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Production of Glycosaminoglycans by Rat Hair Follicle Cells *in vitro*

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Abstract

Cultures of cells from rat dermal tissue, containing a large proportion of cells from the hair follicles, were found to produce glycosaminoglycans. The glycans were associated with the cell aggregates which typically form in such cultures, and also appeared to be present in material which was released from the cells. Examination of the glycosaminoglycan species present in the cultures showed the presence of hyaluronic acid, dermatan sulfate, chondroitin-4-sulfate, heparin and heparan sulfate-C, the first two compounds being secreted into the culture medium. The patterns of synthesis and sulfation of glycosaminoglycans were found to change with continued time in culture.

Introduction

The biology of the mammalian hair follicle has proven to be a difficult field of study, mainly because of the lack of a suitable *in vitro* system. To this end, a cell culture system would be of considerable value in the investigation of the processes controlling such characteristics of the hair follicle as the hair growth cycle and keratin production.

Methods have been described for the isolation and culture of hair follicle cells obtained from rat or mouse skin (Frater 1976, 1979). Some preliminary observations were made of the behaviour and morphological changes occurring in the cultured cells, while recently the synthesis of hair follicle protein by these cells was investigated (Frater and Hewish 1980).

Apart from the initiation of keratin protein synthesis, the growth cycle of hair follicles is also associated with significant changes in both the levels and types of glycosaminoglycan (GAG). These changes occur both within the follicle and also in the extrafollicular structures (Moretti *et al.* 1967; Maekawa 1979).

Histological studies have shown that GAG are present in the dermal papilla, the outer connective tissue sheath of the lower follicle and in the epithelial cells of the bulb surrounding the dermal papilla (Butler 1975). Thus it appears that both dermal and epithelial cells of the hair follicle produce GAG *in vivo*.

In vitro studies on the production of GAG by cultured cells have largely been limited to investigations on fibroblast cell lines (Kraemer 1971*a*, 1971*b*). Studies on GAG production by hair follicles *in vitro* have been confined to histological examination of vitamin A-treated tissue (Bellows and Hardy 1977).

For any cell culture system to be of use in the investigation of processes occurring within the intact follicle, the cells in culture must carry out differentiation processes identical to those which occur in the intact organ. For this reason, and because

preliminary histological studies indicated that cultured cells from follicle tissue gave strong reactions to stains for GAG, the GAG content of the cultures was investigated in order to more fully characterize the differentiation of the follicle cells in culture.

Materials and Methods

Cell Culture

Suspensions of hair follicle cells were obtained from the skin of hooded rats 12–13 days old (all follicles in anagen) by digestion of the lower dermis with trypsin and collagenase as previously described (Frater 1976), and cultured in Falcon petri dishes. Fibroblasts were obtained and cultured from lower-dermal connective tissue by the same procedure. The culture medium was CMRL supplemented with 20% foetal calf serum (both obtained from the Commonwealth Serum Laboratories, Parkville, Vic.).

The culture medium was renewed every second day and the cell layer processed either for histological examination or for GAG analysis at varying times up to 14 days after the initial plating. For extraction of GAG, cells were scraped from the surface of the culture dish, pelleted by low-speed centrifugation, then washed twice with Hanks' balanced salt solution (Commonwealth Serum Laboratories, Parkville, Vic.).

Histology

For *in situ* examination, the cultures were washed three times with phosphate-buffered saline and then stained for 15 min with a solution of 0·1% (w/v) alcian blue 8GS (Gurr) in 3% aqueous acetic acid. The stain was then decanted and the culture washed in running water for 5 min. The stained cells were then examined using a Leitz Ortholux II microscope.

For more detailed examination, the cell layer was washed as before and then fixed in 4% (w/v) paraformaldehyde buffered at pH 7·2 with 0·02 M phosphate. Following dehydration in a graded series of ethanol–water mixtures (30–70–90–100% v/v), the cultures were embedded in acid-free glycol methacrylate (Frater 1980) and, after polymerization, discs of polymer were removed with a metal boring tool. The lower layer of polystrene (from the petri dish) was removed and the upper disc containing the embedded culture sectioned at 3 μm using a glass knife. Sections were stained with alkaline toluidine blue and examined at medium magnification.

Epithelial- and fibroblast-type cells were identified in specimen cultures by the leucine aminopeptidase stain of Jacquemont and Pruniéras (1969), and also by the differential dichrome stain of Everett and Miller (1978). Neither procedure can be applied to living cultures, and can give good estimates of the relative proportions of the two cell types during the early stages of culture, as distinctions between the cell types can disappear with time (Everett and Miller 1978). Quantitation of the results with both methods was impossible because of the morphology of the cultures.

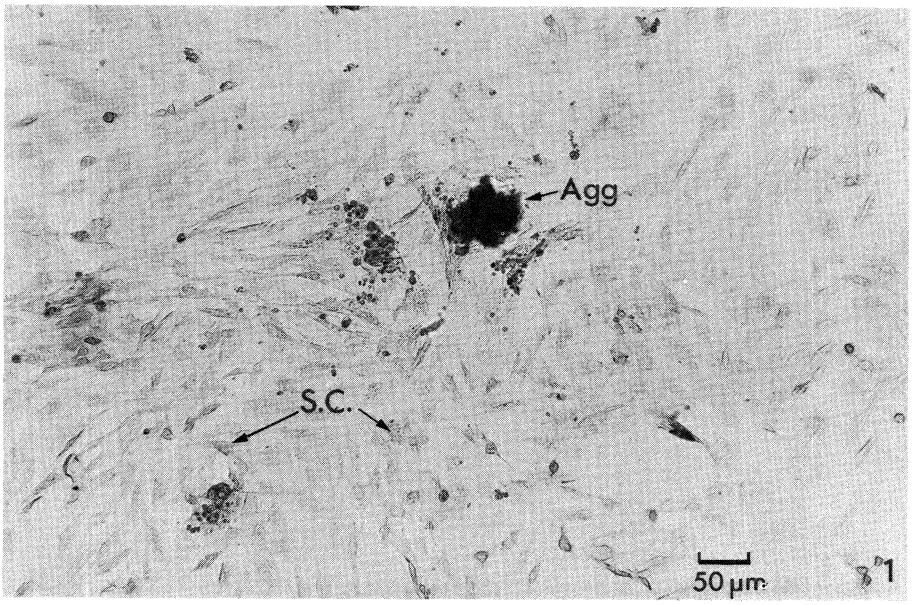
Biochemical Methods

Glycosaminoglycans were extracted from rat skin and cells after culture by pronase digestion, using the method of Svejcar and Robertson (1967). Ethanol-precipitated GAG were washed twice with absolute ethanol, dried and dissolved in distilled water. The GAG contained in ethanol-precipitated material from culture media were extracted by the same method. Cell cultures were labelled for 16 h with [^{3}H]glucose or $Na_2{}^{35}SO_4$ at a level of 20 μCi/ml in the culture medium. The cells were harvested by scraping from the dish surface, washed with unlabelled medium and GAG extracted as above.

Electrophoretic separation of GAG was carried out in the barium acetate buffer system of Cappelletti *et al.* (1979), but with the omission of the final stage in 50% (v/v) ethanol as the extracted GAG from skin and cell cultures were not found to contain any species which electrophoresed at this ethanol concentration. Shandon Celgram cellulose acetate strips were used as the electrophoresis support. This electrophoretic system suppresses the effects of molecular weight and sulfation differences.

An approximation of quantities of GAG within cell culture material was obtained by a modification of the procedure of Whiteman (1973) adapted for microquantities. Cell cultures were harvested, washed with saline and acetone and dried. A known weight of material (300 μg) was suspended in 20 μl of water and digested for 24 h at room temperature with 0·5 μg of pronase. Serial twofold

dilutions of the digest (2 μl each) were applied to a cellulose acetate strip. Dilutions of standard GAG (chondroitin sulfate 12·5–200 μg/ml) were also applied to the same strip and dried. The strip was stained with 0·5% alcian blue 8GS (Gurr) in 50 mM acetate buffer, pH 5·8, for 20 min and destained for 30 min in three changes of the acetate buffer. The densities of the unknown spots were compared with the standard GAG spots. This method allows detection and approximate quantitation of as little as 12 ng of GAG.



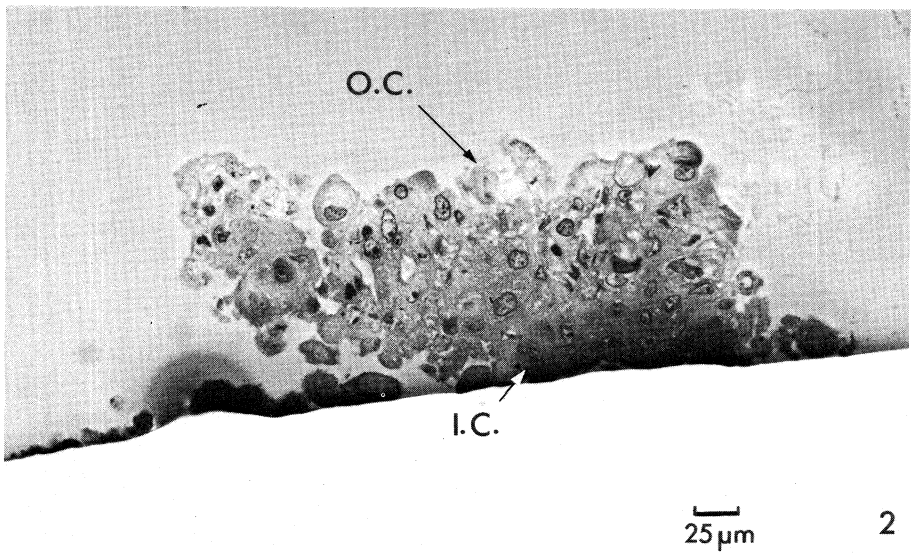
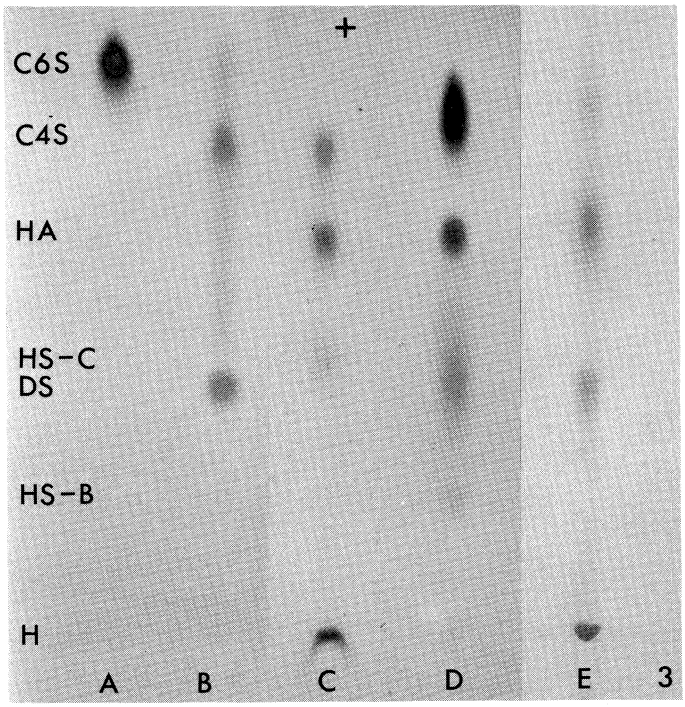


Fig. 1. Eight-day-old follicle hair cell culture stained with alcian blue. *Agg*, large cell aggregate; *S.C.*, single cells.

Fig. 2. Eight-day-old follicle hair culture embedded in glycol methacrylate. A section (3 μm) was stained with toluidine blue, and shows one of the large cell aggregates. *I.C.*, inner layer of cells which were in direct contact with the surface of the petri dish; *O.C.*, outer cell layers.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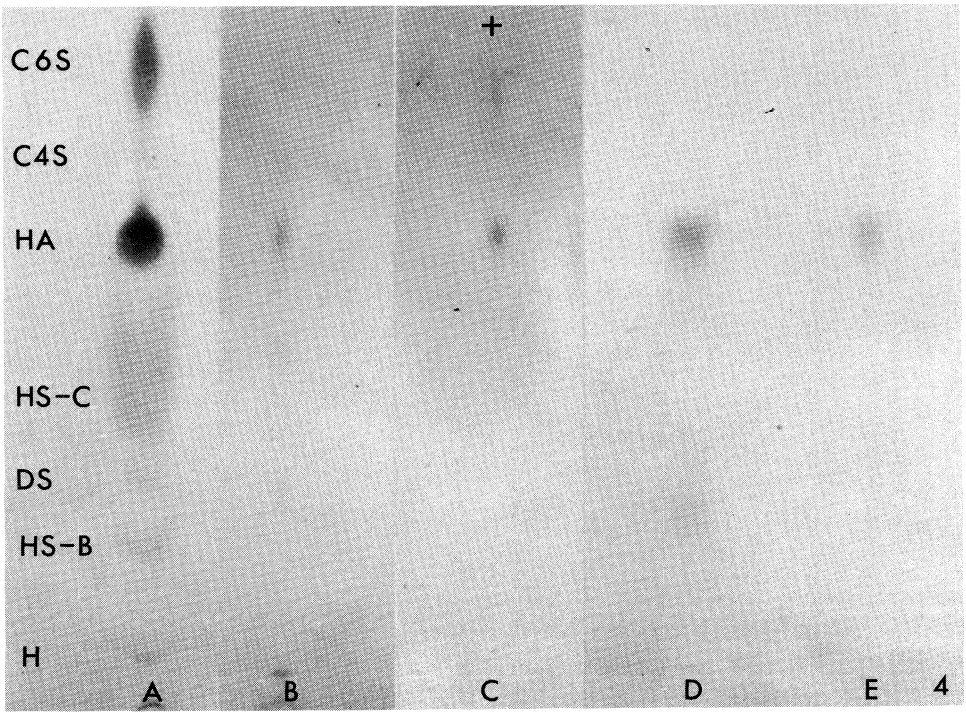
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Chemicals

Standard GAG, chondroitin sulfate A (chondroitin-6-sulfate), chondroitin sulfate B (chondroitin-4-sulfate containing dermatan sulfate) and hyaluronic acid were purchased from the Sigma Chemical Company, St Louis, U.S.A. Heparin (containing heparan sulfates B and C) was obtained from the Commonwealth Serum Laboratories, Parkville, Vic. Radioactive precursors were purchased from the Radiochemical Centre, Amersham, England. Pronase (grade CB) was obtained from Calbiochem. Ltd (Carlingford, N.S.W.).

Results

Cells from the lower dermis of young rats (containing the hair bulbs) formed a monolayer with islands of cell aggregates after 7–8 days in culture. When such cultures were stained directly with alcian blue, the cell aggregates coloured intensely with the dye, while the surrounding layer of single cells only stained very faintly with the dye (Fig. 1).

For more detailed examination, the cultures were embedded in glycol methacrylate and thin sections stained with toluidine blue to detect metachromasia due to acid GAG. Fig. 2 shows a cross-section of one of the cell aggregates. The staining pattern confirms the results obtained with alcian blue. The cell layer in contact with surface of the petri dish showed intense blue staining, whilst the cells above this were stained various shades of red. In addition, it was observed that the outermost layer of cells appeared fragmented, which agreed with the observation that during culture, material was frequently released from such cells (Frater 1979).

Differential staining by the methods of Jacquemont and Pruniéras (1969) and Everett and Miller (1978) indicated that the aggregates were composed largely of epithelial-type cells.

The electrophoretic separation of GAG extracted from the cell cultures was carried out in a buffer system which allows separation on the basis of overall composition. Comparison of the mobilities of the GAG species in the culture extract with standard GAG indicated that hyaluronic acid was the major GAG present (Figs 3 and 4). The pattern of GAG present changed markedly with continuing time in culture. After 5 days in culture the GAG consisted largely of hyaluronic acid and heparan sulfate-C, with minor quantities of chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate and heparin (Fig. 4). After 10 days in culture, the pattern had changed (Fig. 3), although hyaluronic acid remained the major GAG. At this time, chondroitin-4-sulfate and heparin had increased and heparan sulfate-C and dermatan sulfate decreased. The GAG of whole rat skin were found to be, in general, similar to those found in the cultures, with the major exceptions that dermatan sulfate, which was a prominent GAG in the skin, was not seen in large amounts

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Fig. 3. Electrophoretic separation of GAG from follicle cell aggregates. Tracts A, B and D contain mixtures of commercial standard GAG. Tract C contain GAG extracted from a cell preparation after 10 days in culture and E contains GAG extracted from rat skin. H, heparin; HS-B, heparan sulfate-B; DS, dermatan sulfate; HS-C, heparan sulfate-C; HA, hyaluronic acid; C4S, chondroitin-4-sulfate; C6S, chondroitin-6-sulfate.

Fig. 4. Electrophoretic separation of GAG: A, standard GAG mixture; B, GAG extracted from cultures grown for 5 days; C, GAG extracted from pure fibroblasts grown for 5 days in culture; D, extract of follicle culture medium after 7 days of culture; E, extract of fibroblast culture medium after 7 days of culture. Labels as in Fig. 3.

within the cultured material, and heparan sulfate-C was relatively more abundant in the cultures.

Table 1. Relative synthesis of GAG by cultured dermal cells

Cell cultures were labelled for 16 h with the precursors indicated. The GAG were mixed with standard GAG and separated by electrophoresis then stained with alcian blue. The regions containing the various species were cut out and counted by liquid scintillation. The incorporations are expressed as counts per minute per culture and as percentages of the total counts

GAG[A]	Incorporation of label after 4 days				Incorporation of label after 14 days			
	[^{3}H]Glucose		$^{35}SO_4$		[^{3}H]Glucose		$^{35}SO_4$	
	(cpm)	(as % of total)	(cpm)	(as % of total)	(cpm)	(as % of total)	(cpm)	(as % of total)
H	177	4·3	105	2·9	230	12·7	10	0·6
HS-B	134	2·4	69	1·9	19	1·0	10	0·6
DS	348	7·9	163	18·7	55	3·0	53	3·1
HS-C	893	20·7	1550	43·5	155	8·4	213	13·7
HA	1714	43·5	125	3·5	933	51·1	772	2·5
C4S	557	11·4	1020	28·5	423	23·0	1290	77·7
C6S	41	0·9	37	1·0	14	0·8	30	1·8

[A] Abbreviations are as in Fig. 3.

Radioactive labelling of the cells after 4 days in culture with [^{3}H]glucose and $Na_2{}^{35}SO_4$ indicated that the cultures synthesized all of the major species of GAG (Table 1) and that hyaluronic acid and heparan sulfate-C were the major species synthesized. Heparan sulfate-C, chondroitin-4-sulfate and dermatan sulfate were all formed in cell culture with heparan sulfate-C as the major sulfated species. Consistent with the shift in bulk abundances, the pattern was found to be different for the cells after 14 days in culture (Table 1). At this time, hyaluronic acid, chondroitin-4-sulfate and heparin were the major GAG species synthesized; chondroitin-4-sulfate was the major sulfated species. The counts incorporated cannot be taken as giving the precise relative abundances, as they would depend upon the relative rates of synthesis of the various monosaccharide units.

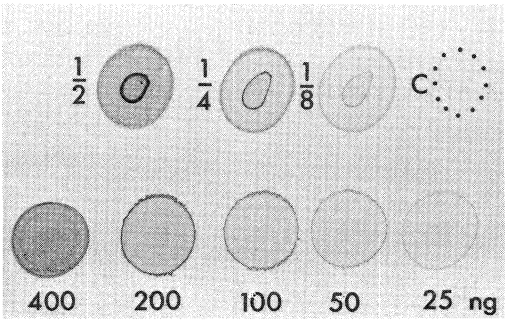

Fig. 5. Approximate quantitation of the GAG content of cell cultures. A pronase digest of cells cultured for 4 days was prepared as described in the Materials and Methods section and dilutions of $\frac{1}{2}$, $\frac{1}{4}$ and $\frac{1}{8}$ applied to a cellulose acetate strip (upper row). Standard GAG (chondroitin sulfate) was also applied (lower row). The strip was stained and destained as described. The control (C) was pronase alone.

Examination of the GAG composition of the culture medium showed the presence of hyaluronic acid as the major GAG, with some dermatan sulfate. The total quantity of GAG secreted into the medium between 4 and 7 days of culture was approximately the same as that contained in the cell pellet.

An approximate quantitation of the GAG content of the cell cultures (see Fig. 5) was obtained by the microprocedure described in Materials and Methods. It was found that after 4 days in culture, the cells contained approximately 1–2% total GAG by weight. The proportion of GAG to the total dry weight of the cells was found to be approximately the same after 14 days in culture.

In order to obtain some information concerning the identity of the cell types responsible for the synthesis of the GAG species, the GAG present in cultured dermal fibroblasts were examined. The presence of hyaluronic acid, chondroitin-6-sulfate and heparan sulfate-C were detected in decreasing order of abundance (Fig. 4). Fibroblast culture medium was found to contain only hyaluronic acid after 7 days of culture (Fig. 4).

Discussion

The cells used in this study were isolated from the dermis of young rats and consist of a mixed population, containing epithelial cells from the hair bulbs and fibroblasts from the dermal connective tissue. It has proven impossible to separate these two cell populations.

Previous studies of such cultures have shown that the cells undergo proliferation and differentiation *in vitro* (Frater 1975, 1976, 1979; Frater and Hewish 1980) although the cells do not appear to synthesize hair cortical keratin (Hewish, unpublished data).

It has been reported that GAG, particularly heparin, heparan sulfate and dermatan sulfate, have specific effects on the growth of hair in the whole animal (Field *et al.* 1961; Meyer *et al.* 1961). In cell culture, therefore, overproduction of particular GAG species or overgrowth by cells producing GAG could alter the differentiation of the remaining cells.

The work described in this paper shows the presence of appreciable quantities of GAG within the cultured cells. The GAG was associated with the aggregates formed by the cultured cells, which appear to be the focus of differentiation (Frater 1979). These aggregates were found to be mainly composed of epithelial-type cells, which suggest that those cells carry out GAG synthesis.

After 4 days in culture, the GAG species found in the cultured cells showed some differences from those which can be extracted from whole rat skin. In particular, the abundance of dermatan sulfate in the cultures was depressed relative to the skin, and the synthesis of heparan sulfate-C elevated. This pattern is complicated by the secretion of dermatan sulfate into the medium by the cultured cells. Hyaluronic acid remained the most abundant GAG within the cultures, as in the skin and fibroblasts. These differences may reflect a selection for specific cell types during the isolation and culture of the cells, or alternatively, may be a changed pattern of GAG synthesis produced in response to the culture conditions. The shift of emphasis in GAG synthesis between 4 and 10–14 days probably indicates the relative increase of a specific cell population during the culture period.

The proportion of cells which gave a positive reaction to specific stains for fibroblast-type cells increased with increasing time in culture, but the observed changes in the GAG cannot be explained simply by an increase in the fibroblast population, because the concentration of chondroitin-6-sulfate, found in pure fibroblasts, did not increase with time. The possibility remains that a specific subpopulation of fibroblasts was

increasing selectively, or that the presence of epithelial cells modified the properties of the fibroblasts.

The presence of relatively high levels of GAG within the cultured cells is a dramatic contrast to the situation which is found within intact hair follicles, where cells containing GAG, as detected by alcian blue staining, are relatively infrequent. This may be an indication that either the differentiation of the cells from the hair follicles is altered *in vitro* from the normal pathways or that overgrowth by minor cell populations occurs. Either of these possibilities may disqualify these cell culture conditions as suitable for the study of hair growth.

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