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In vitro culture of chick down feather bulbi: A tool to obtain proliferating and differentiating keratinocytes in an organotypic structure

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Summary. Chick down feather bulbi can be cultured in different culture systems. Morphological analysis and ^{3}H-thymidine incorporation measurements prove that the majority of cells are viable epithelial cells.
Key words. Organotypical cultures; feather bulbus; keratinocytes.

The majority of cells in organotypic skin cultures are fibroblasts. Using cultures of embryonic chick skin fragments, we previously studied the effects of drugs on the fibroblasts, and to a lesser extent also on the epithelial cells in their interaction with fibroblasts[1,2].

We looked for an experimental model with a population consisting predominantly of epithelial cells, in order to evaluate immediate effects of chemical and physical agents on keratinocytes. Starting from down feather bulbi of a 13.5-day-old chick embryo, we have developed an experimental model which fulfils the criteria mentioned above[3]. The developing rachis and the associated barb system[4] of the bulbus both consist of cells of epithelial origin. These structures are covered with the follicle sheath, a layer of keratin. Fibroblasts are present only in the central pulp region.

As staging is easy in embryonic chicks[5], reproducible bulbi can be prepared and explanted in vitro.

Description of the technique (fig. 1)

Preculture and preparation of the bulbi

Down feathers of the dorsal region of 13.5-day-old chick embryos (white leghorn) were epilated with forceps and collected in Ringer's physiological solution. Intact feathers normally show a proximal dilatation, representing the bulbar region[4]. Feathers which showed this proximal dilatation were selected under a dissecting microscope and transferred into a glass petri dish containing MEM with Hanks' salts, 10% fetal calf serum and antibiotics. At this stage it was impossible to isolate the bulbar region from the shaft of the feather in a reproducible way.

When the feathers were precultured for 5 days at 37 °C, the bulbus could be easily isolated from the shaft. The proximal part of the feather enlarged to form a rounded-up bulbar region during this culture period. The separation of the bulbus was achieved with a watchmaker's needle under a dissection microscope. Bulbi with a diameter of 0.5 mm $\pm$ 0.1 mm were selected. These bulbi will be referred to as precultured bulbi.

Methods for culturing the precultured bulbi

Depending on the aim of the study, the bulbi were incubated either a) on the bottom of a plastic tissue culture flask, or b) on top of a semisolid medium, or c) in a glass tissue-culture flask on a gyratory shaker (fig. 1).

The culture medium used in the different culture systems was MEM with Hanks' salts (when using plastic flasks or semisolid medium) or with Earle's salts (in glass flasks), supplemented with 10% FCS and antibiotics.

a) *Culture on a plastic surface.* Some precultured bulbi were explanted on the bottom of a plastic tissue culture flask, submerged in fluid medium and incubated at 37 °C. They were fixed and stained in toto after different periods of culture. Using this method of culture, the outgrowing cells of the attached bulbi could be examined with the light microscope.

b) *Culture on a semisolid medium.* Semisolid medium was made by mixing the culture medium with premelted agar at a concentration of 0.5%.

Some precultured bulbi were placed on this semisolid medium and incubated at 37 °C for different periods. After fixation, the fragments were prepared for light and electron microscopy. Using this culture method, the ar-

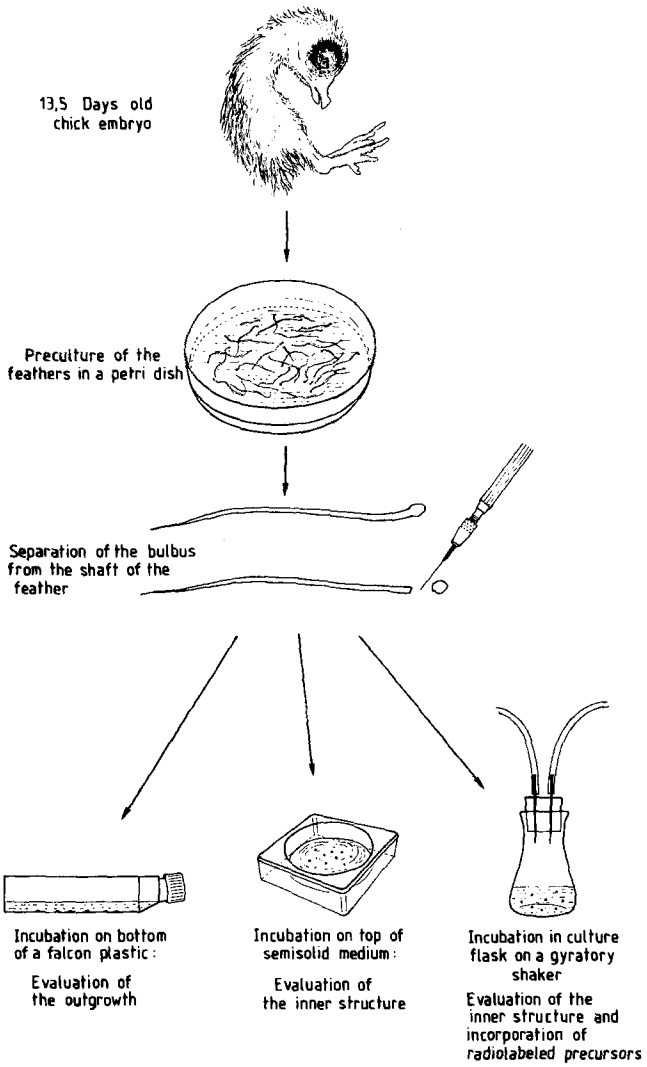

Figure 1. Scheme of the technique. Feathers of a 13.5-day-old chick embryo are epilated, and precultured for 5 days. Then the bulbi are separated from the shaft. Selected bulbi are incubated on a plastic surface, on a semisolid medium, or in culture flasks on a gyratory shaker, depending on the aim of the study.

chitecture of the bulbus remained intact and the different cell layers of the internal structure could be visualized easily.

c) *Culture in culture flasks on a gyratory shaker.* Feather bulbi were also cultured in small culture flasks, shaken on a gyratory shaker at 100 rpm. As the flasks in this culture system were being gassed with air and 5% CO_2, Earle's salts were used in the medium instead of Hanks' salts. Radiolabeled precursors can be added to this system very easily and liquid scintillation counting and autoradiographic analysis can be carried out afterwards.

Evaluation of the technique

To find out whether the precultured bulbi could serve as an experimental model to study effects of physical and chemical agents on keratinocytes in a system with an organotypic architecture, we looked at the viability and the proportion of epithelial cells. For this purpose we carried out a morphological analysis of the bulbus and a ^{3}H-thymidine incorporation experiment.

Morphological analysis

a) *Bulbi fixed and stained in toto: evaluation of the outgrowing cells. Methodology.* After 12, 15 and 20 days of culture of bulbi on a plastic surface, 5 flasks each with at least 10 attached bulbi were fixed as whole mounts with Bouin's solution. The bulbi and their outgrowths were stained in toto with hematoxylin and eosin, or with the pan-anticytokeratin (Boehringer) immunohistochemical procedure, revealed by the PAP technique[6]. The outgrowth of the attached bulbi was examined using a light microscope.

Results. During the culture period, the bulbi attached and the peripherally situated cells gradually grew out. After 20 days of culture, the area covered by the bulbus and the outgrowing cells was 2 mm $\pm$ 0.5 mm in diameter (mean value of 20 bulbi and their outgrowths). Among the outgrowing cells, three morphologically different cell types could be distinguished (fig. 2). We observed many small groups of polygonal cells with strong staining for cytokeratin in the near vicinity of the explanted bulbus. Next to these cells, we found spindle shaped cells with long extensions but which still showed moderate staining for cytokeratin.

Some other spindle-shaped outgrowing cells did not stain for cytokeratin. They appeared at the margins of the outgrowth from the 15th day of incubation onwards.

b) *Bulbi fixed and prepared for LM and EM analysis: evaluation of the inner structure. Methodology.* A series of 5 bulbi was fixed with glutaraldehyde 2% in cacodylate buffer at the end of the preculture period, and after 3, 7 or 10 days of incubation on semisolid medium. Two bulbi of each series were postfixed with osmium tetroxide. The non post-fixed bulbi were embedded in araldite and 2-µm sections were cut. These sections were stained with hematoxylin and eosin, and with keratin staining following Ayoub and Shklar[7]. Pancytokeratin immunohistochemical staining was carried out on paraffin sections of bulbi, cultured for 7 days and fixed in Bouin.

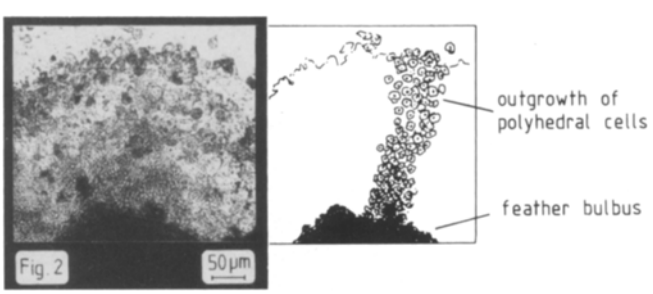

Figure 2. Light micrographic picture of a bulbus cultured for 20 days on a plastic surface and fixed in toto (anti-cytokeratin-pan stain – PAP technique). The most important structures are shown on the accompanying simplified scheme.

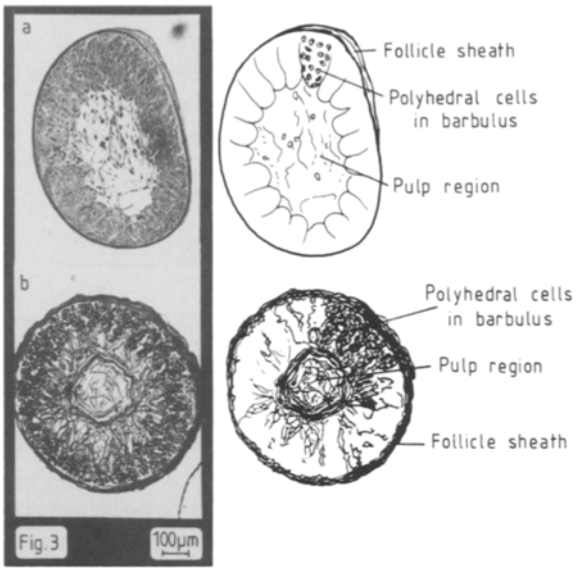

Figure 3. Light micrographic pictures of 2-µm sections of bulbi, *a* immediately after the preculture period and *b* after 7 days of incubation on a semisolid medium (anti-cytokeratin-panstain – PAP technique). The most important structures are shown on the accompanying simplified scheme.

The postfixed bulbi were also embedded in araldite and ultrathin sections (65 nm) were made on an LKB ultramicrotome. They were mounted on copper grids and stained with uranyl acetate and lead citrate. Electron microscopical analysis was carried out with a Siemens Elmiskop A1.

Results

a) *Light microscopy*. Cross sections of bulbi, fixed immediately after the preculture period, show the follicle sheath surrounding the developing feather, as described by Filshie[8] (fig. 3a). The pulp is situated in the center of the bulbus. The epithelial cells of the rachis and the associated barb system show a strong staining for cytokeratin, whereas the central pulp cells do not stain for cytokeratin.

When the fragments are cultured for 7 and 10 days (fig. 3b), the amount of cells/unit of surface decreases in the pulp region. In the meantime, this region gradually gets filled with fibrillar structures and debris. These fibrillar structures stain for cytokeratin. The epithelial zone consists of several layers of cells which stain positively for cytokeratin, separated in several regions by the barb system.

b) *Electron microscopy*. Electron microscopic examination of bulbi cultured for 10 days on a semisolid medium reveals specific cell types in the different layers of cells (fig. 4).

In the center we find the pulp cells. Between the pulp cells a lot of melanin granules can be seen as homogeneous dense structures.

The most central epidermal cells of the barb system (the medulla of the barb) are lying on a clearly visible basement membrane. These cells contain keratin granules and fusing keratin fibrils and they are connected by desmosomes.

Still more superficially we find enlarged vacuolated cells with a clearly visible cell membrane but without cellular organelles, except ribosomes. These cells probably belong to the rachis or shaft of the feather[4,8]. In these cells we find cross-sections and longitudinal sections of keratin fibrils at different stages of lateral aggregation.

The outermost cell layer of the bulbus again consists of flattened cells, without nuclei or cell organelles. A lot of fibrillar keratin can be seen.

^{3}H-thymidine incorporation experiment

1. *Methodology*. In a glass tissue culture flask containing 12 bulbi, ^{3}H-thymidine was added at a concentration of 5 µCi/ml (185 kBq). After 7 and 14 days of incorporation, the bulbi were rinsed twice with saline and cultured for 6 h more in cold medium without ^{3}H-thymidine. After the rinsing procedure, the 12 bulbi of each series were dissolved in 0.5 ml Soluene 100 (Packard).

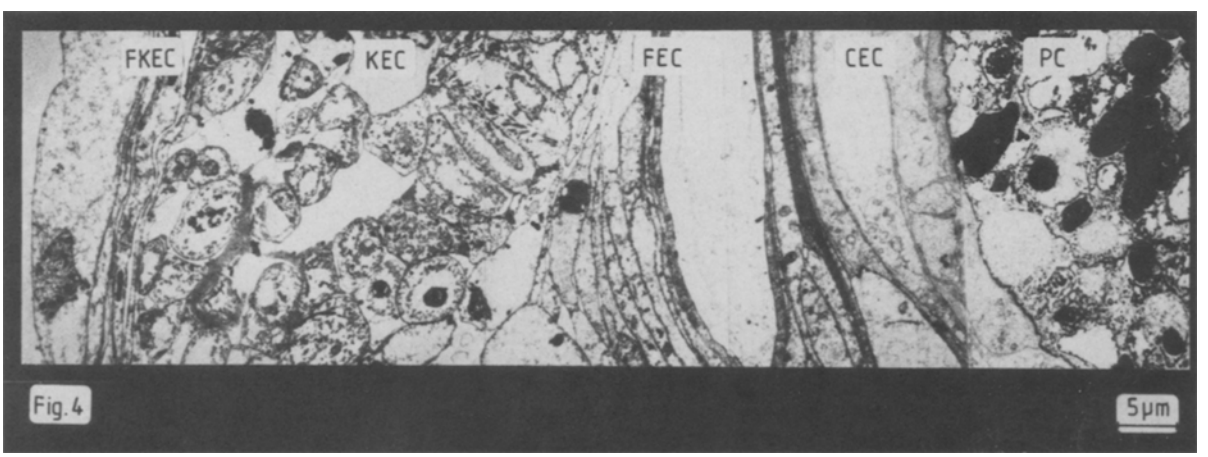

Figure 4. Electron micrographic picture of a 65-nm section of a bulbus cultured for 10 days on a semisolid medium.
PC, pulp cells; CEC, central epithelial cells; FEC, flattened epithelial cells; KEC, keratinized epithelial cells; FKEC, flattened keratinized epithelial cells.

Instafluor (Packard) was added as a liquid scintillation cocktail and counting was performed with a Packard Tricarb Liquid Scintillation Analyser.

The tritium activity of the cold medium used in the rinsing procedure was also measured, in order to determine the amount of weakly attached ^{3}H-thymidine compared to really incorporated molecules. For each duration of incubation (7 days and 14 days), 5 independent series of bulbi were measured.

2. *Results.* The tritium activity of a bulbus cultured in a 5 µCi/ml ^{3}H-thymidine medium for 7 days was $7.5 \cdot 10^2 \pm 1.5 \cdot 10^2$ dpm. The activity of the bulbi cultured for 14 days with ^{3}H-thymidine was $10 \cdot 10^2 \pm 2 \cdot 10^2$ dpm. The activity of the medium used in the rinsing procedure was negligible compared to the incorporated activity in the bulbi themselves.

Autoradiographical analysis revealed that the greater part of the labeling was localized in the outer cell layers of the bulbus. Almost no labeling could be seen in the central pulp cells. Higher magnification showed that the ^{3}H-thymidine was localized in the nuclei of the cells.

Discussion

Our aim was to develop an organotypic experimental model with a majority of keratinocytes and in which fibroblasts only play a minor role. Such a model would be complementary to the fibroblast-rich organotypical skin model which we have used up till now[1,2].

The model we have developed is based on the use of precultured chick down bulbi in different in vitro culture systems. It was necessary to preculture the feathers in order to obtain reproducible bulbi. It is known from the literature that the majority of cells in the bulbus of a developing feather are keratinocytes[4].

We have tried to find out whether bulbi that have been cultured for some time in one of our culture systems still mainly consist of epithelial cells. Next to the typical polyhedral shape of the cell, the presence of desmosomes and a positive staining for cytokeratin are currently used as criteria for defining epithelial cells[9].

In the evaluation of the technique, we have found considerable evidence that the majority of cells in the bulbus are indeed epithelial cells.

Light microscopical analysis of bulbi cultured on the plastic surface of tissue culture flasks revealed that a large number of the outgrowing cells were polyhedral in shape.

Electron microscopic analysis showed the presence of several desmosomes between the cells of the rachis and the barb system of the developing feather.

Using a pan-anticytokeratin antibody, we demonstrated a strong staining for cytokeratin in the majority of outgrowing cells of bulbi cultured on a plastic surface.

In the histological sections of bulbi fixed immediately after the preculture period, only a few layers of peripherally situated cells stained positively for cytokeratin. In the sections of bulbi cultured for 7 or 10 days on a semisolid medium or in the gyratory shaker culture system, several layers of cells at the periphery of the bulbus stained strongly for cytokeratin. This staining increased during the culture period.

Liquid scintillation counting results showed an increased incorporation of ^{3}H-thymidine as a function of time, and this was confirmed by autoradiographic analysis[10,11]. On the other hand, a loss of cells and an increase in the amount of debris-like material and of fibrillar structures was observed in the pulpar region of bulbi cultured for a long time. This loss of cells cannot have been of great importance, as there was still a net increase in ^{3}H-thymidine incorporation in the fragments cultured for a long time, which suggests that there was no progressive starving of the central cells. A process of programmed cell death, together with the formation of debris-like material and of fibrillar structures in the pulpar region, occurs during the normal in vivo differentiation and keratinization of the bulbus[8]. The suggestion that a similar process is taking place here is supported by the fact that these fibrillar structures in the pulp region strongly stain for cytokeratin.

We can conclude that the majority of cells in our precultured down feather bulbus model are keratinocytes, which still have the capability to proliferate and to differentiate, even in vitro.

Our model shows some analogy with the models of Wells[12,13] and of Weterings et al.[14], who described the outgrowth of predominantly epithelial cells, starting from the human hair bulbus.

In their culture system, they explanted hair bulbi on glass or plastic and they could obtain secondary cultures from the outgrowing bulbi. This culture system, and also the classic keratinocyte monolayer cultures as described by Rheinwald and Green[15], can be used to test effects on cultures of isolated epithelial cells. However, these models cannot be used when one wants to learn something about the interaction of the keratinocytes with the basement membrane and the underlying dermis.

In our model we have also incubated the feather bulbi on a semisolid medium and in culture flasks on a gyratory shaker. These culture systems enabled us to observe the keratinocytes in an organotypic context, together with the basement membrane and some underlying fibroblasts. In such a system, the metabolism of the cells of the bulbus can be studied easily by means of radiolabeled precursors.

We think our model can be used to test effects of physical and chemical agents on keratinocytes.

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Effects of serotonin and ketanserin on the functional morphology of chick down feather bulbi in vitro

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Summary. Chick feather bulbi cultured in vitro showed an increased DNA synthesis and a delayed keratinization after treatment with ketanserin, a serotonin$_2$ antagonist with wound-healing properties. In contrast, serotonin stimulates keratinization of the keratinocytes in the bulbus.
Key words. Organotypic cultures; feather bulbus; keratinocytes; serotonin; ketanserin.

Recent clinical observations have demonstrated that ketanserin, a serotonin$_2$ (S_2) antagonist, can promote the healing of chronic leg ulcers, when applied topically[1,2]. This effect could be explained by its anti-serotonin effect on the vascular system, resulting in a better blood supply[8].

On the other hand, we observed a possible direct effect of ketanserin (K) on the fibroblasts[3,4] and/or the epithelial cells in previous studies, using organotypic cultures of both adult human skin and embryonic chick skin.

From morphological analysis and from ^{3}H-thymidine incorporation studies we concluded that K inhibits keratinization and stimulates DNA synthesis significantly, whereas serotonin (S) stimulates keratinization and inhibits DNA synthesis slightly. The majority of cells in these organotypic skin cultures are fibroblasts. To get a better idea of the effects of K and S on the keratinocytes, we set up a complementary experiment using the precultured down feather bulbi model. In this organotypic model, the cell population predominantly consists of proliferating epithelial cells, which are still in contact with a limited number of dermal cells through the basement membrane[5] (this issue).

Materials and methods

The culture methods are described in the preceding article[5]. Ketanserin und serotonin were added to the MEM in doses of 5 and 10 µg/ml, respectively. The choice of these doses was based on the concentrations in the ointment used for the topical application[1]. Fragments cultured in medium without K or S served as controls (C).

1. *Bulbi cultured on a plastic surface: fixation and staining as total mounts.* 5 series of at least 10 bulbi were explanted on the surface of a Falcon plastic flask, and observed daily. The diameters of the bulbus and its outgrowth were measured using a micrometer eyepiece.

At days 10, 15 and 20, bulbi were fixed with Bouin's fluid and stained as total mounts with H&E or with anti-cytokeratin-pan (Boehringer) (PAP technique)[6].

The outgrowth of the bulbi, cultured in the K, S or C medium was compared, using light-microscopical analysis.

2. *Bulbi cultured on top of a semisolid medium: fixation and sectioning for LM and EM.* 5 series of bulbi, cultured on top of a semisolid K, S or C medium for 7 days, were fixed with glutaraldehyde 2% in cacodylate buffer and embedded in ERL. 2-µm sections were stained with Mayer's Hemalum and examined with the light microscope.

65-nm sections were stained with lead citrate and uranyl acetate and analyzed electron microscopically with an Elmiskop 1A (Siemens).

3. *Bulbi cultured in culture flasks on a gyratory shaker: ^{3}H-thymidine incorporation study.* ^{3}H-thymidine was added in a concentration of 5 µC/ml to the medium of bulbi cultured in culture flasks on a gyratory shaker.

After 7 and 14 days of incorporation, the fragments were rinsed twice and incubated for another 6 h in cold medium. Afterwards, the bulbi were dissolved in Soluene 100 (Packard). Liquid scintillation counting of three separate series of 10 bulbi/flask was carried out with a Packard tricarb liquid scintillation spectrometer.