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PRIMARY CULTURES OF EPITHELIAL CELLS FROM RAINBOW TROUT GILLS

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Summary

A method for obtaining primary cultures of epithelial cells from rainbow trout gills is described. The yield of cells from approximately 1.5g wet mass of tissue was $218\times10^6\pm12\times10^6$ cells with a viability defined by eosin exclusion of 80±6%. Cells were seeded in culture dishes and grown in Leibowitz L-15 medium supplemented with 5% foetal bovine serum. Attachment efficiency after 24h was 35±6%. The cells appeared confluent 10–12 days after seeding and exhibited surface structures similar to those seen on respiratory epithelial cells of trout gills *in vivo*. Growth rate, [^{3}H]thymidine incorporation and attachment efficiency were used to evaluate culture conditions. Epidermal growth factor, insulin, transferrin, hydrocortisone, laminin and collagen did not improve growth and attachment. Similarly, coating the culture dishes with rat tail collagen, trout skin extract, laminin or a mixture of human basement membrane proteins (Matrigel) failed to improve attachment. It is concluded that the cells in culture are respiratory epithelial cells and that this culture system could provide a valuable new approach for studying the physiology of these cells.

Introduction

Fish gills have many physiological functions. They are involved in exchange of respiratory gases, in acid–base and ion regulation and in the excretion of nitrogenous waste. The gill epithelium consists of three cell types: respiratory epithelial cells, chloride cells and mucous cells. Respiratory cells constitute as much as 90–95% of the total epithelial surface area. They contain few mitochondria but display a complex system of microridges and microvilli on their external surfaces. Chloride cells have a large number of mitochondria, an extensive tubular network and a high content of Na^+/K^+-ATPase, features that suggest they may be ion-tranporting cells. Mucous cells produce and secrete the mucus that partly covers the gill surface. They are found dispersed between the other cells, mainly at the leading edges of the filament and the gill arch.

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The gill epithelium is able to respond to environmental changes at the cellular level. Compensation for water, ion and acid–base disturbances in the fish includes morphological and physiological adjustments in the gills involving both chloride and respiratory cells (Avella *et al.* 1987; Avella and Bornancin, 1989; Laurent and Perry, 1990; Goss *et al.* 1992). The cellular mechanisms involved in the multiple functions of the gills are poorly understood, particularly in freshwater fish, for largely methodological reasons. Our present knowledge of gill physiology is based on *in vivo* experiments with intact fish or perfused gill preparations. Such methods do not have the resolution to reveal mechanisms at the cellular level. In saltwater fish the situation is different: studies of the ionoregulatory mechanisms of the chloride cells have been greatly facilitated by the use of opercular membranes from some euryhaline fish species. The use of these membranes, which consist of up to 70% of chloride cells in *Fundulus heteroclitus* and can be mounted flat in an Ussing chamber for electrical and flux studies, has contributed greatly to our knowledge of chloride cell biology (Zadunaisky, 1984). For freshwater fish no such tissue with a nearly pure population of ion-transporting cells is available and, therefore, other approaches have to be used.

One such approach is to isolate epithelial cells from freshwater fish gills and grow them in primary culture, a technique that has already been successful with other epithelia of mammalian and non-mammalian origin (Taub, 1985). Particularly interesting with respect to gill physiology is that epithelial cells in primary culture can be grown on permeable supports on which they differentiate fully and retain their polarized properties (Dickman and Renfro, 1986; Ford *et al.* 1990). Furthermore, such supports can be mounted in Ussing chambers for transepithelial transport studies, as in a recent study of Cl^- transport in shark rectal gland cells (Valentich and Forrest, 1991). For gill epithelia, no such method has been tested. In several studies, freshly prepared cells have been used (Guibbolini *et al.* 1988; Perry and Walsh, 1989) but only in a few cases have gill cells been cultured for longer periods (Naito and Ishikawa, 1980). The closest approach to culture of isolated gill cells is a type of tissue culture where gill filaments and opercular membranes have been kept alive in culture medium for up to 5 days in order to study the development and differentiation of chloride cells (McCormick and Bern, 1989; McCormick, 1990).

In this paper we describe a method of growing epithelial cells from rainbow trout gills in primary culture. The cells are grown as monolayers and, at confluence, show the typical surface characteristics of respiratory epithelial cells.

Materials and methods

Two-year-old rainbow trout, *Oncorhynchus mykiss* (Walbaum), weighing 50–100 g were obtained from a local hatchery. They were kept in 1000l holding tanks in recirculating water (pH=7.1–7.2, ion composition in mmol l^{-1} Na^+=0.06, Cl^-=0.07, Ca^{2+}=0.2, Mg^{2+}=0.04, HCO_3^-=0.4) under light:dark conditions following the natural variation over the year. Temperature varied from 8 to 12°C depending on season. They were kept on a maintenance diet of commercial trout food (Ewos T40).

Leibowitz L15 medium, L-glutamine, phosphate-buffered saline without Ca^{2+} and

Mg^{2+} (PBS), PEST (penicillin, streptomycin) and fungizone were obtained from Flow Laboratories, UK. Foetal bovine serum (FBS) and trypsin were obtained from SVA, Uppsala, Sweden. Gentamicin, laminin, collagen type I, epidermal growth factor (EGF), transferrin, insulin, hydrocortisone hemisuccinate (cortisol), dimethylaminostyryl-methylpyridiniumiodine (DASPMI), sodium dodecylsulphate (SDS) and ethylene-diaminetetraacetic acid (EDTA) were obtained from Sigma Chemicals Inc, USA. Tritiated thymidine was from Amersham, UK. Culture dishes (Nunculon) were obtained from Nunc, Denmark.

Rainbow trout serum was prepared from blood obtained from the ductus cuvieri with a syringe. The blood was allowed to clot in sterile glass tubes overnight at 4˚C, centrifuged for 15min at 800 ***g*** and the serum was filtered through a 0.22 μm sterile filter (Millipore Inc. USA). The serum was used fresh or after storage at −20˚C.

A skin extract from trout was prepared according to Blair *et al.* (1990). Skin and fins (6g) were homogenized in a Waring blender for 5min in 100ml of extraction buffer (100mmol l^{-1} NaCl, 5mmol l^{-1} sodium acetate, pH adjusted to 4.2 with acetic acid). The homogenate was dialyzed against 10l of extraction buffer overnight at 4˚C. It was filtered through glass wool, a 1.2 μm filter (Millipore Inc. USA), and finally a 0.22 μm sterile filter (Acrodisc, Gelman Sciences, Ann Arbor, USA), and then frozen (−20˚C). The extract contained 0.65mgproteinml^{-1}. Culture wells (35mm diameter) were coated with 0.5ml of skin extract, covered and dried at 60˚C for 24h. The wells were rinsed with sterile culture medium immediately before use.

Prior to dissection, the fish was kept for 15min in well-aerated bacteria-free water in order to flush loosely bound bacteria from the gills. The fish was decapitated and the gill arches were dissected with sterile instruments. Gill filaments were excised from the arches and rinsed three times for 15min and thereafter twice for 5min in 5ml changes of PBS without Ca^{2+} and Mg^{2+}, containing 200i.u.ml^{-1} penicillin, 200 μg ml^{-1} streptomycin, 400 μg ml^{-1} gentamicin and 2.5 μg ml^{-1} Fungizone. The filaments were then transferred to 50ml conical tubes containing 2ml of trypsinizing solution (PBS without Ca^{2+} and Mg^{2+}, 0.05% trypsin, 0.02% EDTA) and incubated on a shaker (Mini-shaker, Alfred Kuhn, Basel) at 200revsmin^{-1} for 15min. The cell suspension was aspirated from the tubes and filtered through 80 μm nylon cloth into a stopping solution of PBS containing 10% FBS. New trypsin solution was added to the remaining filaments and the procedure was repeated three times. The pooled cell suspension from the four digestions was centrifuged at 200 ***g*** for 10min, and the cell pellet was washed twice with 5 ml of PBS containing 2% FBS by resuspension and centrifugation. After the second wash, the cells were suspended in 20ml of basal culture medium (Leibowitz L-15 medium supplemented with 100i.u.ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, 200 μg ml^{-1} gentamycin, 2mmol l^{-1} glutamine and 5% FBS). Leibowitz L-15 medium is designed for use in CO_2-free systems and it contains no nominal HCO_3^-. It is buffered by its complement of salts (phosphates) and free base amino acids. The pH of the complete medium including FBS and antibiotics was 7.5–7.7.

Cell counts were obtained in a Fuchs–Rosenthal haemocytometer. Viability was assessed by exclusion of 0.1% eosin. 1ml of the cell suspension was incubated for 45–60min with the fluorophore DASPMI (final concentration 25 μmol l^{-1}). DASPMI

accumulates in actively respiring mitochondria (Bereiter-Hahn, 1976) and has been used to stain mitochondria-rich cells (chloride cells) (Marshall and Nishioka, 1980). The whole preparatory procedure was carried out at room temperature (20–22˚C).

Cells were seeded in 35mm diameter multiwell dishes at a density of 3×10^5 cellscm^{-2} and incubated at 18˚C in air. After 20–24h, the medium with non-attached cells was aspirated. The wells with attached cells were washed twice with PBS and new basal medium was added. Initially the basal culture medium was changed every third day, but a comparison with cultures where the medium was not changed showed identical growth curves for the first 7 days. Thereafter a decline in cell number was observed in cultures where the medium was not changed. Based on this observation the medium was changed every seventh day.

Cells were grown in the basal culture medium for up to 40 days. The culture wells were harvested at regular times in order to establish a growth curve. At harvest, cells were either trypsinised (PBS, 0.05% trypsin, 0.03% EDTA, 2min) and counted, or solubilized by adding 400 μl of 0.3mol l^{-1} NaOH with 1% SDS to the wells, and the protein content of the lysate was measured using the Lowry method with bovine serum albumin as reference.

Since cell counting with a haemocytometer is tedious, we examined whether cell protein content could be used as an estimate of cell numbers. Cells were seeded at different densities, grown for 7 days, trypsinised and counted. After washing, the cells were solubilized and the protein content was measured. A linear relationship between the number of cells in the wells and the protein content (Fig. 1) was found, justifying the use of protein content in the wells when following cell growth.

For scanning electron microscopy, cells were grown on tissue culture coverslips (Thermanox 5410, Miles Laboratories Inc, Naperville, USA) attached to the bottom of the culture wells. At day 12 after seeding, the coverslips with attached cells were

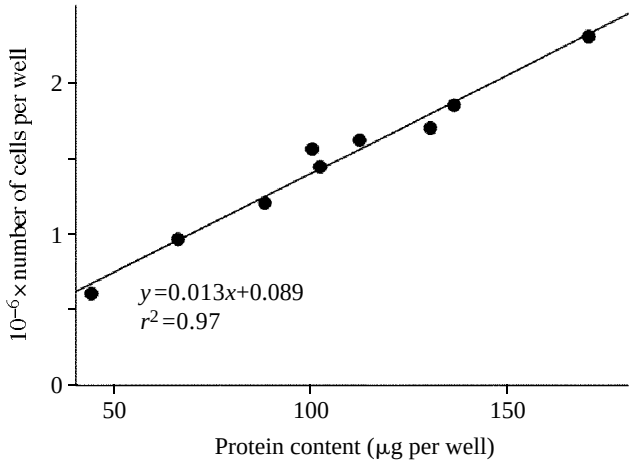


Fig. 1. Relationship between cell number and protein content in culture wells established to validate the use of protein content as a measure of cell number in growth experiments.

removed, rinsed in PBS and fixed in 1.5% glutaraldehyde and 1.5% paraformaldehyde in $0.1 mol\,l^{-1}$ phosphate buffer (pH7.4). They were post-fixed in 1% OsO_4 in $0.1 mol\,l^{-1}$ phosphate buffer, dehydrated, passed through Freon TF (113) and critical-point-dried using liquid CO_2. After drying, the coverslips were mounted on stubs, coated with gold/palladium and examined in a JEOL JSM 35.

[^{3}H]thymidine incorporation into DNA as a measure of DNA synthesis was used to follow cell growth, employing standard methods (Heldin *et al.* 1987). During the establishment of the growth curve, three wells were incubated each day with 37kBq [^{3}H]thymidine per well for 6h at days 5–15 after seeding. To harvest the cells, the medium was removed from the wells and 3ml of ice-cold 5% trichloroacetic acid was added to precipitate proteins and nucleic acids. The acid was aspirated and the wells were extensively rinsed in tap water and dried at room temperature. The precipitate was solubilized with 500 μl of $0.3 mol\,l^{-1}$ NaOH containing 1% SDS for 15min. 200 μl of the lysate was counted for radioactivity in a liquid scintillation counter (Packard) after the addition of 3ml of scintillation cocktail (LKB optiphase MP). The remaining lysate was used for protein measurements.

In another series of experiments, the effects of various growth-promoting factors on [^{3}H]thymidine incorporation were investigated. The following factors were used, with concentrations referring to final concentrations in the culture media: insulin $5\,\mu g\,ml^{-1}$, EGF $10 ng ml^{-1}$, transferrin $5\,\mu g\,ml^{-1}$, laminin $10\,\mu g\,ml^{-1}$ and hydrocortisone hemisuccinate $5\,\mu mol\,l^{-1}$. The cells were grown for 2 days in basal culture medium, the medium was changed to serum-deprived medium (basal medium but with 0.5% FBS) and the cells were grown for 24h in serum-deprived conditions. The various agents were added and after a further 24h [^{3}H]thymidine was added. The incubation with [^{3}H]thymidine lasted for 40h. The cells were then harvested for [^{3}H]thymidine counting and protein measurements.

One critical step in establishing primary monolayer cultures is to get the cells to attach to the culture substratum. We tested two approaches in order to find optimal attachment conditions. In the first, culture wells were treated with trout skin extract ($35\,\mu g\,protein cm^{-2}$) (Blair *et al.* 1990) or the attachment factors collagen type I ($10\,\mu g\,cm^{-2}$), laminin ($2\,\mu g\,cm^{-2}$) or Matrigel ($5\,\mu g\,cm^{-2}$). Matrigel (CR-Human extracellular matrix, lot number 91-0254, Collaborative Res. Inc., Bedford, USA) is a mixture of human basal lamina proteins including laminin, collagen type IV, heparan sulphate proteoglycan and entactin (Kiser *et al.* 1990). The attachment factors were dissolved or suspended in PBS and placed in the culture dishes over-night. Laminin was allowed to dry in air. Dishes were rinsed twice with PBS and once with basal culture medium prior to plating the cells. In the second, the effect of different growth factors together with one attachment factor (laminin) in the culture medium was examined. The growth factors were used at the same concentrations as in the thymidine experiment. The effects of serum levels on attachment were also tested by lowering the serum concentration to 0 or 0.5% or, in one experiment, by replacing foetal bovine serum with 5% rainbow trout serum.

Freshly prepared cells were suspended in basal culture medium (5% FBS) supplemented with the agents to be tested. The cells were seeded in culture wells

and harvested after 24h by trypsination. Cell counts were obtained with a haemocytometer and attachment efficiency was expressed as percentage of attachment in 5% FBS.

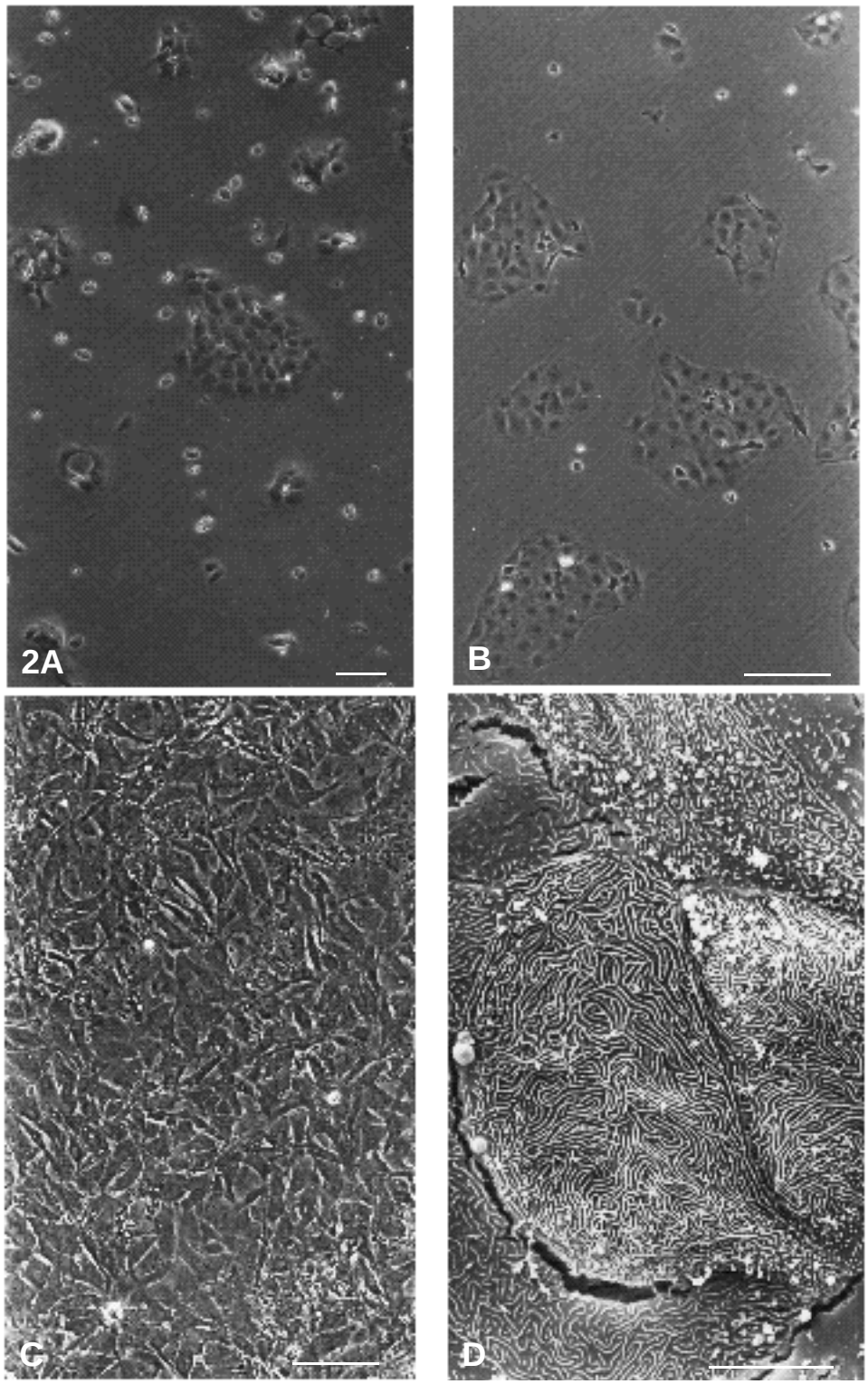
Results

The average yield of cells from approximately 1.5g wet mass of gill filaments was $218\times10^6\pm12\times10^6$ cells (mean ± S.D., N=48), with a viability defined by eosin exclusion of 80±6%. Red blood cells were not included in this cell count. Of the viable cells in the freshly dispersed preparation, 10% (range 7–16%, N=6) were DASPMI-positive, i.e. mitochondria-rich cells. The attachment efficiency in basal culture medium, 24h after seeding, was 35±6%.

The original trypsin digest of the gills contained a mixture of cells, most of which were probably epithelial cells or progenitors of epithelial cells, although red blood cells were also present. When the culture medium was changed after 24h, the unattached cells, including red blood cells, were washed away. Staining for viability showed that more than 50% of the non-attached cells were not viable. Following the medium change, scattered colonies of cells as well as single cells remained attached to the dishes (Fig. 2A). Single cells are mostly elongated whereas cells in colonies have a rounded appearance. At day 3 most of the dish is covered by colonies consisting of 5–50 cells (Fig. 2B). Cells in small colonies are still rounded, whereas cells in larger colonies are flattened. During the following days, the colonies grow larger by division and spreading, to reach confluence approximately 10–12 days after seeding. At confluence, most of the bottom of the dish is covered by a monolayer of cells. The cells have lost their rounded shape. They are flat and display an irregular polygonal shape (Fig. 2C). Scanning electron microscopy of the cell surface shows patterns of microridges (Fig. 2D) very similar to those seen on the surface of intact secondary lamellae from trout gills (Kendall and Dale, 1979; Dunel-Erb and Laurent, 1980), indicating that the cells at confluence have differentiated with their apical surfaces facing the culture medium. Multilayered areas or dome formations caused by solute accumulation between the basolateral membrane and the culture dish (Aleo *et al.* 1989) were rarely seen.

Attached cells, 24h after seeding and at confluence (10–12 days) were incubated with the fluorescent dye DASPMI to stain for chloride cells (Marshall and Nishioka, 1980; McCormick, 1990). No particularly bright fluorescent cells could be observed within the

Fig. 2. Photomicrographs of epithelial cells from fish gills grown in plastic culture dishes or on plastic coverslips. (A) Cells at day 1 after seeding. Scattered colonies of rounded cells as well as single cells are observed. Some red blood cells in suspension are also present. Scale bar, 200 μm. (B) Cells at day 3 after seeding. The colonies have grown larger. Cells in small colonies have a rounded shape, while those in larger colonies are flattened. Scale bar, 200 μm. (C) Cells at day 12 after seeding. Most of the bottom of the culture dish is covered by a monolayer of cells. Both flattened and more elongated cells are observed. Scale bar, 200 μm. (D) Scanning electron micrograph of the surface of cells at day 12 after seeding. The cell in the centre displays a typical pattern of microridges characteristic of respiratory epithelial cells. The cell at the top is probably not fully differentiated and the microridge pattern is still in the process of developing. Scale bar, 10 μm.


2A
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layers of attached cells. It is possible that the impermeable apical membrane might limit entry of DASPMI to the attached cell so cells were trypsinised and the resulting cell suspension was incubated with DASPMI. No DASPMI-positive cells were observed, indicating that chloride cells do not attach under these culture conditions. On the basis of the morphological appearance, we conclude that the cells in the cultures were respiratory epithelial cells.

Cell growth was followed for 38 days by measuring cell protein content in the culture dishes. The protein content increased rapidly for the first 10–12 days after seeding (Fig. 3A). At day 12 a plateau was reached and the protein content stayed constant for 5–7 days. A reduction in protein content was observed from approximately day 17 and by day 38 only some 25% of the original protein content remained. Results from

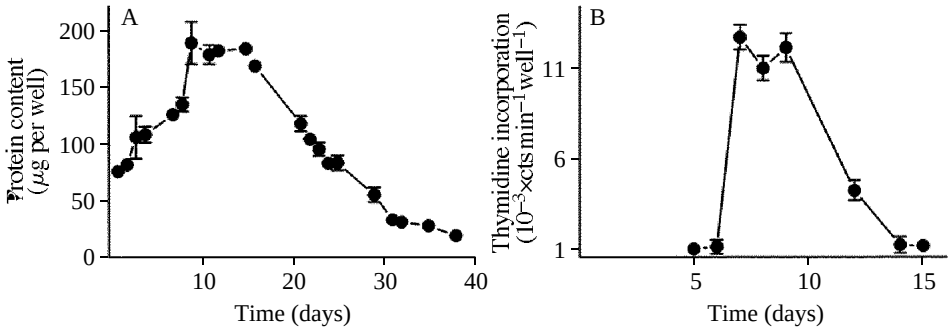

Fig. 3. (A) Growth curve of primary cultures of gill epithelial cells. The curve was established from five cell preparations (days 1–20) and three preparations (days 21–38). Values are mean ± S.D. (B) Incorporation of [^{3}H]thymidine during days 5–15 of the growth curve. The values are means ± S.D. of triplicates for each time point from three different cell preparations.

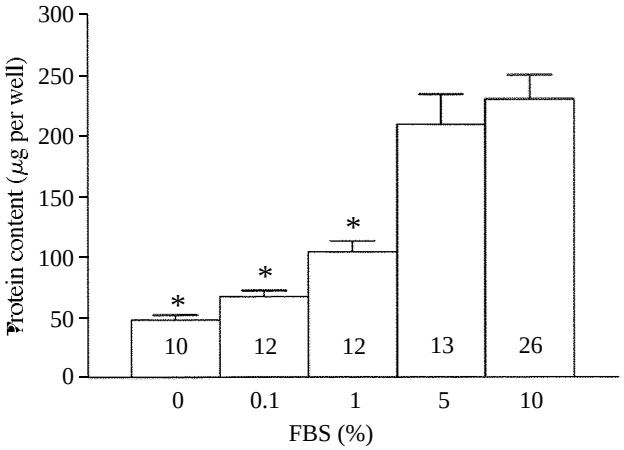

Fig. 4. Serum dependence of growth of primary cultures of gill epithelial cells. FBS, foetal bovine serum. *P<0.05 (Mann–Whitney U-test) compared to 5% FBS. The number of culture wells from 5–7 independent cell preparations is shown. Values are means + S.D.

simultaneous thymidine incorporation experiments (Fig. 3B) showed that most cell proliferation occurred from day 7 to day 10, after proliferation ceased.

Several tests were made to find the optimal conditions for attachment and prolonged culture of the cells. Cell growth was serum-dependent (Fig. 4). Optimal growth rate was obtained in the presence of 5% FBS; a further increase to 10% FBS did not improve growth significantly. Almost no growth was observed in 0 and 0.1% FBS, while a small increase in cell numbers was apparent in 1% FBS.

Treatment of culture dishes with basal laminal proteins is known to improve the attachment of several cell types, including epithelial cells (Taub, 1985; Kleinman *et al.* 1985; Liedke, 1988). Gill cells were seeded into plastic culture dishes treated with attachment factors. When attachment to untreated plastic was set to 100%, attachment after treatment with trout skin extract was 105±12% (mean ± standard deviation) (N=6), with rat collagen type I 102±4% (N=6), with laminin 82±13% (N=6) and with Matrigel 90±20% (N=6), so none of these treatments improved attachment. In addition to plastic, the cells attached to borosilicate glass and we routinely plated cells on glass coverslips.

Growth and attachment factors present in the culture medium have also been shown to improve attachment (Liedke, 1988). The attachment efficiency of the cells was, therefore, evaluated in basal culture medium supplemented with factors known to promote growth and attachment of epithelial cells (Taub, 1985). Attachment efficiency was serum-dependent, being 35±6% with 5% FBS compared to 3±3% in serum-free conditions. Supplements of laminin, insulin, transferrin, EGF or hydrocortisone to medium

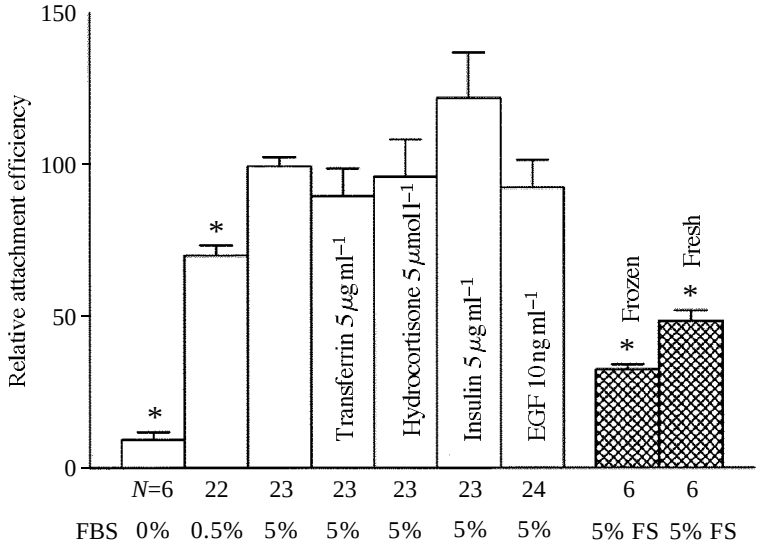

Fig. 5. Attachment efficiency of epithelial cells from fish gills as a method of evaluating essential components in the culture medium. Cells were suspended in media containing 0, 0.5 or 5% foetal bovine serum (FBS) or in medium with 5% FBS and the substances indicated. Attachment efficiencies are normalized to the efficiency in 5% FBS. Values are mean + S.D. from three different cell preparations. The number (N) of wells analyzed is shown. *P<0.05 (Mann–Whitney U-test). EGF, epidermal growth factor; FS, fish serum.

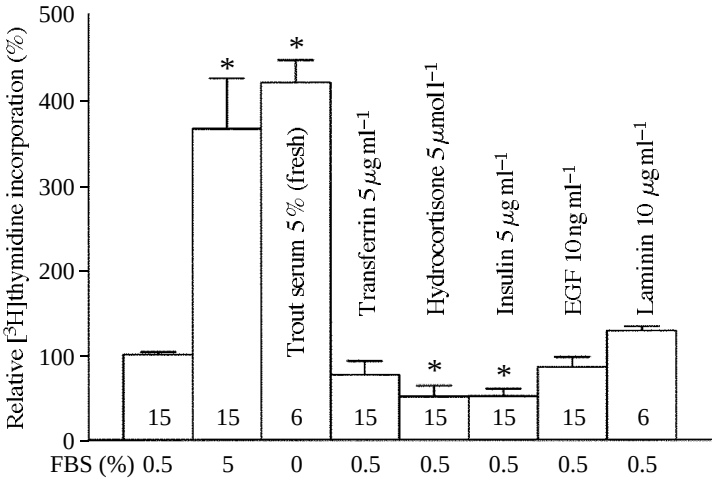

Fig. 6. [^{3}H]thymidine incorporation in epithelial cells from fish gills. At day 3 after seeding, the cells were deprived of serum (0.5% FBS instead of 5%) for 24h, and then serum or other substances was added. [^{3}H]thymidine incorporation was measured after 40h of incubation with [^{3}H]thymidine starting 24h after the addition of sera or the material to be tested. Not shown is a group where 5% frozen rainbow trout serum was included; this proved toxic to the cells, resulting in no [^{3}H]thymidine incorporation. Values are normalized to the incorporation in 0.5% FBS and are presented as means + S.D. The number of wells analysed from three different cell preparations is shown. EGF, epidermal growth factor. *P<0.05 (Mann–Whitney U-test).

containing 5% FBS did not improve attachment (Fig. 5). When FBS was replaced with 5% trout serum, either fresh or frozen, lower attachment efficiencies were obtained.

The effect of the various compounds on cell growth was also evaluated by [^{3}H]thymidine incorporation (Fig. 6). A standard protocol was followed where each factor was tested independently on cells that were quiescent because they lacked growth-stimulating factors (serum starvation) (Heldin *et al.* 1987). Cultures, 2 days old, were starved of serum (0.5% FBS) for 24h, after which sera or the factors to be tested were added and thymidine incorporation was measured (see Materials and methods). Both FBS (5%) and freshly prepared trout serum (5%) greatly stimulated DNA synthesis. A small stimulation (30%) was also observed in the presence of laminin. In contrast, both hydrocortisone and insulin inhibited incorporation by approximatly 50% compared to that in 0.5% FBS. Trout serum stored at −20°C was also tested but it proved to be toxic to the cells; they detached and no thymidine incorporation could be measured. Both the attachment and the [^{3}H]thymidine experiments demonstrated that FBS was necessary for optimal attachment and growth of gill cells and that factors promoting growth in other epithelial cells (Liedke, 1988; Taub *et al.* 1989) had no effect or, in some cases, were even inhibitory.

Discussion

Several reports describe the preparation of isolated cells from fish gills. Such cells in

suspension have been used in biochemical characterization (Kamiya, 1972; Sargent *et al.* 1975; Hootman and Philpott, 1978; Naon and Mayer-Gostan, 1983), in metabolic studies (Perry and Walsh, 1989), in studies of ion transport (Battram *et al.* 1989) and in studies of hormone receptors (Guibbolini *et al.* 1988). However, epithelial cells in suspension lose their polarity and, since the different properties of the apical and the basolateral membranes is a key feature of epithelial cells, this severely limits the conclusions that can be drawn from studies on cells in suspension. Furthermore, the digestion procedures involved in the preparation of cells from intact tissue affect surface proteins, which are likely to be involved in membrane translocation processes. It is, therefore, clearly advantageous to grow epithelial cells in culture where they may re-establish their polarity, repair their surface proteins and restore their normal functions.

We show here that it is possible to isolate viable cells from the gill epithelium of rainbow trout and to establish primary cultures of the cells. The cells grow as a monolayer on the bottom of the culture dishes and reach confluence within 10–12 days. At confluence, the proliferative activity, judged from [^{3}H]thymidine incorporation, is reduced, probably because of contact inhibition. The confluent cells express morphological surface structures similar to those observed in the epithelium from intact secondary lamellae from trout gills and we conclude that the growing cells are respiratory epithelial cells.

The growth curve was established from the protein content of the culture dishes. Although we found a strong correlation between the amount of protein and the number of cells (Fig. 1), the critical assumption is that the amount of protein per cell remains constant over the entire culture period. This assumption has not been tested, but several lines of evidence indicate that the protein content of the dishes reflects the gross dynamics of the cell population. Occasional cell counts, thymidine incorporation experiments and numerous visual observations at different stages of the curve all support the general shape of the growth curve obtained from protein measurements. Moreover, the shape of the curve, with an initial quiescent phase, followed by an exponential growth phase, a plateau phase and a gradual decline, is in accordance with what is generally observed when establishing primary cultures of epithelial cells (Taub, 1985; Liedke, 1988; Aleo *et al.* 1989).

Chloride cells seem not to survive the culture conditions; of the 10% DASPMI-positive cells originally in the tissue digest, none could be observed among the attached cells after 24h or at confluence. It may be that essential factors promoting survival and differentiation of chloride cells are lacking. One such factor could be cortisol, shown by McCormick (1990) to be essential for chloride cell survival in cultured opercular membranes and to stimulate chloride cell development in rainbow trout gills *in vivo* (Laurent and Perry, 1990). We have not tested prolonged incubation with cortisol because it had an inhibitory effect on cell growth in our cultures. Such an effect does not rule out a role of cortisol, which may, for example, act as a differentiation factor rather than a growth factor.

One of our aims was to culture the cells on permeable supports like the systems developed for several other epithelial cells (Dickman and Renfro, 1986; Ford *et al.* 1990). Such a system would permit transepithelial transport studies and permit the apical and

basolateral membranes to be bathed in solutions similar to those encountered by the cells *in vivo*. In culture dishes, the cells are surrounded by culture medium on both apical and basal sides, an unnatural situation for the freshwater gill, where a diluted apical medium would be more appropriate. We have tested most of the commercially available permeable supports (filters), both uncoated or precoated by the manufacturer with collagen I, but have failed to obtain growth of gill epithelial cells on any of these.

Attachment of epithelial cells can usually be improved by coating culture surfaces with extracellular matrix proteins. The most commonly used matrix is collagen (type I) from rat tail tendons or, in one case, from fish skin (Blair *et al.* 1990). Collagen I coating has been shown to promote attachment of fish liver cells (Blair *et al.* 1990), fish kidney tubule cells (Dickman and Renfro, 1986) and shark rectal gland cells (Valentich and Forrest, 1991). However, for gill cells, collagen I does not improve attachment. Apparently gill cells differ from hepatocytes, renal tubule and rectal gland cells in their extracellular matrix requirements. The respiratory cells in the gill lamellae are supported by a basement membrane and one would, therefore, expect coating with basement membrane proteins to facilitate attachment. We tested a commercially available mixture of human basement membrane proteins (Matrigel) as well as laminin – a basement membrane protein with a key role in epithelial attachment and differentiation (Kleinman *et al.* 1985). However, neither Matrigel nor laminin improved attachment to plastic surfaces. We have also coated filters with collagen I, laminin, fish skin extract or a mixture of skin extract and purified fish skin gelatin, but none of the treatments increased the attachment of cells to filters (P. Pärt, unpublished observations). It seems likely that other attachment factors, at present unidentified, may have to be present to enable gill epithelial cells to attach to substrata other than glass and plastic.

Both growth and attachment were serum-dependent, being maximal in the presence of 5 % foetal bovine serum in the culture medium. Kocal *et al.* (1988) found trout serum to be more efficient in promoting attachment of trout hepatocytes than was foetal bovine serum. They used serum that had been frozen at -20°C. We found that rainbow trout serum, frozen or fresh, did not improve attachment of gill cells. Moreover, frozen serum appeared to be directly toxic to the cells since the cells detached in thymidine incorporation experiments. Freshly prepared serum was as efficient as foetal bovine serum in stimulating cell division, as shown by the [^{3}H]thymidine incorporation experiments. Its effects on the promotion of cell survival for longer periods have not yet been tested.

The role of the gill epithelial cells in acid–base and ion regulation in freshwater fish has received considerable attention recently (Goss *et al.* 1992). In particular, the cellular localisation of the different ion-transport processes has been under dispute (Lin and Randall, 1991). The primary cultures described here will provide a useful preparation to investigate the localisation of ion and acid–base exchange mechanisms in the epithelium. Since they are firmly attached to the substratum, their apical membrane can be patch-clamped in order to study the presence of ion channels and transporters. Furthermore, they could be used in studies of intracellular pH and in cell volume regulation, and successful growth on filters would enable transport studies in Ussing chambers. The culture system could also be used to increase our understanding of the factors controlling

growth and differentiation of the gill epithelium, which might also lead to the development of appropriate conditions for primary cultures of chloride cells.

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