Proliferation and Differentiation of Cultured Epidermal Cells from Patients with X-linked Ichthyosis and Ichthyosis Vulgaris

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The growth, differentiation, and regeneration of epidermal cultures from patients with X-linked and autosomal dominant ichthyosis and normal individuals were compared. Cell proliferation was studied by combining the technique of fluorescence-activated cell sorting with [³H]thymidine labelling and autoradiography. As in normal epidermal cultures, a marked heterogeneity in the labelling intensity of S-phase cells was observed in the ichthyotic cultures with totally unlabelled as well as very strongly labelled cells. However, in contrast to normal cultures, by far the largest proportion of S-phase cells in the ichthyotic cultures were very strongly labelled with a corresponding, severe reduction in the proportion of un- and weakly labelled cells. The increased labelling intensity of S-phase cells was observed in primary as well as in regenerating cultures, although it was most pronounced in the latter case. There was no difference between cultures from the two types of ichthyotic skin. The morphologic differentiation in the cultures was assessed by measurement of mean diameter of sorted S-phase cells and by quantitation of cornified envelope formation. Both parameters were reduced in the ichthyotic cultures, compared with normal ones. Taken together, these findings are indicative of a hyperproliferative state in the ichthyotic cultures. *Key words: Keratinocyte cultures; S-phase cells; Hyperproliferation.*

It has recently become possible to propagate human epidermal keratinocytes *in vitro*. Under suitable conditions, the cultured cells will stratify and differentiate, thereby creating a multilayered tissue much like the epidermis *in vivo* (1, 2). By reducing the calcium concentration in the medium it is also possible to strip off all the differentiating, suprabasal cells from the multilayered tissue and to repeatedly study its regeneration under experimentally controlled culture conditions (3). It has moreover proved possible to transfect the basal cells with high efficiency, thus allowing studies on the genetic control of epidermal differentiation (4, own unpublished observations).

These technical achievements have made it relevant to analyse the growth and differentiation of pathological epidermal keratinocytes in culture with the object of testing new strategies for therapeutic intervention.

The ichthyosiform dermatoses are inherited disorders of keratinization in which the skin is dry and accumulates large amounts of scales (5). Clinically evident thickening of the stratum corneum (hyperkeratosis) is characteristic of all these disorders, although the patho-physiological basis for the presence of excess scales may be different in each of them.

In general terms, hyperkeratotic diseases may be considered disorders of epidermal cell renewal and differentiation. Under normal steady-state conditions, the basal cells reproduce at a rate equal to that at which the stratum corneum cells are desquamated. A disruption of the balance between cell proliferation and elimination of scales by either increased production or decreased shedding will result in a thickened stratum corneum. On this basis, two major groups of ichthyosiform dermatosis are usually distinguished: hyperkinetic ichthyosis (epidermolytic hyperkeratosis and lamellar ichthyosis) and normokinetic or 'retention type' ichthyosis (ichthyosis vulgaris and X-linked ichthyosis) (5–7).

In the present study, the cell kinetic behaviour and the differentiation in cultures of epidermal cells obtained from patients with ichthyosis vulgaris and X-linked ichthyosis (steroid sulfatase deficiency) was investigated. In primary culture as well as during regeneration, both types of ichthyoses showed characteristics of hyperproliferation.

MATERIAL AND METHODS

Patients

Skin samples were obtained from 2 patients with X-linked recessive ichthyosis (XL-1 and XL-2), from 2 patients with autosomal dominant ichthyosis vulgaris (AD-1 and AD-2) and from one normal person. The patients were not related. The diagnosis was in each case established by clinical, genetic, and biochemical findings. Both XL-1 and XL-2 had significantly reduced activity of arylsulfatase C (steroid sulfatase), which is deficient in X-linked ichthyosis (8, 9). DNA analysis demonstrated a complete deletion of the steroid sulfatase (STS) gene on the X-chromosome in XL-1, but not in XL-2 (10, 11).

Two samples of affected skin were obtained from the thights of each ichthyosis patient with a keratotome cutting just below the basement membrane. Corresponding samples were obtained from the control person. All biopsies were taken at the dermatologic clinic in Erfurt, GDR and the skin samples were immediately transferred to complete culture medium with antibiotics. They were then transported in this medium to the Institute of Human Genetics, Aarhus, Denmark, where cultures were established 3 days after the biopsies were taken.

Cell culture

Cultures of human epidermal keratinocytes were established as described in detail elsewhere (12). Primary cultures were initiated by an explant method with eight explants per 25 cm^2 tissue culture flask. The cultures were grown in Dulbecco's Modified Eagle medium (DMEM, Gibco) plus 1.68 g/l NaHCO$_3$, 20% v/v fetal calf serum (Gibco), hydrocortisone 0.4 µg/ml, epidermal growth factor (EGF, Collaborative Research Inc., Lexington, Mass.) 10 ng/ml, penicillin 250 U/ml, and streptomycin 25 µg/ml. The cultures were equilibrated with 5% CO$_2$ in air and incubated at 35°C. Medium was changed twice a week.

After 5 weeks of growth in primary culture, half of the cultures were stripped in low calcium medium (3). At this stage the cultures were subconfluent or confluent and extensive keratinization prevailed. The cultures were stripped by incubating in calcium-free minimal essential medium (Gibco) without additives at 35°C. After 72 h all suprabasal cells were detached, leaving a monolayer of basal cells. The cultures were then refed medium with normal calcium concentration to initiate a regenerative response which gradually re-establishes the suprabasal layers (for details, see 3).

[³H]thymidine labelling, cell sorting, and autoradiography

Both primary and regenerating cultures were labelled with [³H]thymidine (³H-TdR) for combined analysis by cell sorting and autoradiography. All labelling experiments were performed with ³H-TdR (New England Nuclear, sp.act. 20 Ci/mmol) at a concentration of 10 µCi/ml for 30 min (13). Primary cultures were labelled after 15 days when extensive lateral growth prevails, whereas regenerating cultures were labelled 7 days after refeeding normal calcium medium (7 days post-stripping). After labelling, a single-cell suspension was obtained by trypsinization and the cells were fixed in absolute ethanol (12, 13). The cells were then stained with a combination of mithramycin and ethidium bromide for flow cytometry of cellular DNA contents. The DNA frequency

distributions were obtained with a fluorescence-activated cell sorter (FACS II) (Beckton Dickinson, Calif.). Cells were sorted from a window of 10 channels in the middle of the S-phase DNA distribution and from a window representing the left (lower) half of the G$_1$ peak. The sorted cells were collected on glass slides and covered with an Ilford K2 emulsion for autoradiography. The autoradiograms were exposed for 8 days, developed, and finally stained with Giemsa.

The number of labelled and unlabelled S-phase cells were counted in the light microscope. 400–700 cells were counted in each experimental group. The labelled cells were further characterized by counting the number of grains per nucleus to obtain a grain count distribution (12, 13). The autoradiographic background was generally ≤ three grains per nucleus. In all experiments more than 99% of cells sorted from the G$_1$ peak were classified as unlabelled.

Morphological differentiation

The morphological differentiation was assessed by measurement of cell size and by quantitation of cornified envelope formation.

Measurement of cell size was performed on young (15 days old) primary cultures and on regenerating cultures 7 days after stripping. In both cases the projected cell diameter of S-phase cells collected on glass slides after cell sorting was determined with the aid of a measure introduced into the eyepiece of the microscope. The measure was calibrated using a micrometer slide. All measurements were performed at ×10^3 magnification (14).

Quantitation of cornified envelope formation was performed on regenerating cultures 7 days after stripping, using the method described by Sun & Green (15). The cells were dislodged from the culture flask by trypsinization, washed in 10 mM Tris-HCl (pH = 7.4), counted, and then heated for 5 min in the 10 mM Tris-HCl with 1% sodium dodecyl sulphate and 20 mM dithiothreitol (dTT). Cells shed into the growth medium were treated together with the trypsinized cells and thus included in the scoring. The numbers of cornified envelopes were scored microscopically (phase contrast) by counting in a haemocytometer and expressed as a percentage of the total number of cells.

RESULTS

Growth and morphology

Generally, the lateral growth rate and morphology in epidermal cultures from X-linked and autosomal dominant ichthyotic skin did not differ significantly from each other or from cultures of normal skin when observed by phase contrast microscopy (3, 12). Occasionally, however, an extensive secondary migration of differentiating cells over the proliferating basal cells was observed from ichthyotic explants. This migration was only seen during the first 2 weeks of growth; during the later phases of growth the differentiating cells were gradually shed, leaving a normal culture morphology. In normal cultures, slight migra-
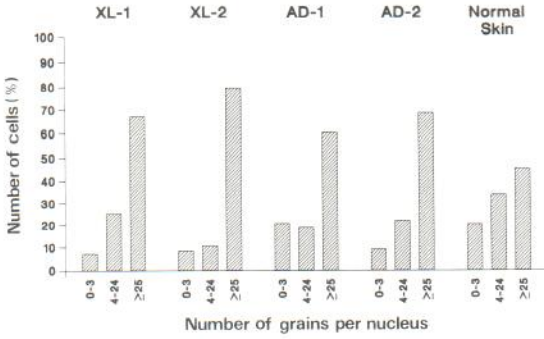
Fig. 1. Grain count distribution of sorted mid S-phase cells in primary cultures of normal skin and of skin from patients with X-linked ichthyosis (*XL-1, XL-2*) and autosomal dominant ichthyosis vulgaris (*AD-1, AD-2*). The cultures were labelled with 10 µCi/ml ^{3}H-TdR for 30 min at day 15.
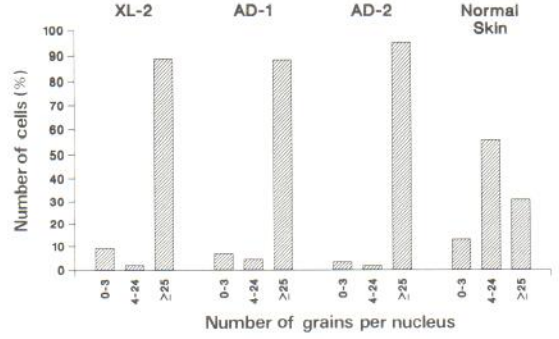
Fig. 2. Grain count distribution of sorted mid S-phase cells in regenerating cultures of normal skin and of skin from patients with X-linked ichthyosis (*XL-2*) and autosomal dominant ichthyosis vulgaris (*AD-1, AD-2*). The cultures were labelled with 10 µCi/ml ^{3}H-TdR for 30 min at day 7 post-stripping.

tion of spinous cells is frequently observed. However, this migration is invariably confined to a narrow rim around the explant (12). Immediately before low calcium stripping, all cultures (both normal and ichthyotic) showed strong keratinization and desquamation.

During regeneration of stripped cultures, a reproducible series of morphological events was observed as described elsewhere (3). Also in this respect, ichthyotic cultures were similar to normal cultures. Within a week after stripping, a gradual re-establishment of a multilayered morphology with minimal lateral growth was observed. However, on day 7 after stripping, keratinization was estimated to be slightly less and the number of mitotic figures larger in ichthyotic cultures vis-à-vis normal cultures.

Flow cytometry and labelling of sorted S-phase cells

Cultured epidermal cells from normal and ichthyotic skin were pulse-labelled with ^{3}H-TdR and prepared for flow cytometry and cell sorting. The relative proportion of cells in the different cell cycle phases was measured from the DNA histograms by a planimetric method (16). Both in primary (15-days old) and regenerating (7 days post-stripping) cultures, cells with S-phase DNA contents amounted to about 11% in cultures of ichthyotic skin; G_2 cells amounted to about 9% and G_1 cells to 80%. There was little variation between cultures from different patients and similar figures for the different cell cycle phases were obtained from normal cultures (S-phase: 10–11%; G_2 phase: 9%; G_1 phase: 80–81%).

When the grain count distribution of sorted S-phase cells was analysed, a marked heterogeneity in the number of grains per nucleus was observed in ich-

thyotic cultures, as has previously been observed in normal cultures (12, 13). The grain count distributions of sorted S-phase cells from primary cultures are shown in Fig. 1. The cells were classified into three groups (12): unlabelled ($\leqslant 3$ grains per nucleus), weakly labelled (4–24 grains per nucleus) and strongly labelled ($\geqslant 25$ grains per nucleus) cells. It appears that the grain count distribution of the S-phase cells from all ichthyotic cultures is clearly different from that of normal cultures. In particular, an increased proportion of strongly labelled S-phase cells is observed; the fractions of un- and weakly labelled S-phase cells are smaller than in normal cultures.

In regenerating cultures, the differences between ichthyotic and normal cultures were qualitatively similar to those observed in primary cultures, but quantitatively even more marked (Fig. 2). In both X-linked and autosomal dominant ichthyotic cultures, about 90% of the S-phase cells were strongly labelled at day 7 after stripping. Many of the strongly labelled S-phase cells were completely studded with grains and looked like black spots in the microscope. Labelling to this extent has previously only been observed when the strong mitogen cholera toxin was added to subcultures of normal keratinocytes grown on 3T3 feeder cells (17). During the peak of proliferative activity in regenerating, normal cultures the proportion of strongly labelled S-phase cells reach a maximum of about 75% (3).

The results of the labelling studies are summarized in Table I. It appears that, on average, the proportion of strongly labelled S-phase cells was doubled in ichthyotic vis-à-vis normal cultures, whereas the proportion of unlabelled S-phase cells was halved in

Table I. *Summary of results of [³H]thymidine labelling of sorted mid S-phase cells[a]*

Culture	Unlabelled (0–3 grains/nucleus)	Mid S-phase cells weakly labelled (4–24 grains/nucleus)	Strongly labelled (≥25 grains/nucleus)
Primary cultures			
Ichthyosis (mean of 4 cases)	7%	4%	89%
Normal skin (mean of 4 cases)	13%	46%	41%
Regenerating cultures[b]			
Ichthyosis (mean of 3 cases)	7%	3%	90%
Normal skin (mean of 3 cases)	16%	39%	45%

[a] Pulse-labelling with 10 µCi/ml [³H]thymidine for 30 min.
[b] 7 days after stripping in low-calcium medium.

ichthyotic cultures. Hardly any weakly labelled S-phase cells were present in the ichthyotic cultures.

Morphological differentiation

The morphological differentiation was assessed by measuring the mean cell diameter of sorted S-phase cells and by quantitation of cornified envelope formation (only in regenerating cultures). An increase in cell diameter is considered an early marker of keratinocyte differentiation, whereas the assembly of the cornified envelope from precursor proteins is thought to represent a very late step of differentiation (1, 18). The results are shown in Table II. It is seen that the mean cell diameter of S-phase cells in both primary and regenerating cultures was significantly smaller in ichthyotic cultures than in normal cultures. Moreover, the proportion of cells with a cornified envelope in ichthyotic cultures was reduced to approximately one-third of the proportion in normal cultures. Both these results indicate that the ichthyotic cultures were more immature than cultures from normal skin.

DISCUSSION

When cultures of normal human epidermal cells are labelled with radioactive DNA precursors a dramatic heterogeneity in the labelling intensity of cells with S-phase DNA contents is revealed. Both unlabelled and very strongly labelled S-phase cells are observed and among the labelled cells there is a more than 100-fold

difference in labelling intensity (13). The biological significance of this heterogeneity is not completely clear. However, studies with keratinocyte differentiation markers have suggested that some of the unla-

Table II. *Mean S-phase cell diameter and proportion of cells forming a cornified envelope in epidermal cultures from normal and ichthyotic skin*

Culture	n	Mean S-phase cell diameter (µm)±SD	Proportion of cells with a cornified envelope[a]
Primary cultures			
XL-1	204	13.22±2.65[b]	n.d.
XL-2	227	13.53±2.64[b]	n.d.
AD-2	116	12.18±2.12[b]	n.d.
Normal skin 1	350	15.03±1.37	n.d.
Regenerating cultures			
XL-2		n.d.	7.3%
AD-1	192	13.28±2.48[b]	5.1%
AD-2	277	14.02±2.93[b]	7.1%
Normal skin	132	16.13±2.87	19.0±2.3%[c]

[a] Determined 7 days after stripping.
[b] Significantly different from normal value ($p<0.001$; Student's *t*-test).
[c] Mean ± SE of 8 separate determinations of the mean (skin samples from 8 different normal persons).
n.d. = not determined.

belled S-phase cells are small (stem?) cells, whereas the majority of un- and weakly labelled S-phase cells are more mature (committed to terminal differentiation) than the strongly labelled S-phase cells (14). It was therefore suggested that the strongly labelled S-phase cells may represent intermediate, so-called transient amplifying cells responsible for rapid expansion of the cell mass.

The present data show that in cultures of ichthyotic skin, the proportion of strongly labelled S-phase cells was much greater than in normal cultures. Simultaneously, a dramatic decrease in the proportion of weakly and a more moderate decrease in the proportion of un-labelled S-phase cells was revealed. The difference between ichthyotic and normal cultures was clearly seen both in primary and regenerating cultures, although it was more marked in the latter case. In contrast, no difference was found between the cultures from patients with X-linked ichthyosis and ichthyosis vulgaris. The results suggest that the ichthyotic cultures were hyperproliferative and more immature (less differentiated) than cultures of normal epidermis. This suggestion was further substantiated when parameters of differentiation were investigated. Compared with normal cultures, the S-phase cells in ichthyotic cultures had a smaller mean diameter and fewer cells had formed a cornified envelope after 7 days of regeneration.

The proportion of cells in the different phases of the cell cycle were found to be very similar for ichthyotic and normal cultures. This indicates that the presumed hyperproliferative state of S-phase cells in the ichthyotic cultures apparently is a balance between a reduced part of the cell cycle time spent in S-phase and a recruitment of new cells to the cycling population, although the proportion of cycling cells in G_1 is not evident from the data.

Very few other studies have been concerned with *in vitro* culturing of ichthyotic epidermis. Kubilus et al. (19) showed that steroid sulfatase was undetectable in cultured epidermal cells from patients with X-linked ichthyosis. Fleckman et al. (20) observed lack of expression of profillagrin and absence of keratohyalin granules in epidermal cultures from ichthyosis vulgaris patients, thus indicating maintenance of some phenotypic characteristics of this disease *in vitro.*

Although the results of the present study are based on simple observations, they clearly indicate that in ichthyotic epidermis *in vitro*, there is a shift in the normal balance between proliferation and differentiation: The ichthyotic cultures taken together showed increased proliferation and reduced differentiation when compared with normal cultures. These findings are somewhat at variance with the *in vivo* situation, where X-linked ichthyosis and ichthyosis vulgaris are both considered normokinetic (5–7). The defect in these disorders is not considered to be that of overproduction of stratum corneum cells but rather an increased retention of scales. Detailed investigations have revealed a defect in keratohyalin granule formation and profillagrin synthesis in ichthyosis vulgaris (21), whereas X-linked ichthyosis is caused by a deficiency of steroid sulfatase leading to an accumulation of cholesterol sulfate in the epidermis, which is thought to be the crucial metabolic abnormality of this disease (8, 22, 23).

The reason for the apparent discrepancy between the *in vivo* and the *in vitro* situation is not clear. As similar results were obtained with cultures from patients with different types of ichthyosis, they may represent a reaction to the *in vitro* situation. Perhaps the observed signs of hyperproliferation in the ichthyotic cultures are a secondary reaction to the incomplete keratinocyte differentiation and lack of hyperkeratosis *in vitro*. In contrast to the *in vivo* condition the cultured cells were not covered by a thick stratum corneum. The denuding of the cultured cells was of course most pronounced in the stripped cultures, where indeed the most dramatic differences between ichthyotic and normal cells were found. Clearly, more studies are needed to elucidate the patho-physiological mechanism underlying the seemingly hyperproliferative state observed in the ichthyotic cultures.

Whether the hyperproliferation is a direct result of the genetic defects or is a secondary reaction to the artificial *in vitro* conditions, the dramatic phenotypic differences between the normal and pathological cultures offer an experimental system for testing the effects of pharmacologic or genetic intervention. As noted above, X-linked ichthyosis is caused by a defect in the gene for steroid sulfatase (the STS gene). By experimental manipulation of the cholesterol metabolism it may be possible to induce or revert the phenotypic characteristics of X-linked ichthyosis in culture (24). The STS gene has been located to the distal end of the short arm of the X-chromosome (Xp22.3) and has recently been cloned (25). If it proves possible to introduce and express the STS gene in the basal cells of X-linked ichthyotic cultures, this might lead to an experimental model for the development of somatic gene therapy.

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