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Induction of Normal and Psoriatic Phenotypes in Submerged Keratinocyte Cultures

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Lesional psoriatic epidermis displays a number of phenotypic changes that are distinct from the differentiation program found in normal interfollicular epidermis. In psoriatic epidermis, keratinocytes are hyperproliferative and several differentiation-associated molecules are expressed that are absent in normal skin (e.g., cytokeratins (CK) 6, 16, and 17, and the epidermal proteinase inhibitor SKALP/elafin). In addition, several molecules which are normally restricted to the stratum granulosum are strongly upregulated in the stratum spinosum (e.g., psoriasis-associated fatty acid binding protein (PA-FABP), psoriasin, involucrin, and transglutaminase). The aim of this study was to develop in vitro culture systems which (a) would allow to study the induction of normal and psoriatic differentiation pathways, and (b) would be amenable for screening of antipsoriatic drugs. Here we have investigated several models for induction of differentiation with respect to the expression of markers for the normal and psoriatic phenotype. Cell cycle parameters and expression levels of CK1, CK10, CK16, SKALP/elafin, transglutaminase, involucrin, psoriasin, and PA-FABP were assessed in these models using flow cytometry, immunocytochemistry, and Northern blot analysis. We observed that induction of differentiation with fetal calf serum resembled the psoriatic phenotype (sustained hyperproliferation; high levels of CK16, SKALP/elafin, transglutaminase, and involucrin; moderate psoriasin expression), whereas differentiation induced by growth factor depletion in a confluent culture resembled the normal differentiation phenotype (low proliferative rate; high expression levels of CK1 and CK10; moderate expression of involucrin and transglutaminase; low expression levels of SKALP/elafin and CK16; absence of psoriasin). We propose that these models can be used to study expression and pharmacological modulation of selected differentiation genes and the coordinated expression of sets of genes associated with epidermal differentiation programs. © 1996 Wiley-Liss, Inc.

Human epidermis is a stratified squamous epithelium with keratinocytes as the main cell type. To maintain normal epidermal integrity, keratinocytes must proliferate at an appropriate rate and differentiate according to an orderly sequence of events, the program of terminal differentiation (reviewed in Watt, 1989; Fuchs, 1990). This program is characterized by an outward migration of keratinocytes from the proliferating basement-membrane-anchored basal layer to the spinous, granular, and finally cornified layers. During their movement through the epidermis, keratinocytes undergo a complex program of terminal differentiation, also known as keratinization. As part of this maturational program, basal keratinocytes express cytokeratins (CK) 5 and CK14. During their movement to the suprabasal spinous layer, keratinocytes start to express differentiation-specific proteins such as CK1 and CK10. Finally, keratinocytes reach the granular layer, where a new set of genes, including involucrin, filaggrin, and loricrin is expressed. The differentiating cells become permeable and transglutaminase is activated by the influx of calcium ions into the cells resulting in biochemically cross-linking the envelope proteins. This highly coordinated process finally leads to a layer of dead cells which form the protective covering of the skin (Fuchs, 1990).

Lesional psoriatic epidermis displays a number of phenotypic changes that are distinct from normal interfollicular epidermis. In lesional psoriatic epidermis, the number of cycling cells is dramatically increased (Van Erp et al., 1989), and a number of molecules that are absent in normal skin are strongly expressed (e.g., CK 6, CK 16, and CK17 (de Jong et al., 1991; Weiss et al., 1984; De Mare et al., 1989), and the epidermal proteinase inhibitor SKALP/elafin which we and others have

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recently described (Alkemade et al., 1994; Molhuizen et al., 1993; Schalkwijk et al., 1990; Wiedow et al., 1990). In addition, several molecules have a restricted expression pattern in normal skin, but are highly upregulated in psoriatic skin. These include psoriasis-associated fatty acid binding protein (PA-FABP) (Madsen et al., 1992), involucrin (Fujimoto et al., 1993; Bernard et al., 1988), transglutaminase (Bianchi et al., 1994; Michel et al., 1992), psoriasin (Madsen et al., 1991), transforming growth factor α (TGF-α) (Turbitt et al., 1990; Elder et al., 1989), amphiregulin (Cook et al., 1992), epidermal growth factor receptor (EGF-R) (Nanney et al., 1992), calgranulins A and B (reviewed in Madsen et al., 1992), interleukin 1ra (Hammerberg et al., 1992), interleukin 1β (Schmid et al., 1993), interleukin 6 and 8 (Nickoloff et al., 1991; Grossman et al., 1989), GRO α/β/γ (Tettelbach et al., 1993), and fibronectin (Bernard et al., 1988). Recently it has been described that $\alpha_5\beta_1$ integrins show a sustained expression in psoriatic epidermis (Hertle et al., 1995; Pellegrini et al., 1992). A few molecules are reported to be downregulated in psoriatic epidermis compared to normal epidermis (e.g., interleukin 1α; Gearing et al., 1990). At present it is not clear whether the altered expression levels of these molecules are causally involved in the pathogenesis of psoriasis, or are mere epiphenomena not directly related to the disease process. The expression patterns of these molecules are not psoriasis-specific and can be found in keratinocytes from normal individuals during pathological conditions (e.g., wound healing and inflammation; Phillips et al., 1990; Hertle et al., 1992) or in cell culture as we described before (Alkemade et al., 1994).

In order to study cell biological processes involved in the transition from normal to psoriatic differentiation (as seen in disease processes) and vice versa (as seen during healing of a psoriatic plaque), we have investigated the profile of differentiation markers in several submerged keratinocyte culture systems. The aim of this study was to develop models that would resemble either the normal or the psoriatic pathway, as defined by the expression of the marker sets described above. In earlier experiments, we observed that the addition of fetal calf serum (FCS) to keratinocytes cultured in keratinocyte growth medium (KGM) resulted in the induction of SKALP/elafin expression (Alkemade et al., 1994). Recently, it was demonstrated that KGM depleted of growth factors induces the expression of CK 1 and CK10 in confluent cultures (Poumay and Pittelkow, 1995). On the basis of these observations, we considered the possibility that distinct keratinocyte culture systems might be used as models for normal and psoriatic differentiation. We therefore investigated the effect of four culture media on confluent monolayers: KGM alone (KGM), KGM with FCS (KGM/FCS), KGM depleted of growth factors (KGM/-GF), and KGM with 1.8 mM calcium (KGM/Ca). Our findings indicate that in these submerged culture systems, it is possible to approach the normal and psoriatic differentiation pathway.

MATERIALS AND METHODS

Biopsies

Biopsies (0.2 mm thickness) from healthy volunteers were taken with a keratome as previously described (Schalkwijk et al., 1990) and used for primary keratinocyte cultures.

Keratinocyte primary culture

Human epidermal keratinocytes were initially cultured according to the Rheinwald-Green system (Rheinwald and Green, 1975). Primary cultures of keratinocytes were seeded on lethally irradiated (3,000 rad, 3 min) Swiss mouse 3T3 fibroblasts in DMEM/F12 (3:1) (v/v) (Flow Laboratories, Irvine, UK) supplemented with 0.4 μg/ml hydrocortisone (Collaborative Research, Inc., Lexington, MA), isoproterenol (10^{-6} M) (Sigma, St. Louis, MO), 100 U/ml penicillin plus 100 μg/ml streptomycin (Gibco, Breda, The Netherlands), 6% fetal calf serum (FCS) (Seralab, Nistelrode, The Netherlands), and 10 ng/ml epidermal growth factor (EGF) (Sigma). Cells were grown at 37°C, 95% relative humidity, and 8% CO_2 in air. EDTA-treated, trypsinized, and liquid nitrogen-stored keratinocytes from the primary culture were used in further experiments.

First passage and induction of keratinocyte differentiation

For the experiments the human keratinocytes were seeded at 10^5 cells in KGM in 60 mm culture dishes as described before (van Ruissen et al., 1994). KGM was composed of KBM (Clonetics, San Diego, CA; 0.15 mM calcium) supplemented with ethanolamine (0.1 mM) (Sigma), phosphoethanolamine (0.1 mM) (Sigma), bovine pituitary extract (BPE; 0.4% v/v) (Clonetics), epidermal growth factor (EGF; 10 ng/ml) (Sigma), insulin (5 μg/ml) (Sigma), hydrocortisone (0.5 μg/ml) (Collaborative Research, Inc.), penicillin (100 U/ml) (Gibco), and streptomycin (100 μg/ml) (Gibco).

Keratinocyte differentiation was induced by switching confluent keratinocyte cultures for 48 h to KGM supplemented with FCS (KGM/FCS), KGM depleted with growth factors (BPE, EGF, hydrocortisone, and insulin; KGM/-GF), and KGM containing high calcium (1.8 mM; KGM/Ca). As a control, we switched confluent keratinocyte cultures to KGM alone (KGM) for another 48 h.

Northern blotting

Total RNA was isolated from cultured human keratinocytes and frozen keratome biopsies derived from normal healthy epidermis and psoriatic lesional epidermis. The normal volunteers had no personal or family history of psoriasis. The isolation was performed using RNAzol™, as suggested by the manufactor (Cinna/Biotex Laboratories, Inc., Houston, TX). RNAzol™ was added to either harvested and frozen (−80°C) keratinocyte cultures, viable keratinocyte cultures, or frozen keratome biopsies.

RNA concentrations were determined from the absorbance at 260 and 280 nm and equal quantities (10 μg) of total RNA were electrophoretically separated in a 1% agarose gel (dissolved in 10 mM sodium phosphate buffer; pH = 7.0), as described in Maniatis (Sambrook et al., 1989). Gels were run submerged in 10 mM sodium phosphate buffer. The pH of the electrophoresis buffer was kept within acceptable limits by constant circulation of the buffer. The gels were blotted using 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate). After transfer, membranes were washed in 2× SSC, and RNA was crosslinked using ultraviolet irradi-

ation (312 nm, 0.2 J/cm^2). Membranes were incubated with ethidiumbromide (1 μg/ml) for 15 min prior to photography.

Filters were (pre)hybridized at 65°C according to Church and Gilbert (1984). Hybridizations were performed overnight, filters were washed for 30 min using 125 mM sodium phosphate buffer and 40 mM sodium phosphate buffer. Autoradiography was performed on X-Omat S films (Kodak) at −80°C with intensifying screens. Filters were hybridized with ^{32}P-labelled random primed probes for SKALP, psoriasin, CK1, PA-FABP, and 28S ribosomal RNA. Processing of the autoradiographs was performed using the Imagemaster^{TM} data image system (Pharmacia LKB Biotechnology AB, Uppsala, Sweden).

Immunocytochemical staining of human keratinocytes cultured on coverslips

Immunocytochemical stainings were performed using the Vectastain ABC kits for monoclonal and polyclonal antibodies purchased from Vector Laboratories, Inc. (Burlingame, CA). For these stainings, we used the following polyclonal antibodies; anti-SKALP (SSK-9201), anti-human involucrin (BT-601; Biomedical Technologies, Inc., Stoughton, MA) and monoclonal antibodies; anti-keratin 10 (RKSE-60), anti-keratin 16 (LL025), and anti-human keratinocyte transglutaminase (BT-621, Biomedical Technologies, Inc.). In brief, human keratinocytes were cultured on tissue culture coverslips (Thermanox, LAB-TEK Division, Miles Laboratories, Inc., Naperville, IL) using different culture conditions. Cells were fixed using either 1% formaldehyde (freshly prepared from paraformaldehyde) or aceton/methanol (50/50) as determined by previous experiments. Cells were stored at −20°C. Before usage, the cells were transferred to 4°C for 30 min and then air-dried at room temperature. Paraformaldehyde fixed cells were incubated for 15 min in 50 mM NH_4Cl (in PBS, pH = 7.5), and aceton/methanol fixed cells were incubated for 15 min in PBS. Immunocytochemical stainings were performed according to the description of the manufactor (Vector Laboratories Inc.).

Immunocytochemical staining for flow cytometry

SKALP. About 10^5 formaldehyde (1%) fixed cells were washed once with PBS containing 1% newborn heat inactivated calf serum (NHICS). After sedimentation, the cells were incubated with a polyclonal antibody against SKALP (1:500) for 30 min at room temperature. After washing, the cells with PBS/NHICS cells were incubated for 30 min at 37°C with goat-anti-rabbit-biotinylated antibody (GARBIO; 1:200). Following this incubation, cells were washed and incubated with a complex of avidine and biotinylated phycoerythrin (PE) (1:100) (Vectastain ABC-Phycoerythrin kit, Vector Laboratories, Inc.) for 30 min at room temperature. Finally, cells were resuspended in 300 μl PBS containing 10^{-6}M TO-PRO-3 (as previously described by van Hooijdonk et al., 1994) and incubated for 15 min with 50 μl of 1% (w/v) RNAse A.

Transglutaminase. The staining for transglutaminase was performed as described above with the exception that we used a monoclonal antibody against transglutaminase (1:15) and a rabbit anti-mouse fluoresceine isothiocyanate (RAM-FITC) antibody to visualize transglutaminase. The DNA content was determined with propidium iodide (PI) instead of TO-PRO-3.

Flow cytometry. Cells stained with propidium iodide or TO-PRO-3 and fluoresceine isothiocyanate (FITC) or phycoerythrin (PE) were analyzed on the Epics® Elite flow cytometer (Coulter Corporation, Hialeah, FL) equipped with a 25 mW argon-ion laser set at 15 mW and a 10 mW He/Ne laser. FITC and PI signals were separated by a 550 nm dichroic mirror. The FITC signals (green fluorescence) were detected through a 630 nm long pass filter. PE and TO-PRO-3 signals were separated by a 625 nm dichroic mirror. The PE signals (orange fluorescence) were detected through a 575 nm band pass filter, and the TO-PRO-3 signals (pink/deep red fluorescence) were detected through a 675 nm band pass filter.

Usually 10^4 cells were measured at a flow rate of approximately 50 cells per second. The data were recorded in listmode and analyzed on the Epics® Elite workstation. The ratio area/peak of the red fluorescence is an excellent discriminator between artifacts due to doublets of diploid cells and real single tetraploid (or late S) cells when intact cells are used (Bauer and Boezeman, 1983).

RESULTS

First passage normal human keratinocytes were cultured using serum-free KGM. When cultures reached confluence, they were switched to KGM/FCS, KGM/-GF, or KGM/Ca and cultured for another 48 h. For comparison, confluent cultures were kept in KGM alone for 48 h, which does not allow a significant degree of keratinocyte differentiation. Expression of SKALP/elafin, CK10, involucrin, transglutaminase, and CK16 was studied by immunocytochemistry. SKALP/elafin, CK1, psoriasin, and PA-FABP were studied on Northern blots. Flow cytometry was used to measure cell cycle parameters, and to quantify and further characterize the SKALP/elafin-positive cell population.

Immunocytochemistry

In the differentiated compartment of normal human epidermis involucrin, CK10, and transglutaminase are expressed whereas SKALP/elafin and CK16 are absent. In lesional psoriatic epidermis there is an increased expression of involucrin and transglutaminase due to a premature expression in the spinous layers. In contrast to normal epidermis, SKALP and CK16 are expressed at high levels in the suprabasal layers of lesional psoriatic epidermis, as shown previously. When confluent keratinocyte cultures grown in KGM were maintained in KGM for another 48 h, only a limited amount of cells is induced to differentiate. In this system, CK10 expression is absent (Fig. 1a), while involucrin and SKALP are expressed only by a few scattered cells (Figs. 2a, 3a). CK16 and tranglutaminase are expressed by a small population of cells that show a rather intense staining (Figs. 4a, 5a).

Switching confluent keratinocyte cultures to KGM/FCS for 48 h results in a strong upregulation of SKALP and involucrin expression (Figs. 2b, 3b) in a distinct population of large polygonal cells on top of the basal cell layer. Also, the number of cells expressing transglutaminase and CK16 is strongly increased (Figs. 4b, 5b), although the staining intensity in the positive cells is less than in the small population of transglutaminase

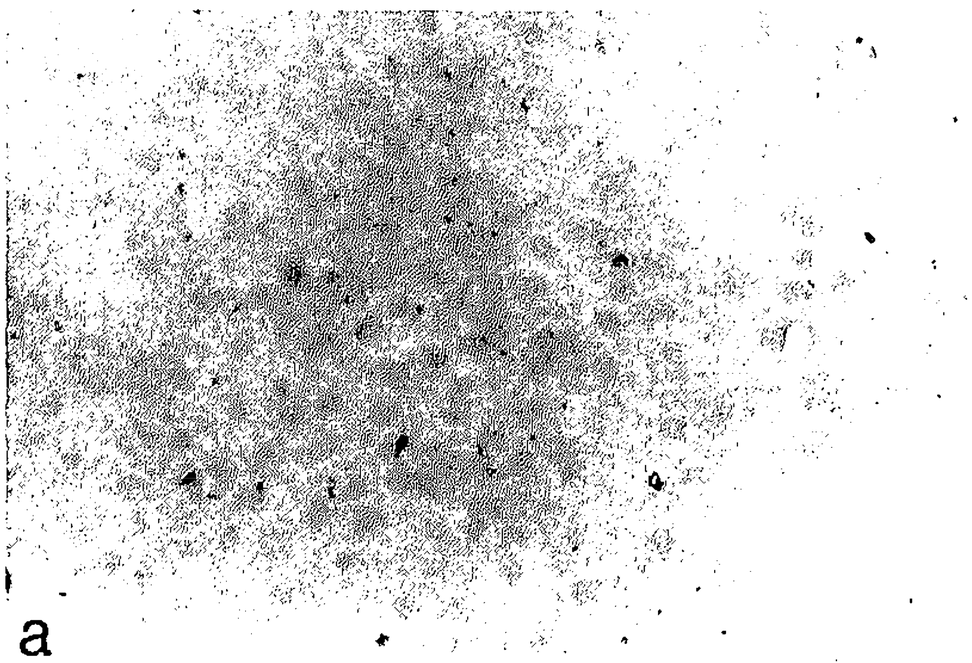

Fig. 1. Immunocytochemical staining for CK10 on human keratinocytes cultured on coverslips under four different culture conditions. KGM (**a**), KGM/FCS (**b**), KGM/-GF (**c**), and KGM/Ca (**d**). Only in KGM/-GF a significant number of CK10 positive colonies were found at 48 h. At 72 h, the entire suprabasal layer was positive for CK10 (not shown).

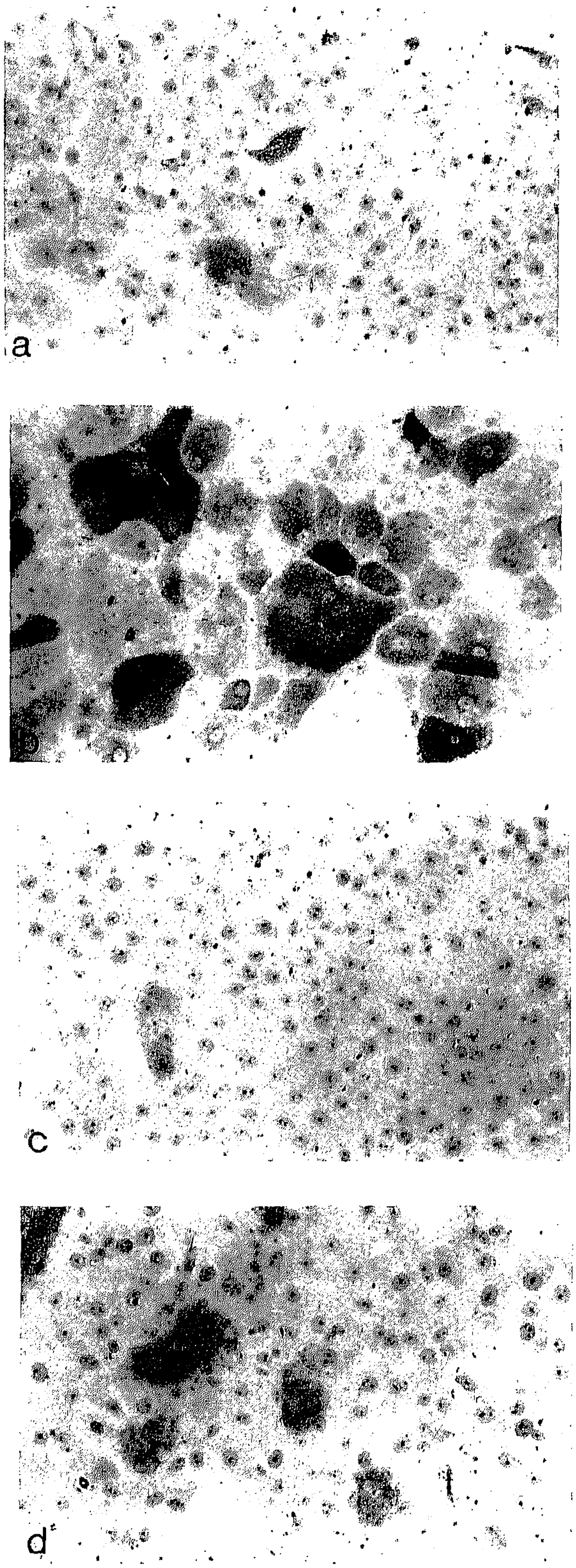

Fig. 2. Immunocytochemical staining for SKALP on human keratinocytes cultured on coverslips under four different culture conditions. KGM (**a**), KGM/FCS (**b**), KGM/-GF (**c**), and KGM/Ca (**d**). Note the strong staining for SKALP in the large polygonal cell population cultured in KGM/FCS.

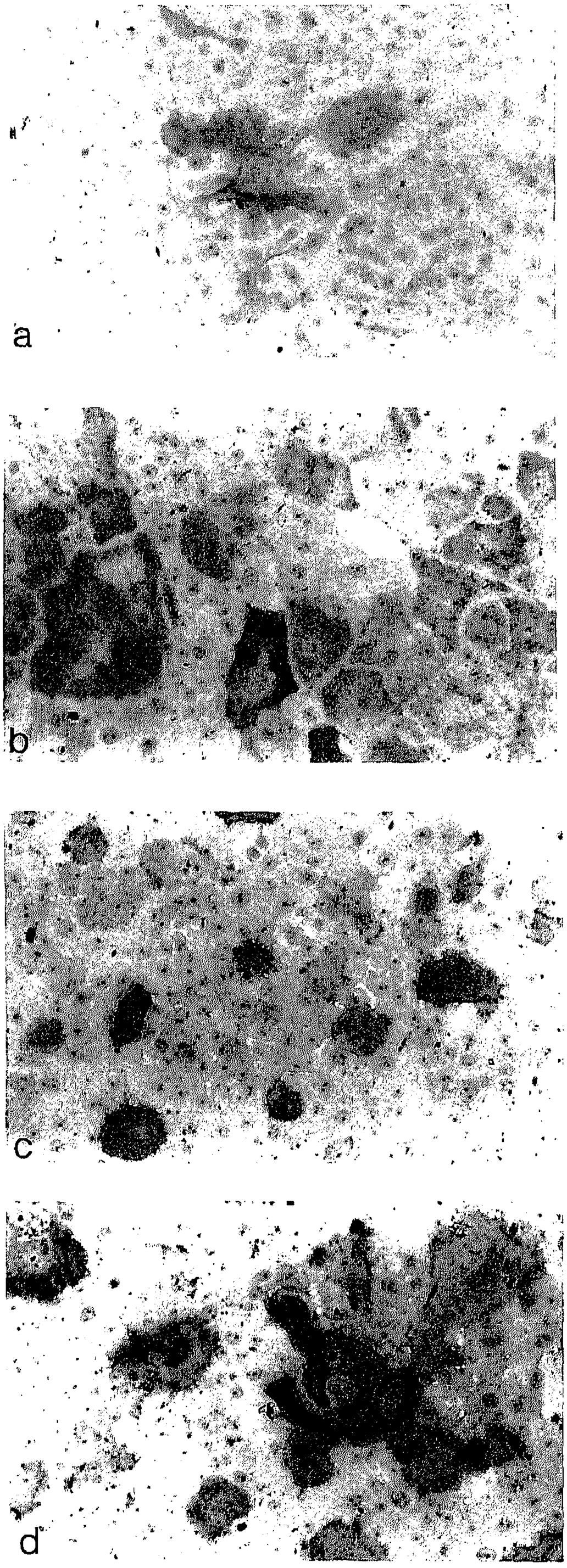

Fig. 3. Immunocytochemical staining for involucrin on human keratinocytes cultured on coverslips under four different culture conditions. KGM (**a**), KGM/FCS (**b**), KGM/-GF (**c**), and KGM/Ca (**d**). Involucrin-positive cells were found in all three models for differentiation, whereas only a few scattered involucrin-positive cells were found in KGM alone.

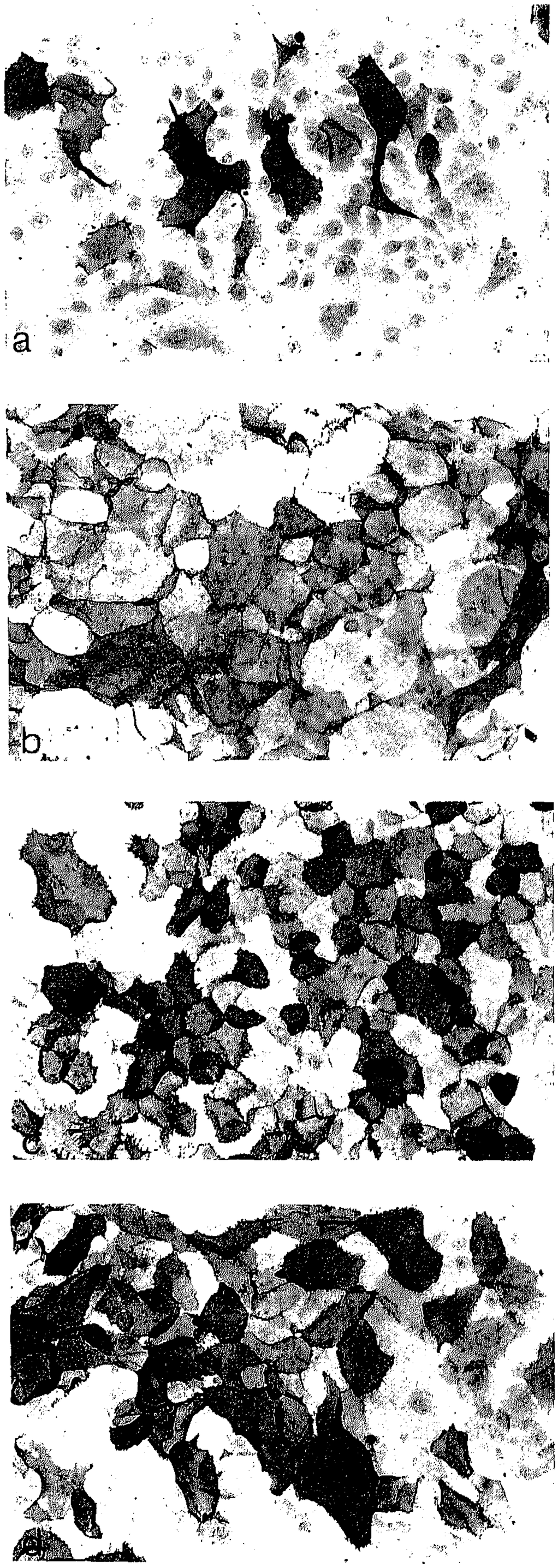

Fig. 4. Immunocytochemical staining for transglutaminase on human keratinocytes cultured on coverslips under four different culture conditions. KGM (**a**), KGM/FCS (**b**), KGM/-GF (**c**), and KGM/Ca (**d**). Note that transglutaminase is abundantly expressed in all three differentiation models, whereas in KGM alone, only a few cells are positively expressed.

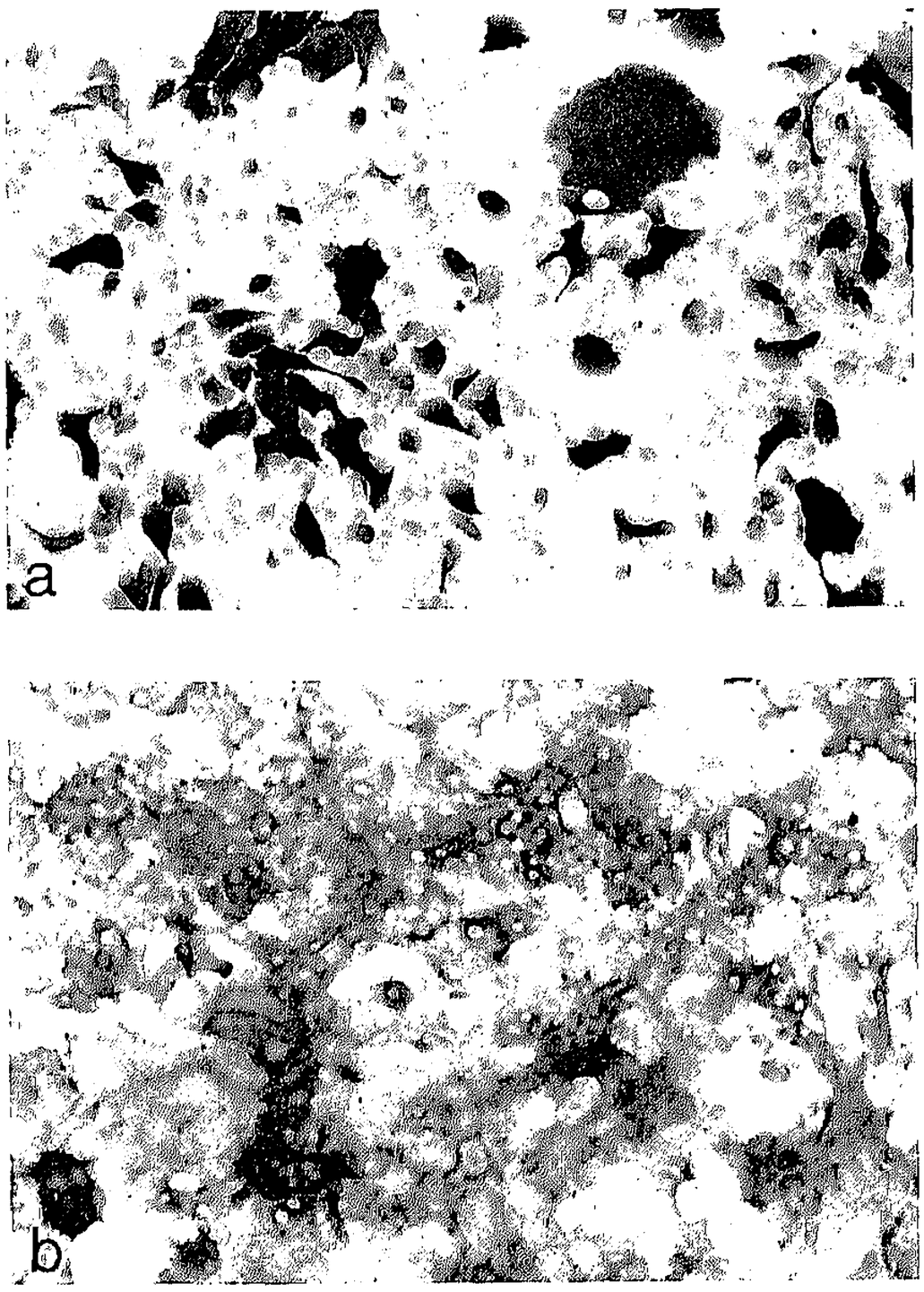

Fig. 5. Immunocytochemical staining for CK16 on human keratinocytes cultured on coverslips under four different culture conditions. KGM (**a**), KGM/FCS (**b**), KGM/-GF (**c**), and KGM/Ca (**d**). Note that in KGM/FCS and KGM/Ca nearly the entire plate is covered by moderately CK16-positive cells, whereas in KGM and KGM/-GF the number of cells is much smaller but the staining is more intense.

or CK16 positive cells in KGM alone (compare Figs. 4a–b and 5a–b). Addition of FCS does not induce expression of CK10 (Fig. 1b).

When confluent cultures were switched to KGM/-GF, only a few cells expressed SKALP (Fig. 2c). The expression of SKALP in this system is comparable to cultures switched to KGM alone, but is strongly decreased compared to cultures switched to KGM/FCS. The number of cells that express involucrin is far lower than in KGM/FCS, and is only slightly higher than in KGM alone (Fig. 3c). It was noted that the intensity of involucrin staining increased in cultures switched to KGM/-GF compared to KGM/FCS, and that cells differ morphologically from cells cultured in KGM alone. Growth factor depletion results in striking differences compared to the other culture systems. After 48 h, a population of cells expresses CK10 (Fig. 1c), and after 72 h, nearly all suprabasal cells are CK10 positive (not shown). Cytokeratin 16 is expressed by a somewhat larger population of cells compared to KGM alone (Fig. 5c). Although the number of CK16-positive cells in KGM/-GF is smaller than in KGM/FCS, the intensity of CK16 staining in the positive population is markedly higher (compare Fig. 5b–c).

The expression level of involucrin and transglutaminase in keratinocytes cultured in KGM/Ca was strongly increased compared to KGM alone and was comparable to cultures switched to KGM/FCS (Figs. 3d, 4d). The number of cells expressing SKALP, however, was low and is comparable to culture conditions with KGM alone. The expression of CK16 (Fig. 5d) is increased compared to cultures switched to KGM and very few cells are expressing CK10 (Fig. 1d). Results from the immunocytochemical stainings are summarized in Table 1. Data on expression levels in vivo are taken from the literature, but were independently confirmed by us (not shown).

Northern blot analysis

In order to confirm and extend these immunocytochemical observations, Northern blots were hybridized using probes for SKALP, CK1, psoriasin, and PA-FABP (results are summarized in Table 2). Using these different markers it is clear that in normal epidermis (Fig. 6, lane 1) expression of SKALP, PA-FABP, and psoriasin is low to undetectable, and CK1 is present. In contrast to normal epidermis, psoriatic lesional epidermis (Fig. 6, lane 2) shows a strong expression of SKALP, PA-FABP, and psoriasin.

Switching confluent human keratinocyte cultures to KGM for another 48 h (Fig. 6, lane 3) shows moderate expression of SKALP. The expression of SKALP is partly dependent on the confluency of the culture, since subconfluent cultures are negative for SKALP (data not shown). These cultures show a faint expression of PA-FABP and do not allow the expression of psoriasin or CK1.

In KGM/FCS (Fig. 6, lane 4) a strong upregulation of SKALP expression is seen. Using this system, a weak expression of PA-FABP and psoriasin is detectable. Under these culture conditions, CK1 is not expressed.

In contrast to keratinocytes cultured in KGM or KGM/FCS, CK1 expression is strongly induced when confluent cultures were switched to KGM/-GF for 48 h (Fig. 6, lane 5). This system further results in the downregulation of SKALP expression to nearly unde-

TABLE 1. Summary of the immunocytochemical stainings performed on human keratinocytes cultured on coverslips[1]

	KGM	KGM/FCS	KGM/-GF	KGM/Ca	Normal epidermis[2]	Lesional psoriatic epidermis[2]
SKALP	+/−	++	+/−	+/−	−	++
Involucrin	+/−	++	+	++	+	++
CK10			+	+/−	+	+
CK16	+[3]	+++	++[3]	+++	−	+
Transglutaminase	+[3]	+++	++[3]	++	+	++

[1]The results are semiquantitative on a 4-point scale indicating absence of positive cells (−), few positive cells (+), many positive cells (++), nearly complete covering of the plate with positive cells (+++).
[2]Data taken from the literature and confirmed by us.
[3]In these cultures, a relatively small population of intensely stained cells is present.

TABLE 2. Summary of the Northern blot analyses performed on total RNA isolated from keratinocytes cultured under the various conditions

	KGM	KGM/FCS	KGM/-GF	KGM/Ca	Normal epidermis[1]	Lesional psoriatic epidermis[1]
SKALP	+	+++	−/+	+	−	++
CK1	−	−	+++	+/−	++	+++
Psoriasin	−	+/−	−	−	−	+++
PA-FABP	+/−	+/−	+++	−/+	−/+	+

[1]Data taken from the literature and confirmed by us.

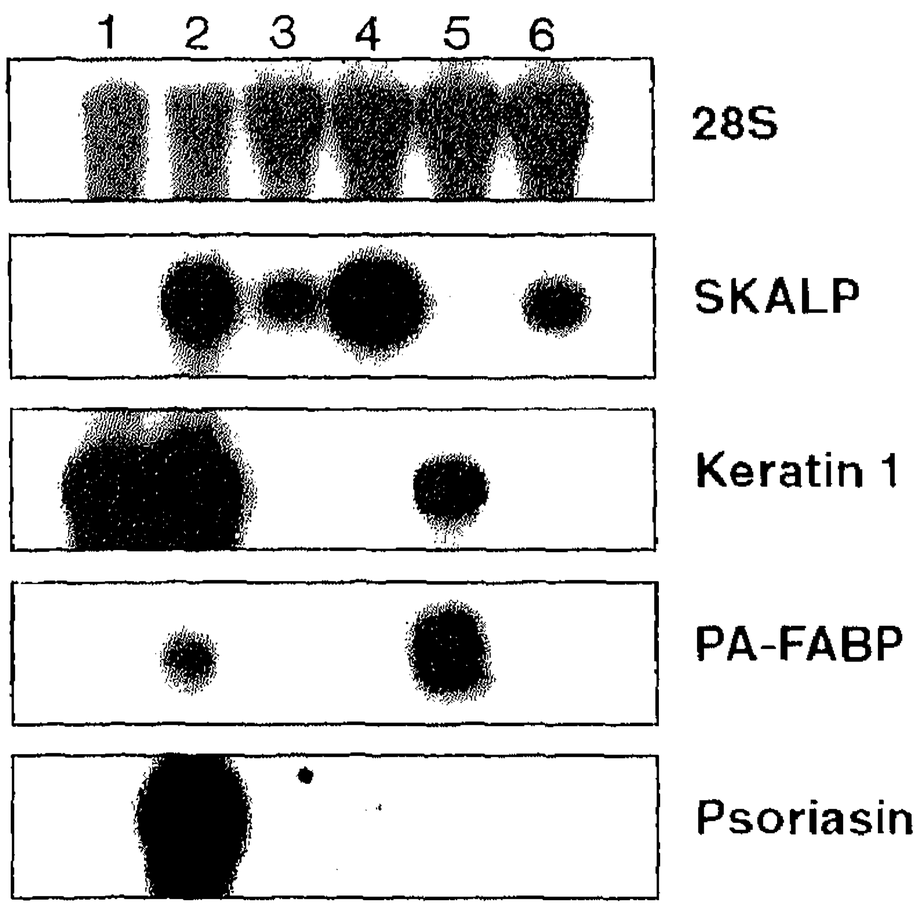

Fig. 6. Northern blot analysis of cultured human keratinocytes compared to normal epidermis and lesional psoriatic epidermis. Total RNA was isolated from normal epidermis (**lane 1**), lesional psoriatic epidermis (**lane 2**), keratinocytes cultured in KGM (**lane 3**), keratinocytes cultured in KGM/FCS (**lane 4**), keratinocytes cultured in KGM/-GF (**lane 5**), and keratinocytes cultured in KGM/Ca (**lane 6**). Hybridizations were performed using probes for PA-FABP, CK1, SKALP, Psoriasin, and 28S ribosomal RNA to check for equal loading.

tectable levels when compared to KGM alone. Furthermore, we see a strong upregulation of PA-FABP in cultures depleted of growth factors. The high expression level of PA-FABP in KGM/-GF is the only marker which does not match the pattern found in vivo.

Using culture conditions with high calcium (Fig. 6, lane 6) there is moderate expression of SKALP, which is comparable to the expression of SKALP in cultures switched to KGM. In this system, a very weak expression of CK1 and PA-FABP is detectable.

Flow cytometry

In addition to immunocytochemical stainings and Northern blot analysis, we applied flow cytometry to study cell cycle parameters under these four culture conditions. Keratinocytes in KGM, KGM/FCS, and KGM/Ca had similar relative DNA distributions as determined by propidium iodide staining. The percentages of cells in S-phase were 20.9, 18.1, and 24.2%, respectively, indicating that these cultures were still hyperproliferative. However, when using KGM/-GF the percentage of cells in S-phase dropped to 11.2%, indicating that these cultures were relatively quiescent compared to the other models.

The immunocytochemical data presented in Figures 1–5 only provide semi-quantitative data on expression of the selected antigens. In order to obtain quantitative data, we performed flow cytometric measurements of SKALP and transglutaminase. Figure 7b shows a dot plot of SKALP positive cells (phycoerythrin fluorescence on the Y-axis) against the right angle scatter (X-axis) which is a measure for cell size. From this figure, it can been seen that the mean right angle scatter of the SKALP-positive cells (window R3) is shifted to the right compared to the negative cells (window R2) indicating that the SKALP positive cells are, on average, bigger than the SKALP negative cells. The SKALP positive population (window R3) comprises about 22% of the total population (windows R2 + R3). Figure 8 demonstrates the transglutaminase expression under our culture conditions. These figures show that when subconfluent cultures were cultured to post confluence, their transglutaminase expression increased from 1.3 to 6.5%. Switching confluent cultures to KGM/-GF or KGM/Ca results in a population of transglutaminase positive cells of 8.6 and 12.4%, respectively. The largest increase in transglutaminase expression was obtained when confluent cultures were switched to KGM/FCS. In this system, we see a population of transglutaminase positive cells that comprises 28.4% of the total cell pop-

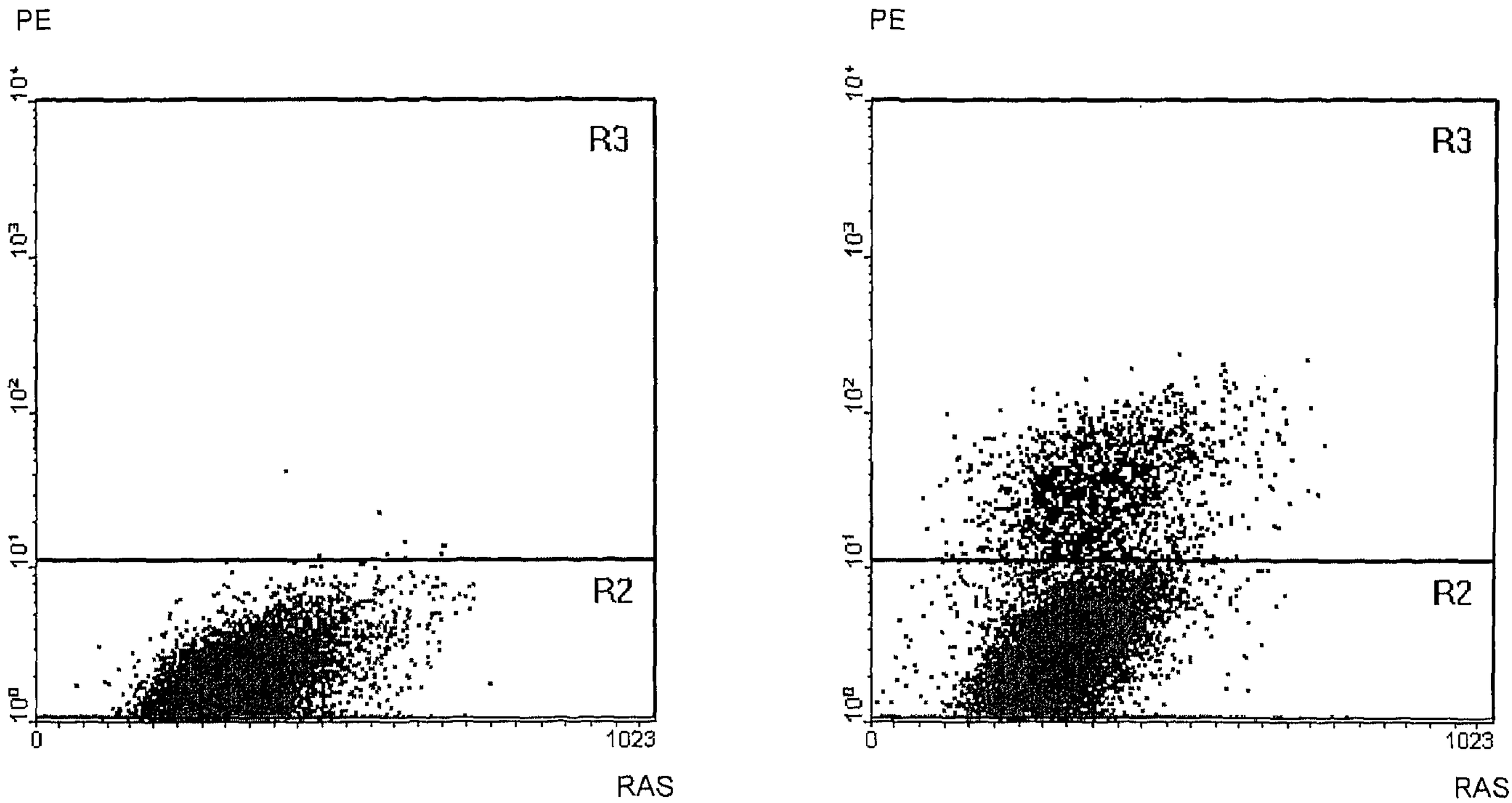

Fig. 7. Flow cytometric characterization of SKALP positive keratinocytes. Human keratinocytes were cultured in KGM/FCS, trypsinized, fixed in paraformaldehyde and immunocytochemically stained for SKALP. The control staining (left) was used to discriminate between negative (window R2) and positive cells (window R3) in the population stained for SKALP (right). On the Y-axis: fluorescence of the phycoerythrin signal; on the X-axis: the right angle scatter which is an indicator for cell size. Note that the SKALP-positive population is slightly shifted to the right compared to the negative cells, indicating that the SKALP positive cells are, on average, bigger than the SKALP-negative cells.

ulation. The quantitative data are in accordance with the immunocytochemical results.

DISCUSSION

Culture systems for human keratinocytes have provided useful models to study cellular control of epidermal growth and differentiation in vitro. However, none of the systems that are commonly used, either submerged or air-exposed, exactly matches the phenotype found in normal human epidermis. Submerged cultures such as the Rheinwald-Green system or the serum-free KGM system allow exponential growth, but only a limited amount of normal differentiation. Air-exposed cultures, although they provide a stratified morphology which resembles normal epidermis, are phenotypically abnormal in that they are characterized by the expression of cytokeratin 6/16, SKALP/elafin, and presence of involucrin and transglutaminase in the spinous layers, as we and others have previously shown (Alkemade et al., 1994; Ponec, 1991). The air-exposed models are, therefore, more similar to hyperproliferative epidermis (as found in psoriasis and injured skin) than to normal epidermis. Although the air-exposed systems are elegant models for reconstructing the tissue in vitro, a serious drawback is that they are not suitable for large scale screening purposes and for experimental manipulation such as transient transfection.

The aim of our study was to obtain keratinocyte culture models that could be used to study the normal and the psoriatic differentiation pathway. Since we intended to use these models for large scale cell biological and pharmacological studies, we opted for submerged culture conditions in the KGM system. Using three models for induction of differentiation (addition of FCS, depletion of growth factors, and high Ca^{++}), we have studied proliferative rates and the expression levels of differentiation-related molecules associated with the normal and the psoriatic phenotype. All three models share a number of differentiation characteristics, as assessed by morphological criteria (appearance of a suprabasal layer of large polygonal cells) and the expression of involucrin and transglutaminase type 1. However, we found that none of the models exactly follows the normal or the psoriatic profile of differentiation markers, and each model displayed a distinct combination of differentiation-related molecules. However, two models stand out that appear to be suitable for further study, as they closely follow the expression of markers that are typical for either differentiation pathway. Cytokeratin 16, SKALP/elafin, and psoriasin are molecules that are not expressed in normal epidermis, but are highly expressed in psoriatic epidermis. As shown in Table 1, induction of differentiation by the addition of FCS is characterized by high expression levels of these molecules compared to undifferentiated cultures (KGM alone) and compared to the other differentiation models. We, therefore, suggest that induction of differentiation by FCS could be a useful model for the psoriatic phenotype. When differentiation is induced by depletion of growth factors, a model which was recently described by Poumay and Pittelkow (1995), SKALP expression is very low, psoriasin expression is undetectable, whereas cytokeratin 1 and 10 are expressed. The marker profile of these cells is similar to that found in

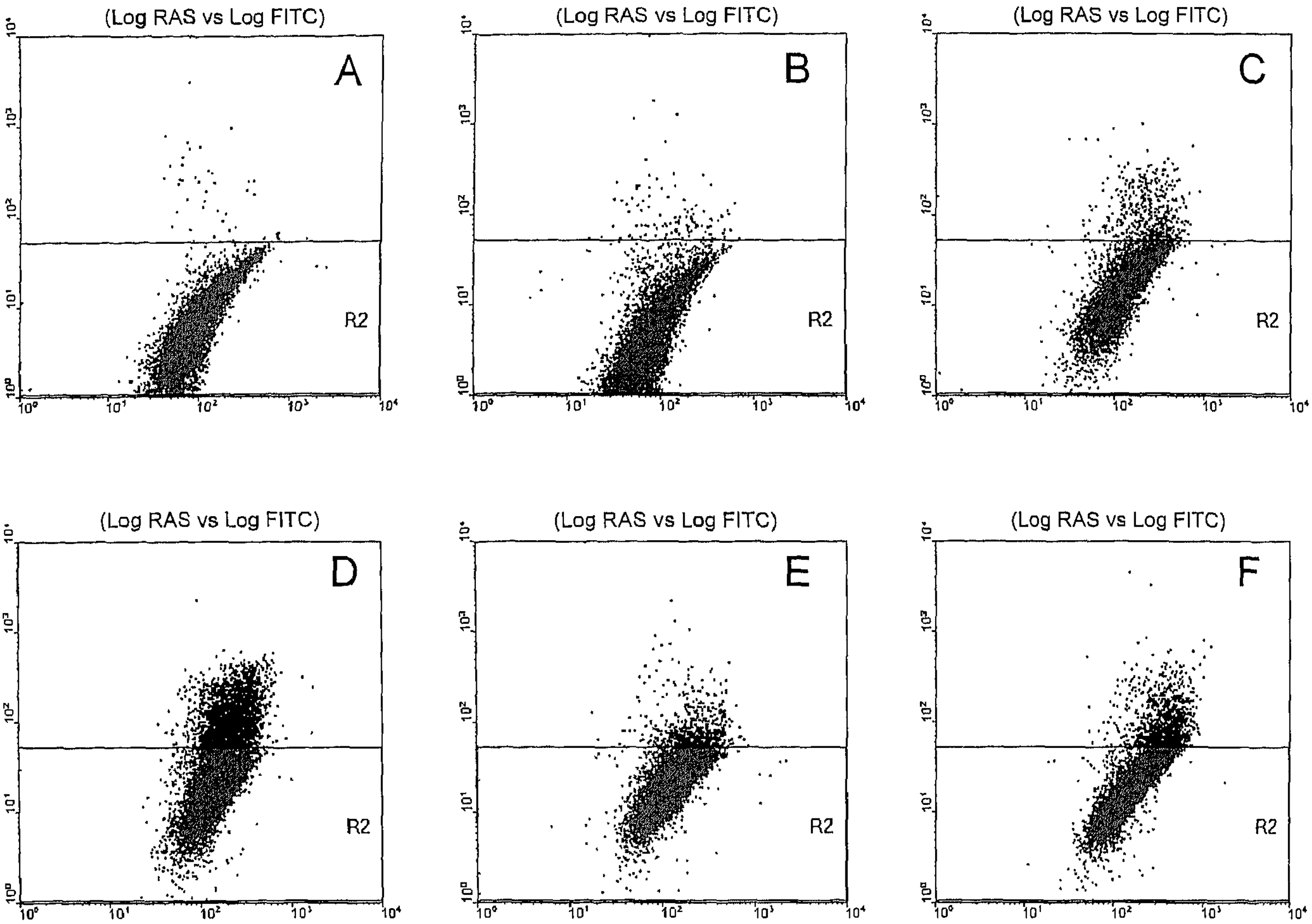

Fig. 8. Flow cytometric characterization of transglutaminase positive keratinocytes. Human keratinocytes were cultured in different models, trypsinized, fixed in acetone/methanol and immunocytochemically stained for transglutaminase. The control staining (panel A) was used to discriminate between negative (window R2) and positive cells (upper window) in the population stained for transglutaminase). On the Y-axis: fluorescence of the FITC signal; on the X-axis: the right angle scatter which is an indicator for cell size. The percentage of transglutaminase positive cells was determined in the following cultures: (**A**) subconfluent culture in KGM (control panel), (**B**) subconfluent culture in KGM; the following panels represent confluent cultures switched to (**C**) KGM for another 48 h, (**D**) KGM/FCS for another 48 h, (**E**) KGM/-GF, and (**F**) KGM/Ca.

normal epidermis, and we suggest that this could be a useful model to study the normal differentiation pathway. In addition to the profile of differentiation markers, the proliferative status of these two models also resembles the in vivo situation. In KGM/FCS, a high proliferative rate is maintained, whereas in KGM/-GF cells appear to be quiescent. Quiescence in this model, however, has to be interpreted with some caution since a number of cells do not accumulate in G_0, but are scattered randomly over the cell cycle phases, as we have shown before (van Ruissen et al., 1994). The third model, using a high Ca^{++} concentration, is the least specific and displays a marker profile which is intermediate between the other two models. In addition to the aforementioned markers, we also studied the expression of PA-FABP, which is a lipid transport molecule expressed in the stratum granulosum of normal epidermis, and throughout the suprabasal layers of psoriatic epidermis. PA-FABP expression does not follow the pattern found for most other markers, as it is highly expressed in the growth factor depleted cultures and expressed at low levels in the FCS treated cultures. A possible explanation for the induction of PA-FABP could be that the growth factor depleted cultures have no lipid source in the medium, whereas the other two media have lipids or lipid precursors present (FCS and/or BPE). Previous studies by others have documented the relationships between epidermal differentiation and lipid metabolism (Brod et al., 1991; Boyce and Williams, 1993; Schurer et al., 1994; Larsen et al., 1994). As shown in figure 5 and Table 1, a considerable number of cytokeratin 16 positive cells was present in confluent cultures in KGM alone and in all three differentiation models. Expression of cytokeratin 16 appears to be a function of cell density rather than being under control of the culture media used (not shown). It was recently described by others that control of keratinocyte commitment to terminal differentiation, and the expression of selected differentiation genes are mediated by cell confluence and by specific culture factors. It also shows that induction of differentiation was independent of the calcium concentration, and the presence

of growth factors inhibited cytokeratin 1 and 10 gene expression (Poumay and Pittelkow, 1995). In this study, we confirmed these findings.

The finding that addition of serum could induce expression of psoriasis associated markers could very well be relevant for the in vivo situation. We and others have found that during inflammation (wound healing, psoriasis) the epidermis becomes more permeable to plasma derived proteins (Andriessen et al., 1995; Verschoore et al., 1990). Speculatively, epidermal hyperproliferation and abnormal differentiation as seen in psoriasis could be induced by the presence of growth factors and cytokines from the interstitial fluid and plasma. In this study, we have used FCS for induction of psoriatic differentiation; we have found, however, that human serum is equally effective in inducing SKALP expression both at the protein and mRNA level (data not shown).

In vitro systems for keratinocyte culture can be used to study the cell biology of epidermal differentiation and to characterize the factors that regulate expression levels of individual genes or coordinated expression of sets of genes. Alternatively these systems can be used for large scale screening of drugs. We are currently using the models on a smaller scale (96-well plates) to test the effect of drugs on selected differentiation genes. Preliminary results indicate that, in this system, the effect of retinoids and retinoid-like compounds can be easily screened using downregulation of SKALP and transglutaminase as a read-out in a direct cell-ELISA as described by others (Daneels et al., 1994). Ideally, we would like to obtain culture systems with predictive power concerning the effects of drugs on keratinocyte differentiation in vivo. Future studies will be aimed at further refining of the models by adjusting the cell density and the lipid availability in order to match the marker profiles of the in vitro system more closely to the in vivo differentiation pathways.

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