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0014-4754/90/101053-05$1.50 + 0.20/0

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

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Received 25 October 1989; accepted 25 April 1990

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.

Results

1. *Bulbi fixed and stained as total mounts: morphological analysis of the outgrowth.* From the first day of culture onwards, a marked difference was seen between the bulbi cultured in C, K or S medium. After 5 days, more than 75% of the bulbi attached to the bottom of the Falcon plastic flask in the K and C medium; in the S medium, on the other hand, less than 50% of the bulbi attached. The relative increase in diameter of the attached bulbus with its outgrowth was twice as high in K-containing medium than in the C medium (fig. 1). On the other hand the S-treated fragments hardly showed any outgrowth, even after 20 days of incubation. In the K-treated bulbi fixed and stained as whole mounts after 15 days of culture (fig. 2), the majority of outgrowing cells were polyhedral in shape and stained positively for cytokeratin. In the C cultures, there were fewer cytokeratin-positive cells than in the K-treated cultures, and at the edge of the S-treated bulbi, we found hardly any outgrowing cells.

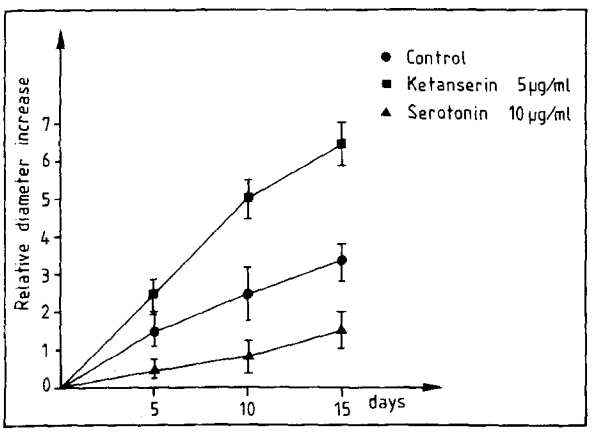

Figure 1. The relative increase in diameter ($\frac{\Delta\varnothing}{\text{original }\varnothing}$) of bulbi incubated in control (C), ketanserin (K) or serotonin (S) medium, measured on days 5, 10 and 15, shown as a function of time (mean value of 5 series). Error bars indicate standard deviations.

2. *Bulbi fixed and sectioned for LM and EM: evaluation of the cell-layers of the inner structure*

a) *Light microscopy* (fig. 3). After 7 days of culture in control medium (fig. 3a), we found a bulbus surrounded by a clearly visible follicle sheath. Underneath this keratinized layer, several layers of cytokeratin (+) staining cells were observed, divided into different regions by the barb system. In the center of the pulp zone, we only observed a few cells, not staining for cytokeratin. The major part of this region is filled with amorphous material and fibrillar structures, which stain positive for cytokeratin.

In the bulbi treated with K (fig. 3b), several layers of cytokeratin (+) staining cells can be seen outside the follicle sheath. Underneath the follicle sheath, the majority of cells also stain positive for cytokeratin. The surface of the pulp region not staining for cytokeratin is still reduced in comparison with the C bulbi.

In the S-treated bulbi (fig. 3c), on the other hand, there is a thick keratinized layer surrounding the bulbus. The number of underlying cells was rather limited and the majority of these cells were vacuolated. In the center of the bulbus, we found a large quantity of fibrillar structures which stained strongly for cytokeratin.

b) *Electron microscopy*. The results of the electron microscopical analysis confirm those of the light microscopy.

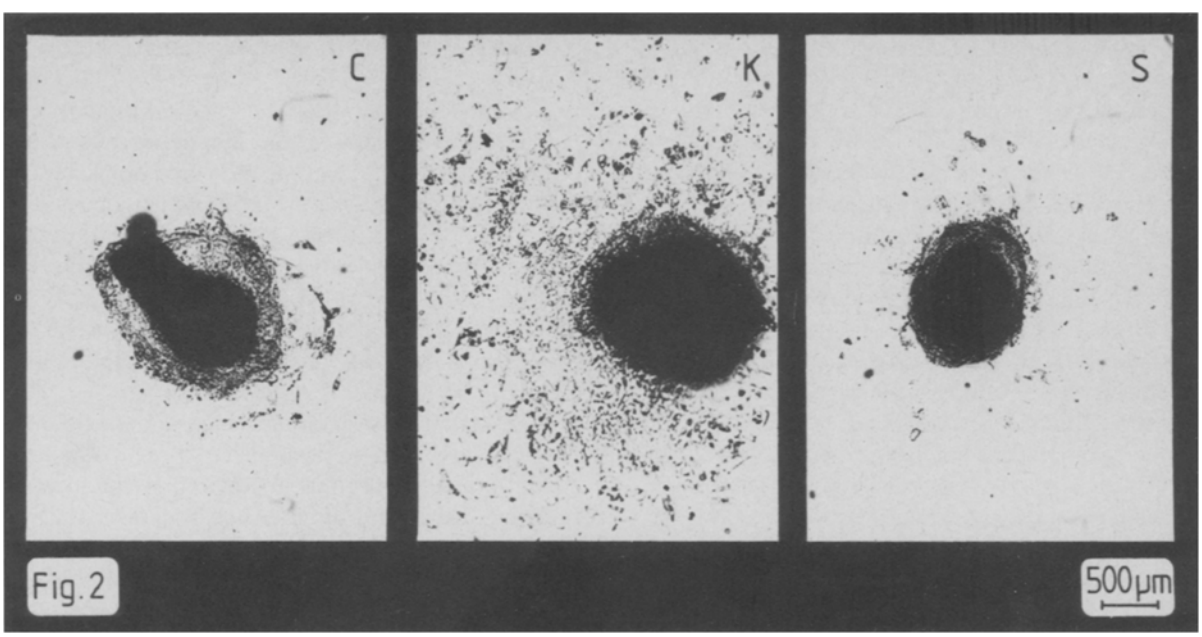

Figure 2. Light micrographic picutres of total mounts of bulbi cultured on a plastic surface for 15 days in control (C), ketanserin (K) or serotonin (S) medium (anti-pancytokeratin stain – PAP technique).

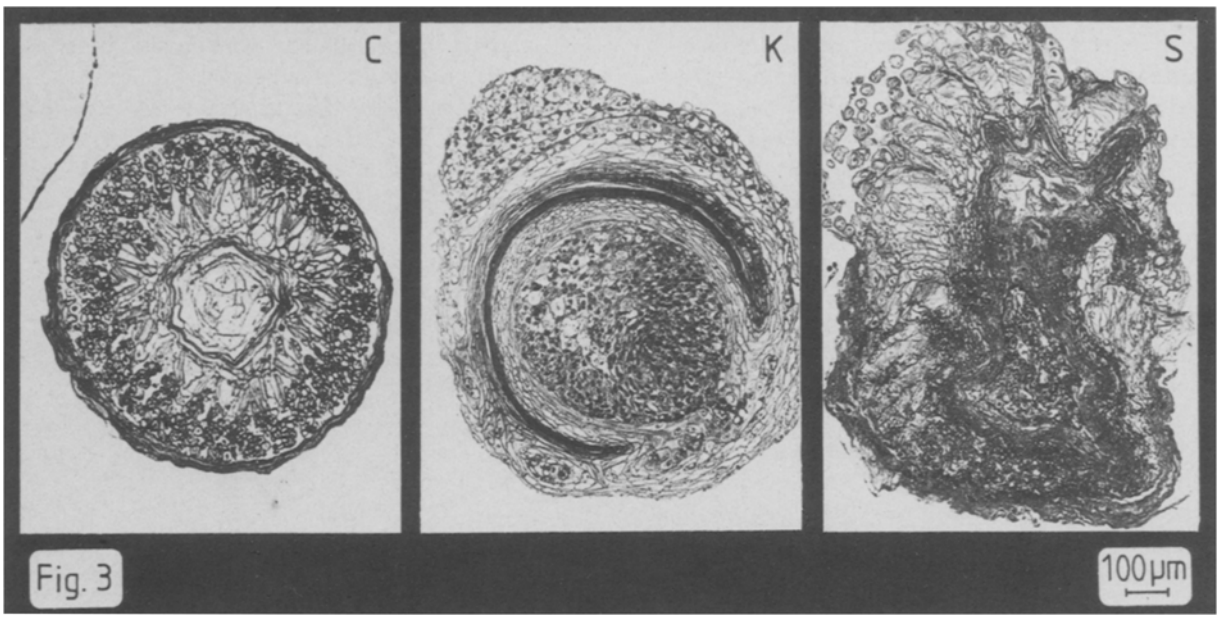

Figure 3. Light micrographic pictures of 2-µm sections of bulbi cultured for 7 days on a semisolid control (C), ketanserin (K) or serotonin (S) medium. (Mayer's Hemalum stain.)

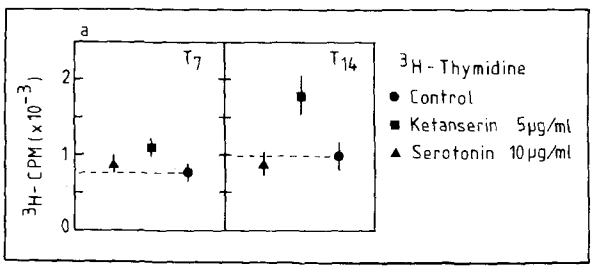

Figure 4. Incorporation of ^{3}H-thymidine of bulbi cultured for 7 days (T7) and 14 days (T14) in culture flasks with control (C), ketanserin (K) and serotonin (S) medium, containing 5 µC/ml ^{3}H-thymidine. (Mean value of 3 separate series.) Error bars indicate standard deviations.

In the K-treated bulbi, the number of pulp cells and the amount of debris-like material is less than in the control bulbi. On the other hand, more superficially, we found many layers of epidermal cells with a clearly visible nucleus and a lot of mitochondria and rough endoplasmic reticulum, and with only a few single keratin filaments. Only a few keratinized cells, with cross-sections and longitudinal sections of bundles of keratin filaments at different stages of lateral aggregation, as described in the most superficially situated regions of the control bulbi, could be observed.

In the S-treated bulbi, the pulp region seemed to be enlarged. We found a lot of keratin, cell debris and melanin granules in the central zone. Among the cells surrounding the pulpar zone, we found hardly any with clearly visible cell organelles or nucleus. The majority of the cells were flattened and filled with keratin filaments.

3. *Incorporation of radiolabeled precursors: liquid scintillation counting.* The tritium activity of bulbi cultured in the presence of ^{3}H-thymidine is shown in figure 4.

The ^{3}H-thymidine incorporation of the bulbi treated with K for 7 or 14 days was significantly higher than that of the control and the S-treated bulbi.

In all cases, the activity of the medium used in the rinsing procedure was negligible (less than 0.05 nCi (1.85 Bq)), indicating that the ^{3}H-thymidine was not only attached to but really incorporated in the cells.

Discussion

Wound healing results from an appropriate interaction of effects in both the dermal and epidermal zones of the skin [7]. Clinical observations suggest that ketanserin promotes the healing of chronic wounds [1, 2].

In previous papers, we have concluded that these wound healing effects can, at least partially, be explained by a direct effect of ketanserin on the skin. We have observed an enhanced cell number, an increased DNA-synthesis and a decreased keratinization in organotypical skin fragments in vitro. The opposite effects were seen in fragments treated with serotonin [3, 4].

As the majority of cells in these organotypical skin cultures are fibroblasts, it was impossible to deduce from these experiments whether there was a stimulatory effect of ketanserin on the keratinocytes, be it directly, or indirectly via the dermal layer.

To find out more about a possible direct effect of K and S on the keratinocyte compartment of the skin, we set up a complementary experiment, using the precultured

down feather bulbi model[5]. Morphological analysis showed marked differences in the outgrowth and in the organization of the inner structure of the bulbi.

In the bulbi treated with K, we found a broad margin of outgrowing cells. Hardly any outgrowth could be seen in the serotonin-treated bulbi.

In the K-treated bulbi, the pulpa region is reduced in comparison with control sections. On the other hand, we found several layers of cells in which the appearance of the cellular ultrastructure was normal, but with only minimal signs of keratinization. In the S-treated bulbi, the cells, very limited in number, contained hardly any cell organelles. Meanwhile, keratin accumulation was enhanced.

The tritium activity of the K-treated bulbi was significantly higher than that of the C- and S-treated bulbi, especially after a longer culture period. The higher incorporation of ^3H thymidine reflected a stimulation of the DNA synthesis, as the medium used in the rinsing procedure hardly showed any tritium activity. We can deduce that K directly stimulated the DNA synthesis in epithelial cells, as the majority of cells in our model were keratinocytes[5].

When we combine these findings with the results of previous experiments[3,4], we can conclude that K probably stimulates DNA synthesis and inhibits keratinization in organotypic skin cultures with a majority of fibroblasts, as well as in the precultured down feather bulbi model, with a majority of keratinocytes.

These findings suggest that the wound-healing properties of K can be attributed, at least partially, to a stimulating effect on both dermal and epidermal cells.

Acknowledgments. This investigation was financially supported by the National Fund of Scientific Research, Belgium, Grant No 3.0022.87. H.B. is a research assistant of the National Fund for Scientific Research. Serotonin and ketanserin were kindly provided by Janssen Pharmaceutica, Beerse, Belgium.

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0014-4754/90/101057-04$1.50 + 0.20/0

In vitro morphological characterization of the bursal reticuloepithelial (REp) cells of chicken

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Received 6 February 1990; accepted 8 May 1990

Summary. The REp cells of the bursa follicle medulla of chicken were isolated in vitro. Culture of the REp cells was maintained over a period of 10 days and the cells were observed at 3 and 10 days by means of transmission electron microscopy (TEM) and immunofluorescence. The use of an anticytokeratin monoclonal antibody confirmed their epithelial nature. TEM observations showed the presence of desmosomes and tonofilaments, which are characteristic of epithelial cells. Furthermore, to some extent the cells regenerated in vitro the network they form in vivo. Though the growth rate becomes slower with time, the features of the REp cells do not significantly change.
Key words. Bursa of Fabricius; cell culture; cytokeratin; REp cells; electron microscopy.

The Bursa of Fabricius, which is present only in birds, is made up mainly of lymphoid tissue. It is commonly considered to be the primary lymphoid organ for B lymphocyte differentiation. However, in spite of the widespread acceptance of its role in B lymphocyte maturation, the hypothetical factors involved in this maturation are not fully understood. A differentiative microenvironment is thought to promote the maturation of bursal stem cells towards mature B lymphocytes; this point of view has long since been accepted[1-6]. The REp cells of the Bursa of Fabricius are involved in forming the pattern of lymphoid follicle medulla. They are star-shaped when observed in vivo and are linked to one another by processes connected by desmosomes[7-9]. They give rise to a three-dimensional network whose meshes contain medullary lymphocytes[10]. The REp cells possess numerous filaments which react to an anticytokeratin monoclonal antibody[10]. They have an oval nucleus, which is sometimes indented, and often a nucleolus may be clearly observed inside it. The cytoplasm contains small vacuoles, a number of mitochondria and free ribosomes and a few vesicles of the endoplasmic reticulum.