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Research Article

Primary culture of dispersed skin epidermal cells of rainbow trout *Oncorhynchus mykiss* Walbaum

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Abstract. This is the first report on a primary culture of dispersed skin epidermal cells of rainbow trout *Oncorhynchus mykiss* Walbaum. These primary cells revealed a low seeding efficiency after 3 days (11.6 ± 4.6%), whereas subcultured cells had a higher seeding efficiency at the same time point (75.5 ± 34.0%) and increased in cell number (150–200% of seeded cells after 20 to 30 days). The cells were characterized applying histological, immunocytochemical and ultrastructural methods. The culture consisted of undifferentiated keratinocytes. Mucous cells as well as differentiated epithelial cells were absent. To date the cells were cultured for maximally 9 passages and 402 days and therefore provide the possibility for long-term studies.

Key words. Epidermis; primary cell culture; rainbow trout; growth curve; histology; immunofluorescence; electron microscopy.

Introduction

Fish skin is in direct contact with the aqueous environment and functions as a barrier to protect the organism against biotic and abiotic stressors. The skin has been proposed to participate actively in regulation of important ions, for example Ca^{2+}, and in acid-base balance through Cl^-/HCO_3^- exchange, at least in some species [1–3]. The skin contributes substantially to the exchange of lipophilic substances [4]. Dermal absorption ranges from 1.6 to 50% of total absorption depending on the nature of the substance, concentrations and exposure times, the species and the age of the fish [5]. Additionally, the skin is a metabolically active tissue that quickly responds to stressors, for example acidified water [6].

In vitro systems are useful to study normal and disturbed functions of cells and tissues. Established cell cultures form the basis for a variety of investigations on the biology and pathological state of the tissues as well as its involvement in disease and toxicology [7–12]. For fish epidermis, primary cell and tissue culture technology is rather underdeveloped [13]. In vitro studies using explants were carried out to examine motility and structure of keratinocytes [14–16] or to establish tissue cultures to study infection mechanisms [13].

Culture of dispersed skin epidermal cells is common practice in mammalian and bird models [17]. To our

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knowledge, it has not been described for fish until now. However, dispersed gill epidermal cultures have been established [18–21].

In this study primary skin epidermis cultures were developed from rainbow trout *Oncorhynchus mykiss* Walbaum. To characterize origin, growth and morphology of the cells, growth curves were calculated, and histochemical, immunocytochemical and ultrastructural investigations were performed.

Materials and methods

Cell preparation and cultivation. Juvenile (4–16 months old) rainbow trout, *Oncorhynchus mykiss* Walbaum, were randomly selected from the stock culture located at the Centre for Fish and Wildlife Health. After a 2-day period without feeding, the fish were killed by cutting the neck. The preparation was carried out according to standard methods [11], modified as follows. The skin of the whole body was cut into strips ($\leq 1 \times 2$ cm^2), lifted carefully from the underlying muscles and incubated in antibiotic solution (100 IU ml^{-1} of penicillin, 100 µg ml^{-1} of streptomycin-sulphate, 0.25 µg ml^{-1} of amphotericin B (fungizone) (Gibco BRL Life Technologies, Gaithersburg, MD, USA) in William's Medium E (WME, Sigma, St. Louis, MO, USA) + 20% fetal calf serum (FCS, BioConcept, Allschwil, Switzerland) for 2 h at 4 °C and in dispase solution [10 mg ml^{-1} dispase II (Boehringer Mannheim, Mannheim, Germany) in WME + 10% FCS] for 2 h at 4 °C. Dispase removes the hemisdesmosomal junctions which connect the keratinocytes to the basal lamina. While holding the tissue strips upside down with a pair of tweezers, dermal and muscle tissue remnants were scraped off easily. The epidermis was minced into pieces (~ 1 mm^2) and digested 8–10 times with 6–8 ml of trypsin 0.25% in phosphate-buffered saline (PBS; Seromed, Biochrom, Berlin, Germany) for 5 min each time at room temperature (RT). One millilitre of FCS was added to the supernatant. The cell solution was centrifuged at 800 rpm for 10 min at 22 °C. The cells were counted (hemocytometer, crystal violet staining) and seeded in culture flasks (25 or 75 cm^2) [polystyrene, Techno Plastic Products (TPP), Basel, Switzerland] at a density of 10^5 cells cm^{-2}. WME was supplemented with 10% FCS, 5 µg ml^{-1} L-glutamine (Seromed), 10 IU ml^{-1} of penicillin (Seromed), 10 µg ml^{-1} of streptomycin (Seromed), 10 ng ml^{-1} of epidermal growth factor (EGF, Gibco BRL), 5 µg ml^{-1} of insulin (Sigma), 0.32 µg ml^{-1} of hydrocortisone (Sigma) and 10 ng ml^{-1} of choleratoxin (Sigma), pH $\approx$ 7.0. Alternatively, Minimal Essential Medium (MEM, Gibco BRL) with the same supplements, pH $\approx$ 7.0, was used. In addition, cells were grown in 24- and 96-well plates (polystyrene, TPP). Cells were cultured at 19 °C, 5% CO_2 in air for buffering the medium to pH $\approx$ 7.0 [11] and relative humidity 98%. The medium was changed twice a week.

After reaching confluence, the cells were grown for an additional 1 to 2 weeks followed by a passaging procedure using trypsin/EDTA 0.05/0.02% in PBS (Seromed) (15–20 min, RT). Cells were reseeded at a density of approximately 3×10^4 cells cm^{-2}. Primary cells were trypsinized after 30 to 40 days, subcultured cells after 20 to 30 days.

In total, cells of 35 fish were cultivated. Cells originating from different fish were never mixed.

Cell number and growth curves. During cultivation cells were counted twice a week using a calibrated ocular grid. To reduce counting mistakes, a quantity of approximately 20–100 cells within the area covered by the grid was counted each time. Depending on cell density, different numbers of grid units were counted. For example, at a low cell density the whole grid (100 units) was counted, at a high density 10 units or even less. Ten randomly chosen areas were counted per flask. A coefficient of variation of CV = 0.1 for the counting method was accepted.

Since detaching cells were observed during the first days, counts were carried out starting day 3 after seeding. Accordingly, seeding efficiency was not calculated until 3 days after seeding, when in some cases proliferation had begun.

Histology, immunofluorescence and electron microscopy. Cells were grown on sterile noncoated glass coverslips and snap-frozen in precooled isopentane in liquid nitrogen. For the investigation of the freshly prepared primary cells in suspension, the pellet was snap-frozen and sectioned (cryostat sections).

For histological examination, samples were stained with haematoxylin-eosin (H-E) or periodic acid-Schiff (PAS).

For immunofluorescent keratin labelling, cultures on coverslips and sections of the pellet were rinsed in PBS with 0.1% bovine serum albumin (BSA, Sigma) (10 min). Cells were permeabilized in TritonX-100 (Boehringer Mannheim), 1% in PBS (10 min). Samples were incubated with the monoclonal anti-mammalian-cytokeratin antibody AE1 (Progen, Heidelberg, Germany), dilution 1:100 or 1:50 in PBS for 1 h, RT, washed with PBS and incubated with the fluorescein isothiocyanate (FITC)-conjugated secondary antibody (DAKO A/S, Glostrup, Denmark), dilution 1:50 in PBS or in Bio-X dilution solution (Bio-X, Brussels, Belgium) for counterstaining (1 h, RT). As control, the primary antibody was omitted. The fibroblast-like cell line RTG-2 served as a negative control culture [22], while dog skin was the positive control. Samples were examined with a Leitz Laborlux S fluorescence microscope.

For electron microscopy, cells were fixed in ice-cooled 2% glutaraldehyde in 0.1 M Soerensen buffer, pH 7.6,

for at least 1 h. Preparation for transmission electron microscopy (TEM) was carried out as described by Burkhardt-Holm et al. [23]. Ultrathin sections were examined in a Zeiss EM 902. For scanning electron microscopy (SEM) osmicated samples were dehydrated through an ascending ethanol series, critical point-dried in a CPD 030 (Bal-Tec, Baltzers, Liechtenstein), sputter-coated with approximately 20 nm of gold in an SCD 004 (Bal-Tec) and examined in a Leo DSM 982 (Leo, Oberkochem, Germany).

Statistics. Data for cell number in one culture flask were expressed as mean of 10 randomly chosen areas. One data point of the growth curve was expressed as mean $\pm$ SD (n), in which n was the number of fish. The coefficient of variation CV is a relative value for the variation ($CV = SD \times mean^{-1}$). Agreement between cell numbers obtained by countings with ocular grid and hemocytometer was assessed by evaluation at R-square value (R^2).

Results

Growth and morphology of primary and passaged cells in culture. Growth of cells was influenced by the medium. WME was chosen because it supports long-term culture of mammalian keratinocytes. For the same reasons, appropriate supplements were used [24, 25]. MEM, a common medium in culture of fish cell lines, led to cell attachment, but the cells did not effectively proliferate and many of them swelled, even when the same additives were used as in WME. The best results were thus obtained using supplemented WME. When one of the supplements was removed, slower spreading and/or prolonged time for reaching confluence was observed. Increasing concentrations of FCS to 20% during seeding or subsequent cultivation did not result in a visible improvement of cell growth. Five percent FCS resulted in a lower attachment efficiency, and ten percent FCS was thus found to be optimal. Cells grew slightly faster in flasks compared with growth in 24- and 96-well plates. A seeding density below 8×10^4 cells cm^{-2} was not sufficient for starting a primary culture. Cell freezing and storage in liquid nitrogen as well as thawing and reseeding were possible using standard methods.

Cells were counted by grid directly before passaging and by hemocytometer before reseeding. Reasonable agreement between both counting methods was obtained ($n = 17$, $R^2 = 0.84$, $p < 0.001$). The seeding efficiency of primary cells after 3 days in culture was $11.6 \pm 4.6\%$ ($n = 7$) (table 1, fig. 1a). After enzyme treatment the cells attached in the first few hours. Most of them developed a flat multipolar shape. Some contacted each other via small long filopodia (fig. 2a, b). Some cells were round and pancake-like or half-moon-shaped with filopodia. Seven to 10 days after seeding, cell proliferation led to a net increase in cell number (fig. 1a) with a doubling time of 6.2 ± 2.2 days ($n = 6$). When confluence was reached after ~ 3 weeks, intercellular contacts were common (fig. 2c). Later on islands of large round cells were seen among walls of smaller elongated cells (fig. 2d). The latter were multilayered cell aggregates. Nuclear shape varied from round to oval or kidney-shaped (fig. 2e).

The growth pattern of subcultured cells differed from that of primary cells (fig. 1b). On average, passaged cells had a seeding efficiency after 3 days of $75.5 \pm 34.0\%$ ($n = 23$). Culture over 20 to 30 days resulted in a net increase of about 150 to 200% of the seeded cell number (fig. 1b, table 1).

A doubling time of 5.2 ± 4.0 days ($n = 17$) was calculated. A seeding density of fewer than 10^4 cells was too low for successful subculturing. Because of faster growth, intervals between passaging procedures could be decreased after several passages (table 1). Directly after passaging, the shape of the single cells was more homogeneous than in the primary culture, mostly multi-

Table 1. Comparison of growth parameters of primary and passaged cells.

Culture	Seeding density (number $\times$ 10^4 cm^{-2})	Seeding efficiency after 3 days (attached cells in % of seeded cells)	Harvested cells (in % of seeded cells)	Duration (d)	n
Primary	11.2 ± 2.6	11.6 ± 4.6 (7)*	31 ± 19	33 ± 7	35
Passage 1	2.9 ± 1.1	74.5 ± 37.8 (5)*	131 ± 78	28 ± 12	20
Passage 2	2.5 ± 0.6	51.5 ± 4.7 (4)*	157 ± 52	29 ± 9	11
Passage 3	2.6 ± 0.9	78.8 ± 30.4 (6)*	220 ± 102	26 ± 10	9
Passage 4	2.8 ± 0.4	104.2 ± 20.9 (3)*	149 ± 91	22 ± 8	4
Passage 5	2.1 ± 0.5	157.8 (1)*	231 ± 247	22 ± 9	4

*The seeding efficiency was determined from cell-counting data with the grid, which was carried out for a limited number of fish, given in parentheses. A seeding efficiency of more than 100% might be calculated, because cell counts had to be carried out 3 days after seeding, when proliferation already had started partly.

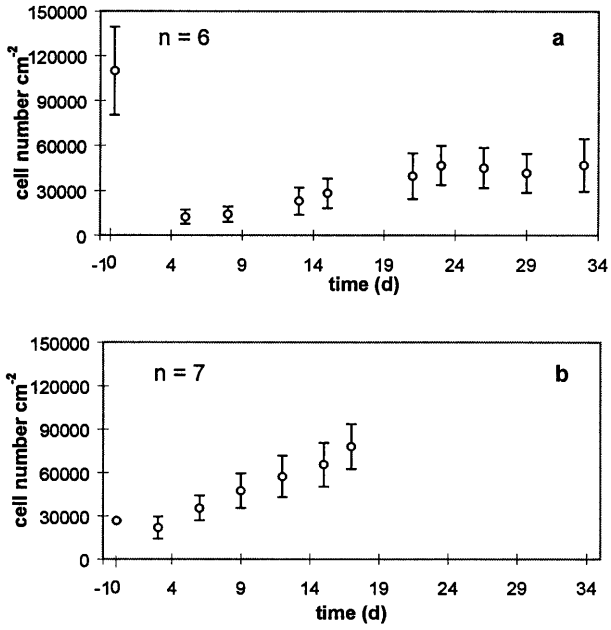

Figure 1. Cell count of (*a*) primary cells and (*b*) cells of the third passage, representative for the course of growth of passaged cells in general, plotted against the time. Cells were seeded on day 0. Error bars indicate standard deviation of cells from different fish: $n = 6$ for primary cells and $n = 7$ for cells of third passage. The seeding efficiency after 3 days of primary cells is low ($11.6 \pm 4.6\%$), and the cell number after 30 to 40 days reached $31 \pm 19\%$ of the seeded cells. Cells of passage 3 have a seeding efficiency after 3 days of $78.8 \pm 30.4\%$, and after about 20 to 30 days the cell count reaches $220 \pm 102\%$ of seeded cells.

polar shaped. After reaching confluence, two morphs of cells were present similar to that described for primary cells (fig. 2d, e).

For primary as well as subcultured cells, it was not favourable to passage them immediately after reaching confluence, because seeding was followed by a phase of reduced growth. Continued growth was, therefore, allowed for at least 1 to 2 weeks before subculturing. The cells could successfully be cultured over a time period of 402 days, and maximal nine passages were reached. Without passaging, the round cells died after 5 to 6 weeks, and the open space was resettled 1–2 weeks later. The pattern consisting of walls of elongated cells and islands of round cells was maintained. The elongated cells were dying after 5 to 6 weeks as well, but because of multiple cell layers present in these areas, it was not possible to describe a time course of dying and resettlement. The full course of the cell culture had not yet been obtained. A maximum cultivation time of 3 months was observed.

The cell number gained from different fish of the same size was variable. In general cell growth was better from younger and smaller fish, though on average, a higher cell number was obtained from older fish with more body surface. Seasonal variability may occur, but it seems to be eclipsed by variability resulting from size (and age) of fish. Taking cells from fish older than 14 months gave high cell numbers and led to a high amount of attaching cells (about 30%). However, these cells hardly proliferated, so confluence was not reached.

The total cell number obtained from one fish was ~ 4–5 million on average. After five passages, for example, about 9 to 10 million cells were obtained.

Ultrastructure. Examination of the cell suspension by TEM revealed single cells and epidermal fragments as well as remnants of cells, and necrotic and apoptotic cells. Single keratinocytes were spherical. Microridges, the typical surface structures of apical epidermis cells in vivo, could hardly be found (fig. 3a). Ultrastructurally intact cells showed nuclei of lobulated shape without heterochromatin. Most nucleoli were located eccentrically. A perinuclear organelle- and a peripheral filament-containing zone could be distinguished. The nuclei were surrounded by a rim of dictyosomes, several small vesicles, cisternae of the endoplasmic reticulum (ER) and small round to spherical mitochondria. Sometimes mitochondria had slightly irregular surfaces and distended intermembranous spaces. The ER was distributed in small irregular distended cisternae. Intact mucous cells were detected only in epidermal fragments. These fragments sometimes showed microridges. Extremely distended intercellular spaces as well as intracellular electron lucent vacuoles and a high number of myelin bodies were observed. In freshly seeded cells, various states of adhesion were seen with SEM. Spherical cells with a small adhesion area were found as well as partially and completely flat cells which showed a multipolar shape resembling the cells after a longer culture time. None of those cells showed microridges. When the cells grew out, they increased in diameter, but remained flat. After 6 days of culture, cells measured ~ 20–40 μm in diameter.

Confluent cells examined by TEM were very flat with a height of 80 to 2000 nm (fig. 3b). Up to four cell layers occurred. The cell surfaces were mostly smooth, in some cases forming small extensions. The intercellular spaces were sometimes distended and filled with fibrouslike material. Some cells were very close to adjacent cells without being connected by desmosomes. Within the cell a peripheral compartment consisting of filaments and some vesicles could sometimes be distinguished from the perinuclear part of the cytoplasm that contained all organelles and the nucleus. In most cases the nucleus was flat with a smooth surface and rather elongated. Part of the chromatin was condensed at the

inner rim of the nucleus. Nucleoli could hardly be observed. Mitochondria were of spherical to ovoid shape, occasionally very elongated, and showed few, irregularly arranged cristae. The Golgi apparatus consisted of several piles of flat cisternae, budding off numerous small vesicles containing material of medium electron density. Smooth ER occurred in single, slightly distended cisternae and vesicles. In some cells extensive stacks of rough ER occurred. Mitochondria and lysosomes containing myelin bodies were often visible.

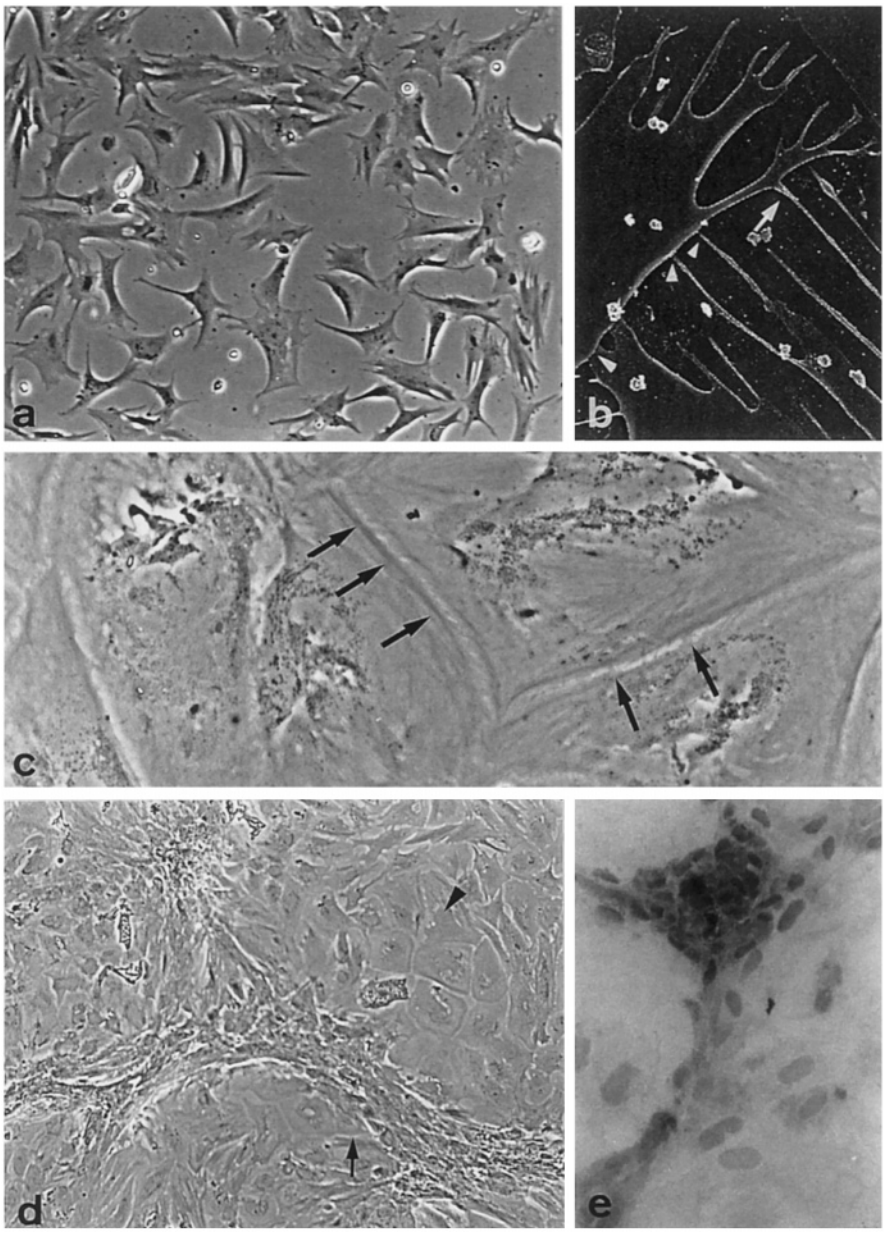

Figure 2. Primary epidermal cells in culture. (*a*) Phase-contrast microscopy after 10 days. Most cells are single and of multipolar shape, sometimes attached to each other by filopodia (102×). (*b*) SEM of filopodia. Sometimes filopodia connect cells (arrow); in other cases cells grow on top of each other (arrowheads) (2706×). (*c*) Phase contrast, confluence, after 20 to 30 days. Arrows point out intercellular contacts (271×). (*d*) Phase contrast, postconfluence, after 40 days. Islands of big round cells (arrowhead) lying among walls of small elongated cells (arrow); the latter grow in up to four layers above each other (68×). (*e*) PAS staining, postconfluence. Nuclei are round, oval or kidney-shaped. Areas with multilayered cells show strong positive staining (271×).

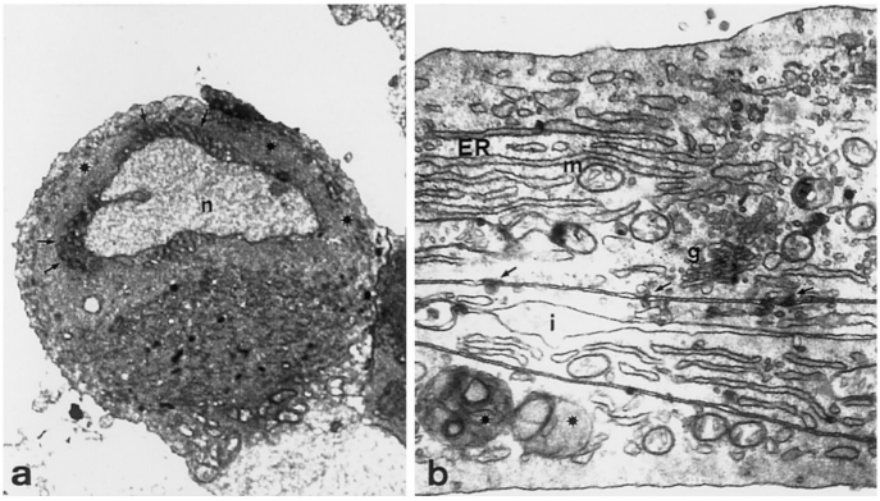

Figure 3. TEM of (*a*) cells in suspension and (*b*) postconfluent cells of passage 3. (*a*) Cells in suspension are spherical without microridges. Perinuclear organelle-containing zone (arrows) and peripheral filament-containing zone (asterisks). Nucleus (n) lobulated, nucleolus eccentrical (5325 ×). (*b*) Several cell layers with very flat cells (80 nm–2 μm). Intercellular spaces (i) distended or narrowed, with a type of junction that could not be identified (arrows). Mitochondria (m), endoplasmic reticulum (ER) as well as Golgi apparatus (g) similar to in vivo. Large lysosomes with myelin bodies (asterisks) (21 300 ×).

Caveolae, invaginated vesicles of uniform diameter that are formed at cell surfaces [26], were often observed. No basal lamina was found.

Keratin labelling. To determine the epithelial origin of the cells, keratin labelling was performed by immunofluorescence. Keratin was detected in primary cells, subcultured cells as well as in the spherical cells in suspension. The cytoplasm was labelled diffusely, whereas typical filamentous structures could not be seen (fig. 4a, b). The nuclear region was unstained. The filopodia attaching to neighbouring cells were keratin-positive (fig. 4b). Unlabelled cells were not found.

Mucous cells. In the suspension, both single spherical cells, which were supposed to be keratinocytes due to keratin labelling, and PAS-positive mucous cells, were observed.

No PAS-positive cells were observed in the cell culture. In postconfluent cultures strong PAS-positive areas were found occasionally in areas of multiple cell layers (fig. 2e), probably due to the view onto many slightly stained cell layers or to intercellular spaces artifactually filled with PAS-positive substance.

Discussion

This is the first report on the establishment of a primary culture out of dispersed skin epidermal cells from rainbow trout and – following the definition of Freshney [11] – the production of a cell line with up to nine passages.

The skin epidermis of fish consists in all layers of viable keratinocytes [27–29]; high mitotic activity appears, however, in deeper cell layers [29]. The low seeding efficiency of our primary cells after 3 days indicates that basal cells are more likely to attach successfully to an

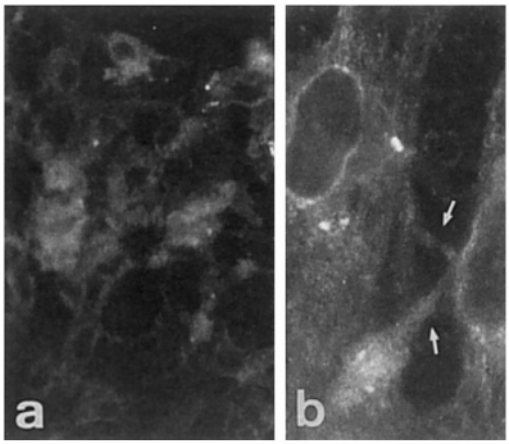

Figure 4. AE1 antikeratin immunofluorescence labelling on cells of passage 1. (*a*) Cytoplasm with diffused labelling, nuclei unstained (99 ×). (*b*) Cell-cell contacts labelled (3300 ×). All cells of different shapes are labelled.

artificial substrate. Similarly, the terminal differentiation of mammalian keratinocytes in vivo is assumed to account for the observed low seeding efficiencies (1–20%) in those cultures [24, 30]. The higher attachment efficiency of $35 \pm 6\%$ found for isolated gill epithelial cells [18] could be due to the high relative amount of basal cells in the bilayered gill epithelium compared with the multilayered skin epidermis.

The observed doubling time of 5 to 6 days corresponds to the findings of the epidermal cell cycle of the plaice *Pleuronectes platessa* in vivo that exceeds 108 h [31]. In studies on explants of fish epidermis, six cell doublings were concluded in 3 weeks [13]. The high migration velocity of epidermal cells out of explants was, however, obviously not considered [14, 15]. The low proliferation rate of our cells in comparison with epithelial gill cell cultures [18, 32 and own unpublished studies] is assumed to be influenced by different media compositions or/and culture conditions. We obtained the best results with supplemented WME, which supports long-term culture of mammalian keratinocytes [24, 25]. Modifications in the composition of the supplements and FCS concentrations were tested. Since P_{CO_2} values in fish tissues are about 10 mmHg or less (compared with P_{CO_2} values between 30 and 40 mmHg in mammalian tissues), we plan to test a lower CO_2 concentration of 1 or 2% that will be closer to the physiological state of the fish. Changes in the CO_2 concentration have not yet been tested due to the dependence of CO_2, O_2 and pH, which have to be controlled carefully during the experimentation period. A stable pH of the medium is important for long-term culture, because pH changes may lead to known and unknown chemical reactions in cell metabolism. In our opinion it is favourable to use CO_2 to achieve a stable pH of the medium. Furthermore, Mothersill et al. [9, 13] have been successfully culturing fish skin epidermis explants at 5% CO_2. An additional effect of a lowered P_{CO_2} is the rise in the medium pH to ≈ 7.4 and 7.6, respectively [11]. Medium pH of 7.5 to 7.8 is often used in gill epithelial cell culture [18, 20, 32].

A possible reason for the lack of differentiated keratinocytes with microridges as well as differentiated mucous cells is that seeding dispersed cells leads to loss of differentiated cells, since they do not attach to the artificial substrate. This hypothesis is supported by the absence of mucous cells as well as differentiated chloride cells in gill epithelial cells after cell isolation [18], and by the occurrence of differentiated epithelial cells with microridges in explant culture [32, 33].

Isolation of cells is assumed to lead to loss of polarization, which means that spherical cells in suspension did not have microridges, a characteristic of cell polarity in pavement cells. Microridges were found on epidermal fragments only. We suppose that they might be maintained when intercellular contacts are preserved during preparation, for example when using the explant method [13, 14, 16, 33]. Immediately after isolation of rainbow trout hepatocytes, at least a temporary loss of the cell polarity was observed [34]. Hence, microridges on keratinocytes in culture had to be formed de novo, which obviously did not occur, even after a 55-day culture period. The same circumstances may have led to an absence of mucous cells. Differentiation into mucous cells did not occur after seeding. Perhaps a reduction of the drastic trypsin treatment, which damages many surface proteins, might lead to diminished dedifferentiation.

Regular microridges were, however, detected in 6 to 9- and 12-day-old gill cultures after isolation of cells [18, 20]. Number and arrangement of microridges may depend on the type of serum used. Using FBS, Avella and Ehrenfeld [20] found irregular or reduced microridges in cultures of dispersed gill cells. Using fish serum from the sea bass *Dicentrarchus labrax*, the species the authors used as donor for gill cells, however, a regular and concentric microridge arrangement was reported [20]. In contrast to these findings, other studies revealed no effects on microridge formation, but even toxic effects on the cells by replacement of FBS by fish serum [18, 32]. Therefore, factors triggering and influencing microridge formation remain to be defined.

Ultrastructurally, cells in suspension showed typical compartmentalization in a perinuclear organelle- and a peripheral filament-containing zone similar to in vivo keratinocytes [35]. The nuclei of cytologically viable cells were lobulated, displaying a high degree of similarity to their appearance in native cells [35]. The organelles closely resembled the corresponding in vivo structures as well [35]. Most nucleoli were located eccentrically, reflecting metabolic activity [26]. In monolayered cells, the most obvious difference to in vivo cells was their flat shape. This is consistent with the description of all fish epithelial cell and tissue cultures so far [14, 16, 20, 21, 32, 33]. It may influence a flattening of the nuclei and the intracellular arrangement of organelles. Occasionally, peripheral filament-containing zones were distinguished, but no regularity was observed. Besides the cellular arrangement, the ultrastructure of smooth and rough ER, mitochondria and dictyosomes were similar to that in native epidermis [35, 36] and indicate the viability of the cells. Intercellular contacts were found, but a typical desmosome structure was not unambiguously observed.

Our cells are similar ultrastructurally to cultured gill epithelial cells as described by Fernandes et al. [33] for the explant method and Avella and Ehrenfeld [20], and Wood and Pärt [21], for disaggregated primary gill cells and cells of the first passage grown on filter inserts. This is a strong indication for the epithelial origin of our cells. The observed different shapes of single cells is similar to other descriptions of fish keratinocytes [15]. A

possible explanation for the half-moon shape of epithelial cells is that they are in movement [14, 15, 37–39]. Thus, and although the morphology of our cells points to a possible epidermal origin, the lack of differentiated features makes it likely that we are working with a type of more general epithelial cells.

In the postconfluent cultures our cells grew in up to four layers. Cultures of stratified epithelial cells of mammals and birds also continue to proliferate after reaching confluence, although at a reduced rate, resulting in multilayered cells [11]. This is in accordance with our results and with all studies on fish surface epithelial cell cultures carried out so far [18, 20, 21, 33].

In general, keratins are markers for epithelial cells. To test the origin of our cells in culture, we applied immunofluorescence. The occurrence and distribution pattern of keratins in fish is different from that of mammals, since keratins in fish are not limited to epithelial tissues [22, 28]. The monoclonal antibody AE1 against mammalian cytokeratins labels keratinocytes and mucous cells in the epidermis of rainbow trout, whereas dermal cells are negative [22, 28]. Immunofluorescence labelling of keratin in our monolayer cells was diffuse. Whether it reflects the distribution of accessible and inaccessible forms of the specific epitopes is not clear. Even if the observed pattern deviates from that often reported (e.g. ref. 40), diffuse labelling of cytokeratin is not rare [41]. It could also be an effect of the labelling of cells in culture as described for RTG-2 cells with the monoclonal cytokeratin antibody CO4 [28]. Because we never found unlabelled cells, we conclude that the culture consists only of cells from one type, that is undifferentiated keratinocytes.

Primary cultures of epidermal cells are in general threatened by contamination with fibroblasts [11]. We cultured dermis explants from rainbow trout, which contained extremely slender cells, assumed to be fibroblasts, and cells microscopically similar to those of the epidermis culture (unpublished results). The latter were assumed to originate from small epidermal explants. Using WME plus all additives including 10% FCS, fibroblasts did not overgrow the epithelial cells; in contrast, epithelial cells seemed to displace fibroblasts. Thus the medium appears to preferentially support keratinocytes and to suppress fibroblast growth. In cultures of dispersed cells, the only area where fibroblasts might hide is the multilayered areas (fig. 2d). It is very unlikely, however, that immunofluorescence labelling of keratin in this area of multiple cell layers did hide single unstained fibroblasts between many labelled keratinocytes, since somewhere on the coverslip these unstained cells should have been observed. Dispersed epithelial monolayer cells are ultrastructurally similar to keratinocytes. We therefore conclude that the cultured cells consist of keratinocytes, though we were not able to prove the absence of fibroblasts beyond all doubt.

Other cell types can be isolated from the epidermis of rainbow trout [42]. We assume that these are taken into culture as well, but do not survive under the given conditions. At least, ultrastructural investigation gives no indication of the presence of other cell types.

Our cells can be cultured and subcultured over a long period. On average, primary cultures can be maintained over 3–5 passages, which means a minimum of 90 days. The maximal length of cultivation time was 402 days (passage 9). Until now, the longest culture period of fish skin epidermal cells reported was 22 days [13]. Differences in the reported culture lengths may be due to the use of various media and supplements. WME especially is known to support long-term growth of epithelial cells [43]. The possibility that our cells became somehow transformed was not investigated. However, morphological similaritiy of cells from different fish and different culture durations as well as the reproducibility of the preparation and culture suggest that this was not the case. Transformation or senescence may occur at any time, but it is most likely to occur between 20 and 80 population doublings [11]. With our observed cell doubling time of 6 days (lag phases not included) and the maximum cultivation time of 402 days, $\sim$67 population doublings were reached. Thus the 'normal range' is not exceeded, but that still does not exclude transformation.

Subculturing cells offer the possibility of increasing the number of cells and reducing the number of fish needed for experiments. Furthermore, a higher cell count is available for high numbers of replications in an experiment. Cells originating from one fish are more homogeneous, thus leading to results with lowered variance. This can be especially useful for toxiocological investigations. An additional important advantage is that long-term studies become easier. To our knowledge, this is the first report on subculturing fish skin epidermal cells. However, the disadvantage of undifferentiated cells, which may not fulfill all functions, should not be neglected. Whereas the explant method allows culturing of differentiated cells, it obviously implies a limited culture time of $\sim$22 days [13]. Furthermore, topical application on explant cultures seems to be difficult, since overall growth is rarely reached (own unpublished results). In cultures of dispersed gill epithelial cells, a higher degree of differentiation is reached as those cells develop microridges [18, 20]. In previously reported experiments, cells were used on days 6–9 [20] or could be grown for up to 40 days [18], which is still shorter than our system.

Finally, the suitability of each system depends on the aim of the study. Experiments are underway which will probably lead to differentiation of the cells in our culture.

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