Label-Retaining Cells in Human Embryonic and Fetal Epidermis

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Human embryonic and fetal epidermis was examined and labeling indices (LIs) for basal, intermediate, and periderm cells were determined. The LI for fetal basal cells was 8–11% and the LI for fetal intermediate cells was 7.5–9%. The total fetal epidermal LI was 16–20%, which equaled the basal LI for embryonic epidermis. After 21 days in organ culture, only basal cells in the fetal epidermis labeled with tritiated thymidine, while both basal and intermediate cells in the embryonic epidermis labeled and the total LI for fetal and embryonic epidermal cells was the same as the adult epidermal LI (7%). The LI for periderm decreased with increasing estimated gestational age (EGA) from 9.5% at 49 days EGA to 0.4% at 85 days EGA. A subpopulation of epithelial cells that retained tritiated thymidine label and that have some of the attributes associated with stem cells has been previously demonstrated in rodents. In order to examine human embryonic and fetal epidermis for the presence of such cells, epidermis from various gestational ages were labeled and grown in organ culture for 21 days. The mean percent label-retaining cells (LRCs) for embryonic and fetal epidermis was determined. Approximately 4% of the embryonic and 2% of the fetal epidermal cells retained label for 21 days in organ culture. Embryonic LRCs were found in the basal and suprabasal layers, but fetal LRCs were found only in the basal layer. The presence of LRCs in human embryonic and fetal epidermis suggests that epithelial cell proliferation in these tissues may be regulated via a stem cell pattern of proliferation. *J Invest Dermatol 88:42–46, 1987*

Adult epidermis is a continuously renewing tissue in which cell production equals cell loss [1,2] and cell proliferation is confined to the "basal compartment" [2–5]. Cells that leave this compartment and move into the "differentiation compartment" no longer divide [2,5]. The distinction between proliferative and differentiative compartments in developing human embryonic and fetal epidermis is not clearly understood, nor is it clear whether cell division is confined to a distinct compartment similar to the "basal compartment" in adult epidermis. A previous study of proliferation in human embryonic [53-day estimated gestational age (EGA)] and fetal (74-day EGA) epidermis showed that intermediate and periderm, as well as basal, cells took up tritiated thymidine label in organ culture [6].

Cells that retain tritiated thymidine for a long period of time [label-retaining cells (LRCs)] and that have some of the attributes proposed for stem cells [7] have been demonstrated previously in rodent epidermis and oral mucosa [8–10]. In order to examine developing human epidermis for the presence of such cells and to evaluate the proliferative potential of cells in all epidermal layers, we used an organ culture system in which human embryonic and fetal skin proliferated and differentiated for up to 63 days [6].

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Abbreviations:
EGA: estimated gestational age
LI: labeling index
LRCs: label-retaining cells

MATERIALS AND METHODS

Tissues Embryonic and fetal skin specimens were obtained from the trunk region of normal human embryos and fetuses in the age range of 7–13 weeks EGA (courtesy of Dr. Thomas Shepard, Director of the Central Laboratory for Human Embryology), with approval of the University of Washington's Human Subjects Committee and according to DHEW guidelines. Adult skin specimens were obtained from the legs of volunteers who were 40–60 years old. The tissues were rinsed 3 times in sterile phosphate-buffered saline containing 100 μg/ml streptomycin and 100 U/ml penicillin. Maintaining sterile conditions, the skin was gently dissected free from the underlying tissues and cut into approximately 2 × 2 mm squares and placed in organ culture medium.

Organ Culture Medium The medium was prepared from Dulbecco's modification of Eagle's medium with glutamine (Microbiological Associates) and supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin, 10 ng/ml epidermal growth factor (Collaborative Research, Inc.), 50 μg/ml ascorbic acid, 10% fetal bovine serum (FBS, Hyclone), pH to 7.2 and buffered with 4.4% sodium bicarbonate. The medium was sterilized through 0.22-μm Millipore filters and stored in 25-ml aliquots at −20°C.

Organ Cultures Culture units were prepared by placing sterilized triangular mesh screens (Falcon) and sterilized filters (Millipore, HAWP 01300) over the central wells of organ tissue culture dishes (Falcon 3037) with 1 ml of medium added to the central wells such that Millipore filters absorbed the medium from beneath. The tissue samples were placed, epidermis up, on top of

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the saturated Millipore filters. Although the tissue received most of its nutrients from the central wells through the screens and Millipore filters, a thin layer of medium was always present over the tissue surface. The medium was changed every 2–3 days. Cultures were harvested by placing the Millipore filter with the explant directly into fixative.

Histologic Techniques Samples of fresh tissue and explants from the organ cultures were fixed in half-strength Karnovsky's fixative at 4°C [11], rinsed 30 min at room temperature in 0.1 M cacodylate buffer, stained en bloc with 10% solution of Ehrlich's hematoxylin overnight, dehydrated through a series of graded ethanols to propylene oxide, and embedded in Epon [12]. Tissues for light microscopy were sectioned at 1 μm, mounted on glass slides, and stained with Richardson's stain [13].

Autoradiographic Techniques All specimens were placed in organ culture and immediately labeled with sterile tritiated thymidine (Amersham, sterile aqueous solution, 2 Ci/mM) at a concentration of 1 μCi/ml medium. After 1 h of labeling, 5 cultures from each fetal age (49, 53, 67, 73, and 85 days EGA and adult) to be examined for initial labeling indices were sampled and processed for histology. The cultures to be examined for LRCs were rinsed 5 times with medium (without tritiated thymidine) over a period of 2 h and allowed to remain in culture until sampled 7, 14, and 21 days after labeling. Autoradiographic techniques were followed according to Rogers [14]. Sections for light microscopic autoradiography were cut at 1 μm thickness and mounted on glass slides, dipped dry in Kodak NTB_2 nuclear track emulsion (diluted 2:1 with 2% glycerin in water) in the dark, dried, and stored in the dark with silica gel as a desiccant for 2 and 4 weeks' exposure time. Appropriate controls for positive and negative chemography were included. Slides were developed in Kodak Dektol developer (diluted 1:1) at 20°C for 5 min, rinsed in stop bath (9 g chromium potassium sulfate + 18 g anhydrous sodium sulfate + 300 ml water), fixed in 30% sodium thiosulfate, washed in water, air dried, stained with Richardson's stain [13], and coverslips mounted with Permount.

Determination of Labeling Indices (LIs) The numbers of labeled and unlabeled cells were counted on 5 discontinuous sections from each of 5 organ cultures sampled from each specimen of each age. A cell was considered labeled if 5 or more silver grains lay over the nucleus (background was less than 1 silver grain per cell). To determine LIs and percent LRCs, the number of labeled basal cells and labeled intermediate cells was expressed as a percentage of the total number of epidermal cells counted and the number of labeled periderm cells was expressed as a percentage of the total number of periderm cells counted. For adult LIs, the number of labeled basal cells was expressed as a percentage of the total number of basal cells counted. Standard deviations were determined and analysis of variance (ANOVA) was performed to determine statistically significant differences.

RESULTS

The specimens were grouped into 3 categories: embryonic, in which the epidermis consisted of 1 layer of basal cells and 1 layer of periderm cells; fetal, in which the epidermis consisted of basal, intermediate, and periderm cell layers; and adult, in which the epidermis consisted of basal, spinous, granular, and cornified cells layers. In all embryonic and fetal specimens labeled immediately after dissection, basal, intermediate, and periderm cells took up tritiated thymidine label. In the adult specimens and 21-day-old cultures of fetal specimens, only basal cells took up label. Although, in the 21-day-old cultures from embryonic specimens both basal and intermediate cells took up label, 90% of the labeled cells were basal.

Labeling Indices The mean percents of 1-h LIs for each of 5 cultures from skin specimens from fetuses of various ages of gestation were determined. The number of specimens for each age and the mean percent LIs are shown in Table I.

Table I. Mean Percent One-Hour Epidermal Labeling Indices (± SD)

Estimated Gestational Age	Total	Basal Cells	Intermediate Cells
Embryonic			
49 d (n = 3)	15.7 (1.2)	15.7 (1.2)[a]	NA
53 d (n = 12)	19.7 (6.2)	19.7 (6.2)[a]	NA
Fetal			
67 d (n = 4)	17.8 (2.6)	10.3 (2.1)	7.5 (1.4)
73 d (n = 10)	20.4 (6.5)	10.8 (3.0)	9.6 (2.3)
85 d (n = 4)	15.8 (2.7)	8.3 (1.4)	7.5 (2.1)
Adult (n = 3)	6.7 (1.2)[b]	6.7 (1.2)	NA

[a]Significantly different from the other labeling indices (LIs) in the column ($p < 0.01$), but not from each other.
[b]Significantly different from the other total LIs ($p < 0.01$), which are not significantly different from each other.

In the embryonic specimens (49 and 53 days EGA), the total mean percentage of labeled epidermal cells equaled the mean percent labeled basal cells which ranged from 16% for 49-day EGA specimens to 20% for 53-day EGA specimens. In the fetal specimens the total mean percentage equaled the sum of the percent labeled basal cells plus the percent labeled intermediate cells and ranged from 16% for 85 days EGA specimens to 20% for 73 days EGA specimens. The mean percent labeled fetal basal cells ranged from 8% for 85 days EGA specimens to 11% for 73 days EGA specimens and none of the figures differed significantly from the mean percent labeled adult basal cells (approximately 7%). The mean percent labeled embryonic basal cells differed significantly ($p < 0.01$) from the adult and the fetal. The mean percent labeled fetal intermediate cells ranged from 7% for 85 days EGA specimens to 10% for 73 days EGA specimens.

The total mean percent labeled basal and intermediate cells for 1-h and 21-day cultures are shown in Table II. The LIs for the embryonic and fetal epidermal 1-h cultures range from 16% for 49 and 85 days EGA specimens to 20% for 73 days EGA specimens and were not significantly different from each other. The LIs from the embryonic and fetal 21-day cultures were approximately 7% and did not significantly differ from the 1-h adult culture.

The mean percent labeled periderm cells is shown in Fig 1. The periderm LIs decreased with increasing age of the specimens from 9.5% for 49 days EGA specimens to 0.4% for 85 days EGA specimens. Each point was significantly different from each other except the 67- and 73-day points which were not different from each other.

Label-Retaining Cells The mean percent labeled epidermal cells and LRCs are shown in Table III. One hour after labeling, 16–20% of the embryonic and fetal epidermal cells were labeled. Seven days later the percentage of labeled cells had increased to 40–60% in the embryonic specimens (49–53 days EGA) and approximately 30% in the fetal specimens (67–85 days EGA). Four-

Table II. Mean Percent Total Labeling Indices (± SD)

Estimated Gestational Age	1-Hour Cultures[a]	21-Day Cultures[b]
Embryonic		
49 d (n = 3)	15.7 (1.2)	8.2 (1.7)
53 d (n = 12)	19.7 (6.2)	7.7 (1.2)
Fetal		
67 d (n = 4)	17.8 (2.6)	7.5 (1.4)
73 d (n = 10)	20.4 (6.5)	7.0 (1.9)
85 d (n = 3)	15.8 (2.7)	7.5 (2.1)
Adult (n = 3)	6.7 (1.2)[b]	ND

[a]Not significantly different from each other, except Adult ($p < 0.01$).
[b]Not significantly different from each other.

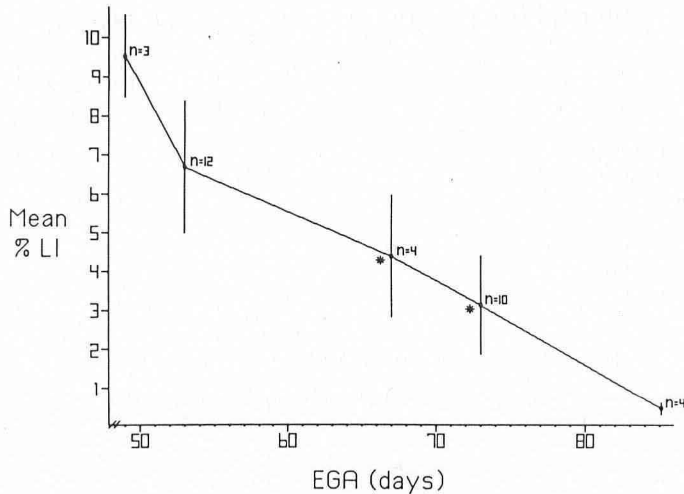

Figure 1. Mean percentage of labeled periderm cells (± SD) at various estimated gestational ages (EGA). Each point represents the mean of 5 cultures from each specimen (n = number of specimens of each age category) and are significantly different ($p < 0.01$) except* which do not differ from each other.

teen days after labeling, the percentage of labeled epidermal cells was 12–17% in the embryonic specimens and 9–12% in the fetal specimens. By 21 days after labeling, the percentage of epidermal cells, which had retained label, had decreased to approximately 4% in the embryonic specimens and approximately 2% in the fetal specimens, but the mean percent embryonic and fetal LRCs were not significantly different from each other.

DISCUSSION

Two studies of proliferation of human fetal skin have been reported previously [15,16] and in both studies the skin was grown submerged in plastic Petri dishes for 1–8 days. The results vary from a LI of 5% at 126 days EGA [15] to 1–12% for skin 60–85 days EGA, 5% for 85–105 days EGA, and 3% for 105–135 days EGA [16]. No data were presented to indicate what cells were included in the proliferating population and our data indicate that basal, intermediate, and periderm cells all divide during this period of development (Table I, Fig 1); therefore, it is difficult to compare our results with theirs. In the study presented here, the total LIs between the embryonic and fetal specimens were not significantly different. However, when only the basal cells were considered, embryonic LIs were significantly higher than the fetal LIs which were similar to those seen in the adult (Table I), suggesting that although the epidermis throughout the first trimester of development must maintain a high proliferative rate for the massive tissue expansion taking place, the fetal basal cells are already beginning to slow their proliferative rates to those seen in adult epidermal basal cells. This correlates with previous results in which it was demonstrated that once the specimen passed the embryonic–fetal transition period (approximately 60 days EGA) and stratified, the epidermis expressed a number of the adult markers of differentiation, such as the high-molecular-weight keratins [17,18].

The periderm remains as a single layered epithelium until 24 weeks EGA and undergoes its own series of morphologic changes, then is sloughed into the amniotic fluid [19]. Occasionally, mitotic figures have been seen in periderm cells from specimens younger than 93 days EGA [19], but the proliferative rate of this epithelium had not been determined. Using our organ culture system, we examined the LIs of periderm cells from embryonic and fetal specimens of various ages and found that with increasing age, the periderm's ability to divide was decreased (Fig 1) and by the end of the first trimester, < 1% of the periderm cells labeled with tritiated thymidine. The periderm appeared to die in this culture system, as indicated by complete loss of the periderm cells in the cultures from fetal specimens and decline in proliferative activity in the cultures from embryonic specimens.

Leblond et al [1,20] examined cell proliferation and migration from the basal layer of adult rat esophageal epithelium and concluded that division occurred randomly. It has since been shown that epithelia with spatially organized structures, such as tongue [21] and palatal papillae [22], intestinal villi [23], monkey palm epidermis [24,25], and columns in mouse ear epidermis [26–29] have foci of proliferative activity. Gelfant [30] has suggested that in human epidermis subpopulations of proliferating cells are blocked in the cell cycle in G_0, G_1, or G_2 and upon injury they reenter the cell cycle to repopulate the damaged tissue. Briggaman and Kelly [31] have reported that in adult human epidermis grown on nude mice only 56% of the basal cells were labeled after 16 days of pulses of tritiated thymidine given 6 h apart. The unlabeled basal cells may be blocked in the cell cycle, as Gelfant suggests [30] or, as it has been more recently reported, some cells in S-phase of the cell cycle do not take up tritiated thymidine [32]. Alternatively, these cells may be cycling, but at a very slow rate [33,34]. It has been previously demonstrated that a subpopulation of basal keratinocytes in mouse and hamster epithelia can be marked with a tritiated thymidine label [8–10] and that such cells have some of the characteristics proposed for stem cells [7]. It has also been suggested that such cells are not blocked in the cell cycle, but cycle at a slow rate [10,34].

It has been assumed that epithelial stem cells would form a proliferative subpopulation with a slower cell cycle than the other proliferating epithelial cells [7]. Therefore, a single pulse of tritiated thymidine would infrequently label such slowly proliferating cells, but if they were labeled, they would not be expected to dilute their label through cell division as rapidly as the other proliferating cells, and would thus remain labeled for a longer period of time. Label-retaining cells have been identified following single or repeated pulse labeling of neonate and adult rodent epithelia [8–10,34]. The numbers of LRCs and the distribution of silver grains over these cells decreased with increasing time after labeling, indicating that LRCs are dividing very slowly [10,34]. We have applied the same reasoning and a modified technique to

Table III. Mean Percent Labeled Epidermal Cells and Label-Retaining Cells (± SD)

Estimated Gestational Age	1 Hour[a]	7 Days	14 Days	21 Days[a]
Embryonic				
49 d (n = 3)	15.7 (1.2)	67.2 (2.1)[b]	17.1 (2.6)[b]	4.1 (1.8)
53 d (n = 12)	19.7 (6.2)	40.8 (7.4)	11.7 (3.1)	3.7 (2.4)
Fetal				
67 d (n = 4)	17.8 (2.6)	32.6 (2.7)	9.3 (2.7)	1.2 (0.9)
73 d (n = 10)	20.4 (6.5)	33.0 (2.9)	9.5 (2.2)	1.5 (0.7)
85 d (n = 4)	15.8 (2.7)	38.1 (1.7)	12.3 (1.4)	2.0 (0.9)

[a]Indices in the same column are not significantly different from each other.
[b]Significantly different ($p < 0.01$) from the rest of the indices in the same column, which are not significantly different from each other.

human embryonic and fetal skin and found a population of basal and suprabasal cells that retained label for 21 days (Table III). The majority of LRCs in the fetal specimens were basally positioned and were assumed to be keratinocytes, but in the embryonic specimens approximately half of the LRCs were suprabasal and with the presence of Langerhans cells in human embryonic and fetal specimens [35], the suprabasally positioned LRCs may not all be keratinocytes. However, Langerhans cells account for only 0.6% of the embryonic epidermal cells (calculated from 35) and it is unlikely that all of the Langerhans cells would be labeled. Therefore, some of the suprabasal LRCs must be keratinocytes. It is not known whether suprabasal LRCs are derived from basal LRCs which moved suprabasally without dividing or whether they are intermediate cells which, after initial labeling, remained in the suprabasal position and retained label. It is unlikely that these cells are unable to divide or migrate due to radiation damage. Leblond and Cheng [23] demonstrated that few cells died due to tritiated thymidine damage, but that when cells were damaged they died within a few days and were either observed as pyknotic cells or were phagocytized by surrounding cells, and Bickenbach [8] showed that LRCs in mouse epidermis could be stimulated to divide.

It has been suggested that stem cells may play a role in the regulation of epithelial cell proliferation and that as such, these cells may be target cells in diseases which involve altered patterns of cell proliferation [36–38]. Among these hyperproliferative disorders are psoriasis [39] and many genetic disorders, such as some of the ichthyoses [40,41]. Increased cell proliferation is also characteristic of some premalignant and malignant epithelia [42] and epidermal repair during wound healing [43]. Some of the ichthyoses are expressed as early as 19 weeks of gestation [44,45] and it is assumed that an altered pattern of cell proliferation is a component of the epidermal pathology in utero. An understanding of the processes of epithelial proliferation and their control is related to normal tissue development and maintenance and to pathologically or genetically altered disease processes. Information about the distribution and behavior of LRCs in growing epithelia might provide valuable insight into the control and regulation of human fetal epidermal growth.

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