

Effects of Intradermally Injected and Topically Applied Mouse Epidermal Growth Factor on Wool Growth, Skin and Wool Follicles of Merino Sheep

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Abstract

Twice daily intradermal (ID) injections of mouse epidermal growth factor (mEGF) in sterile saline for 1-4 days into delineated areas of skin of Merino sheep produced dose-dependent changes in wool follicles and fibres, ranging from slight reduction in follicle bulb size and transient disturbance of cuticle formation on some fibres to the induction of catagen of follicles and shedding of fibres with distorted, tapered ends. Regeneration of follicles commenced by day 7. By contrast, ID injections of saline did not affect follicle activity. The epidermis became thicker and more parakeratotic after multiple injections of mEGF than after injection of saline, but was almost normal again by day 14. Persistent small increases in sebaceous gland size, additional to those induced by ID injections of saline, and delayed small increases in sweat gland size also occurred after multiple injections of mEGF.

Daily topical applications of mEGF in 50% (v/v) aqueous propylene glycol 5 days each week for 4 weeks did not affect wool growth or the follicles and other skin components. The only effect observed, due to application of the aqueous propylene glycol, was an increase in the number of layers of cornified cells in the stratum corneum of the epidermis, with the cells arranged in clearly discernible stacks.

The effects produced by ID injections of mEGF indicate that mEGF acts directly on the pilosebaceous and epidermal components of skin.

Introduction

Administration of mouse epidermal growth factor (mEGF) by daily subcutaneous injection to newly born mice or by subcutaneous or intravenous infusion to adult and fetal sheep produces the paradoxical effects of stimulating the epidermis and sebaceous glands while inhibiting hair and wool growth (Levi-Montalcini and Cohen 1960; Cohen and Elliott 1963; Moore *et al.* 1981; Thorburn *et al.* 1982; Hollis *et al.* 1983; McDonald *et al.* 1983). Infusion of mEGF into isolated cutaneous sites on sheep does not induce changes similar to those produced by systemic administration (McDonald *et al.* 1983). Intradermally injected mEGF has been reported to inhibit wool growth at the site of injection, but no detail has been given (Moore *et al.* 1981), while it is unknown whether topical application of mEGF has any effect on sheep skin.

This paper, some details of which have been presented previously in abstract form (Hardy and Chapman 1986), describes histological changes produced in Merino sheep skin and wool follicles by intradermally injected mEGF, and reports the lack of effects of topically applied mEGF.

Materials and Methods

Three adult castrated male Merino sheep were used in each of the two studies. They were housed indoors in individual pens and fed a maintenance ration of pelleted lucerne chaff and oats (3:2, w/w) with access to water *ad libitum*.

Intradermal (ID) Injections of mEGF

Mouse EGF was prepared from the submaxillary glands of adult male mice by the method of Savage and Cohen (1972) and tested for biological activity as in Cohen (1962). A solution of mEGF (10 µg mEGF ml⁻¹ sterile 0.9% (w/v) saline) was injected intradermally in 0.5 ml quantities twice daily (at 0900 and

1600 h) for 1, 2, 3 or 4 days into 13 sites *c.* 5 cm apart in a rectangular area of *c.* 30 × 20 cm on the right side of the trunk which had been closely clipped on three of the sheep. Sterile saline was similarly injected into four additional sites. On the first occasion that each site was injected, the area of skin raised by the injection (*c.* 2 cm in diameter) was outlined with a waterproof marking pencil. For subsequent injections, the tip of the hypodermic needle was positioned at the centres of the outlined areas.

Skin samples, 1 cm in diameter, were biopsied under local anaesthetic from an untreated control site anterior to the treated sites, from the mEGF-treated sites at days 1, 4, 7 and 14 after the first injection, and from the saline-treated sites at days 1–4. The skin samples were fixed in Serra's fluid for 4 h then transferred to 70% (v/v) ethanol for processing and embedding in paraffin. Serial sections, 8 µm thick, were cut longitudinal to the follicles through the centre of each sample and mounted on microscope slides. They were stained with haematoxylin, eosin and picric acid and examined by light microscopy for effects of saline or mEGF on the wool follicles and other skin components. The effects observed were mainly in the centres of the skin sections and did not extend uniformly across the full widths of the sections, even though the areas raised by the ID injections were larger than the 1 cm diameter of the skin samples. The percentages of affected follicles were therefore determined from counts across diametrical sections through the skin samples, and were not based on sample area.

At the time of the first injections, dyebands (Chapman and Wheeler 1963) were applied at skin level at four additional sites, which were injected with mEGF for 1, 2, 3 or 4 days, on each sheep. The dyebanded wool was carefully clipped from these sites with fine animal clippers 5 weeks later and degreased in Shell X4 solvent. Ten individual fibres were withdrawn from the centre of each sample under a stereomicroscope and were mounted on scanning electron microscope stubs with double-sided adhesive tape. They were sputter coated with gold and examined in an I.S.I. Super IIIA scanning electron microscope.

Topically Applied mEGF

Seven areas, 7.5 cm square, were closely clipped in two horizontal rows of four and three sites, respectively, on the right sides of the three other sheep, and were outlined with tattooed lines. The areas were treated in an anterior-posterior order as follows: (1) nil; (2 and 5) 2.5 ml of 50% (v/v) propylene glycol (PG) in water (vehicle); (3 and 6) 14 µg mEGF in 2.5 ml vehicle (i.e. 0.25 µg mEGF cm⁻²); (4 and 7) 140 µg mEGF in 2.5 ml vehicle (i.e. 2.5 µg mEGF cm⁻²). The treatments were applied once daily, 5 days per week for 4 weeks.

Wool growth on the dorsal row of four patches was harvested with fine clippers at fortnightly intervals commencing 4 weeks before treatments began and continuing until 2 weeks after treatments ceased. The patches were finally clipped 4 weeks later (i.e. 6 weeks after treatments ceased). The wool samples were cleaned in warm aqueous neutral detergent solution, rinsed with water and dried to constant conditioned weight in a controlled atmosphere at 20°C and 65% relative humidity.

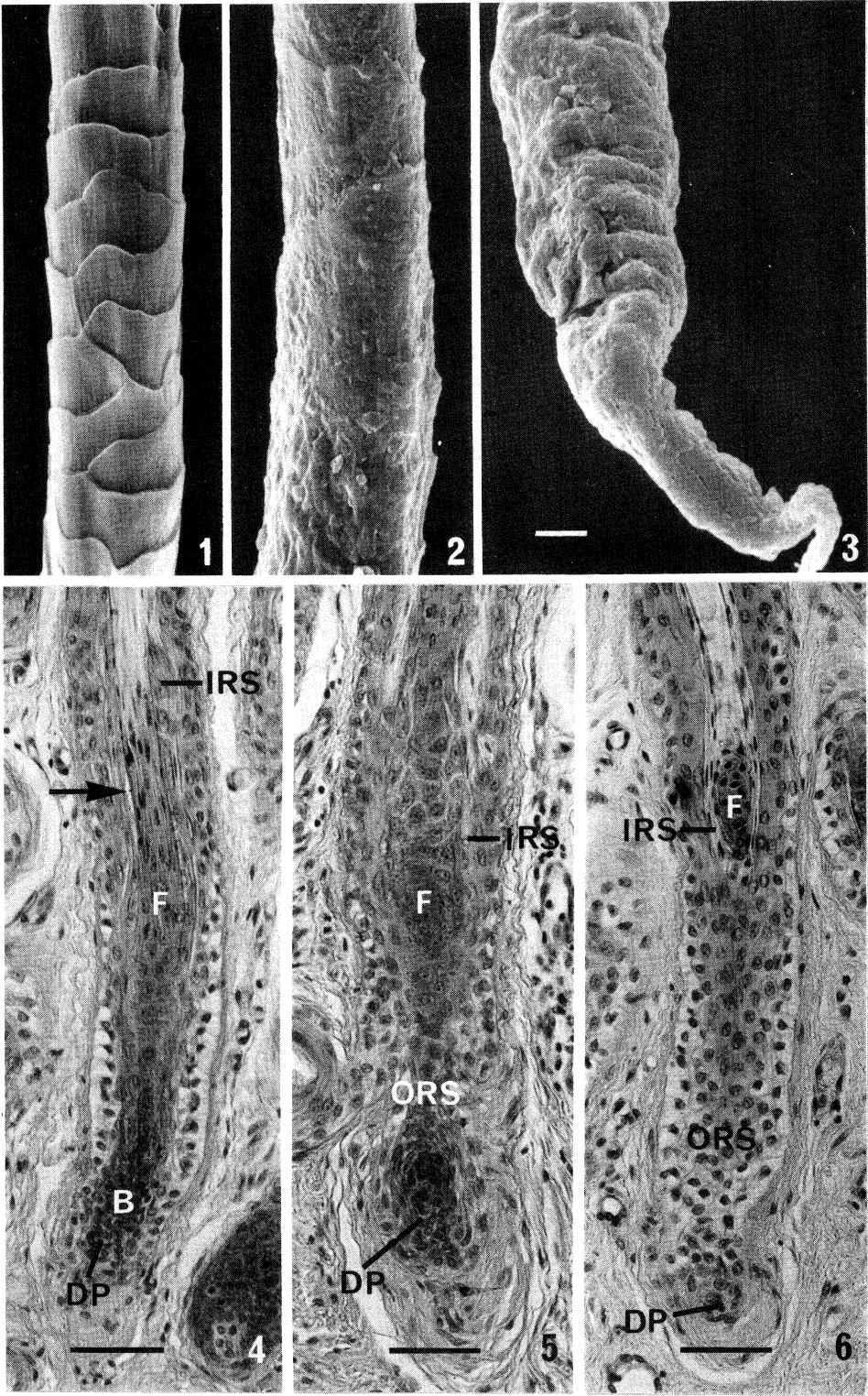
Before treatment, a control skin sample was biopsied under local anaesthetic from an untreated site anterior to the ventral row of patches on each sheep. Further skin samples were biopsied from within the ventral sites at weekly intervals during treatment and for a further 4 weeks after treatment. Final skin samples were taken 5 weeks later (i.e. 9 weeks after treatments ceased). The skin samples were fixed and processed for light microscopy as described above.

Figs 1–3. Scanning electron micrographs of portions of wool fibres showing (1) normal cuticle pattern before intradermal (ID) injections of mEGF, (2) impaired cuticle formation after two twice-daily ID injections, and (3) the distorted tapered end on a fibre shed subsequent to four twice-daily ID injections. Scale bar, 10 µm.

Fig. 4. Longitudinal section of a wool follicle in early catagen of mEGF-induced regression. The follicle bulb (B) is decreasing in size because of progressive withdrawal of fibre and inner root-sheath cells from around the dermal papilla (DP). Slight thickening of the fibre (F) in the keratogenous zone is associated with delayed hardening of the inner root sheath (IRS). Areas of separation (→) are also present between the inner root sheath and fibre. Scale bar, 50 µm.

Fig. 5. Longitudinal section of a wool follicle in mid-catagen of mEGF-induced regression. Fibre (F) and inner root-sheath (IRS) cells have withdrawn from around the dermal papilla (DP) and are separated from the dermal papilla by a short stalk of outer root-sheath cells (ORS). The fibre end and surrounding inner root-sheath cells are unhardened. Scale bar, 50 µm.

Fig. 6. Longitudinal section of a wool follicle in late catagen of mEGF-induced regression. The end of the fibre (F), which has not completely hardened, and the last of the surrounding inner root sheath (IRS), which has hardened, have moved up the follicle away from the dermal papilla (DP), from which they are separated by a longer stalk of outer root-sheath cells (ORS) than in Fig. 5. Scale bar, 50 µm.



Results

Effects of ID Injected Saline and mEGF

Wool follicles

The 2–8 ID injections of saline produced no observable effects on the follicles, all follicles appearing to remain active (i.e. in anagen), as in the uninjected skin.

After two ID injections of mEGF (i.e. 10 μ g mEGF), a slight reduction in the size of the follicle bulbs was detectable in c. 20% of follicles in one sheep, lasting from days 1–7, and there was disturbance of cuticle formation (Fig. 2, compared with Fig. 1) along short lengths of some fibres equivalent to a few days' growth.

After four ID injections of mEGF (i.e. 20 μ g mEGF), c. 5% of follicles had entered early to mid-catagen (Figs 4, 5) by day 4 in two of the sheep, the remaining follicles being in anagen. Areas of separation had formed between the inner root sheath and fibre at the level of the keratogenous zone of the fibre in some of the follicles in early catagen (Fig. 4). By day 7, up to 10% of follicles had regressed to mid- to late catagen (Figs 5, 6) in all the sheep and a further small number (4%) were maximally regressed (Fig. 7) in one sheep. By day 14, up to 15% of follicles had maximally regressed in the three sheep and no follicles remained in catagen, while a further small number (up to 7%) in two sheep were regenerating and were in early F2–F4 stages (Hardy and Lyne 1956) of anagen of new growth (Fig. 8). Some maximally regressed follicles contained no fibre because of shedding. The shed fibres could be found amongst the clipped fibres in the fleece samples. The ends of the shed fibres had very distorted cuticle patterns and were irregularly tapered (see Fig. 3.).

After six ID injections of mEGF (i.e. 30 μ g mEGF), up to 20% of follicles were in early to late catagen in all of the sheep by day 4, and further 1–2% were maximally regressed in two of the sheep. In the keratogenous zone of some of the remaining anagen follicles, the cells and nuclei of the fibre cortex failed to elongate normally. This produced a slight thickening of the fibre. By day 7, the numbers in maximal regression had increased slightly in all sheep, ranging up to 8%, while the numbers in catagen decreased. At day 14, no follicles remained in catagen; up to 15% were maximally regressed in the three sheep and an additional 2–12% had progressed to early anagen in two.

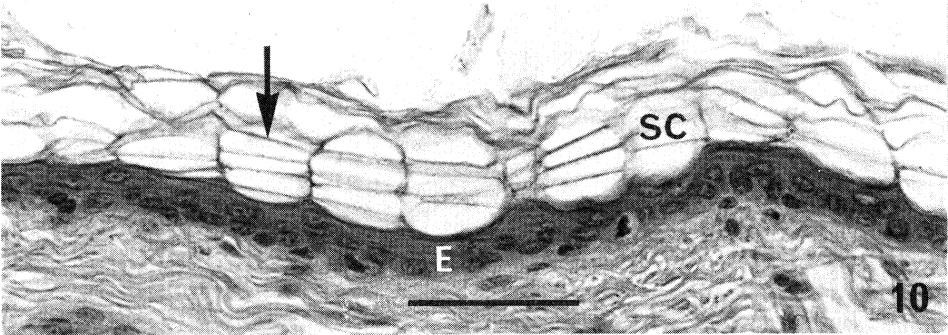
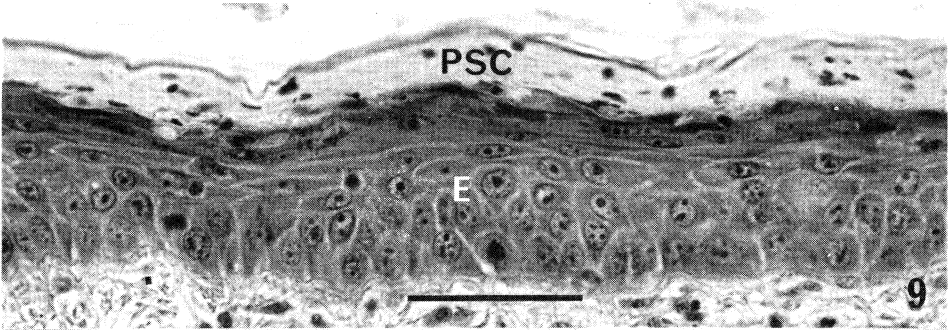
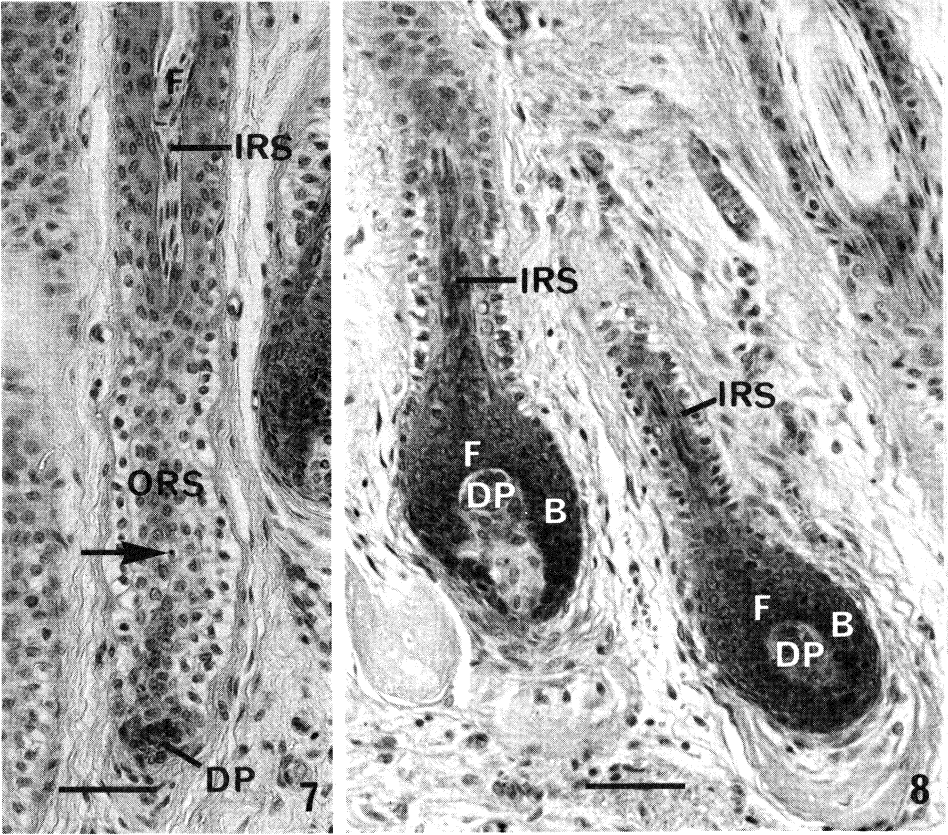
After eight ID injections of mEGF (i.e. 40 μ g mEGF), up to 60% of follicles were in early to late catagen in all sheep by day 4 and a further 3% were maximally regressed in one. Some anagen follicles showed the same abnormalities in the keratogenous zone as after six injections. By day 7, up to 12% of follicles were maximally regressed in all sheep and the numbers in catagen had decreased to 40% or less. An occasional follicle had progressed through to early anagen of regeneration in one sheep. By day 14, virtually no follicles remained in catagen in any sheep; up to 20% were maximally regressed, and similar percentages were regenerating and were in stages of anagen up to F6, at which newly formed fibres were beginning to keratinize.

Fig. 7. Longitudinal section of a wool follicle in mEGF-induced maximal regression. The end of the fibre (F) has hardened within the surrounding hardened inner root sheath (IRS), the last of which in this illustration extends below the fibre end. Some apoptotic bodies ($\rightarrow$) are present in the stalk of outer root-sheath cells (ORS) above the now round dermal papilla (DP). Signs of follicle regeneration are not yet obvious. Scale bar, 50 μ m.

Fig. 8. Slightly oblique longitudinal sections of two follicles in early stages of regeneration (anagen) after mEGF-induced regression. New bulbs (B) have invested the dermal papillae (DP), and cones of unhardened inner root-sheath (IRS) and fibre (F) cells are forming above the dermal papillae. Scale bar, 50 μ m.

Fig. 9. Vertical section of the thickened epidermis (E) of a Merino sheep with a thick parakeratotic stratum corneum (PSC) after eight twice-daily intradermal injections of mEGF. Scale bar, 50 μ m.

Fig. 10. Vertical section of stacks of cornified cells ($\downarrow$) in the stratum corneum (SC) of the epidermis (E) of a Merino sheep following five daily topical applications of 50% (v/v) aqueous propylene glycol. Scale bar, 50 μ m.



Epidermis

Using the number of layers of living cells as a measure of epidermal thickness there was no change in the thickness after two ID injections of saline, compared with the uninjected skin, although slight parakeratosis developed in one sheep at the saline-injected site. Epidermal thickness increased slightly in one sheep after 4-8 ID injections of saline, and transient parakeratosis developed in two sheep.

Two ID injections of mEGF increased epidermal thickness slightly in one sheep and parakeratosis developed in two and persisted for 14 days in one. Four ID injections produced slight to moderate epidermal thickening, together with parakeratosis, in two sheep by day 4. The thickening almost disappeared by day 7, although the parakeratosis was still evident in one sheep at day 14. Six and eight ID injections of mEGF caused epidermal thickness to increase two-fold, together with marked parakeratosis by day 4 (Fig. 9). The parakeratotic stratum corneum was being sloughed at day 7, and epidermal thickness had almost returned to normal by day 14.

Glands

After two and four ID injections of saline, the sebaceous glands were slightly larger in the injected sites than in uninjected skin, but this was less evident after six and eight injections.

Only after six and eight ID injections of mEGF was there a slight increase in sebaceous gland size compared with sites similarly injected with saline. This effect disappeared in 7-14 days after six injections of mEGF, but still persisted at day 14 after eight injections.

Sweat glands tended to be smaller following ID injections of saline than in uninjected skin in two of the sheep. However, the sweat glands were slightly larger at 7-14 days in the sites injected 4-8 times with mEGF in these two sheep.

Dermis

Dilatation and rupture of some of the blood vessels deep in the dermis were evident after ID injections of both saline and mEGF, although these features were more extensive in the sites injected with mEGF. Changes in the thickness of the dermis from the epidermis to the level of the follicle bulbs after injections of saline and mEGF were small and variable.

Effects of Topically Applied Aqueous PG and mEGF

All the follicles in the untreated skin were in anagen, and the follicles remained active in the areas treated with PG/water either with or without mEGF. There were no changes in follicle morphology, similar to those induced by ID injections of mEGF, in any of the sites treated topically.

After adjusting the wool weights grown on the tattooed patches for the variation in growth rate on the untreated patch in the several periods, there was no pattern in any of the sheep in the small differences (all < 10%) between the wool growths on the patches treated with PG/water and with the two concentrations of mEGF.

The size of the sebaceous glands remained virtually unchanged during and after the topical treatments. Variations in sweat gland size were inconsistent and appeared unrelated to any particular treatment.

The only consistent morphological change observed was an effect of PG/water on the stratum corneum of the epidermis in causing an increase in the number of layers of cornified cells, which were dilated and arranged in clearly discernible stacks (Fig. 10). This effect was evident after five or more applications and persisted until 7-14 days after applications ceased. It was also present in the sites treated with mEGF in the vehicle. However, at no time was there thickening of the epidermis below the stratum corneum comparable to that induced by intradermally injected mEGF (Fig. 9).

Discussion

The present study has shown that intradermally injected mEGF produces dose-dependent changes in the wool follicles and fibres, epidermis and skin glands. The effects ranged from

variable, transient disturbance of fibre cuticle formation at low doses to regression of follicles, thickening of the epidermis and slight enlargement of sebaceous and sweat glands at higher doses. The effects at the higher doses were similar in many respects to those produced by intravenous (IV) infusion of a depilatory amount of mEGF (Hollis *et al.* 1983; Hollis and Chapman 1987). However, separation of the inner root sheath from the fibre at the level of the keratogenous zone of the fibre was less extensive after ID injection than after IV infusion. Some follicles regressed in 4 days after ID injection, which was as quickly as after IV infusion, but the majority of those follicles that regressed maximally took longer to regress and to start regenerating after ID injection (7–14 days) than after IV infusion (4–8 days). A feature of maximally regressed follicles was the presence of inner root-sheath material around and sometimes below the distorted and tapered ends on the fibres which had ceased growing. This is in contrast to the withdrawal of inner root-sheath cells from around the fibre end during late catagen and the absence of any inner root sheath around the brush end of the fibre in the telogen phase of normal cyclic hair or wool growth (Straile *et al.* 1961; Montagna and Parakkal 1974; Chapman, unpublished data). The maximally regressed state induced by mEGF bears some resemblance to the catagen stage CVI of Straile *et al.* (1961), in that a long strand of epithelial cells separates the fibre end from the rounded dermal papilla. But there the similarity ends, because in mEGF-regressed follicles the fibre end is not brush-like and inner root-sheath material has not withdrawn from around the fibre end, as in catagen CVI. These differences presumably arise from an interrelationship between inhibition of cell proliferation and cell migration in the fibre and inner root sheath in mEGF-induced catagen which differs from the corresponding interrelationship in catagen of cyclic hair growth.

The maximally regressed follicles are, in effect, in the telogen (i.e. end state) of mEGF-induced regression even though they do not have features associated with telogen of cyclic hair growth. In the broader sense of the term, there can be various morphological forms of follicles in telogen depending on whether fibre growth ceases as part of cyclic hair growth (Montagna and Parakkal 1974), or because of administration of chemicals (Flesch 1963; Rook 1965; Chapman 1980) or a growth factor (Hollis *et al.* 1983).

Increase and subsequent decrease in epidermal thickness occurred more rapidly after ID injection of mEGF than after IV infusion. The associated parakeratosis after ID injection was not a feature of epidermal thickening after IV infusion (Hollis *et al.* 1983). It appears to be a disturbance of epidermal function by the ID injection procedure, because a lesser degree of parakeratosis was also present on some of the sites that received ID injections of saline.

The increase in sebaceous gland size following ID injection of mEGF, and subsequent decrease, resembled the effects of IV infusion (Hollis *et al.* 1983). The action of EGF on sebaceous glands appears to be direct, as has been shown to occur in hamsters following subcutaneous injection (Matias and Orentreich 1983).

Topically applied mEGF, by comparison, had no effect on epidermis or follicles, presumably because insufficient mEGF penetrated the skin to have any effect. The accumulation of stratum corneum in stacks of swollen cells following topical application of propylene glycol/water was unusual, because the stratum corneum of Merino sheep is normally thin, with little obvious cell stacking. The dilated appearance of these cell stacks was reminiscent of the swelling effect of sodium hydroxide on the stacks of cells in mouse-ear stratum corneum (Mackenzie 1969).

The lack of effect of local infusions of 300–400 μ g mEGF into isolated cutaneous patches supplied by a caudal branch of the deep circumflex iliac artery was interpreted to mean that the effects observed after systemic administration of mEGF resulted from changes in the sheep's hormonal profile rather than a direct effect of mEGF itself (McDonald *et al.* 1983). However, the changes induced by ID injections of mEGF in the present study, together with the localization of receptors for mEGF in sheep skin and wool follicles (Wynn *et al.* 1985) would indicate direct action of mEGF.

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