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# The Effects of Fibroblast Growth Factors 1 and 2 on Fibre Growth of Wool Follicles in Culture

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**The effects of fibroblast growth factor-1 (FGF-1) and FGF-2 on fibre growth and follicle function were examined using a previously described procedure to culture wool follicles. Because the FGFs bind to glycosaminoglycan components of the extra-cellular matrix, we also investigated interactions between FGF-1 and FGF-2 with heparin on wool fibre growth.**

**Individual follicles were microdissected from Merino sheepskin and transferred to culture. Follicles increased in length for 6 days, and in all groups, no significant differences in follicle length were observed. Increase in follicle length was associated with fibre growth, follicles maintained normal anagen morphology and incorporated [$^3$H]thymidine into the bulb and outer root sheath cells. Follicles in all treatments continued to produce fibre keratins, as detected by immunohistochemistry. However, the patterns of fibre and cytoskeletal proteins incorporating [$^{35}$S]methionine in control and treated follicles were significantly different. We found a considerable decrease in the intermediate filament keratins or low sulphur proteins in follicles cultured in the presence of FGF-1 and FGF-2 compared to controls. The majority of proteins detected in these samples were acidic high sulphur proteins. These studies provide evidence for a specific role for the fibroblast growth factors in the regulation of fibre differentiation.**

***Key words: wool follicle culture; fibre growth; FGF-1; FGF-2; electrophoresis.***

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Studies of wool follicles in culture have suggested that the growth factors EGF and TGFα are directly involved in regulating fibre growth and differentiation of the follicle root sheaths (1). Members of the fibroblast growth factor family also appear to be involved: FGF-1, FGF-2 and their receptors have been detected in the anagen follicle (2–4), their distributions suggesting roles in bulb cell proliferation and fibre and root sheath differentiation. In the mature wool follicle, FGF-2 was localized in the outer root sheath and in the basement membrane surrounding the proliferative zone of the bulb matrix by immunohistochemistry (2). By contrast, strong staining for FGF-1 was also observed in the suprabulbar cells of the keratogenous zone of the fibre.

Different members of the FGF family can bind to a single fibroblast growth factor receptor (FGFR). The human FGFR1 (flg) and FGFR2 (bek) receptors bind both FGF-1 and FGF-2 with similar affinities (5). In the hair follicle, FGFR2 is expressed in the root sheaths and bulb, whereas FGFR1 expression is associated with the dermal papilla and dermal sheath (3, 4). In addition, FGFs interact with proteoglycans of the extra-cellular matrices and the basal lamina. FGF-1 and FGF-2 bind to cell surface heparan sulfate proteoglycans (HSPGs), which are thought to be low-affinity receptors mediating binding to high-affinity receptors (6, 7).

Early work on the role of FGF in the skin concentrated on its effects on cultures of individual skin cells, in which the FGFs stimulate the proliferation and differentiation of keratinocytes and fibroblasts (8). FGF-2 has also been shown to stimulate dermal papilla and dermal sheath cells and capsular and dermal fibroblasts of sheep (9). In mice, injections of FGF-2 after birth delayed the first and subsequent hair cycles when compared to control animals injected with bovine serum albumin only (10). However, a study on the direct effects of FGF-1 and FGF-2 on fibre growth and differentiation in mature follicles has not been reported. Here, we have examined the responses of isolated wool follicles in culture to FGF-1 and FGF-2 and heparin on fibre length growth in isolated wool follicles.

## MATERIALS AND METHODS

### *Isolation culture and assessment of wool follicles*

Wool follicles were microdissected from midside skin sampled from Merino sheep and maintained in culture as described previously (1). Follicles were examined daily using an inverted microscope (TMS, Nikon, Japan) and follicle length was defined as described (1). The diameter of the follicle bulb was also measured at its widest point, at right angles to the axis of the dermal papilla.

### *Culture medium and supplements*

Williams' E culture medium, insulin, hydrocortisone, and transferrin were obtained from Sigma (St. Louis, MO, USA), a trace element mixture from Gibco (MD, USA) and antibiotic-antimycotic solution from Flow Laboratories (Costa Mesa, CA, USA). Bovine FGF-1 and FGF-2 were purchased from Sigma (St. Louis, MO, USA), Upstate Biotechnology Incorporated (Lake Placid, NY, USA) and R & D Systems (MN, USA). Heparin was obtained from Sigma. The culture medium was supplemented with FGF-1 and FGF-2 on day 1 of culture and the supplemented medium changed on day 4.

### *Experimental design and analysis*

Follicles were obtained from four medium wool (fibre diameter 23 ± 1 μm) Merino wethers, 3–4 years of age. A total of four skin samples were collected from each sheep. Each day, a total of 24 follicles was dissected from one skin piece collected from each of 3 sheep. Follicles from each skin biopsy were distributed evenly among each of the six treatment groups (control, heparin 10 ng/ml, FGF-1 10 ng/ml, heparin 10 ng/ml and FGF-1 10 ng/ml, FGF-2 10 ng/ml, heparin 10 ng/ml and FGF-2 10 ng/ml) in a 24-well tissue culture plate. Follicles were distributed to culture plates in a balanced randomized block design, and the data analysed using a generalized linear model (11). Follicle survival and measurements were assessed as described previously (1). The

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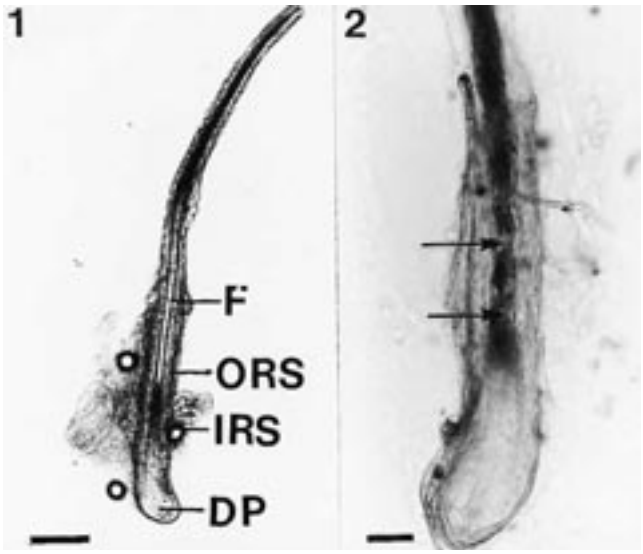


*Fig. 1.* Light micrograph of a follicle cultured with FGF-1 (10 ng/ml) for 6 days of culture. Normal anagen morphology is retained. The dermal papilla (DP) is a lenticular structure and the cells surrounding the growing fibre (F; above line) are arranged in concentric layers (ORS; IRS). Follicles cultured with FGF-2 (10 ng/ml) for 6 days displayed the same morphological features as control and FGF-1 treated follicles. Bar = 200 μm.

*Fig. 2.* A light microscope photomicrograph of a follicle grown in the presence of FGF-1 for 6 days of culture showing an intermittent pattern of keratinization (arrows) in the suprabulbar region of the emerging fibre. Bar = 50 μm.

analysis of fibre growth and bulb diameter is based on follicles which exhibited greater than 200 μm growth after 6 days of culture.

*Histology and immunochemistry*

Freshly isolated and cultured wool follicles were fixed, cut and stained as described previously (1). For immunohistochemical studies, the tissue sections were then treated as described (1) prior to primary antibody binding with monoclonal antibody αK6 to hard keratin (12). Binding was then visualized following incubation with a secondary antibody conjugated to a fluorescent anti-sheep IgG marker (Wellcome, UK).

*[$^3$H]-thymidine labelling of follicles*

The distribution of mitotically active follicle cells was examined by incubating follicles for 8 h with 2 μCi/ml [$^3$H]thymidine (Amersham, Buckinghamshire, UK). Radioactive incorporation was assessed on days 2 and 6 of culture. To trace the movements of cells, follicles were pulse labelled for 12 h with [$^3$H]thymidine (2 μCi/ml) on day 2 of culture, the unincorporated label removed and the follicles harvested on day 6. Labelled follicles were processed for autoradiography following embedding in araldite, as described previously (1).

*2-dimensional electrophoresis of extracts of cultured follicles*

Cultured follicles (30 per sample) were labelled with [$^{35}$S]methionine (50 μCi/ml, Amersham, UK) for 12 h on day 3 of culture. The synthesis of keratins by the follicles in the absence and presence of FGF-1 (10 ng/ml) or FGF-2 (10 ng/ml) was detected following extraction overnight in a 0.05m Tris, 0.05m dithiothreitol, 8m urea (pH 9.3) buffer, and sonication for 10 min. Dissolved proteins were then S-carboxymethylated with iodoacetamide (Flow laboratories), to convert them to ScamK derivatives. Unreacted iodoacetamide was removed by the addition of 2-mercaptoethanol. The alkylated extract was then desalted by ultracentrifugation in a centricon 3 (MW cut-off 3,000; Amicon, Danvers, MA, USA) against three changes of Milli-Q water and lyophilized. The proteins were resuspended in a solution of 2% bio-lyte (pH 3 – 10; Biorad, Hercules, CA), 100 mm Tris, 4% CHAPS (3(3-cholamidopropyl) dimethyl-ammonio-1 propane sulfonate (Flow laboratories) and 2% mercaptoethanol and separated by 2D gel electrophoresis.

Isoelectric focussing in the first dimension was carried out in an immobilized pH gradient (pH 3 – 10), using a Multiphor II electrophoresis unit (Pharmacia, Uppsala, Sweden). Immobilized pH gradient dry strips (Pharmacia) were rehydrated overnight in 8m urea, 0.5% (w/v) CHAPS, 13mm dithiothreitol (DTT) and bio-lyte 3 – 10 (Biorad). Samples were loaded onto the strips in a paraffin-wetted flatbed with the cooling plate set at 20°C. Running conditions for the three phases of isoelectric focussing were 500V, 1mA, 5W for 5 h; 1,900V, 1mA, 5W for 5 h and 3,500V, 1mA, 5W for 15 h. After isoelectric focussing, unused strips were stored in a freezer at – 80°C.

Prior to electrophoresis in the second dimension, the strips were equilibrated first in a stock solution of 5mm Tris-HCl buffer, pH 6.8, containing 6M urea, 30% (w/v) glycerol, 2% (w/v) SDS and 0.5% (v/v) 2-mercaptoethanol for 10 min. This was followed by 15 min in the same stock solution, but with iodoacetamide (0.012m) in place of 2-mercaptoethanol, for 15 min. Electrophoresis in the second dimension was carried out on a separating gel composed of a 10% acrylamide gel underlain by an 18% acrylamide gel. Both portions of the separating gel were polymerized together (adapted from (13)). The two-phase (10 – 18%) separating gel was overlain by a 4% stacking gel, in which the strip was embedded for SDS-PAGE of the proteins, using a PROTEAN II electrophoresis unit (Biorad). Following electrophoresis, the proteins were detected by fluorography. Identification of particular proteins was based upon theoretical pI points and molecular weights of sequenced keratins and by comparison with electrophoretic profiles of wool proteins and cultured follicles (14 – 16).

## RESULTS

*Isolation and culture of wool follicles*

Follicles cultured in the absence of growth factors grew for 6 days, retained their morphological integrity and increased in fibre length linearly during culture. The mean size of the follicle bulb increased from days 1 – 2 and on day 4 of culture, but this was not significant and did not persist for the remaining 2 days of culture.

*Effects of FGF-1 and FGF-2 and heparin on follicle length, bulb diameter and morphology*

As in previous experiments (1, 17), treatment effects were small compared to the sheep and sampling effects. In the presence of heparin, FGF-1 and FGF-2 there was no significant differences in follicle length compared to controls. A slight increase in length observed with FGF-2 was not confirmed in a replicate experiment. Similarly, inclusion of heparin with FGF-1 or FGF-2 had no effect on follicle length when compared to the control group. Increases in follicle length were associated with fibre growth, and no obvious degenerative morphological changes were observed in any treatment group (Fig. 1). A small number of the follicles treated with FGF-1 or FGF-2 displayed an intermittent pattern of keratinization in the suprabulbar zone (Fig. 2).

Significant differences in bulb diameter between the control and treatment groups occurred on days 2 and 3 of culture, but these treatment effects did not persist for the duration of the culture period.

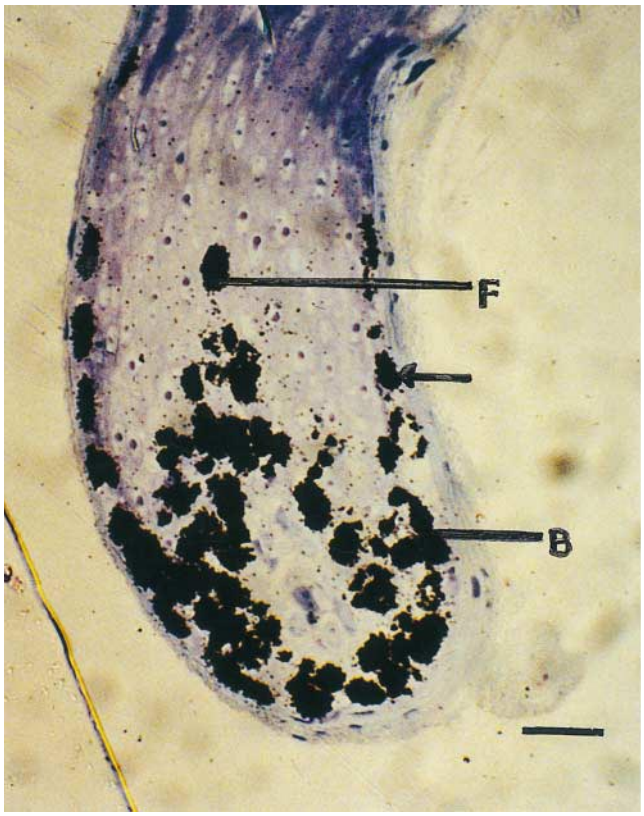


*Fig. 3.* An autoradiograph of a follicle grown in the presence of FGF-1 labelled on day 2 of culture, the unincorporated label removed and harvested on day 6. Labelled cells are localized in the follicle bulb (B) and cortical cells of the fibre (F) and ORS (arrow). Bar = 40 μm.

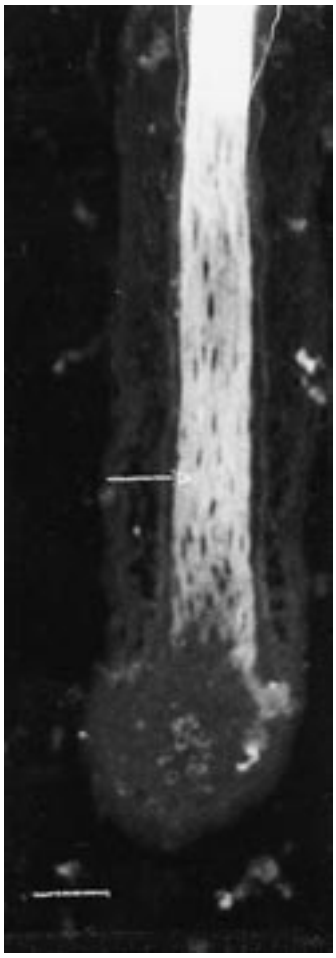

*Fig. 4.* Immunohistochemical demonstration of intermediate filament keratins using monoclonal antibody αK6 in a control follicle on day 6 of culture. Fluorescent labelling in the suprabulbar and cortical cells (arrow) indicates that keratin synthesis continued for 6 days. Bar = 40 μm.

*Distribution of cells incorporating [$^3$H]thymidine*

Autoradiography revealed that cells of the outer root sheath and bulb of cultured follicles incorporated [$^3$H]thymidine on day 2. The distribution of labelled cells was similar when follicles were labelled on day 6 of culture. Follicles were also labelled for 12 h on day 2 of culture and harvested on day 6 to determine the location of proliferating cells. In control incubations, labelling was confined to the bulb matrix, fibre cortical cells and outer root sheath. In heparin, FGF (Fig. 3; FGF-1) and FGF and heparin-treated follicles, the pattern of labelled cells was similar to that of controls (Fig. 3).

*Patterns of keratin synthesis*

*Immunohistochemistry.* The keratin antibody, αK6, which binds to intermediate filament keratins of the fibre, was localized by immunofluorescence in suprabulbar cortical cells and the fibre of control follicles after 6 days in culture (Fig. 4). Similar patterns of staining were observed in follicles cultured with either FGF-1 or FGF-2.

*2D electrophoretic patterns of proteins in wool and follicles in vitro.* 2D electrophoresis was used to detect changes in the composition of proteins synthesized in the fibre and follicle structure between treatments in culture. The patterns of proteins obtained using immobilized pH gradient-isoelectric focussing 2D electrophoresis of wool samples were compared with those found using previously described techniques. The pI and molecular weight of sequenced keratin genes (14, 15) were used to identify individual keratin components and classes in the fibre. However, these do not represent the complete array of proteins found in follicle extracts, since other keratins exist in the root sheaths of the follicle.

We used the procedure to detect the synthesis of keratins from cultured follicles labelled with [$^{35}$S]methionine from day 3 to day 4 of culture. When compared with wool extracts, a larger number of proteins was detected by immobilized pH gradient-isoelectric focussing (pH 3 – 10; 10 – 18% SDS-PAGE) in extracts of cultured follicles (Fig. 5). The major protein classes (low sulfur; LS, high sulfur; HS, high glycine/tyrosine;HGT) are labelled on the fluorogram. The majority of proteins detected were highly acidic (pI 3 – 4.5); only a few proteins fell into the basic end of the gradient (pI 6). The large numbers detected indicated even greater heterogeneity in all classes of proteins synthesized compared to wool extracts alone. Intermediate filament keratins of the fibre were observed in the size range between 40 – 65 kDa, and fell into two groups: an acidic group (pI 3 – 5) and a basic group (pI 6). Some of the proteins in this group had lower molecular weights (30 – 40 kDa) than

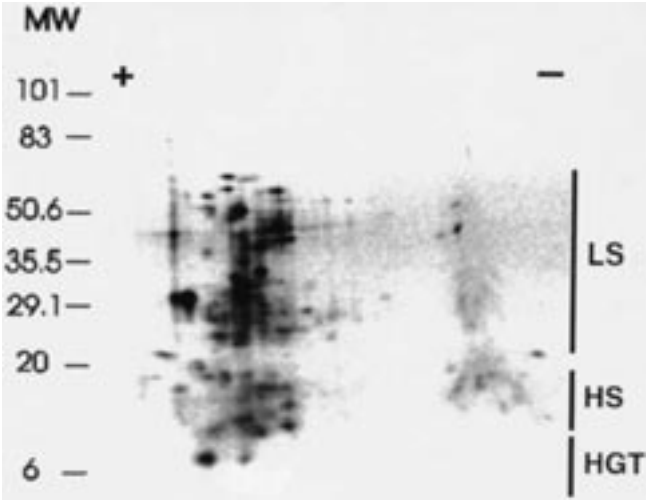


*Fig. 5.* Fluorogram of S-carboxymethylated keratins from cultured follicles ($n=30$) grown in culture for 4 days using [$^{35}$S]methionine to detect keratin proteins. Left: Prestained molecular weight markers. Right: 2D electrophoretic pattern of proteins from control wool follicles in culture. Immobilized pH gradient-isoelectric focussing in the first dimension was carried out on pH 3–10 Immobilize Drystrips. SDS-PAGE in the second dimension was carried out on a two-phase separating gel (10% and 18% acrylamide). Approximate regions corresponding to intermediate filament keratins (low sulfur; LS), high-sulfur (HS), and high-tyrosine/glycine (HGT) proteins are indicated. The same number of cpm was loaded onto each gel (Figs. 5, 6 and 7) with an exposure time of 96 h.

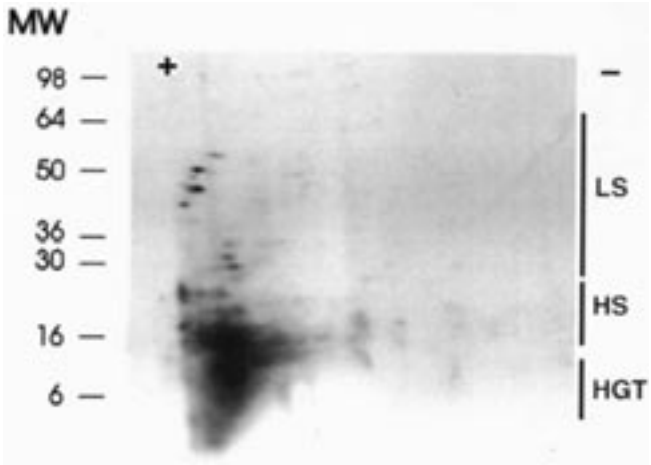


*Fig. 6.* Fluorogram of the 2D electrophoretic pattern of proteins labelled with [$^{35}$S]methionine from wool follicles ($n=30$) grown in the presence of FGF-1 (10 ng/ml) in culture for 4 days. Left: Prestained molecular weight markers. Right: 2D electrophoretic pattern of proteins.

identified previously for the intermediate filaments. Perhaps surprisingly, a larger number of proteins corresponding with the HS wool proteins of the fibre were detected in cultured follicles than in wool samples. The proteins in this group (10–26 kDa) were resolved as a clear pattern of spots in 2D gels. Few of the components of the fibre have been sequenced, and those described have molecular weights of 10–26 kDa and pI 6.0–8.0 (14, 15). The majority of the proteins detected in this class were acidic, although a discrete group was visualized at the basic end of the gradient. A few proteins with size (6–9 kDa) corresponding to the HGT group were also detected. These have similar molecular weights, and their heterogeneity was related almost entirely to charge (pI 3.5–4.5; 7.5 and >8).

*2D pattern of proteins in follicles grown in the presence of FGF-1 and FGF-2.* The patterns of proteins found in control follicles differed significantly from those cultured in the presence and absence of FGF-1 and FGF-2. Intermediate filament or LS keratins, which are among the first to be synthesized in the suprabulbar region of the fibre in control cultures, were reduced in the presence of FGF-1 (Fig. 6). All proteins in this group fell within the acidic pI range of 3.5–4.5. Only three proteins with a molecular weight of 30–40 kDa and pI of 4.0–4.5 were observed in the smaller size range, whereas numerous spots were observed in this region in the control. In the presence of FGF-2, the LS proteins were very faintly labelled or completely absent (Fig. 7).

Most proteins visualized in the presence of FGF were a heterogeneous group of HS and HGT keratins. Some of the large spots may have contained several components with a similar charge. Unlike control samples, which had a distinct acidic and basic pattern of proteins in the HS class, FGF-1-treated follicles had a diffuse pattern of spots in the basic pI range (pI >5–9) within the molecular weight range of 15–30 kDa (Fig. 6). In FGF-2-treated follicles, no proteins >pI 5 were detected in this size range (Fig. 7). Individual HGT proteins were difficult to distinguish compared to controls.

## DISCUSSION

Members of the FGF family have been shown to influence the hair cycle and hair growth. Injections of FGF-2 in mice *in vivo* after birth delayed the first and subsequent phases of active hair growth when compared to control animals (10). The effects of FGF-1 on follicle function and the hair cycle *in vivo*, however, have not been reported.

We did not observe any significant effects of FGF-1 and FGF-2 on the length of wool follicles grown in serum-free culture conditions compared to the controls. Similarly, while heparin may protect FGF-1 and FGF-2 from degradation *in vitro* (6), it did not alter the responsiveness of follicles to these growth factors. Follicles in all treatments retained their anagen morphology for 6 days in culture, and continued to incorporate [$^{3}$H]thymidine into bulb and outer root sheath cells. Jindo et al. (18) reported that FGF-1 and FGF-2 did not affect fibre growth of mouse follicles in culture. In addition, they did not detect any difference in the percentage of [$^{3}$H]thymidine incorporated into DNA of bulb cells in FGF-treated and control follicles, indicating that cell proliferation in the bulb occurred at similar rates to those observed in controls.

The identification of specific receptors for FGF-1 and FGF-2, FGFR-1 and FGFR-2 in the follicle (3, 4) suggest, however, that these peptides are of functional significance. Perhaps the most surprising result was the failure of heparin to alter the biological response to FGF in follicles grown in culture. In CHO cells lacking glycosaminoglycans (19), the interaction between heparan sulphate proteoglycans (HSPG) and FGF-2 results in a positive mitogenic response. It is possible the lack of any measurable mitogenic response to heparin and FGF by the follicles in culture was related to the amount of glycosaminoglycans already present in the cells.

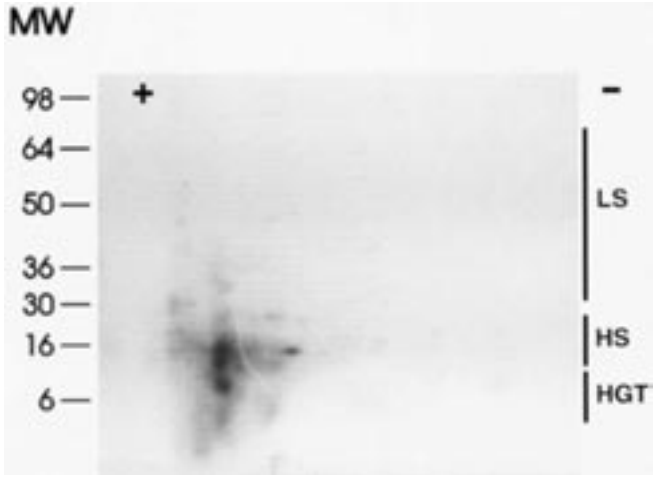


*Fig. 7.* Fluorogram of the 2D electrophoretic pattern of proteins incorporating [$^{35}$S]methionine from wool follicles ($n = 30$) grown in the presence of FGF-2 (10 ng/ml) in culture for 4 days. Left: Prestained molecular weight markers. Right: 2D electrophoretic pattern of proteins.

While cellular kinetics play an important role in the growth and co-ordinated movement of the fibre through the sheaths, fibre composition is determined by keratin synthesis. Intermediate filament keratins are synthesized at an early stage of fibre production (20). These provide the scaffold around which the matrix (HS) proteins form in the suprabulbar region of the fibre. These are followed by the appearance of high glycine/tyrosine proteins of the fibre. Accompanying these processes, the follicle sheaths undergo a process of differentiation which increases progressively towards the skin surface. Changes in the composition of proteins produced in the fibre (21) and incomplete hardening during oxidation and cross-linking of the keratins result in a weakened or abnormal hair (22).

The most striking effect of FGF-1 and FGF-2 in culture we observed was the suppression of intermediate filament protein synthesis. This was revealed in the pattern of proteins following incorporation of [$^{35}$S]methionine. Immobilized pH gradient-isoelectric focussing 2D electrophoresis resolved a larger number of proteins with varying charges and molecular weights than did previously described techniques (14). In control treatments, an equimolar distribution of acidic and basic keratin pairs incorporating [$^{35}$S]methionine was not found, which is a characteristic pattern of cytokeratins extracted from human epithelial cells (23). FGF-1 and FGF-2 considerably decreased the quantity of intermediate filament proteins synthesized compared to controls, while the production of HS matrix proteins was maintained or stimulated *in vitro*. The LS and HS proteins detected in the presence of FGF-1 and FGF-2 were almost entirely acidic (pI 3–4). Basic HS and HGT keratins were only faint spots in extracts of treated follicles. Presumably, these keratins are synthesized at a later stage of keratinization, as we did not detect them by labelling with [$^{35}$S]methionine for 12 h.

Although fibre growth continued for 6 days in culture, FGF-1 and FGF-2 interrupted the normal pattern of keratins synthesized compared to the control group. Our results suggest that the distribution of FGF-1 and FGF-2 in specific regions of the follicle (2) does not correlate with proliferation in the follicle bulb. Instead, FGF-1 and FGF-2 are involved in differentiation of cells into fibre and root sheaths. The changes induced by FGF-1 and FGF-2 resembled changes in the pattern of wool protein synthesis induced by infusion with cysteine (24) or treatment with EGF (25). Relatively little is known about how these changes in fibre composition are related to the physical properties of the fibre, but it is likely that they affect tensile strength.

These studies provide further evidence for specific roles for different growth factors in the control of follicle metabolism. The procedure we have developed should facilitate future studies of the effects of other growth factors, hormones and nutrients on fibre growth in anagen follicles in culture.

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