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Keratin 19 as a biochemical marker of skin stem cells in vivo and in vitro: keratin 19 expressing cells are differentially localized in function of anatomic sites, and their number varies with donor age and culture stage

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SUMMARY

This study was undertaken to evaluate keratin 19 (K19) as a biochemical marker for skin stem cells in order to address some long standing questions concerning these cells in the field of cutaneous biology. We first used the well-established mouse model enabling us to identify skin stem cells as [^{3}H]thymidine-label-retaining cells. A site directed antibody was raised against a synthetic peptide of K19. It reacted specifically with a 40 kDa protein (K19) on immunoblotting. It labelled the bulge area of the outer root sheath on mouse skin by immunohistochemistry. Double-labelling revealed that K19-positive-cells were also [^{3}H]thymidine-label-retaining cells, suggesting that K19 is a marker for skin stem cells of hair follicles. K19-expression was then used to investigate the variation in mouse and human skin stem cells as a function of body site, donor age and culture time. K19 was expressed in the hair follicle and absent from the interfollicular epidermis at hairy sites (except for some K18 coexpressing Merkel cells). In contrast, at glabrous sites, K19-positive-cells were in deep epidermal rete ridges. K19 expressing cells also contained high levels of $\alpha_3\beta_1$ integrin. The proportion of K19-positive-cells was greater in newborn than older foreskins. This correlated with keratinocyte culture lifespan variation with donor age. Moreover, it could explain clinical observations that children heal faster than adults. In conclusion, K19 expression in skin provides an additional tool to allow further characterization of skin stem cells under normal and pathological conditions in situ and in vitro.

Key words: Stem cell, Skin, Intermediate filament, Keratin, Epithelium, Aging

INTRODUCTION

Keratinocytes, epithelial cells of the epidermis, are organized into multiple layers, and undergo terminal differentiation (stratum corneum) as they move through the suprabasal layers toward the skin surface. Squames from the stratum corneum are continually shed or lost in the environment. The maintenance of this self-renewing tissue is possible through stem cells which are responsible for skin homeostasis. Therefore, stem cells must be present throughout life. However, the variation in the number of stem cells after birth remains to be determined. Based on previous in vivo studies of several tissues, stem cells are expected to possess many of the following properties: they are relatively undifferentiated, both ultrastructurally and biochemically; they retain a high capacity for self-renewal throughout adult-life and have a large proliferative potential; they are normally slow-cycling, but can be stimulated to proliferate in response to injuries and to certain growth stimuli; finally they are usually found in a well-protected, highly vascularized and innervated area (Hall and Watt, 1989; Lajtha, 1979; Lavker and Sun, 1982; Leblond, 1981; Potten and Loeffler, 1990).

One of the most distinguishing features of stem cells is their slow-cycling nature. They divide infrequently and have a long cell cycle. Following [^{3}H]thymidine uptake, they retain the radioactive label over a long period of time (Bickenbach, 1981; Potten, 1981). In mouse skin, slow-cycling cells were initially localized in the basal layer of the epidermis (Bickenbach, 1981; Potten, 1981). Then, stem cells of the pilosebaceous unit were thought to reside in the matrix cells of the bulb area because of: (i) the high proliferation rate of these cells; (ii) their role in the growing phase of hair follicles; and (iii) the importance of the dermal papilla in the activation of hair growth (Kligman, 1959; Oliver and Jahoda, 1988; Pinkus, 1978; Reynolds and Jahoda, 1991). However, recently, thymidine-label-retaining cells of the hair follicle

were shown to reside in the bulge area, the portion of the outer root sheath localized under the sebaceous gland to which the arrector pili muscle attaches (Madsen, l964; Mehregan and Hashimoto, 1986), indicating that stem cells are not in the bulb but in the bulge region (Cotsarelis et al., 1990; Lavker et al., 1993).

As the characterization of any tissue stem cell population remains a difficult task, a new strategy to reach this goal consists in developing phenotypic markers distinguishing differentiated cells from progenitors. Interesting results have been obtained in several tissues: hepatic, neuronal, and mainly hematopoietic tissues, using many monoclonal antibodies (Brill et al., 1993; Germain et al., 1988a; Lendahl et al., 1990; Marceau et al., 1989; Spangrude et al., 1988; Thorgeirsson, 1993; Uchida et al., 1993). In skin, stem cells could be distinguished from differentiated keratinocytes according to their higher expression of β1 integrins. The β1 integrin-bright cell population contains stem cells, but remains heterogeneous and other markers, such as keratins, could help to better define the stem cell compartment (Bata-Csorgo et al., 1995; Jones and Watt, 1993; Jones et al., 1995).

The main proteins of skin epithelial cells are the keratins which assemble into 10 nm filaments that are part of the cytoskeleton (Coulombe and Fuchs, 1990). There are over twenty distinct keratins expressed in soft epithelial tissues, many of which are present in various skin epithelial cells (Moll et al., 1982a). The keratin distribution is specific for each epithelial tissue and varies according to the differentiation status of the cells (Fuchs and Green, 1980; Lane et al., 1985; Osborn and Weber, 1982; Sun et al., 1983). In the epidermis, the basal cells express keratins 5 and 14 whereas the suprabasal cells, which are more differentiated, express keratins 1 and 10 (Fuchs, 1990). In the hair follicle, the keratin distribution pattern is more complicated, reflecting the structural complexity of this skin appendage. Beside the above-mentioned epidermal keratins certain regions of hair follicles express keratins 6, 16 and 17 (Heid et al., 1988; Moll et al., 1982b; Stark et al., 1987).

The skin also contains minor keratins, which are only present in a small proportion of cells, such as keratin 19 (K19). Hardly detectable biochemically, these keratins can be visualized by histolocalization. The K19, a protein of 40 kDa, is the smallest member of the family and has a wide tissue distribution (Bartek et al., 1985; Heid et al., 1988; Moll et al., 1982a; Stasiak et al., 1989; Su et al., 1993). In foetal human skin, K19 is observed in cells of the basal layer (Dale et al., 1985; Oliver, 1990). In contrast, in adult human skin, K19 is restricted to the outer root sheath of the hair follicle and has not been detected in the epidermis (Stasiak et al., 1989). The particular localization of K19 positive cells in various epithelia led to the hypothesis that it could be a marker for epithelial progenitors (Bartek et al., 1986). In the skin, this idea was suggested (Lane et al., 1991; Stasiak et al., 1989) based on the presence of K19 in the bulge area of the hair follicle where the [^{3}H]thymidine-label-retaining cells have been identified.

In the present study, we tested the possibility that K19 could be a marker of skin stem cells, and addressed a number of important issues regarding this cell population. Our results show that K19 expressing cells in the skin are: (i) slow-cycling cells; (ii) localized in the bulge area of the hair follicle; (iii) coexpressing a high level of $\alpha_3\beta_1$ integrin; (iv) absent from the interfollicular epidermis at hairy skin sites (except for some keratin 18 (K18) coexpressing Merkel cells) but present in deep rete ridges at glabrous skin sites; (v) decreasing in number from newborn to adult; (vi) present in keratinocyte culture and their number in situ was proportional to the in vitro culture lifetime of the epithelial cells. Taken together, these data strongly suggest that K19 is a potential biochemical marker for cutaneous stem cells. Moreover, these results would imply a differential skin stem cell localization in function of the anatomic site and a decrease in stem cell number with donor age.

MATERIALS AND METHODS

Antibodies

A site-directed polyclonal antibody was raised in guinea pig against the specific amino acid sequence 391-404 (NH_2-Glu-Ala-His-Tyr-Asn-Asn-Leu-Pro-Thr-Pro-Lys-Ala-Ile-OH) of keratin 19 (Lussier et al., 1989). This peptide was synthesized by solid-phase methodology using a scheme of synthesis based on tert-butyloxycarbonyl chemistry/acid labile amino acid protecting groups and purified by preparative reverse-phase HPLC as previously described (Gaudreau et al., 1992). Its purity was 99% as assessed by analytical HPLC (μ-Bondapak C_{18} column, Waters, 10 μm particle size, 0.39 cm × 15 cm) using two binary solvent systems (0.01% aqueous trifluoroacetic acid (TFA), pH 2.9, and acetonitrile (CH_3CN)-0.01% TFA; 0.01 M ammonium acetate, pH 6.9, and CH_3CN). The peptide synthesis yielded the predicted amino acid composition with a peptide content of 83% by quantitative amino acid analysis after acidic hydrolysis in vacuo (6 M HCl, 110°C, 18 hours) and phenylisothiocyanate derivatization (Heinrikson and Meredith, 1984). The pure peptide was adsorbed onto methylated bovine serum albumin (BSA) as carrier protein, at a ratio of 5 mg peptide/1 mg BSA and injected subcutaneously in male guinea pig, initially at a dose of 0.5 mg/kg in complete Freund's adjuvant. Preimmune serum was collected in order to use it as control. The animals were subsequently boosted five times once every three to four weeks at a peptide dose of 0.17 mg/kg, in incomplete Freud adjuvant (Benoit et al., 1987). Blood was obtained at two week intervals following each injection. Serum were tested as described below and used at dilutions of 1:500 to 1:2,000.

The following antibodies were also used: mouse monoclonal anti-human keratin 20 (IT-Ks 20.10, APR, Belmont, MA), mouse monoclonal anti-human K19 (Ks 19.1, IBL, Cambridge, MA), mouse mono-clonal anti-human keratin 18 (Ks 18.174, Progen-IBL, Cambridge, MA), fluorescein-isothiocyanate (FITC)-tagged mouse monoclonal anti-α_3 integrin subunit (VM2; Kaufman et al., 1989), FITC-tagged goat anti-guinea pig Ig (Jackson ImmunoRes Lab., West Grove, PA), FITC-tagged goat anti-mouse Ig (Cederlane, Ontario, Canada), rhodamine-tagged goat anti-mouse Ig (Chemicon, Temecula, CA), and alkaline phosphatase tagged goat anti-guinea pig IgG (H+L) (Kirkegaard and Perry Lab, Gaithersburg, MD).

Gel electrophoresis and immunoblot analysis

Total proteins of KLN 205, a cell line derived from mouse squamous cell carcinoma (Kanedo and LePage, 1978), were separated by one- and two-dimensional gel electrophoresis (SDS-PAGE and NEPHGE, 10% acrylamide). Proteins were fixed and visualized by Coomassie blue staining. For immunoblot analysis, proteins from unfixed gels were electroblotted onto nitrocellulose sheets according to methods described before (Laemmli, 1970; Marceau et al., 1986; O'Farrell et al., 1977; Towbin et al., 1979). The blots were stained with Ponceau red, photographed, destained, blocked in 5% skimmed milk, and incubated with the K19 antiserum followed by alkaline phosphatase-

conjugated goat anti-guinea pig IgG antibodies and revealed with NBT and X-Phosphate (Boehringer Mannheim Biochemica, Laval, Qc). In the controls, the first antibody was replaced by the pre-immune serum. For protein identification, the position of reference proteins (BSA, actin, 3-phosphoglycerokinase) was determined by co-electrophoresis in parallel.

Tissues

SENCAR adult and newborn mice (Harlan Sprague-Dawley, Indianapolis) were purchased from Charles River. Human skin was obtained after plastic reconstructive surgery (finger, breast) or circumcision (foreskin). Skin samples taken from different anatomic sites were embedded in OCT Compound (Miles Inc., Elkhart, IN), frozen in liquid nitrogen and kept at −70°C until use.

Cell culture

Normal human keratinocytes were isolated from foreskin, breast or finger tissues, and cultured on a feeder layer of irradiated 3T3 mouse fibroblasts as previously described (Germain et al., 1993; Rheinwald and Green, 1975). Cells were cultured in the following medium originally described by Simon and Green (1985). This medium is a combination of the DME with Ham's F12 medium in a 3:1 proportion (Flow Laboratories, Miss, Ont.), supplemented with 24.3 μg/ml adenin (Sigma Chemicals, St Louis, MO), 5 μg/ml insulin (Sigma), 5 μg/ml human transferrin (Sigma), 2×10^{-9} M 3,3′,5′-triiodo-L-thyronine (Sigma), 0.4 μg/ml hydrocortisone (Calbiochem, La Jolla, CA), 10^{-10} M cholera toxin (Schwartz/Mann, Cleveland, OH), 10% FCS (Flow Laboratories), 10 ng/ml human EGF (Chiron Corp., Emeryville, CA), 100 i.u./ml penicillin G (Sigma) and 25 μg/ml gentamicin sulfate (Scheering Inc, Pointe-Claire, Canada). Cells were plated on Petri dishes or on glass coverslips and grown in a humidified 8% CO_2 atmosphere at 37°C. Medium was changed 3 times a week.

Newborn mouse keratinocytes were isolated and cultured as previously described (Rouabhia et al., 1992), except that an irradiated fibroblast A31 3T3 feeder layer was added. For immunofluorescence study, cells were plated on glass coverslips and grown in a humidified 5% CO_2 atmosphere at 32°C.

Double-labelling procedure: co-localization of K19 and [^{3}H]thymidine-label-retaining cells

[^{3}H]Thymidine-labelling experiment was done according to the method of Cotsarelis et al. (1990). [Methyl-^{3}H]thymidine (specific activity 81.6 Ci/mmol, NEN-Dupont, Miss, Ont.), 5 μCi per gram of body weight, was injected subcutaneously into SENCAR mice twice daily for the first 7 days of post-natal life. The mice were killed after 28 days. Pieces of back skin were excised, frozen and kept as described above (see Tissues). Following immunofluorescence labelling of skin sections, autoradiography was performed as previously described (Germain et al., 1988b).

Immunofluorescence microscopy

Indirect immunofluorescence assay was performed on acetone-fixed (10 minutes at −20°C) cryostat sections (4 μm thick) or ethanol-fixed cultured keratinocytes (10 minutes at −20°C) as previously reported (Bouvard et al., 1991; Germain et al., 1988b). For the K19-K20 double labelling procedure, the sequence of the antibodies was as follows: mouse anti-human K20, rhodamine-tagged goat anti-mouse IgG, mouse anti-human K19, FITC-tagged goat anti-mouse IgG. For the K19-α_3 integrin subunit double labelling procedure, the sequence of the antibodies was as follows: mouse anti-human K19, rhodamine-tagged goat anti-mouse IgG, FITC-tagged mouse monoclonal anti-α_3 integrin subunit (VM2). Cell nuclei were also labelled with Hoechst reagent (33258) following immunofluorescence staining. They were then examined under a Nikon Optiphot microscope, equipped with epifluorescence, and photographed with a Kodak Tmax 400 ASA film.

The percentage of K19 positive cells in the basal layer was determined on foreskin tissue sections. Total basal cell number was evaluated by Hoechst-labelled nuclei counting. Over 2.5×10^4 basal cells from 9 neonate (2-7-day-old boys), 7 child (2-5 years) and 11 youth and adult foreskins (7-42 years) were counted. An average of two blocks from the same skin specimen was evaluated.

Flow cytometry analysis

Subconfluent cell cultures were trypsinized and resuspended in 0.9% NaCl at 4°C. Freshly isolated cells or trypsinized cultures were fixed in a solution of 70% ethanol and stored at −20°C until use. Cells were washed in phosphate-buffered saline (PBS) containing 1% BSA, then incubated 45 minutes at room temperature with mouse anti-K19 antibody or PBS for control. After washing, the second antibody was added to the cell suspension and incubated 30 minutes at room temperature. Cells were then washed and resuspended in PBS before analysis. Cells were analyzed on a Becton-Dickinson fluorescence-activated cell sorter (FACS®). A number of 10,000 events were acquired in list mode for each sample (except for one sample of freshly isolated keratinocytes from newborn: 5,000 events acquired), according to three parameters: forward scatter (FSC), side scatter (SSC), and green fluorescence (FL1). The FSC was gated at 200 (arbitrary units) to exclude false positive differentiated cells (a variable background staining of differentiated keratinocytes was obtained with unrelated antibodies such as anti-digoxigenin). The percentage of K19 positive cells present in the small and medium size keratinocyte populations (less differentiated, lower FSC) was evaluated by subtracting the control from the labelled cell profile in a FSC gated (channels 0-200 kept and 200-256 excluded) population, using the Consort 30 program, overlaid histogram (Becton-Dickinson) and evaluating the proportion of the cells under the peak or shoulder.

RESULTS

K19 expressing cells of the bulge area are [^{3}H]thymidine-label-retaining cells

To determine if K19 expressing cells of the bulge area were [^{3}H]thymidine-label-retaining cells, we chose to work on mice. An antiserum was raised against 14 amino acid residues of the C-terminal sequence of mouse K19. To verify the antiserum specificity, total proteins of KLN 205, a cell line derived from mouse squamous cell carcinoma, known to express K19, were separated by SDS-PAGE and then subjected to immunoblot analysis. Fig. 1A shows that the antiserum reacted specifically to a 40 kDa protein. Fig. 1B establishes that this 40 kDa antigen behaved like an acidic protein, corresponding to K19 according to the numerical classification of Moll et al. (1982a).

Immunohistological staining of frozen sections of adult mouse skin from various body sites (back, abdomen, muzzle with vibrissae) with this K19 antiserum indicated that K19 was present exclusively in the outermost layer of a defined zone of the outer root sheath of the hair follicle, located below the sebaceous gland and corresponding to the bulge area. The lower part of the hair follicle, the bulb area, and the interfollicular epidermis were not labelled (Fig. 2a). The number of K19 expressing cells was lower in smaller hair follicles compared to larger ones (back skin versus vibrissae). This K19 distribution corresponds to that reported in human skin with various antibodies (e.g. Bartek et al., 1986; Stasiak et al., 1989).

Since one of the most distinguishing features of stem cells

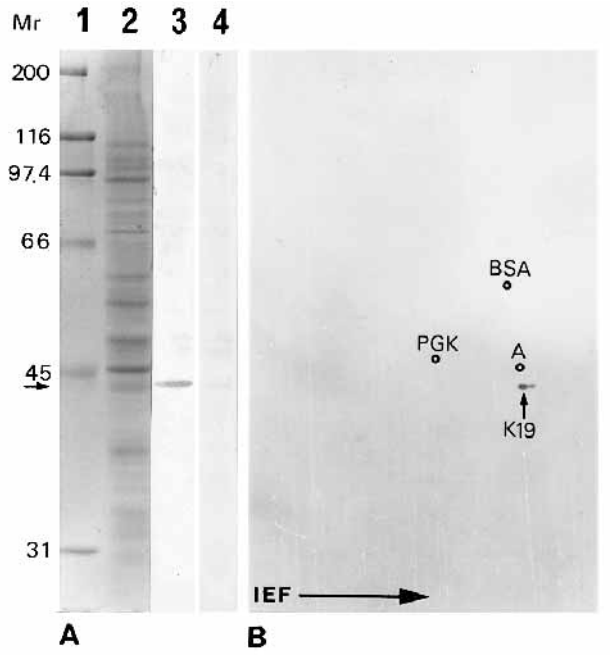

Fig. 1. Western blot analyses of the specificity of guinea pig polyclonal K19 antiserum. Coomassie blue staining of SDS-PAGE (A, lane 2) and western blotting of KLN 205 cell lysate separated by one-dimensional (A, lanes 3 and 4) or two-dimensional (B) gel electrophoresis. In A, lane 1 is Coomassie blue stained molecular mass standards separated by SDS-PAGE. Their molecular mass is indicated on the left. Small arrow indicates the position of 40 kDa. Nitrocellulose papers were incubated with guinea pig anti-K19 (A, lane 3; B) or pre-immune (A, lane 4) antisera. In B, the position of protein standards BSA, actin (A) and 3 phosphoglycerokinase (PGK) determined by co-electrophoresis in parallel, and Coomassie blue staining, is indicated. IEF, direction of isoelectric focusing.

is their slow-cycling nature, we took advantage of the method of Cotsarelis et al. (1990) to label mouse slow-cycling cells to identify hair follicle stem cells. [^{3}H]Thymidine was injected subcutaneously twice daily, during the first week of postnatal life, a period of synchronized hair growth (Chase, 1954; Wolbach, 1951). The label was chased from mice during 4 weeks and biopsies from back skin were frozen and processed for the double labelling of K19 (by immunofluorescence) and [^{3}H]thymidine (by autoradiography). On transverse sections through the bulge area, the K19 expressing cells were easily detected (Fig. 2c,e,g), and the double labelling revealed that they were [^{3}H]thymidine-label-retaining cells (Fig. 2d,f,h). Keratinocytes from the bulb region were not labelled with grains nor fluorescence (data not shown). Therefore, there is a strong correlation between K19-labelling and label-retaining cells into the hair follicle. Although label-retaining cells are observed in the interfollicular epidermis of hairy skin, the presence of stem cells at this location remains controversial (Lane et al., 1991; Yang et al., 1993). The double labelling experiment revealed that the rare [^{3}H]thymidine-label-retaining cells observed in the interfollicular epidermis did not express K19 (data not shown). Taken together, these results are consistent with the fact that K19 would be a marker of at least a proportion of the stem cells, those located in the bulge area of the hair follicle.

Differential localization of K19 expressing cells in hairy and glabrous skin

In order to determine if K19 expressing cells were always restricted to the bulge area of the hair follicle, we analyzed adult human tissues from various anatomic sites. At all hairy skin sites studied, K19 expressing cells were localized in a well defined zone of the hair follicles below the sebaceous gland, the bulge area (Fig. 3a). This is the deepest region of the permanent portion of the hair follicle. K19 expressing cells were detected throughout the hair cycle, in telogen follicles (the resting phase that follows the degeneration phase; Fig. 3a) as well as in anagen follicles (the growing phase of the hair follicles; Fig. 3a). The number of cells expressing K19 varied with hair size. A greater number of K19 expressing cells was detected in the large hair follicles of scalp compared to small follicles from breast or abdomen (data not shown).

In order to confirm that these K19 expressing cells were not Merkel cells, labelling with an anti-K18 antibody was performed. Merkel cells are specialized cells, containing typical neurosecretory granules, and intermediate-size filaments of the keratin family, specifically K18, K19, and K20 (Moll et al., 1984; Moll, 1994). In the hair follicle, Merkel cells were found above the bulge area (Fradette et al., 1995). K19 expressing cells of the bulge area did not coexpress K18 as detected by Ks 18.174 antibody, indicating that they were not Merkel cells (Fradette et al., 1995). In contrast, the rare K19 positive cells detected in the interfollicular epidermis also coexpressed K18, indicating that they were all Merkel cells (Fig. 3c,e). Since K19 seems to be a marker of stem cells of the bulge area of the hair follicle but only labels Merkel cells in the interfollicular epidermis at hairy sites, we decided to examine skin at glabrous sites where stem cells have been identified in the epidermis, at the bottom of the deep rete ridges (Lavker and Sun, 1982).

In human glabrous skin, small K19 positive cells were present in the basal layer of palm (Fig. 4a) and sole (not shown) epidermis. They were localized in the deep part of the rete ridges (Fig. 4a,b). These K19 positive cells did not contain K18 (Fig. 4c), indicating that they were not Merkel cells. Merkel cells coexpressing K18 and K19 were also observed. Thus, in contrast to hairy skin, a population of cells expressing K19 but not K18, was observed in the epidermis of glabrous skin at a location similar to that expected for stem cells (Lavker and Sun, 1982, 1983).

In skin, stems cells are known to be within the cell population expressing high levels of $\alpha_2\beta_1$ and $\alpha_3\beta_1$ integrins (Jones and Watt, 1993; Jones et al., 1995). To determine if K19 expressing cells were in the proportion of keratinocytes expressing high levels of β_1 integrins, we have performed double labelling of K19 and α_3 integrin subunit. In hairy skin, K19 expressing cells (Fig. 5c,e) of the bulge area of the outer root sheath were also integrin-bright cells (Fig. 5d,f). Similarly, in glabrous tissue, a few small K19 positive cells (Fig. 5a) were also integrin bright cells (Fig. 5b). Thus, this is consistent with the idea that K19 expressing cells would be a sub-group of the integrin-bright cells.

Similar results were obtained for the K19 distribution in mouse tissues. We took advantage of the synchronized hair growth occurring during the first wave of follicular cell prolif-

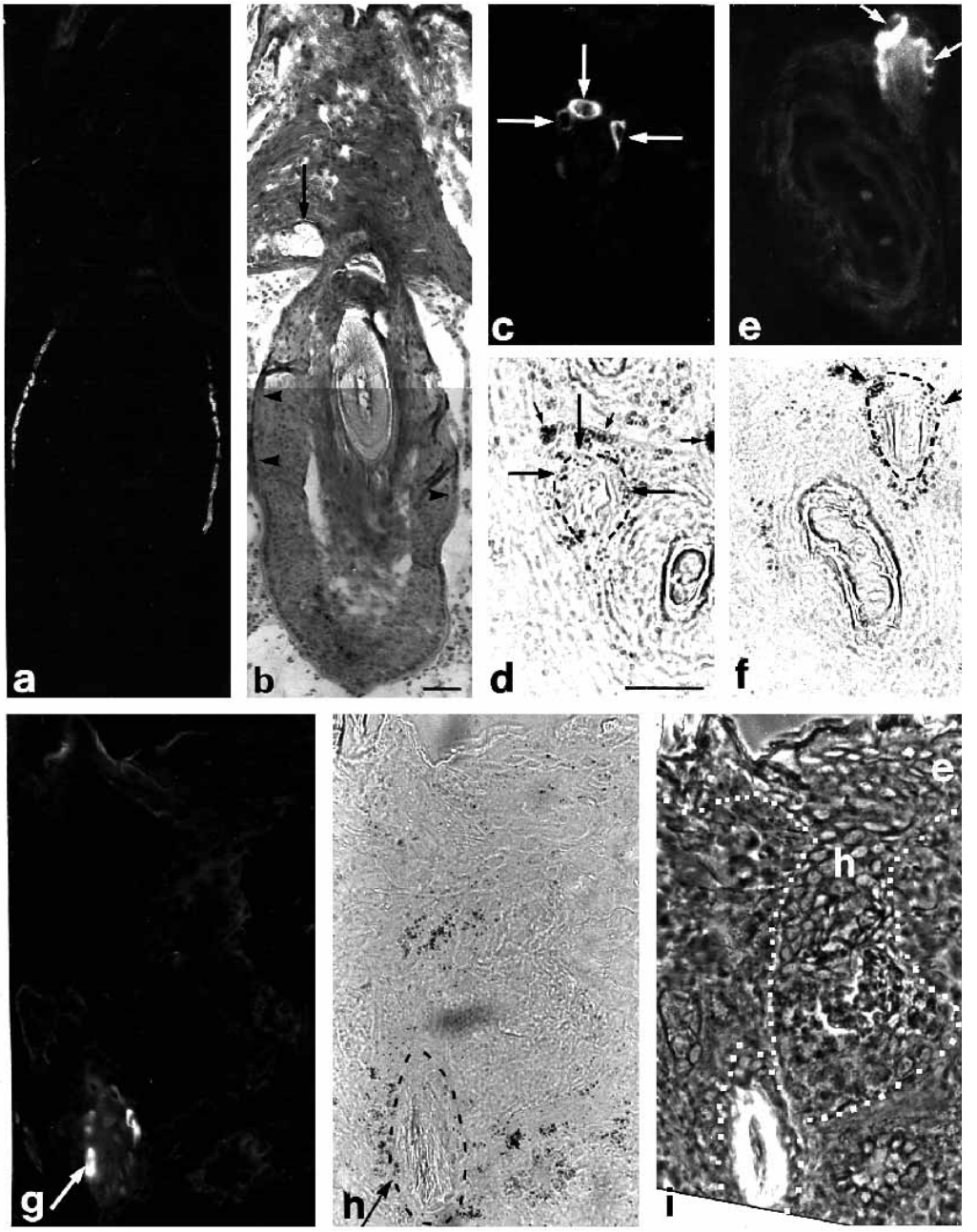

Fig. 2. K19 expressing cells of the bulge area are [^{3}H]thymidine-label-retaining cells. Sections (4 μm) were prepared from freshly frozen mouse skin at hairy anatomic sites: mouse vibrissae (oblic section; a,b) or back skin (transverse section; c-i) and processed for immunofluorescence labelling of mouse K19 (a,c,e,g) and autoradiography (d,f,h). (b) Hematoxylin, phloxine and saffron staining of a section consecutive to (a). (d,f,h) Bright field micrographs of c, e and g, respectively. (i) A phase contrast micrograph of g. Note that K19 expressing cells are localized in a specific region of the outer root sheath (arrowheads in b) located under the sebaceous gland (arrow in b) and identified as the bulge area. They were absent from the interfollicular epidermis. (c and d, e and f, g and h) Double localization of K19 expressing cells (by immunofluorescence) and [^{3}H]thymidine-label-retaining cells (by autoradiography) as described in Material and Methods. Note that K19 expressing cells (c,e,g, arrows) also retained [^{3}H]thymidine-labelling (d,f,h, arrows). The black broken lines in d, f and h indicate the junction between the epithelium and the mesenchyme surrounding hair follicles. Small arrows indicate mesenchymal cells retaining [^{3}H]thymidine labelling (d). The number of K19 positive cells varies with hair size, being lower in the small hair follicles of back skin. The broken white lines in (i) represent the dermo-epidermal junction. h, hair; e, epidermis. Bars: (a,b) 50 μm; (c-i) 25 μm.

eration after birth to demonstrate that K19 staining of bulge cells was detected throughout the whole hair cycle. In contrast, hairless skin from sole contained few K19 expressing cells localized at the bottom of the rete ridges.

The proportion of K19 expressing cells varies with donor age

To evaluate if the distribution of K19 expressing cells varied with the age of the person, immunofluorescence labelling was performed on foreskin from 2-day-old to 42-year-old donors. The proportion of positive cells present in the basal layer was quantified on these tissue sections. In neonatal foreskin (2-7-day-old boys), K19 expression was observed in most of the cells of the epidermal basal layer (84%, s.d.=17%; Fig. 4d). These cells did not contain K18 (Fig. 4f). In the epidermis of older donors the K19 expressing cells were restricted to the basal layer in the shallow region of the papilla. They represented 9% (s.d.=13%) and 0.1%, (s.d.=0.3%) of the basal layer in 2-5-year-old children and 17-42-year-old men, respectively. K19 positive cells were also present in the few hair follicles observed in these two age groups whereas foreskins from 2-7-day-old boys were glabrous.

To better quantify the age-related variations in the proportion of K19 expressing cells present in these tissues, cells were isolated from epidermis and hair follicles by a double digestion method with thermolysin and trypsin. These single cell populations were analyzed by flow cytometry after K19 labelling. The results show an age dependent decrease of the K19 positive cells in foreskin. In adults, less than 1% of the cells expressed K19 (Table 1).

Table 1. Percentage of K19 expressing cells in function of the anatomic site, age and culture time*

	Anatomic sites			
	Foreskins		Breast	
Passage	2-7 Days	2-10 Years	17-42 Years	16-64 Years
Fresh cells	7.5 (3.5)† (*n*=3)	1.5 (1.7) (*n*=2)	0.2 (0.2) (*n*=5)	0.7 (1.0) (*n*=4)
P0	21.3 (12.9)‡ (*n*=10)	5.4 (5.7) (*n*=5)	1.7 (1.8) (*n*=5)	1.2 (0.9) (*n*=4)
P1	24.8 (13.0) (*n*=10)	9.3 (5.3) (*n*=4)	1.7 (1.5) (*n*=6)	1.9 (1.7) (*n*=4)
P4-P5	17.1 (9.4) (*n*=6)	10.3 (5.8) (*n*=2)	1.9 (0.4) (*n*=2)	1.2 (1.0) (*n*=4)
P6	11.8 (7.9) (*n*=2)	ND	ND	ND
P7-P8	3.6 (5.5) (*n*=4)	ND	0.6 (0.6) (*n*=3)	ND

*Cell suspensions were fixed and analyzed by FACS®. The percentage of K19 positive cells was determined in a FSC gated (0-200) cell population as described in Materials and Methods.

†Cells were isolated by the double digestion method with thermolysin and trypsin, fixed and stained as described in Materials and Methods. Mean (s.d.).

‡For primary culture (P0) or serial subcultures (P1 to P8), cells were trypsinized at 70-80% of confluence, fixed and stained as described in Materials and Methods.

The proportion of K19 expressing cells varies during cell culture

In order to evaluate the fate of K19 expressing cells upon keratinocyte culture, epidermal cells from neonatal human foreskin were stained with the anti-K19 antibody. One day after plating freshly isolated epidermal cells, most of the attached keratinocytes were still round. The K19 expressing cells were found primarily as single cells (Fig. 6a). After 2 days, keratinocytes had spread onto the surface. Several K19 positive cells were arranged in pairs. The characteristic filament pattern was detected in each cell of the doublet (Fig. 6b). After one week in culture, numerous colonies were formed. Most of them contained several positive cells. K19 expressing cells were mainly observed in the small keratinocytes at the periphery of the colonies. In the following subcultures, similar patterns of K19 expression were detected, cells harboring filaments being at the border of the colonies (Fig. 6c). These results suggest that K19 expressing cells proliferate in culture for several passages.

To insure that K19 expressing cells present in culture were not Merkel cells, double-labelling of K19 and K20 was performed on primary culture or first subculture of neonatal foreskin epidermal cells. K19 expressing single cells, as well as doublets and cells at the periphery of the colonies did not contain K20, indicating that they were not Merkel cells.

The fate of K19 expressing cells in culture was also evaluated in cell populations isolated from mouse skin. In primary culture of mouse epithelial cells, K19 expressing cells were observed as single cells after 2 days, pairs after 2 to 4 days, and colonies after one week. Therefore, the similar results observed with mouse and human skin, clearly establish that the number of K19 positive cells increases during the first subcultures.

The lifespan of keratinocyte culture decreases as the age of

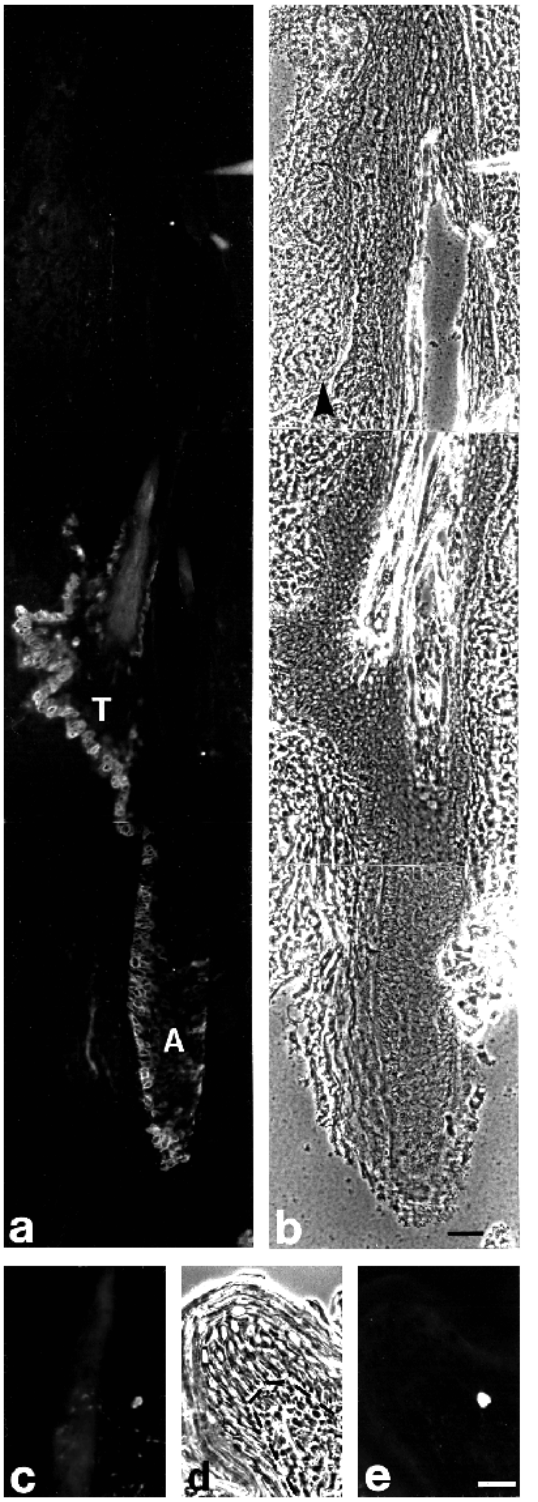

Fig. 3. K19 and K18 expression in hairy anatomic sites of human skin. Indirect immunostaining of K19 (a,c) and K18 (e) of breast skin. (b,d) Phase contrast micrographs of a and e, respectively. (a) Note that K19 expressing cells are present in a specific region of the outer root sheath under the sebaceous gland (arrowhead in b). This longitudinal section of a new hair shows that K19 expressing cells are present in anagen (A) as well as telogen (T). The few K19 expressing cells present in the basal layer of the epidermis are also K18 positive indicating that they are Merkel cells. Bars: (a,b) 50 μm; (c-e) 100 μm.

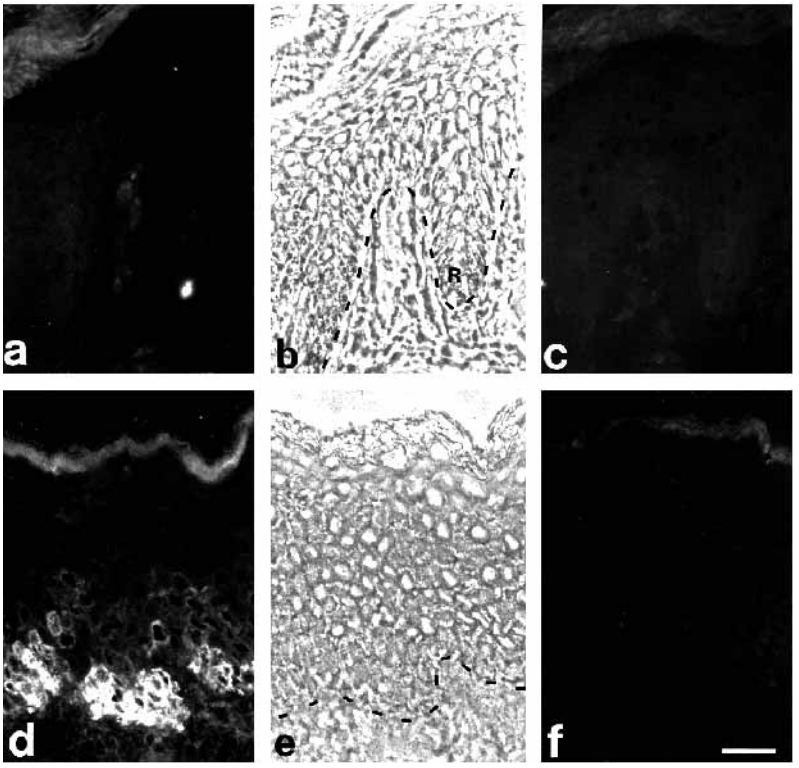

Fig. 4. K19 and K18 expression in cells from glabrous anatomic sites of human skin. Indirect immunostaining of K19 (a,d) and K18 (c,f) on consecutive 4 μm thick sections of finger skin of a one-year-old child (a-c) and from foreskin of a 2-day-old child (d-f). (b and e) Phase contrast micrographs of c and f, respectively. Note the presence of a small K19 positive cell in the deep part of the rete ridge (R) (a), that does not contain K18 (c). In newborn foreskin, the expression of K19 is present in most of the basal cells (d), the absence of K18 labelling (f) indicates that they are not Merkel cells. The dermo-epidermal junction is indicated by a broken line. Bar, 25 μm.

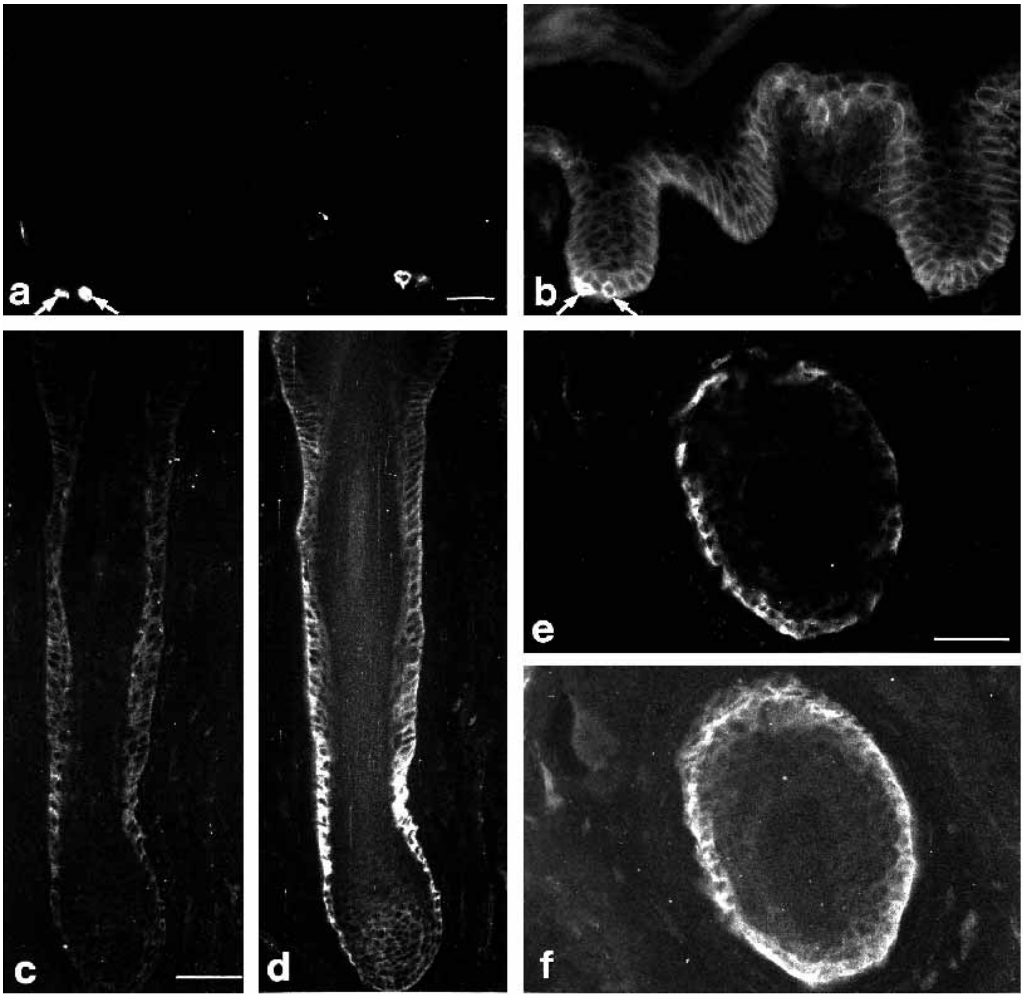

Fig. 5. K19 and $\alpha_3\beta_1$ integrin expression in cells from hairy and glabrous anatomic sites of human skin. Epidermis from palm skin (a,b) or hair follicle from breast skin (c,d,e,f) were double labelled with an antibody to K19 visualized with rhodamine-conjugated second antibody (a,c,e) and an FITC-conjugated α_3 integrin subunit (b,d,f). Note that in glabrous skin (a,b), small K19 positive cells of the deep part of the rete ridge are also integrin-bright cells (arrows); another K19 positive cell, which is not integrin bright, is not round and probably represents a Merkel cell (a, bottom right). In hairy skin (c-f), the K19 expressing cells are also integrin-bright cells. Bars: (a,b,e,f) 25 μm; (c,d) 50 μm.

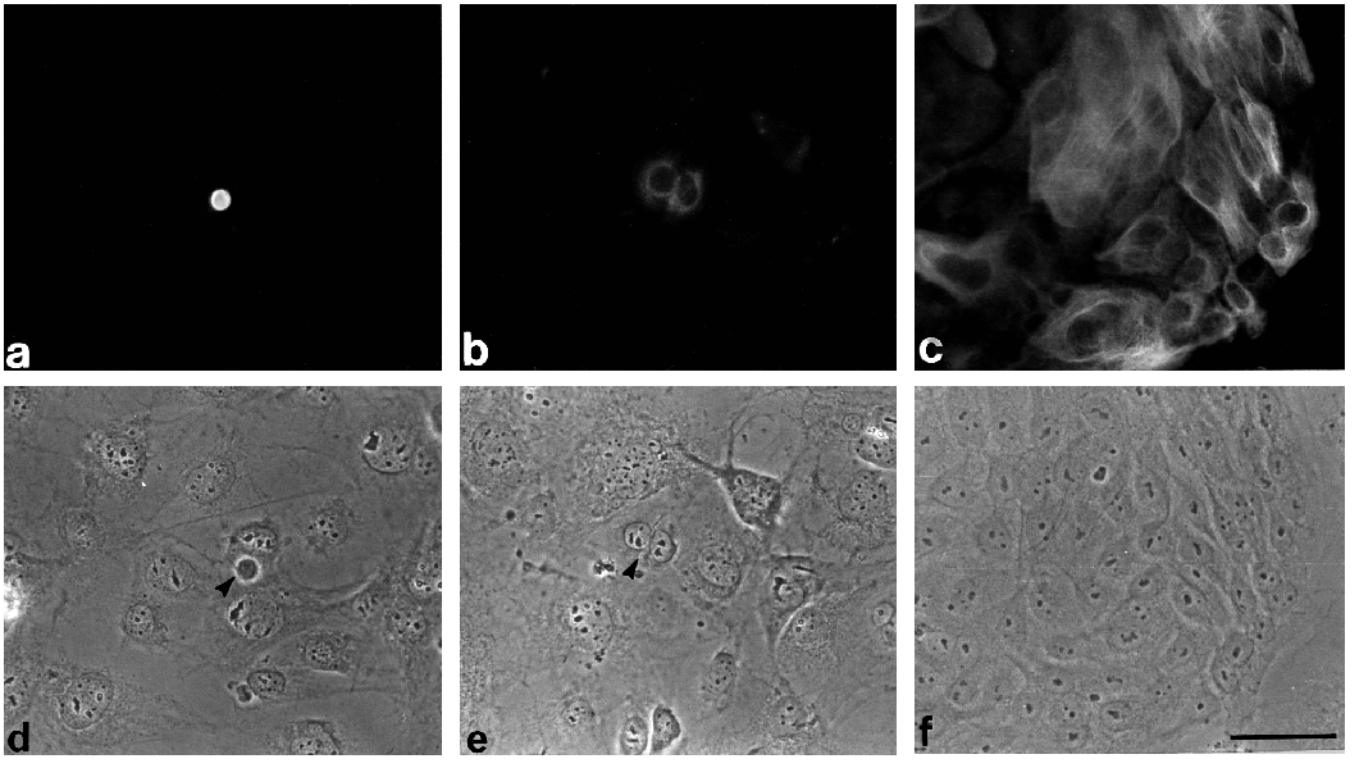

Fig. 6. Detection of K19 expressing cells in culture. Indirect immunostaining of K19 on human keratinocytes, isolated from foreskin of a 2-day-old child, and cultured in vitro (a-c). (d-f) Phase contrast micrographs of a-c, respectively. Keratinocytes ($1\times10^4/cm^2$) and 3T3 feeder layer ($6\times10^4/cm^2$) were seeded on glass slides. (a,b,d,e) Freshly isolated keratinocytes, (c,f) second subculture of keratinocytes. Note that 24 hours after the seeding of freshly isolated cells, K19 is expressed in round cells (a). After 2 days in culture, cells are spread, and doublets of K19 expressing cells are observed (b), indicating that these cells proliferate. After one subculture, K19 expressing cells are small and mainly located at the periphery of the large colony (c,f). Arrowheads (d,e) point to labelled cells in a and b, respectively. Bar, 50 μm.

the donors increases (Rheinwald and Green, 1975, 1977; M. Michel and L. Germain, unpublished observations). To evaluate if the fate of these cultures was related to the number of K19 expressing cells, freshly isolated cells and various passages of newborn, children and adult foreskin keratinocyte cultures were analyzed by flow cytometry with a FACS®. Fig. 7 shows the fluorescence intensity (FL1) and the forward light scatter (FSC) of newborn keratinocytes (passage 1) stained with an anti-K19 antibody. A population of K19 positive cells (Fig. 7b) can be easily distinguished from control unlabelled cells (Fig. 7a). The labelling of large keratinocytes (identified by FSC values over 200) was considered as non specific since differentiated keratinocytes were also variably stained by unrelated antibodies (anti-digoxigenin). Therefore, data were gated for FSC (0 to 200) in further analysis. The percentage of K19 positive cells was determined by subtracting the control from the labelled cell profile and evaluating the proportion of the cells under the peak or shoulder. The results show that the percentage of K19 positive keratinocytes in cell populations freshly isolated from foreskin decreased when the age of the donors increased. When these epithelial cell populations were cultured, the percentage of K19 positive keratinocytes significantly increased in primary culture, remained high for the first subcultures and decreased before senescence of the cultures. These variations were observed in all age groups (Table 1).

Breast skin is a common source of adult starting material for cell culture. At this anatomic site, the percentage of K19 expressing cells, after tissue dissociation and in serial cultures, was comparable to that of adult foreskin (Table 1).

DISCUSSION

Using different labelling techniques to further characterize the K19 expressing cell population, we provided evidence that this cell population contains skin stem cells. In skin, the presence of pluripotent stem cells in hair follicles was expected from empiric observations. Indeed, in partial thickness wounds, the regeneration of interfollicular epidermis results from the migration of keratinocytes of the hair follicle (Argyris, 1976; Eisen et al., 1955; Stenn and DePalma, 1988). However, in most studies the identification of skin stem cells is a difficult task and rests on indirect evidences. The most reliable feature of stem cells is their slow-cycling nature (Potten, 1981). In animal models, they are identifiable as cells retaining [^{3}H]thymidine-labelling over a long period of time (Bickenbach, 1981; Potten, 1981). Using this method, Cotsarelis et al. (1990) identified the bulge area as the exclusive localization of [^{3}H]thymidine-label-retaining cells of the mouse hair follicle. This led to the conclusion that follicular stem cells reside in this region (Cotsarelis et al., 1990; Lavker et al., 1993) and that they are absent in the bulb.

Previous studies have reported a K19 expression pattern in

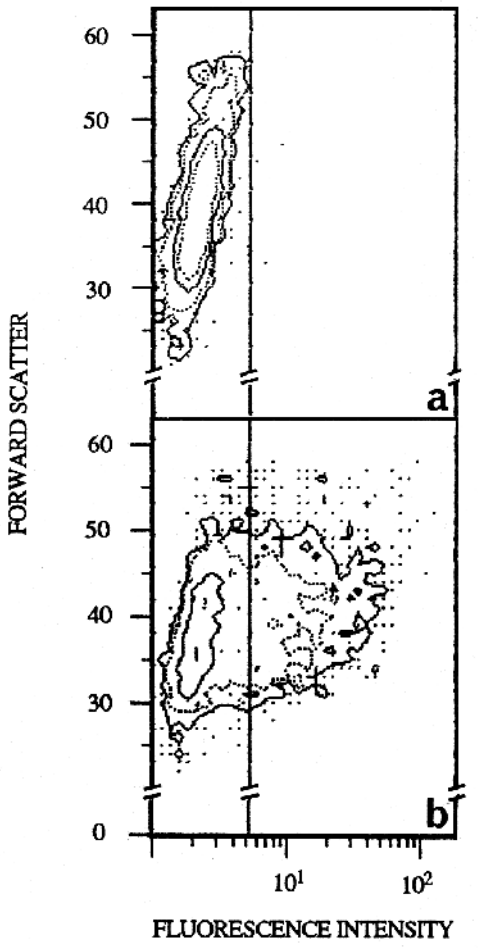

Fig. 7. Flow cytometry analysis of K19 expression in a population of human keratinocytes extracted from foreskin of a 2-day-old child. FSC and K19 immunostaining (b) or control (a) are represented as dot plots. After the second subculture, cells were labelled with anti-K19 antibody (b) detected with an FITC-conjugated second antibody and examined with a flow cytometer. FSC and fluorescence were measured in arbitrary units on a linear and logarithmic scale, respectively. In controls, the primary antibody was replaced by PBS (a). The percentage of K19 expressing cells was 35% in the experiment shown.

human hair follicle, finding K19 positive cells in the bulge area (Narisawa et al., 1994; Stasiak et al., 1989). In order to assess the possibility that K19 could be a biochemical marker for skin stem cells, we used the well established mouse system to identify [^{3}H]thymidine-label-retaining cells in combination with K19 detection in a double labelling procedure. We produced a site directed polyclonal antibody specific to mouse K19. The K19 expression pattern found in mouse skin corresponds to the distribution reported in human (Bartek et al., 1986; Narisawa et al., 1994; Stasiak et al., 1989; Wilson et al., 1994). In hair follicles, K19 is consistently expressed in several cells of the outer root sheath, in the area of the bulge. Moreover, our results agree with previous observations showing that K19 expressing cells were present through the hair cycle (Fig. 3a for human, and data not shown for mouse; Wilson et al., 1994). They were observed in anagen as well as telogen follicles, which represent the growth phase and the rest phase following the degeneration of the lowest part of the follicle. Thus, K19 positive cells do not degenerate, since they are located in the deepest permanent portion of the hair follicle, as expected from a stem cell population.

The double-labelling experiment shows that K19 expressing cells of the hair follicle are [^{3}H]thymidine-label-retaining cells, indicating that they are slow-cycling, the most reliable property of stem cells. This supports the hypothesis that K19 could be a biochemical marker for skin stem cells. But K19 does not label interfollicular [^{3}H]thymidine-label-retaining cells. This raises the question whether K19 labels all stem cells. The putative presence of stem cells in the interfollicular epidermis is based upon the detection of slow-cycling cells in this location (Bickenbach, 1981; Potten, 1981). However, this idea was called into question by recent findings. Indeed, recent in vitro studies comparing the growth potential of various subpopulations of follicular keratinocytes to that of interfollicular epidermis have shown that cells isolated from the bulge area or slightly lower in the hair follicles had a significantly greater proliferative potential and were more clonogenic than cells from the bulb (Rochat et al., 1994) or interfollicular epidermis (Yang et al., 1993). These results lend strong support to the idea that skin stem cells are located in the bulge area or slightly lower in the hair follicle and that they are absent in the interfollicular epidermis. Moreover, a characteristic usually associated with stem cells, their location in a well protected site is lacking from the interfollicular epidermis since the tips of the papilla can easily be removed by abrasion. Nonetheless, additional studies will be necessary to elucidate the question of whether the interfollicular epidermal slow-cycling cells are true stem cells or if they could rather be regarded as amplifying cells as suggested by Lane et al. (1991). Thus, because this issue has yet to be resolved, it is difficult to determine whether K19 is expressed in all cutaneous stem cells.

Additional data support the hypothesis that K19 could constitute a biochemical marker for skin stem cells. First, our identification of K19 expressing cells at the bottom of deep rete ridges in glabrous skin (palm and sole) is consistent with previous work identifying stem cells in this region based on their morphological and functional characteristics (Lavker and Sun, 1982, 1983). Second, the expression of high levels of the $\alpha_3\beta_1$ integrin at the surface of K19 positive cells present in hairy and glabrous skin sites is consistent with the previous demonstration that skin stem cells express higher amounts of β_1 integrin than differentiated keratinocytes (Jones et al., 1993, 1995). The β1 integrin-bright cells encompass a much larger compartment than the K19 expressing cells, based on the higher percentage of cells expressing high levels of β1-integrins (e.g. 25% bright cells in the basal layer of palm) compared to K19 (Fig. 5). Our results are consistent with the fact that K19 positive cells are a subset of the $\alpha_3\beta_1$ integrin-bright cell population, which is otherwise too important to be uniquely specific to stem cells.

If the hypothesis that K19 labels all cutaneous stem cells is true, our results imply a differential stem cell localization depending on the anatomic site. In glabrous skin, they would be present in deep rete ridges of the epidermis, whereas in hairy skin they would be restricted to the bulge area or slightly lower portion of the hair follicle.

The distribution of K19 among epithelia is fairly wide (Moll et al., 1982a; Sun et al., 1984) and its expression pattern is

variable in stratified, pseudo-stratified and simple epithelia (Bartek et al., 1985; Moll et al., 1982a; Stasiak et al., 1989). Different type II keratins probably associate with K19 to form filaments in intestine (e.g. K8; Baribault and Oshima, 1991) and skin (e.g. K5). This is in contrast to other keratin pairs, K5/K14, K1/K10, K3/K12, K4/K13 which are expressed in a similar fashion in a variety of stratified epithelia (Coulombe et al., 1989; Moll et al., 1982a; Osborn and Weber, 1982). Therefore, it is possible that K19 would be restricted to stem cells especially in other stratified epithelia but additional experiments will be necessary to get a definitive answer.

In the skin, a minor cell population, the Merkel cells, have been shown to express K19 (Moll et al., 1984). Merkel cells can be selectively identified by their expression of K18 or K20 (Moll et al., 1984; Moll, 1994). To confirm that the K19 expressing putative stem cells are not Merkel cells, we localized K18 by immunohistochemistry on consecutive human skin sections. At hairy skin sites, we found that rare Merkel cells were located in the interfollicular epidermis and in the area above the bulge in the pilosebaceous unit while the bulge region was negative for K18 (Fig. 3; Fradette et al., 1995). A similar distribution of Merkel cells was reported by Moll (1994) using an anti-K20 antibody. In glabrous skin, several cells located at the tips of the deep rete ridges are K19 positive (Figs 4a,d, 5a). Only a proportion of these cells coexpress K18. These results indicate that in every anatomic site examined, hairy as well as hairless, there are two distinct populations of K19 positive cells which can easily be distinguished since only Merkel cells also coexpress K18 or K20. Since K19 is not restricted to putative cutaneous stem cells, we have chosen foreskin where the Merkel cell density is relatively low for the following experiments (Lacour et al., 1991; J. Fradette and L. Germain, unpublished observations).

In order to examine some long-standing questions in the field of skin biology, we exploited the concept that K19 could be a marker for cutaneous stem cells to perform in situ and in vitro studies. Whether the number of stem cells varies during life remains a matter of debate. In order to evaluate age-associated changes in the number of human stem cells, we analyzed cell populations from the same body site, foreskin, in newborns, 2-5- and 7-42-year-old individuals. Our study clearly established that the number of K19 expressing cells decreased after birth. The two methods used to quantitate the proportion of K19 expressing cells in situ on tissue sections, and in vitro after cell dissociation, led to the same conclusion: the proportion of K19 expressing cells diminishes from neonates to children to adults. Based on the hypothesis that K19 labels stem cells, this would indicate a decrease in the number of stem cells after birth.

The well documented empiric observation that newborn foreskin keratinocytes can generate a larger cell number than adult trunk keratinocytes in culture is still unexplained. The number of passages that newborn cells can undergo in culture is also higher (Gilchrest, 1983; Rheinwald and Green, 1975, 1977). The results of our study suggest that the cultured keratinocyte lifespan depends on the number of stem cells initially seeded in primary culture. Indeed, we observed a correlation between the percentage of K19 expressing cells in freshly isolated keratinocytes and the number of subcultures that could be generated in vitro from these cell populations. Based on the notion that stem cells have, by definition, an extensive capacity for self-renewal in vivo (Hall and Watt, 1989; Potten and Loeffler, 1990), it seems likely that the greater the number of stem cells is, the longer will the lifetime be. Therefore, considering the hypothesis that K19 is a marker for stem cells, the number of K19 expressing cells in freshly isolated populations would be a good parameter to predict the lifetime of in vitro cultures.

Cultures of keratinocytes can be divided into three clonal types (holoclone, meroclone, paraclone) with different capacities for self-renewal, the holoclones being the cells with the highest proliferation potential (Barrandon and Green, 1987b). Interestingly, the percentages of K19 expressing cells that we found in cultured keratinocyte populations are similar to the percentages of holoclones generated by these cultures previously reported by Barrandon and Green (1987b). Indeed, newborn foreskin contained 28% holoclones (24.8% of K19 positive cells) whereas adult breast skin contained 3.1% holoclones (1.9% of K19 positive cells). Thus, a lower number of K19 positive cells corresponds to a lower number of holoclones and a decline in culture lifetime, as donor age increases. Therefore, we propose that holoclones are generated by K19 positive cells.

Based on the hypothesis that K19 labels stem cells, we took advantage of K19 labelling to assess the fate of stem cells in culture. We observed that the frequency of K19 expressing cells increased in primary culture compared to cells freshly isolated from the epidermis. This suggests that compared to other keratinocytes the proliferation rate of these cells is higher. Moreover, following their isolation from the tissue, K19 positive keratinocytes attached to the culture dish, spread and formed doublets after a few days. This kinetic is similar to that described by Morris and Potten (1994) for mouse [^{3}H]thymidine-label-retaining cutaneous epithelial cells. Later, K19 expressing cells were present at the periphery of several large colonies where proliferating keratinocytes have been localized (Barrandon and Green, 1987a). This slow increase in K19 positive cell number suggests that they proliferate in culture. A decrease in the proportion of K19 expressing cells was observed after several subcultures when the number of differentiated cells increased, before the senescence of the culture. Therefore, we suggest that K19 expressing cells in vitro represent the progeny of the in vivo stem cell population. However, the finding that in another culture system, skin equivalent, cells expressing K19 tend to be not in the basal layer, as expected for stem cells, but in the suprabasal layers (Kopan et al., 1987) seems to disagree with our hypothesis. In this organotypic system, keratinocytes stratify, differentiate and form a stratum corneum when cultured at an air-liquid interface (Auger et al., 1995; Michel et al., 1995a; Pruniéras et al., 1983). However, our recent results indicate that in these skin equivalents, keratin 19 positive cells are initially present in the basal layer early after keratinocyte seeding, and then seem to migrate towards the stratum corneum upon culture (Michel et al., 1995b). This cell migration in vitro could be the result of an ‘incomplete’ basement membrane that does not provide an optimal anchorage for putative stem cells. On the other hand, unlike in vivo, K19 is present in suprabasal cells. This might be due to the presence of retinoic acid in the culture medium which has been shown to stabilize the K19 mRNA (Crowe, 1993). This K19 expression in skin equivalents is only observed when newborn foreskin keratinocytes are seeded on

the equivalents. Indeed, no K19 positive cells are observed in skin equivalents produced from cell populations containing few K19 positive cells e.g. adult keratinocytes (Michel et al., 1995b).

In conclusion, based on the hypothesis that K19 labels cutaneous stem cells, our results showing a diminution of K19 expressing cell number with increasing age are of particular interest considering the expected role of stem cells in wound healing. They could explain the clinical observations that the repair rate decreases with the age (Grove, 1982; Levenson et al., 1950). Indeed, wound repair of second degree burn patients is faster in children than in adults (Levenson and Lund, 1945). Moreover, K19 will be of great interest for further cutaneous stem cell characterization under normal and pathological conditions, using in situ as well as in vitro studies.

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