

# Fibronectin and $\alpha 5$ Integrin Regulate Keratinocyte Cell Cycling

## A Mechanism for Increased Fibronectin Potentiation of T Cell Lymphokine–driven Keratinocyte Hyperproliferation in Psoriasis

Zsuzsanna Bata-Csorgo,\* Kevin D. Cooper,<sup>†§</sup> Kathleen M. Ting,<sup>‡</sup> John J. Voorhees,\* and Craig Hammerberg<sup>‡</sup>

\*Department of Dermatology, University of Michigan Medical School, Ann Arbor, Michigan 48109; <sup>‡</sup>Department of Dermatology, Case Western Reserve University, University Hospitals of Cleveland; and <sup>§</sup>Department of Dermatology, Veterans Administration Medical Center, Cleveland, Ohio 44106

### Abstract

In addition to being T lymphocyte–driven, psoriasis may be due in part to abnormal integrin expression. Normal-appearing (uninvolved) skin from psoriatic patients was examined to determine whether altered fibronectin or its receptor expression is detectable before development of psoriatic lesions. In contrast to skin from normal subjects, we detect by immunofluorescence the abnormal presence of plasma fibronectin in the basal cell layer of the epidermis of psoriatic uninvolved skin. Furthermore, increased fibronectin exposure superinduces the *in vitro* cell cycle induction and expansion of psoriatic nonlesional keratinocytes in response to a cocktail of T cell lymphokines. Fibronectin alone also appeared to increase cell cycle entry among uninvolved but not normal keratinocytes. Concordantly, the  $\alpha 5$  integrin fibronectin receptor, but not  $\alpha 2$  or  $\alpha 3$ , is overexpressed in the *in vivo* nonlesional psoriatic epidermis. The involvement of  $\alpha 5\beta 1$  in the early outgrowth of clonogenic keratinocytes in the *ex vivo* culture was demonstrated by the ability of anti- $\alpha 5$  mAb to inhibit keratinocyte growth on fibronectin. Thus, the fibronectin receptor appears to be one of the components required for the development of the hyperresponsiveness of psoriatic keratinocytes to signals for proliferation provided by lymphokines produced by intralesional T lymphocytes in psoriasis. (*J. Clin. Invest.* 1998. 101:1509–1518.)

**Key words:** psoriasis • fibronectin •  $\alpha 5\beta 1$  integrin • keratinocytes • T lymphokines

### Introduction

Psoriasis is a chronic inflammatory skin disease with very distinct hyperproliferation of the normally quiescent basal keratinocyte population which contains the keratinocyte stem cells (1). Because epidermal hyperplasia is a key pathologic phenomenon in psoriasis, uncovering its triggering mechanisms is fundamental to research on pathomechanisms and new therapeutic strategies in this disease. That skin-infiltrating T lymphocytes play an important role in the induction and maintenance

of keratinocyte hyperproliferation in the lesion is strongly supported by numerous observations (for a review, see reference 2). We have found previously that lesional psoriatic CD4<sup>+</sup> T cells produce a balance of lymphokines that are growth-stimulatory for keratinocytes (3, 4) and in particular, psoriatic uninvolved quiescent keratinocytes are hyperresponsive to lesional T cell lymphokines relative to normal keratinocytes (4). Our data have indicated that IFN- $\gamma$  in the context of GM-CSF and IL-3 plays a critical role in this growth-regulatory effect.

In addition to soluble mitogens (growth factors, lymphokines), growth of anchorage-dependent cells is regulated by insoluble extracellular matrix molecules (5–7). Growth factors and extracellular matrix molecules could interact in several ways to regulate cell growth (8). It has long been known that basement membrane fenestration (or basal keratinocyte herniation) is an integral part of the psoriatic lesion formation (9, 10), and it has been suggested that (plasma-originated) fibronectin could leak into the epidermis, simulating a micro-wound situation for basal keratinocytes (11, 12). In addition to functions in keratinocyte adherence and migration (for a review, see reference 13), fibronectin, through its main receptor  $\alpha 5\beta 1$  integrin, can provide anchorage-independent direct cell cycle regulatory signals (14, 15). Expression, topography, and function of integrin receptors appear severely altered not only in involved but also in uninvolved skin of psoriatics (16, 17). In normal skin, the  $\alpha 5\beta 1$  integrin is poorly expressed and diffusely distributed on basal keratinocytes, and is not organized into defined adhesive structures. By contrast, psoriatic involved and uninvolved keratinocytes show clear  $\alpha 5\beta 1$  fibronectin receptor staining *in vivo*, and the  $\alpha 5\beta 1$  molecules are organized into discrete focal contacts *in vitro*, without obvious increase in expression and synthesis (16). Recently, Carroll et al. have constructed transgenic mice in which they induced suprabasal epidermal expression of human integrins  $\alpha 2$ ,  $\alpha 5$ , and  $\beta 1$ , which are not normally expressed on terminally differentiated keratinocytes. Interestingly, mice with overexpressed suprabasal integrin expression exhibited epidermal hyperproliferation, perturbed keratinocyte differentiation, and skin inflammation, all characteristics of psoriasis (18).

In this study, using a quantifiable model in which fresh *ex vivo* human undifferentiated (keratin 1/keratin 10 [K1/K10]<sup>+</sup>)<sup>1</sup> quiescent (proliferating cell nuclear antigen [PCNA]<sup>−</sup>) keratinocytes can be stimulated to exit G0 and enter the proliferative phase of cell cycle almost synchronously, we show that (a) induction of quiescent psoriatic uninvolved but not normal ke-

Address correspondence to Dr. Kevin D. Cooper, Department of Dermatology, University Hospitals of Cleveland, 11100 Euclid Avenue, Cleveland, OH 44106-5028. Phone: 216-844-3111; FAX: 216-368-0212; E-mail: kdc@po.cwru.edu

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keratinocytes into cell cycle is increased significantly by recombinant IFN- $\gamma$  only in the context of other lymphokines (GM-CSF and IL-3), which models the lymphokine profile of *in vitro*-derived lesional psoriatic T cell supernatants; (b) fibronectin potentiates the growth-stimulatory effect of T cell lymphokines (IFN- $\gamma$ , GM-CSF, and IL-3) in psoriatic uninvolved keratinocytes; (c) fibronectin (compared with uncoated tissue culture plastic) increases cell cycle entry, without increase in adhesion, among uninvolved but not normal keratinocytes, even in the absence of T cell lymphokines. Additional evidence of abnormal and relevant psoriatic uninvolved keratinocyte fibronectin reception includes the following: (d) plasma-derived fibronectin could be found around basal keratinocytes in uninvolved psoriatic skin from the close proximity of a lesion; (e)  $\alpha 5$  integrin is overexpressed on psoriatic uninvolved keratinocytes relative to normal keratinocytes *in vivo*; and (f) fibronectin can regulate keratinocyte growth through the fibronectin receptor ( $\alpha 5\beta 1$ ). These results support the possibility that psoriatic keratinocytes may indeed have disturbed integrin functions which precede and may precipitate the hyperproliferative state.

## Methods

**Human subjects.** Keratome biopsies were taken from normal-appearing symptom-free uninvolved or lesional buttock area skin of psoriasis vulgaris patients and normal volunteers. Lesional skin was characterized by inflamed but stable psoriatic plaques. A medication-free period of 1 mo for oral and 2 wk for topical treatments was required in both groups before the procedures. Informed consents were obtained from all donors.

**Tissue staining.** Frozen sections (4  $\mu$ m) in OCT (Tissue Tek II; Miles Laboratories, Inc., Elkhart, IN) were fixed in acetone and stained with anti-human fibronectin (Sigma Chemical Co., St. Louis, MO) at 1:100 dilution and an isotype control rabbit IgG at similar concentration (Endogen, Inc., Boston, MA). FITC-conjugated anti-rabbit IgG was used as secondary antibody (Caltag Laboratories, Inc., San Francisco, CA) at 1:20 dilution.

**Flow cytometric analysis of freshly separated epidermal cells.**  
**Staining procedure.** The epidermis was separated from the dermis after overnight dispase (Collaborative Biomedical Products, Bedford, MA) incubation at 4°C. Epidermal cell suspensions were prepared using 0.25% trypsin (USB Biologicals, Cleveland, OH) and 0.01% DNase (Sigma Chemical Co.) as described previously (4). For integrin staining, cells were fixed in 1% formalin for 15 min on ice; for PCNA and K1/K10 staining, they were fixed in -20°C cold 70% ethanol and kept at -20°C at least overnight before staining. The following antibodies and immunoglobulins as isotype controls were used in conventional indirect staining procedures: anti- $\alpha 2$  (CD49b; PharMingen, San Diego, CA) 1:100 dilution, anti- $\alpha 3$  (CD49c; Oncogene Science Inc., Uniondale, NY) 1:20 dilution, anti- $\alpha 5$  (CD49e; PharMingen) 1:50 dilution, anti-PCNA (Boehringer Mannheim Biochemicals, Indianapolis, IN) 1:100 dilution, anti-K1/K10 (AE2; ICN Biomedicals Inc., Costa Mesa, CA) 1:50 dilution, and mouse IgG1 and mouse IgG2a (Sigma Chemical Co.) at identical concentrations as the antibodies. Secondary antibodies used were anti-mouse IgG1-FITC and anti-mouse IgG2a-phycoerythrin (PE) (Boehringer Mannheim Biochemicals) at 1:100 and 1:80 dilutions, respectively. After staining with anti- $\alpha 5$  antibody, cells were fixed in -20°C cold ethanol and kept at -20°C overnight. All samples were stained for DNA content with propidium iodide (50  $\mu$ g/ml) and RNase (100 U/ml) (Sigma Chemical Co.) as described previously (1).

**Flow cytometry.** Flow cytometry was performed using a flow cytometer (Epics Elite; Coulter Corp., Hialeah, FL). Forward and 90° light scatter were used for gating out debris, and cell aggregates were

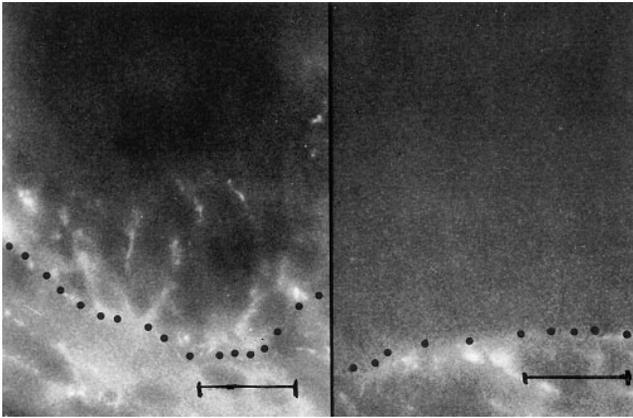
eliminated based on the ratio of integrated to peak fluorescence of propidium iodide. Listmode data were analyzed using Elite software (Coulter Corp.).

**Short-term *ex vivo* keratinocyte stem cell growth assay.** As described previously in more detail (4), epidermal cell suspensions were prepared from keratome biopsies after dispase and trypsin treatment. Cells were cultured in keratinocyte basal media (KBM) (keratinocyte-serum-free media without bovine pituitary extract and EGF; GIBCO BRL, Gaithersburg, MD) supplemented with 1% FBS (Hyclone, Logan, UT) on tissue culture plastic or on 20  $\mu$ g/ml fibronectin (bovine plasma)-coated plastic, for 24 h for measurement of longer term keratinocyte stem cell adherence and for 3–4 d for measurement of cell growth. Cells were then briefly trypsinized (0.025% trypsin plus 0.01% EDTA; Sigma Chemical Co.), collected, fixed in ethanol, and stained with the following antibodies: anti- $\beta 1$  integrin (4B4; Coulter Corp.), anti-PCNA (Boehringer Mannheim Biochemicals), anti-vimentin (Boehringer Mannheim Biochemicals), anti-AE1/AE3 (Boehringer Mannheim Biochemicals), and anti-K1/K10 (AE2; ICN Biomedicals Inc.). Isotype controls were mouse IgG1, mouse IgG2a (Sigma Chemical Co.), and secondary antibodies used were anti-mouse IgG1-FITC and anti-mouse IgG2a-PE (Boehringer Mannheim Biochemicals). Recombinant human lymphokines were added to the cultures 2 h after plating, the medium was supplemented with IFN- $\gamma$ , GM-CSF, and IL-3 (Collaborative Biomedical Products) alone or in combination at 100 U/ml, 1 ng/ml, and 0.3 ng/ml, respectively. (Concentrations were chosen based on the activity range of the recombinant lymphokines determined by the manufacturer and on the ELISA-determined lymphokine content of the psoriatic T cell supernatants found to be growth promoting in our previously reported work [4].) To determine the criticality of  $\alpha 5\beta 1$  in stem cell outgrowth, anti- $\alpha 5$  mAb (clone IIA1) or mouse IgG1 isotype control (both from PharMingen) was added to cells, cultured on 20 mg/ml fibronectin-coated plastic, 24 h after plating at 25  $\mu$ g/ml final concentration. All the initially plated cells were then harvested after 3 or 4 d in culture (and a corresponding control culture at the same time), stained with the combination of antibodies to  $\beta 1$  integrin and vimentin or PCNA, and analyzed by flow cytometry to quantitate basal keratinocyte cell entry into cell cycle and proliferation.

**Statistical analysis.** Paired two-tailed Student's *t* test was used to compare cell growth under different conditions.

## Results

**Fibronectin penetrates the basement membrane zone and is present between basal keratinocytes in perilesional psoriatic uninvolved skin.** Normal skin showed no staining with anti-plasma fibronectin polyclonal antibody above the basement membrane relative to the isotype control. However, normal skin did exhibit distinct and specific fluorescence in the dermis and underneath the basement membrane, indicating the presence of plasma fibronectin in the dermis of normal skin (Fig. 1, *right*). In the perilesional uninvolved psoriasis skin sections, staining in the dermis was more pronounced relative to normal skin, and in distinct contrast to normal skin, fibronectin staining extended into the epidermis, appearing as patchy fine lines around the basal cells (Fig. 1, *left*), thus demonstrating the presence of plasma fibronectin surrounding the basal cells of uninvolved psoriatic epidermis. On some sections from lesional skin samples, we have observed a similar staining pattern of fibronectin. Uninvolved skin from sites far distant from lesions (also mainly in patients with less disease) showed staining similar to sections of normal skin; however, the staining at the dermoepidermal junction was more pronounced (data not shown). Our observations extend, to uninvolved skin, the reports of others suggesting the presence of plasma fibronectin



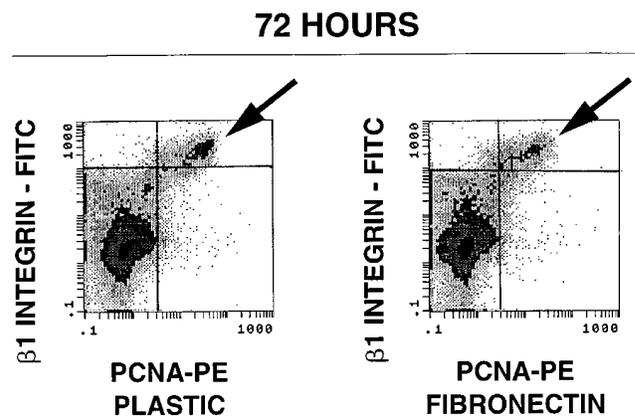
**Figure 1.** Staining of normal and nonlesional psoriatic epidermis with antibodies against plasma fibronectin. In normal skin, positive staining is restricted to the dermis and the basement membrane (*right*), whereas in nonlesional psoriatic skin, fluorescence extends into the epidermis surrounding the basal layer cells (*left*). Bar, 20  $\mu\text{m}$ .

in the basal hyperproliferative epidermis of psoriatic lesions (11, 19).

*Characterization of in vitro assay for measurement of quiescent (PCNA<sup>-</sup>), clonogenic keratinocyte induction into cell cycle for modulation by plasma fibronectin;  $\beta 1$  integrin<sup>bright</sup> cell, PCNA<sup>+</sup> cell expansion does not include melanocytes or other vimentin<sup>+</sup> keratin<sup>-</sup> cells.* We hypothesized that a functional effect of fibronectin on the cell cycle responsiveness of the hyperproliferative basal, K1/K10<sup>-</sup> cells in psoriatic lesions could be addressed in an assay which measures K1/K10<sup>-</sup> epidermal keratinocyte outgrowth upon primary ex vivo culture. In previous work using flow cytometric analysis of primary ex vivo keratinocyte cultures, we demonstrated that  $\beta 1$  integrin<sup>+</sup> K1/K10<sup>-</sup> PCNA<sup>-</sup> keratinocytes established such cultures (4). As these cells transit from cell cycle quiescence (G0 phase, PCNA<sup>-</sup>) into cell cycle, they express PCNA, a DNA polymerase auxiliary protein that arises in G1, and characteristically express  $\beta 1$  integrin brightly without undergoing differentiation in the early culture (do not yet express the differentiation keratin, K1/K10<sup>-</sup>) (Fig. 2 A, *arrow*). K1/K10<sup>+</sup> cells, which have committed to terminal differentiation but are still proliferating ( $\beta 1$  integrin<sup>+</sup> K1/K10<sup>+</sup> PCNA<sup>+</sup>), continue their terminal differentiation in the early culture to a senescent state (become PCNA<sup>-</sup> and  $\beta 1$  integrin<sup>-</sup>). Whereas PCNA is likely to play an essential role in cell proliferation as a cell cycle regulatory element (20, 21), the  $\beta 1$  integrin<sup>bright</sup> expression may not relate directly to cell proliferation. Their concomitant expression in the clonogenic keratinocytes of the early culture does not necessarily indicate the existence of a common regulatory pathway for their expression. In fact, it has been shown using primary keratinocyte cultures that rapamycin blocks PCNA expression in the K1/K10<sup>-</sup> clonogenic keratinocytes and stops them in the G1 phase of the cell cycle without affecting  $\beta 1$  integrin<sup>bright</sup> expression (22). Therefore, we checked whether keratinocytes grown on fibronectin would behave similarly to cells grown on tissue culture plastic. Normal ex vivo epidermal cells plated on plasma-derived fibronectin-coated tissue culture plastic exhibited behavior similar to cells grown on tissue culture plastic (Fig. 2 B). As observed with

epidermal cultures grown on tissue culture plastic (4), PCNA<sup>+</sup> expression precedes  $\beta 1$  integrin<sup>bright</sup> expression in the cultures on fibronectin-coated plastic (data not shown). In addition, the appearance of  $\beta 1$  integrin<sup>bright</sup> expression was detected only on PCNA<sup>+</sup> cells in cultures grown on either plastic or fibronectin-coated plastic (Fig. 2), as well as in the presence of human recombinant T cell lymphokines.

Using multiparameter flow cytometric analysis of the different cell characteristics (K1/K10,  $\beta 1$  integrin, and PCNA expression) and cell cycle analysis based on DNA staining, we have shown in previous work that the number of PCNA<sup>+</sup> or  $\beta 1$  integrin<sup>bright</sup> cells represent cell cycle entry and subsequent division of quiescent clonogenic cells in the early (48–72 h) culture (4). In those previous experiments, we noted that in addition to keratinocytes, melanocytes could survive, grow, and express  $\beta 1$  integrin in the culture. Because we wanted to monitor only keratinocyte growth, we determined the degree to which melanocyte growth contributes to the calculation of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells. Vimentin is expressed by melanocytes but not by keratinocytes in vivo, and should therefore be a good marker. However, it has been reported (and we also observed) that in long-term multiply passaged cultures, keratinocytes can express vimentin. Therefore, we double-stained our short-term cultures with vimentin and a mixture of anti-keratin antibodies that would stain all keratinocytes in the skin. We found that in the early culture, keratinocytes are still vimentin-negative (Fig. 3 A, quadrant 4), validating the use of vimentin to effectively differentiate keratinocytes from melanocytes. This result allows us to determine whether the number of  $\beta 1$  integrin<sup>bright</sup> cells appearing during early cell outgrowth represents only vimentin negative keratinocytes. Upon covisualization of vimentin expression with  $\beta 1$  integrin expression, the nonkeratinocytic vimentin<sup>+</sup> cells did not express bright  $\beta 1$  integrin (Fig. 3 B, *lower right cell cluster*). Thus, the  $\beta 1$  integrin<sup>bright</sup> population under these culture conditions seems to be a good estimation of the induction of the clonogenic, undifferentiated (K1/K10<sup>-</sup>), in vivo quiescent (PCNA<sup>-</sup>) keratinocyte entry into cell cycle upon primary ex vivo culture.



**Figure 2.** Culture of ex vivo normal epidermal cells shows similar growth behavior on pre-coated fibronectin relative to noncoated tissue culture plastic. 72-h cultured epidermal cells were analyzed by flow cytometry. Proliferating cells in the early cultures simultaneously express PCNA (x axes, PE fluorescence) and bright  $\beta 1$  integrin (y axes, FITC fluorescence), both on plastic and on fibronectin-coated plastic. *Arrows*, Double positive population.

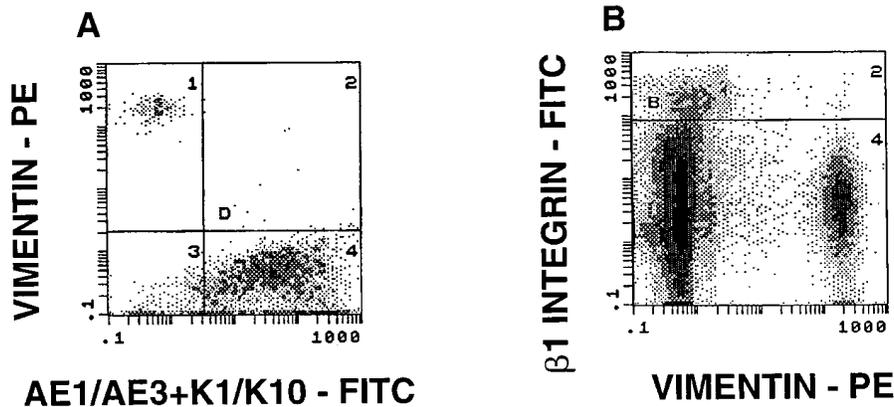


Figure 3. Short-term ex vivo cultured keratinocytes remain vimentin<sup>-</sup>. Vimentin differentiates keratinocytes from melanocytes in the early culture. Vimentin<sup>+</sup> cells (A, upper left) show no keratin expression and are not included in the  $\beta 1$  integrin<sup>bright</sup> population. Antibodies against keratins expressed in both undifferentiated and differentiated keratinocytes are visualized by FITC; the keratin-FITC fluorescence of vimentin<sup>+</sup> (PE) cells falls below the isotype control level. The PE fluorescence (y axis) of the keratin-expressing cells (lower right) is below the fluorescence of the isotype control, indicating that keratinocytes do not express vimentin in the early epidermal cell culture. The PE fluorescence intensity (x axis) of the  $\beta 1$  integrin<sup>bright</sup> cells (B, upper left) falls below the isotype control level, indicating vimentin negativity.

Establishment of defined conditions to model psoriatic T cell lymphokine effects in the context of fibronectin; recombinant IFN- $\gamma$  induces psoriatic uninvolved K1/K10 keratinocyte growth only in the presence of IL-3 and GM-CSF. In previous work, we have demonstrated that supernatants of CD4<sup>+</sup> T cells cloned from psoriatic lesions could provide a growth-stimulatory environment for psoriatic uninvolved keratinocytes (4). Neutralization experiments have indicated that IFN- $\gamma$  in the presence of IL-3 and GM-CSF was responsible for the observed growth stimulation (4). To determine whether these observations could be used to create a defined model of the specific growth-stimulatory lymphokine milieu for psoriatic uninvolved keratinocytes, we tested the effect of recombinant human IFN- $\gamma$ , IL-3, and GM-CSF separately and in combination on psoriatic uninvolved epidermal cells in the in vitro assay. No changes in the percentage of  $\beta 1$  integrin<sup>bright</sup> cells were observed upon incubation with IL-3 ( $21 \pm 19\%$ ,  $n = 5$ ) or GM-CSF ( $-16 \pm 15\%$ ,  $n = 5$ ) alone, and an inhibition was observed with the addition of only IFN- $\gamma$  ( $-28 \pm 9.7\%$ ,  $n = 6$ ) (Fig. 4). By contrast, the combination of the three lymphokines resulted in an  $86 \pm 36\%$  ( $n = 5$ ) increase in the number of  $\beta 1$  integrin<sup>bright</sup> cells, indicating a positive and synergistic effect on the cell cycle induction of psoriatic uninvolved keratinocytes (Fig. 4). This cell expansion effect of the three lymphokines was found to be significant relative to medium containing cultures in a separate set of experiments using psoriatic uninvolved keratinocytes (Fig. 5,  $n = 7$ ,  $P = 0.005$ ). However, a similar growth-enhancing effect was not observed in normal ex vivo epidermal cell cultures (Fig. 5,  $n = 6$ ,  $P < 0.871$ ). These results allowed us to use a more defined model of psoriatic T cell-basal keratinocyte interactions for subsequent fibronectin experiments, without potential confounding interactions with undefined released or membrane ghost products of T cells used to generate lymphokines.

Fibronectin increases cell cycle entry among psoriatic uninvolved but not normal keratinocytes relative to tissue culture plastic, and potentiates the growth-stimulatory effect of the lymphokines (IFN- $\gamma$ , GM-CSF, and IL-3). We next carried out a set of experiments using normal and psoriatic uninvolved epidermal cells to compare the effect of these lymphokines on cell

outgrowth from normal or psoriatic uninvolved epidermis grown on fibronectin-coated plates. When cells were plated on fibronectin as the substrate, the T cell lymphokines had a significant stimulatory effect for expansion of psoriatic uninvolved keratinocytes ( $P < 0.002$ ) and no significant effect on normal epidermal cells ( $P < 0.562$ ) (Fig. 5).

To determine if psoriatic uninvolved keratinocytes are hyperresponsive to fibronectin even in the absence of lymphokines, cultures on fibronectin and plastic without lymphokines were compared. We detected consistent growth induction again in all of the psoriatic uninvolved cultures on fibronectin relative to plastic, although the difference in the matched paired  $t$  test did not prove to be significant at the 95% confidence level ( $P < 0.101$ ) (Fig. 6). By contrast, normal epidermal cells were inconsistent in their response to fibronectin ( $P < 0.675$ ) (Fig. 6).

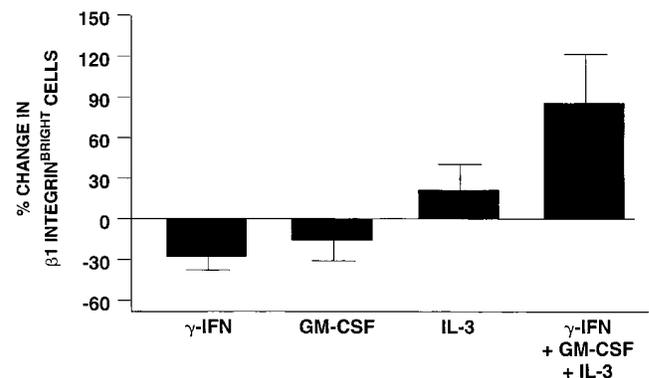


Figure 4. Growth-stimulatory effect of human recombinant T cell lymphokines on nonlesional psoriatic basal keratinocytes. Cells were grown in KBM plus 1% FBS media with or without individual lymphokines or their mixture. The number of  $\beta 1$  integrin<sup>bright</sup> cells was determined in each culture, and the lymphokine-supplemented cultures were compared to the media-containing cultures, in which the number of  $\beta 1$  integrin<sup>bright</sup> cells represented 100% growth. Bars, Average  $\pm$  SE ( $n = 5$ ), except for IFN- $\gamma$  alone ( $n = 6$ ).

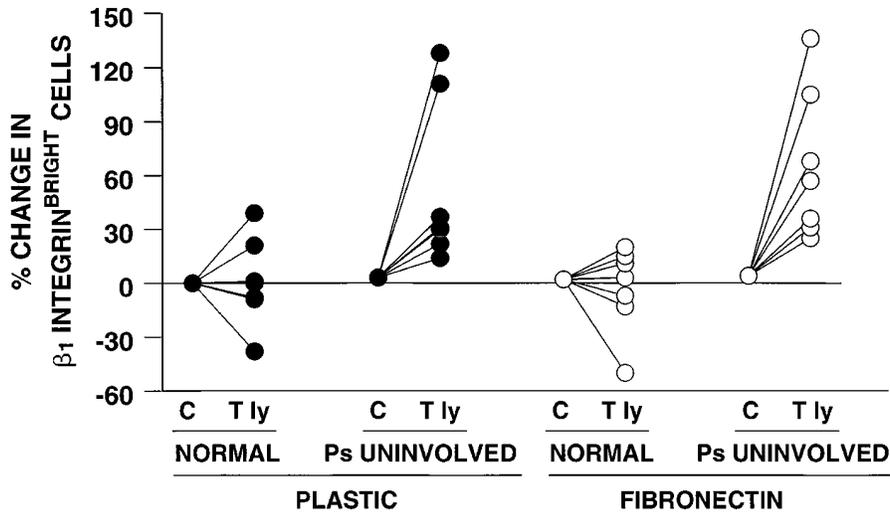


Figure 5. Keratinocyte stem cell growth upregulation by T cell lymphokines. Psoriatic nonlesional epidermal cells show significantly higher numbers of  $\beta 1$  integrin<sup>bright</sup> cells in the early epidermal cell cultures both on plastic and on fibronectin-coated plastic if lymphokines were present in the media. *C*, Control culture. *T Ly*, Media supplemented with IFN- $\gamma$ , GM-CSF, and IL-3. In each experiment, the number of  $\beta 1$  integrin<sup>bright</sup> cells in the lymphokine cultures are compared with the control cultures, and the differences are expressed as percentage of the control culture. For normal cultures ( $n = 6$ ),  $P < 0.871$  on plastic and  $P < 0.562$  on fibronectin. For nonlesional cultures ( $n = 7$ ),  $P < 0.005$  on plastic and  $P < 0.002$  on fibronectin. *Ps*, Psoriatic.

Examination of primary data from uninvolved epidermis of a patient with psoriasis is illustrative of the relative effect of fibronectin, lymphokines, and combined fibronectin and lymphokines upon the clonogenic cell outgrowth (Fig. 7). The outgrowth of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells in the ex vivo short-term culture showed modest increase in this population from 13 to 22% of total epidermal cells when cultured in the presence of fibronectin alone (Fig. 7 C) and from 13 to 16.4% in the presence of T cell lymphokines alone (Fig. 7 B) relative to control culture conditions (Fig. 7 A). However, cultures containing both fibronectin and T cell lymphokines (Fig. 7 D) resulted in an increase of 152% in the outgrowth of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells relative to cells in control cultures (Fig. 7 A), reaching 32.7% of cells in the culture. Comparison of the average percent increases in  $\beta 1$  integrin<sup>bright</sup> cells among seven different cultures in response to the fibronectin and the lymphokines alone showed comparable growth stimulation of psoriatic uninvolved keratinocytes ( $71 \pm 32\%$  increase with fibronectin and  $53 \pm 17\%$  increase with the lymphokines). The

group mean of the combination of fibronectin with the lymphokines demonstrated greater cell cycle induction than either stimulus alone, indicating at least an additive and potentially a synergistic effect ( $178 \pm 50\%$ ).

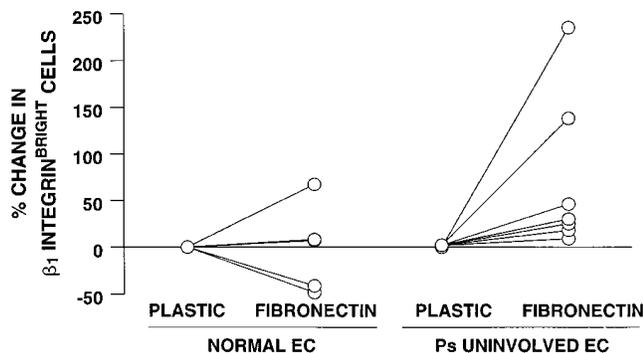


Figure 6. Fibronectin effect on keratinocyte stem cell growth. In nonlesional epidermal cell cultures, the number of  $\beta 1$  integrin<sup>bright</sup> cells is consistently higher when cells are grown on fibronectin-coated plastic relative to plastic alone ( $n = 7$ ,  $P < 0.10$ ); among normal epidermal cells, no consistent change can be detected ( $n = 6$ ,  $P < 0.675$ ). Differences in  $\beta 1$  integrin<sup>bright</sup> cell numbers are expressed as percentage of the number of cells measured in cultures of the tissue culture plastic. *EC*, Epidermal cells. *Ps*, Psoriatic.

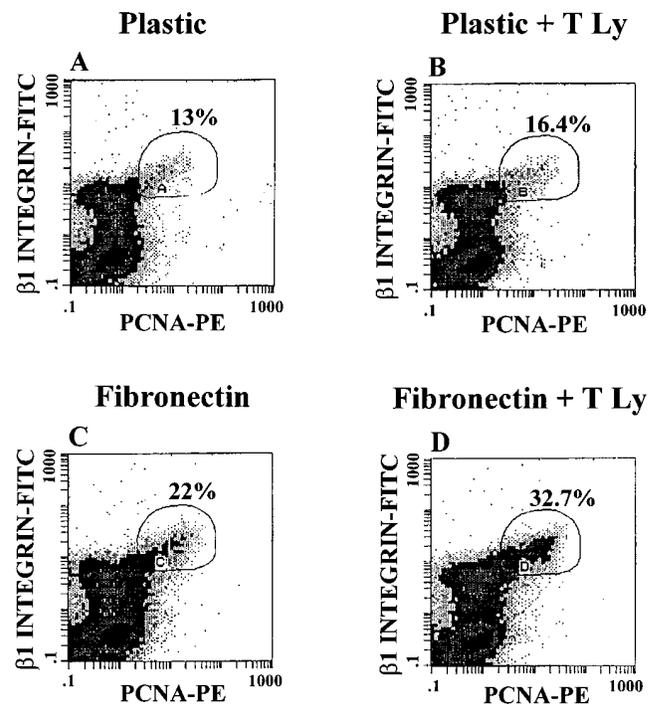


Figure 7. Fibronectin potentiates the growth-stimulatory effect of T cell lymphokines on ex vivo nonlesional psoriatic keratinocytes. Epidermal cells from a nonlesional psoriatic keratome biopsy were cultured on noncoated tissue culture plastic (A and B) and on fibronectin-coated tissue culture plastic (C and D) in KBM plus 1% FBS medium (A and C) or in the same medium supplemented with T cell lymphokines (B and D). The presence of either the fibronectin (C) or the lymphokines (B) alone resulted in increased numbers of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells (22 and 16.4%, respectively; encircled area) in the cultures by 72 h. Relative to the control culture (A, epidermal cells grown on noncoated tissue culture plastic in medium alone), even higher keratinocyte outgrowth was detected if cells were exposed to both fibronectin and T cell lymphokines (T Ly) (32.7%  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells, D, compared with 13%, A).

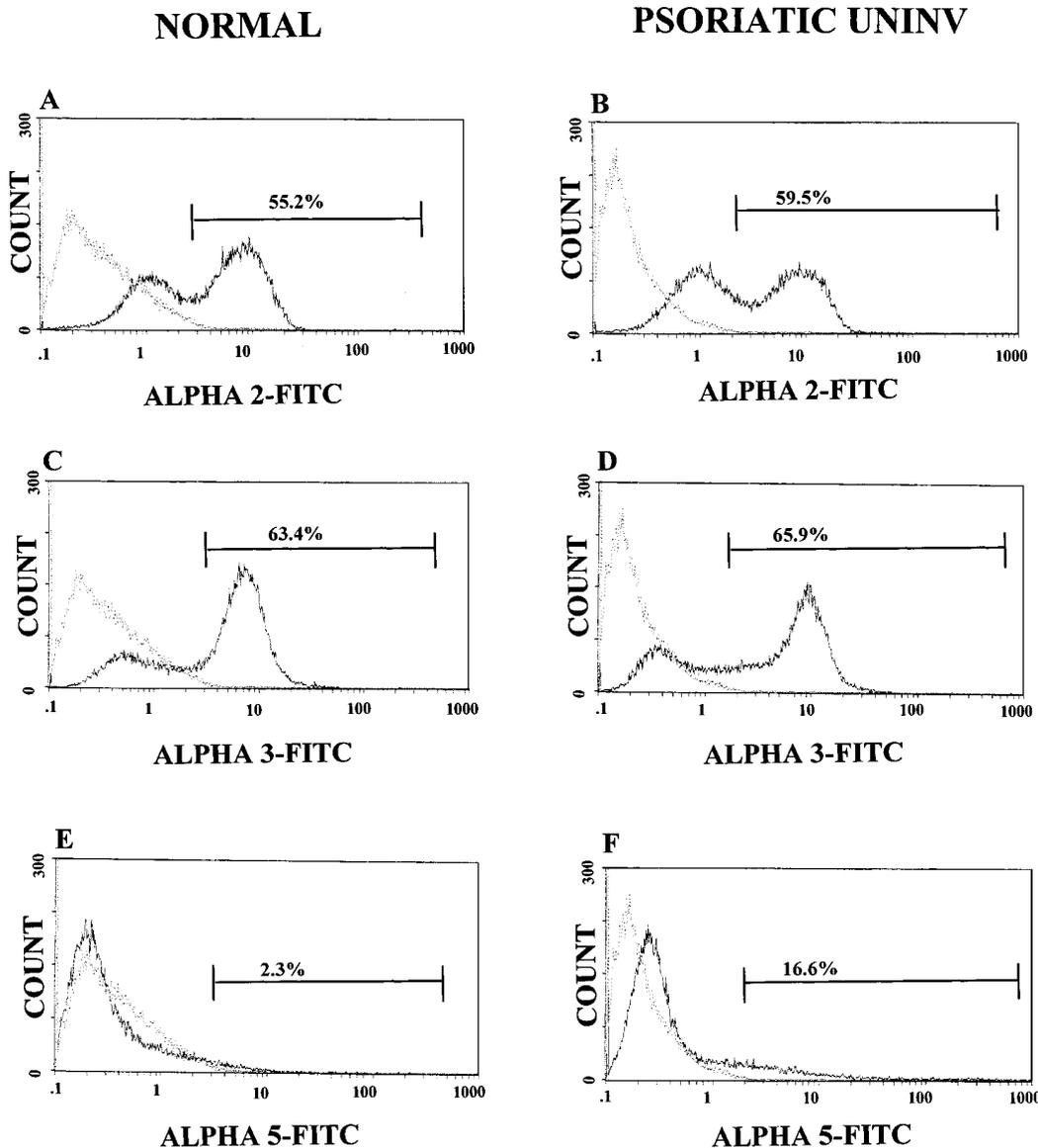
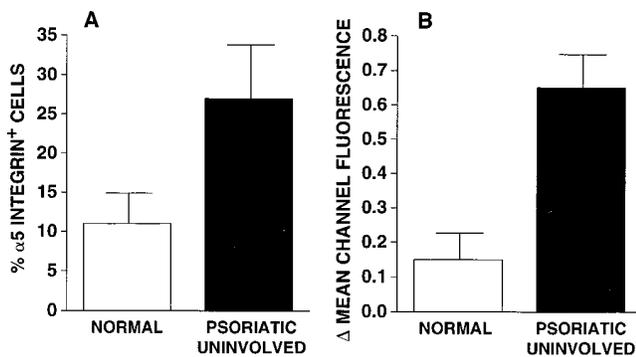


Figure 8.  $\alpha 5$  but not  $\alpha 2$  or 3 is abnormally expressed in psoriatic uninvolved epidermal cells. Freshly obtained normal (A, C, and D) or psoriatic uninvolved (UNINV) (B, D, and F) epidermal cells were stained for either  $\alpha 2$  (A and B),  $\alpha 3$  (C and D), or  $\alpha 5$  (E and F). Broken line, Isotype control. Solid line, Antibody staining. Percent positive staining is indicated within each histogram.

$\alpha 5$  integrin (fibronectin receptor) expression is upregulated on *in vivo* psoriatic uninvolved keratinocytes relative to normal keratinocytes. Because extracellular fibronectin has been reported to upregulate the fibronectin-binding receptor ( $\alpha 5\beta 1$ ) (23), we initially screened several integrins that are commonly expressed by keratinocytes,  $\alpha 2$ ,  $\alpha 3$ , and  $\alpha 5$  (24), to determine whether changes in integrins may be associated with the increased presence of fibronectin in uninvolved epidermis of skin from psoriatic patients (Fig. 8). Of the three integrins, only  $\alpha 5$  expression was clearly upregulated on epidermal cells from psoriatic uninvolved skin (16.6% positive) compared with normal skin (2.3% positive) (Fig. 8). Examination of  $\alpha 5$  expression in a larger group of normal and psoriatic patients (Fig. 9) revealed that in both groups (normal and psoriatic uninvolved), the  $\alpha 5$  integrin expression varied, from 0 to 23% positive cells in the normal group ( $n = 5$ ), and from 6 to 71% in the psoriatic uninvolved group ( $n = 10$ ). However, overall mean  $\alpha 5$  integrin expression was higher among psoriatic uninvolved than among normal keratinocytes ( $11 \pm 4\%$  of all epi-

dermal cells in normal patients and  $27 \pm 7\%$  in nonlesional psoriatics,  $P = 0.06$ ) (Fig. 9 A). Lesional psoriatic skin also appeared to exhibit elevated percentages of  $\alpha 5$  integrin<sup>+</sup> keratinocytes, with  $27.5 \pm 1.0\%$  positive epidermal cells (data not shown,  $n = 2$ ), consistent with the prior immunohistology observations of Pellegrini et al. (16). Of perhaps more importance, the intensity of the expression of  $\alpha 5$  on individual psoriatic uninvolved epidermal cells was significantly higher than on normal cells. The change in mean channel fluorescence of normal epidermal cells was  $0.151 \pm 0.08$ , whereas the change in mean channel fluorescence of psoriatic uninvolved epidermal cells was  $0.649 \pm 0.1$  ( $P < 0.004$ ) (Fig. 9 B).

*The stimulatory effect of fibronectin on psoriatic uninvolved keratinocytes is not a direct result of differential adherence.* In the short *in vitro* keratinocyte growth assays, cell binding is probably not limited to fibronectin, due to relatively low fibronectin concentration (20  $\mu\text{g/ml}$ ). Moreover,  $\alpha 5$  integrin expression is induced on normal keratinocytes shortly after plating into culture (in a normal culture, the percentage of  $\alpha 5$



**Figure 9.**  $\alpha 5$  integrin expression is upregulated in the psoriatic nonlesional epidermis. The number of cells as a percentage of total epidermal cells expressing  $\alpha 5$  integrin is higher in the psoriatic nonlesional skin (black bar, average  $\pm$  SE,  $N = 10$ ) relative to normal skin (white bar,  $n = 5$ ) (A). The higher difference in the change in mean channel fluorescence between normal and nonlesional psoriatic samples indicates that individual cells express more  $\alpha 5$  integrin molecules (B).

integrin<sup>+</sup> cell number rose from 10 to 22% in 2 h on plastic). Thus, an enriched adherence of the K1/K10<sup>-</sup> clonogenic keratinocyte population may have occurred and could have resulted in the observed higher numbers of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells in the early in vitro growth assays of psoriatic uninvolved keratinocytes. To determine whether better adherence of the K1/K10<sup>-</sup> psoriatic uninvolved cell population alone was responsible for induced cell cycle entry in the early cultures, we plated normal and psoriatic uninvolved epidermal cells on uncoated tissue culture plastic or on 20  $\mu$ g/ml fibronectin-coated tissue culture plastic. After a 24-h incubation, we determined the relative size of the K1/K10<sup>-</sup> culture-establishing cell population among the adherent cells. No enrichment was detected in K1/K10<sup>-</sup> cells on fibronectin relative to the plastic whether using cells from normal skin or psoriatic uninvolved skin. Among the adherent psoriatic uninvolved epidermal cells, 62 $\pm$ 5% were K1/K10<sup>-</sup> on fibronectin and 59 $\pm$ 5% on plastic ( $n = 3$ ). In the normal cultures, 34 $\pm$ 11% of the adherent cells were K1/K10<sup>-</sup> on fibronectin and 36 $\pm$ 6% on plastic ( $n = 3$ ). Thus, the differential clonogenic cell outgrowth response between normal and psoriatic uninvolved keratinocytes in response to fibronectin is not a direct result of preferential initial adhesion of psoriatic uninvolved K1/K10<sup>-</sup> keratinocytes to the substrate. These data also suggest that interaction between fibronectin and the uninvolved basal keratinocytes may provide cell cycle regulatory signals independent of adhesion.

*Fibronectin can influence keratinocyte cell growth without affecting adhesion.* Once we established that in our clonogenic cell outgrowth assays, the presence of low concentration fibronectin did not influence the K1/K10<sup>-</sup> keratinocyte adhesion, we sought to determine experimentally whether fibronectin can influence clonogenic cell expansion in our system independent of adhesion. To abrogate  $\alpha 5\beta 1$  integrin binding to fibronectin, we added an anti- $\alpha 5$  mAb (clone IIA1) that inhibits  $\alpha 5\beta 1$  ligand binding (25), or a mouse IgG1 isotype control, to primary ex vivo cultures of normal keratinocytes 24 h after plating. At this time, cell adherence has already occurred, so the addition of antibody will not influence the outgrowth by inducing differential adherence. Anti- $\alpha 5$  treatment of three

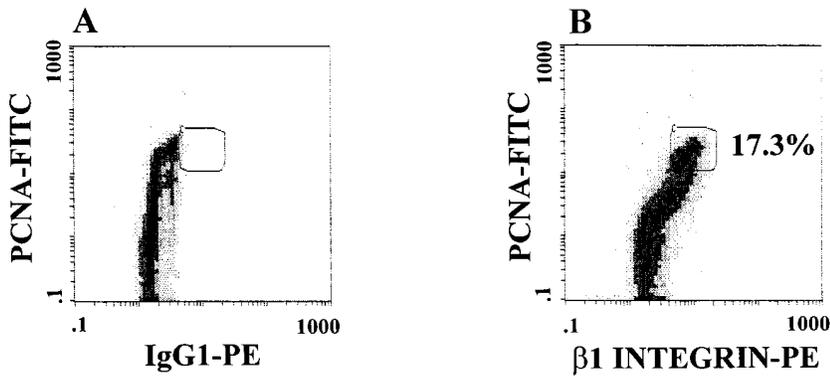
separate ex vivo cultures resulted in a mean reduction of 55 $\pm$ 12% in the outgrowth of  $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> cells relative to the IgG1 isotype treatment. Raw data demonstrating this reduction in cell outgrowth ( $\beta 1$  integrin<sup>bright</sup> PCNA<sup>+</sup> subset) by anti- $\alpha 5$  treatment (from 17.3% in the isotype-treated culture to 5.5% in the anti- $\alpha 5$ -treated culture) are demonstrated in Fig. 10 B, and D. Similar results were observed with treatment using the Arg-Gly-Asp peptide (57% inhibition relative to control peptide; data not shown). These results suggest that fibronectin could influence clonogenic keratinocyte expansion and cell cycle induction through other growth-regulatory integrin pathways that are distinct from adhesion, such as cell spreading (26, 27), focal contact formation (28–30) or a direct cell cycle regulatory pathway (14, 15).

## Discussion

Perturbed keratinocyte proliferation and differentiation accompanied by various degrees of skin inflammation are central pathological features of the lesional tissue in psoriasis. As the typical skin lesion develops in psoriasis, one of the earliest events is the appearance of T cells and macrophages in the lesions. This is followed by excessive basal cell proliferation and perturbed keratinocyte differentiation, which are regarded as central pathologic features of the disease. In previous work, we have shown that the primarily hyperproliferative cell population in the lesional skin is the normally quiescent basal keratinocyte population that contains the keratinocyte stem cells (1). Relative to normal basal keratinocytes, similar cells from nonlesional psoriatic skin are hyperresponsive to supernatants of in vitro-activated T cells derived from psoriatic lesions in their proliferation (in short-term in vitro assays). Previous experiments have indicated that among the lymphokines produced by the T cells, IFN- $\gamma$  plays a key role in the mitogenic effect (3, 4, 31). In the present work, using human recombinant lymphokines, we show that IFN- $\gamma$  exerts its mitogenic effect on psoriatic uninvolved keratinocytes only in the presence of GM-CSF and IL-3, which are also present in the psoriatic lesional T cell supernatant. Psoriatic uninvolved as opposed to normal keratinocytes proved to be hyperresponsive to the combination of human recombinant IFN- $\gamma$ , IL-3, and GM-CSF in their growth. This hyperresponsiveness of the nonlesional basal keratinocytes of psoriatics to T cell lymphokines could be a result of genetic susceptibility or a stimulatory priming of these cells in vivo.

Increasing evidence suggests that in addition to soluble mitogens, extracellular matrix proteins play an important role in cell growth regulation. Of the several molecules found on the cellular surface interacting with the extracellular matrix, integrins constitute a large family. It is well-documented that in the skin, as in other tissues, keratinocyte integrins are important regulatory molecules in epidermal homeostasis. Due to cell-matrix and cell-cell interactions, they are involved in adhesion, migration, growth, and differentiation (for a review, see reference 32). Recently, alterations in expression, topography, and function of integrin receptors have been reported not only in lesional but also nonlesional psoriatic keratinocytes (16, 17). We find the latter most suggestive of an inherent early, critical alteration in the disease. Indeed, using flow cytometric analysis of freshly isolated epidermal cells, we also found higher numbers of psoriatic uninvolved epidermal cells

## IgG1 ISOTYPE TREATED EC



## ANTI-ALPHA 5 TREATED EC

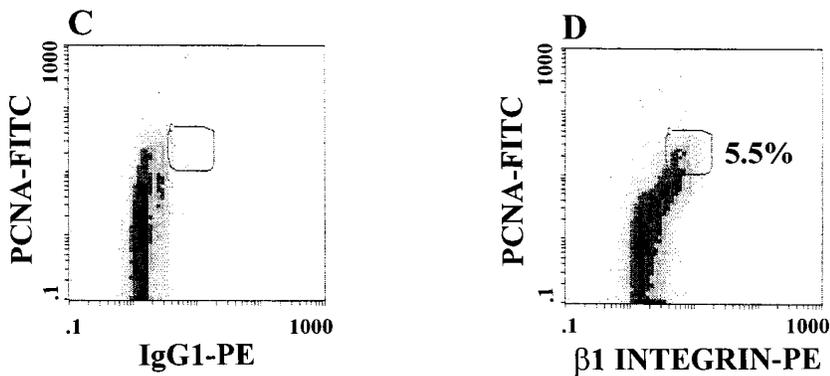


Figure 10. Anti- $\alpha$ 5 mAb inhibits keratinocyte growth in the early culture. Normal ex vivo epidermal cell cultures were treated with either IgG1 isotype control (A and B) or anti- $\alpha$ 5 mAb (C and D) for the last 48 h of a 72-h culture on fibronectin. The numbers of  $\beta$ 1 integrin<sup>bright</sup> PCNA<sup>+</sup> cells are indicated by contour plot C. The background staining by the IgG1 PE-conjugated isotype control was < 0.5%. The percentage of positive cells in terms of total epidermal cells (EC) with a DNA content between 2 and 4N is indicated in each histogram.

expressing  $\alpha$ 5 integrin, relative to normal epidermal cells, as well as in greater molecular density. As in the case of lymphokine hyperresponsiveness, the difference in  $\alpha$ 5 integrin expression in vivo could be related to genetic susceptibility or it could be the result of altered regulation by the in vivo environment.

Normal keratinocytes express  $\alpha$ 5 integrin under certain physiological conditions, such as in wound healing and during tissue development (8, 33–37), as well as under certain in vitro conditions (38). There is good evidence that extracellular matrix binding can modulate integrin expression (39–41). One well-described major alteration in the psoriatic basal keratinocyte environment is the fenestration of the basement membrane (9, 10, 12, 42). It is possible that this change could alter the integrin expression observed in the in vivo skin of psoriatics by changing the composition of the extracellular matrix to which the basal keratinocytes are exposed. For instance, fibronectin, which is normally located below the basal lamina, could penetrate the dermal-epidermal junction and bathe the keratinocytes in much higher than normal levels of fibronectin. Indeed, we report here that fibronectin could be found around basal cells in uninvolved psoriatic skin that is adjacent to a psoriatic plaque. Recently, Mondello et al. reported alteration of laminin 1 distribution in the basement membrane of unin-

involved psoriatic skin relative to normal skin (43). A thorough examination of the basement membrane and the exact composition of the extracellular matrix around the basal keratinocytes in uninvolved psoriatic skin should contribute very useful information to our understanding of the disease.

It is of interest that the higher expression of  $\alpha$ 5 integrin in vivo shows no direct correlation with a higher proliferative state (determined by cell cycle analysis based on DNA staining) of the uninvolved basal keratinocytes in vivo ( $r = -0.363$ , data not shown). This indicates the importance of other factors in triggering hyperproliferation, such as fibronectin and/or lymphokines. In colon carcinoma cells,  $\alpha$ 5 $\beta$ 1 integrin expression in the absence of fibronectin activates a signaling pathway leading to decreased cellular proliferation; ligation of the receptor with fibronectin reverses the signal, thereby contributing to the proliferation (44). Fibronectin itself provoked a higher proliferative response in uninvolved keratinocytes in our short-term in vitro assay that could not be attributed to an initial increase in adherence of basal cells. To address specifically the involvement of the  $\alpha$ 5 $\beta$ 1 fibronectin receptor, ex vivo epidermal cells cultured on fibronectin were treated with anti- $\alpha$ 5 mAb or its IgG1 isotype control. The specific growth inhibition by anti- $\alpha$ 5 in our ex vivo cultures further indicates that fibronectin could influence keratinocyte growth in ways that are

different from adhesion and more similar to the effect upon cell cycling observed when fibronectin–fibronectin receptor interaction was blocked in other systems (26). Although normal basal keratinocyte growth was not influenced by the addition of the low concentration fibronectin to the plastic, their growth was inhibited substantially by anti- $\alpha 5$  mAb treatment, indicating that  $\alpha 5 \beta 1$  integrin and fibronectin binding contribute to the normal basal keratinocyte growth in vitro even when the cells are grown on plastic. That cultured keratinocytes produce and release fibronectin in vitro (45, 46) and show substantial  $\alpha 5$  integrin expression shortly after plating supports the above indication. The increased outgrowth of psoriatic uninvolved keratinocytes relative to normal keratinocytes in the presence of fibronectin suggests that the fibronectin receptor on freshly isolated psoriatic uninvolved keratinocytes may be in a more functionally active state at the initiation of the ex vivo culture.

The fact that both lymphokines and fibronectin can be mitogenic for psoriatic uninvolved basal keratinocytes, and that in combination their effect is at least additive and perhaps synergistic, indicates that these factors are important in the initiation and maintenance of the actual diseased state. It has been reported that extracellular matrix components, in particular heparan sulfate, can bind IFN- $\gamma$ , GM-CSF, and IL-3 and may enhance the biological activity of the lymphokines through conformational changes, resulting in better presentation to their receptor (47–50). The ability of cells to respond to various growth and differentiation factors is also determined to a large extent by the cell's shape and orientation, both of which, in turn, are modulated by components of the extracellular matrix (51–53).

It has been demonstrated recently, by genetically forced suprabasal integrin expression in transgenic mice (18), that proper downregulation of integrins on keratinocytes is essential for normal epidermal homeostasis. It is of significant interest to psoriasis that these mice exhibit almost all the major characteristics of the disease, including the inflammatory reaction. Our data suggest that keratinocytes in the in vivo nonlesional psoriatic epidermis are also hyperplastic in their growth response to T cell lymphokines and to fibronectin and concomitantly exhibit altered expression and functionally active  $\alpha 5$  integrin. These changes could be innate characteristics of the psoriatic keratinocytes, or could be the result of an altered regulatory extracellular matrix milieu.

With respect to the two apparently conflicting models presented recently for psoriasis, altered integrin expression as responsible for the psoriatic state (18), and psoriasis as an immunological disease (54), our data support both. We would propose that in order to develop a psoriatic lesion, the presence of both components is required. Nonlesional psoriatic skin expresses higher levels of  $\alpha 5$  integrin. However, this condition can occur without the keratinocytes undergoing hyperproliferation. Our in vivo and in vitro data both suggest that the presence of T cell lymphokines is required to obtain a hyperproliferative basal keratinocyte population. Due to the increased expression of  $\alpha 5$  integrin and the presence of fibronectin around the basal cells, the nonlesional psoriatic keratinocytes become primed to respond to the presence of T cell lymphokines, causing increased numbers of basal stem cells to leave the quiescent state and enter cell cycle, resulting in a hyperproliferative state. This model has implications both for candidate gene discovery in psoriasis and for novel therapeutic approaches.

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