation of keratinocyte cultures at concentrations of 10^{-5} M and 10^{-6} M and suppressed keratinocyte proliferation at 10^{-3} M. We believe that this explains why keratinocyte locomotion is either unaltered or slightly suppressed when high concentrations of DBcAMP are used. When low concentrations of the DBcAMP are used, we believe that locomotion is not altered because the agent is too dilute to exert a biologic effect. Like keratinocytes, dermal microvascular endothelial cells proliferate in a bi-phasic manner to concentrations of DBcAMP [38]. Our experiments on cell migration show that the effect of DBcAMP on keratinocyte locomotion has a similar bi-phasic effect.

Sarret *et al* [39] have shown that human keratinocyte locomotion and keratinocyte cellular division occur by independent mechanisms. The results here support this notion. The enhancement of

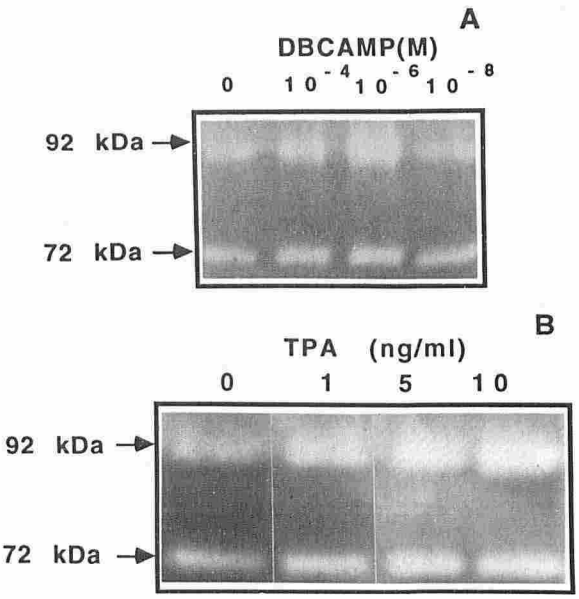

Figure 8. Zymograms of conditioned medium from keratinocytes cultured for 24 h on type I collagen in the presence of DBcAMP *(A)* or TPA *(B)*. The *white bands* on each zymogram are proteins in the medium with metalloproteinase activity. The effects of DBcAMP and TPA on type IV collagenase expression human keratinocytes in zymography. *A,* keratinocytes were cultured on 4 μg/ml of type I collagen in the presence of 10^{-4} M, 10^{-6} M, 10^{-8} M, or no DBcAMP. The media samples (equalized for protein content) were analyzed by zymography. *Arrows,* positions of a 92-kDa and a 72-kDa type IV collagenase. *B,* the expression of a 92- and a 72-kDa type IV collagenase when cells were incubated with 0, 1, 5, or 10 ng/ml of TPA. The 92-kDa and 72-kDa *arrows* indicate the positions of the 92-kDa and 72-kDa type IV collagenases.

keratinocyte migration by the presence of DBcAMP occurs predominantly within the first 24 h. In contrast, DBcAMP does not invoke an augmentation in the proliferative potential of the cells until 48 h. This is in accordance with the studies of Falanga *et al* [10] who demonstrated that the enhancement of keratinocyte growth by DBcAMP occurs at 48 h. Taken together, the parallel locomotion and thymidine incorporation studies suggest that DBcAMP promotes keratinocyte locomotion independently from its effects on cell division. DBcAMP promotes keratinocyte locomotion in the first 24 h of culture, at a time when no effect upon the proliferative potential of the cells can be observed.

In our study, the time-course experiments demonstrated that the DBcAMP enhancement of keratinocyte migration occurred within the first 24 h. Within the first 24-h period, DBcAMP does not alter the thymidine incorporation of the cells (Fig 6). During this period, however, the cells are migrating and the presence of DBcAMP enhances this migration (Fig 5). The effect of DBcAMP upon thymidine incorporation (the proliferative potential of the cells) occurs later, after 24 h, at a time when the cells have stopped migrating (Fig 6). Therefore, the DBcAMP effect upon keratinocyte migration can act independently from the DBcAMP influence upon the proliferative potential of the cells.

Human keratinocyte locomotion on extracellular matrices such as fibronectin, interstitial collagen, and basement membrane collagen may be mediated by integrin receptors [40]. Blocking the $\alpha 2\beta 1$ integrin receptor inhibited keratinocyte migration on collagen matrices but not on fibronectin. Blocking $\alpha 5\beta 1$ integrin receptor inhibited keratinocyte migration on fibronectin but not on collagen [40]. These findings show that the $\alpha 2$ or $\alpha 5$ integrin receptor subunits contribute to the keratinocyte migration on collagen matrices or fibronectin, respectively. The $\alpha 3\beta 1$ integrin uniquely enhanced migration on fibronectin and collagen [40]. Because these studies showed that integrins may play a role in keratinocyte migration, we examined the effect of DBcAMP on the integrin receptor expression using FACS analysis. However, the presence of DBcAMP was found not to alter the expression of integrin receptors on cultured keratinocytes. The enhancement of keratinocyte locomotion by DBcAMP appears to be independent of integrin expression.

Terranova *et al* [30] showed that migration of human oral squamous cell carcinoma cells (SCC-4) was enhanced by type IV collagenase derived from conditioned medium of SCC-4 cells and the collagenase inhibitor, SC-44483, inhibited the motility of SCC-4 cells in a dose-dependent manner. These data suggested that type IV collagenase may directly enhance the motility of tumor cells. This has not been shown to occur in cells with a non-malignant phenotype. However, the experiments reported here show that DBcAMP stimulates the synthesis and secretion of the 92-kDa type IV collagenase although leaving the expression of the 72-kDa enzyme and type I collagenase unaltered. Moreover, the induction of keratinocyte collagenase by TPA that enhances protein kinase C was associated with enhanced keratinocyte migration. Taken together, these experiments suggest that one mechanism by which DBcAMP may stimulate keratinocyte migration is related to the enhanced secretion of type IV collagenase.

Another striking result of these studies was the interaction of the DBcAMP effect with the extracellular matrix effect. For example, DBcAMP could not exert an effect on migration when the keratinocytes were apposed to either a suboptimal (4 μg/ml) matrix of basement membrane (type IV) collagen or an optimal (60 μg/ml) matrix of fibronectin. We do not understand the mechanisms involved in the interactions between the cells, matrix and soluble factors that account for these observations. However, it may be that when cells are maximally stimulated to migrate by an optimal fibronectin matrix that no further migration can be invoked by the addition of DBcAMP on this particular matrix. Alternatively, for DBcAMP to have an effect on the migration of keratinocytes on basement membrane collagen, they must be provided with an optimal matrix. It is conceivable that DBcAMP adsorbs itself to the type IV collagen and in some way becomes more accessible to the cells. Whatever the mechanism involved, it appears that keratinocyte locomotion can be modulated by the interactions of soluble factors and matrix working in the same direction. Moreover, it is likely that *in vivo,* keratinocyte migration is ultimately modulated by a combination of soluble factors and extracellular matrix.

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