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Localisation of transforming growth factor β_1 -like immunoreactivity in foetal and mature skin from Romney and Merino sheep

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ABSTRACT

Skin samples removed from sheep foetuses at weekly intervals during gestation were studied to examine how between breed differences in follicle development are induced. Immunocytochemical methods were used to examine the localisation of the peptide transforming growth factor- β_1 (TGF β_1).

Our observations indicate general localisation of TGF β_1 in the skin between 83 and 104 days of gestation in both Romney and Merino breeds. This coincides with the maturation of primary follicles and the development of sebaceous and sweat glands. TGF β_1 immunoreactivity was maintained in primary follicles (particularly the presumptive inner root sheath cells in lower regions of the bulb), sweat gland and sebaceous gland but by day 134, a more discrete localisation was observed in the inner root sheath (IRS), outer root sheath (ORS) and sebaceous gland with intense immunoreactivity in the sweat gland. The timing of the localisation and the lack of difference between the breeds suggest that variation in the TGF β_1 level is unlikely to have a regulatory role in primary and secondary follicle initiation but may play a role in regulating the diverse differentiation pattern of various cell types within the follicle. The intense immunoreactivity in mature sweat glands suggests a possible osmoregulatory role for TGF β_1 in the skin.

Keywords: TGF β ; wool follicle; development; Romney; Merino.

INTRODUCTION

The initiation and growth of hair follicles is a complex process involving interactions between the foetal epidermis and underlying dermis (Hardy, 1992). The morphological changes which occur during development of pelage, vibrissae and wool follicles have been described in many species including sheep (Hardy & Lyne, 1956). The first sign of hair or wool follicle development is a thickening of the foetal epidermis and an accumulation of cells in the adjacent dermal layer. Tissue recombination experiments (reviewed in Sengel, 1986), have suggested a series of messages which pass in both directions between the dermal and epidermal tissues directing the initiation and growth of hair follicles. As development progresses, the epidermal cells proliferate and penetrate the dermis. Populations of ectoderm-derived cells, the dermal papilla and the dermal sheath, become incorporated into the follicle. Eventually some cells of epidermal origin begin to migrate upwards through the central core of the follicle towards the skin surface. Initially a hair cone is formed, followed in sequence by the inner root sheath (IRS) and wool fibre. Wool follicle initiation can be divided into two phases; the first follicles formed are known as primary follicles and the later initiated are called secondary follicles.

Transforming growth factor beta (TGF β) is a multifunctional cytokine which regulates cell growth and differentiation and plays a role in epithelial-mesenchymal interactions that occur during embryonic development

(Nilsen-Hamilton *et al.*, 1992). TGF β has been demonstrated to stimulate deposition of extracellular tissue and induce mesoderm formation during early embryogenesis (reviewed in Roberts & Sporn, 1991). Further, TGF β inhibits keratinocyte differentiation without altering growth rates (Reiss & Sartorelli, 1987), and stimulates differentiation, but inhibits proliferation, of bronchial epithelial cells (Masui *et al.*, 1986).

TGF β gene expression has been described during the development of mouse whisker follicles (Lehnert & Ackhurst, 1988) with expression of the mRNA transient in the skin during follicle development. Targeted disruption of the mouse TGF β_1 gene produces no gross developmental abnormalities (Shull *et al.*, 1992), however maternal sources of TGF β_1 have been shown to contribute to the normal appearance of TGF β_1 -null newborn mice (Letterio *et al.*, 1994). In ovine skin, TGF β mRNA is expressed at a time which corresponds to follicle initiation (Sutton & Ward, 1991) and immunolocalisation of the polypeptide in the mouse has indicated that there is an involvement of TGF β in pelage and vibrissa formation (Heine *et al.*, 1987; Pelton *et al.*, 1991). We have extended these studies, making a detailed examination of TGF β_1 distribution in developing and mature ovine skin.

MATERIALS AND METHODS

Foetuses were collected at weekly intervals from 63 to 145 days of gestation, from Merino and Romney ewes

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mated to rams of their own breed as described previously (Hocking Edwards *et al.*, 1996). Two ewes of each breed were allowed to proceed to term and skin samples were taken from each lamb at 3 and 12 weeks, and 9 months after birth. Skin biopsies were taken from the midside of foetuses and lambs. The skin samples were placed in Bouin's fixative for 4 to 6h and then removed and stored in 70%(v/v) ethanol.

Skin samples were embedded in paraffin wax and sectioned at 5mm thickness longitudinal to the plane of the follicle. The sections were dewaxed in xylene, rehydrated and then washed in phosphate-buffered saline (PBS; pH 7.2). Non-specific protein binding to tissues was blocked by soaking sections in 10% normal sheep serum (NSS) in 0.01M PBS for 1 hour at room temperature. The sections were then incubated overnight at 4°C with a commercial monoclonal primary antibody (murine anti-human TGF β antibody (IgG1); Serotec Ltd., Oxford, UK) at dilutions of 40 and 20 μ g/ml in 0.01M PBS/1% BSA. Control sections were those in which the primary antibody was replaced with an IgG negative control monoclonal antibody (Dako Corporation, California, USA). For more complete demonstration of the specificity of the antibody used, we blocked the staining by preincubating the anti-TGF β IgG with a 300 μ g/ml TGF β peptide solution. This procedure resulted in a substantial decrease in the intensity of the staining (Figs 3a & 3b). Tissues were then washed in PBS and incubated for 40 minutes at room temperature with biotinylated sheep anti-mouse secondary antibody (Amersham International, Buckinghamshire, UK) in PBS/1% BSA. After further washes in PBS, the sections were exposed to a biotin-streptavidin-peroxidase preformed complex (Amersham International, Buckinghamshire, UK) for 15 minutes at room temperature and washed with PBS. Sections were then reacted with 0.05% (w/w) 3,3'-

diaminobenzidine tetrahydrochloride dihydrate (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) in PBS with 0.1%(v/v) hydrogen peroxide for 3 minutes, washed in deionised distilled water, dehydrated and mounted.

RESULTS

In the present study there was no difference in the distribution of TGF β_1 immunoreactivity during wool follicle development between Romney and Merino sheep, and data have been pooled for presentation (see Table 1).

There was no TGF β_1 immunoreactivity in the samples taken at 38 days of gestation. TGF β_1 -like immunoreactivity was first observed in the upper periderm layer of the epidermis, and in endothelial tissue within the dermis, on day 56 of gestation and was unchanged on days 63, 70 (Fig. 1a) and 76 of gestation. No staining was associated with the developing primary follicles.

By day 83 of gestation, staining remained strong in the epidermis and blood vessels, and there was weak general TGF β_1 -like immunoreactivity throughout the dermis. The nuclei of the follicular cells, dermal fibroblasts, sebaceous gland cells and sweat gland cells were all stained. Distribution of the TGF β_1 -like immunoreactivity remained unchanged on days 90 (Fig. 2a) and 97 of gestation.

By day 100 of gestation, TGF β_1 -like immunoreactivity in the primary follicles was restricted to the cytoplasm of cells in the proximal region of the IRS and the nuclei and cytoplasm of cells in the outer root sheath (ORS), sebaceous gland and sweat gland (Fig. 3a). This pattern of staining remained similar on days 105 (Fig. 4a), 111, 118 (Fig. 5a), 125, 132 and 139 of gestation, and 3 and 12 weeks, and 9 months, after birth. However, the intensity of staining within the sweat glands increased markedly by day 118 (Fig. 5a). Both original and derived secondary

TABLE 1: Distribution of TGF β_1 -like immunoreactivity detected by immunocytochemistry in foetal ovine skin during wool follicle development (CTS - connective tissue sheath; ORS - outer root sheath; IRS - inner root sheath; GE - germinal epithelium; DP - dermal papilla; x - structure not present; o - not detected; + - weak immunoreactivity; ++++ - very strong immunoreactivity).

Stage of Development*	0	1	2	3/4	4/5	6	7	8	8	8	8	8
Day of Gestation	63	70	76	83	90	97	105	111	118	125	132	139
Epidermis	o	+	+	++	++	+++	+++	++	++	++	++	+
Dermis	o	o	o	o	+	+	+	+	+	+	o	o
ORS	x	x	x	x	o	o	++	+++	++	++	++	++
CTS	x	x	x	x	x	o	++	++	++	+	+	+
Hair Cone	x	x	x	x	x	o	x	x	x	x	x	x
GE	x	x	x	x	x	o	+	+	+	+	+	+
DP	x	x	x	x	x	o	o	o	o	o	o	o
IRS :Henle	x	x	x	x	x	x	o	o	o	o	o	o
:Huxley	x	x	x	x	x	x	o	o	o	o	o	o
:Cuticle	x	x	x	x	x	x	o	o	o	o	o	o
Hair :Cortex	x	x	x	x	x	x	+	+	+	+	+	+
:Cuticle	x	x	x	x	x	x	+	+	+	+	+	+
Seb. Gland	x	x	x	x	+	++	++	++	++	++	++	++
Sw. Gland	x	x	x	x	x	o	++	++++	++++	++++	++++	++++
A.P. Muscle	x	x	x	x	x	o	+	+	+	++	+++	+++

* After Hardy & Lyne, (1956). eg. Stage 1 - follicle plug stage.

FIGURE 1A: Longitudinal section through sheep skin at day 70 post-conception. TGF β_1 -like immunoreactivity is confined to the upper periderm layer of the epidermis and endothelial tissue. Magnification 100x.

FIGURE 2A: At day 90 post-conception, weak general TGF β_1 -like immunoreactivity extends throughout the skin. Follicular cell nuclei, dermal fibroblasts, sebaceous gland and epitrachial sweat gland cells are all stained. Magnification 100x.

FIGURE 3: At day 100 post-conception. (a) anti-TGF β_1 -like immunoreactivity occurs in presumptive IRS and epidermal cells. (b) an equivalent aliquot of antibody incubated with an excess of TGF β peptide gives reduced staining. Magnification 50x.

FIGURE 4A: At day 105 post-conception, follicular immunoreactivity is concentrated in the proximal region of IRS, ORS, and in endothelial cells within the dermal papilla. Magnification 100x.

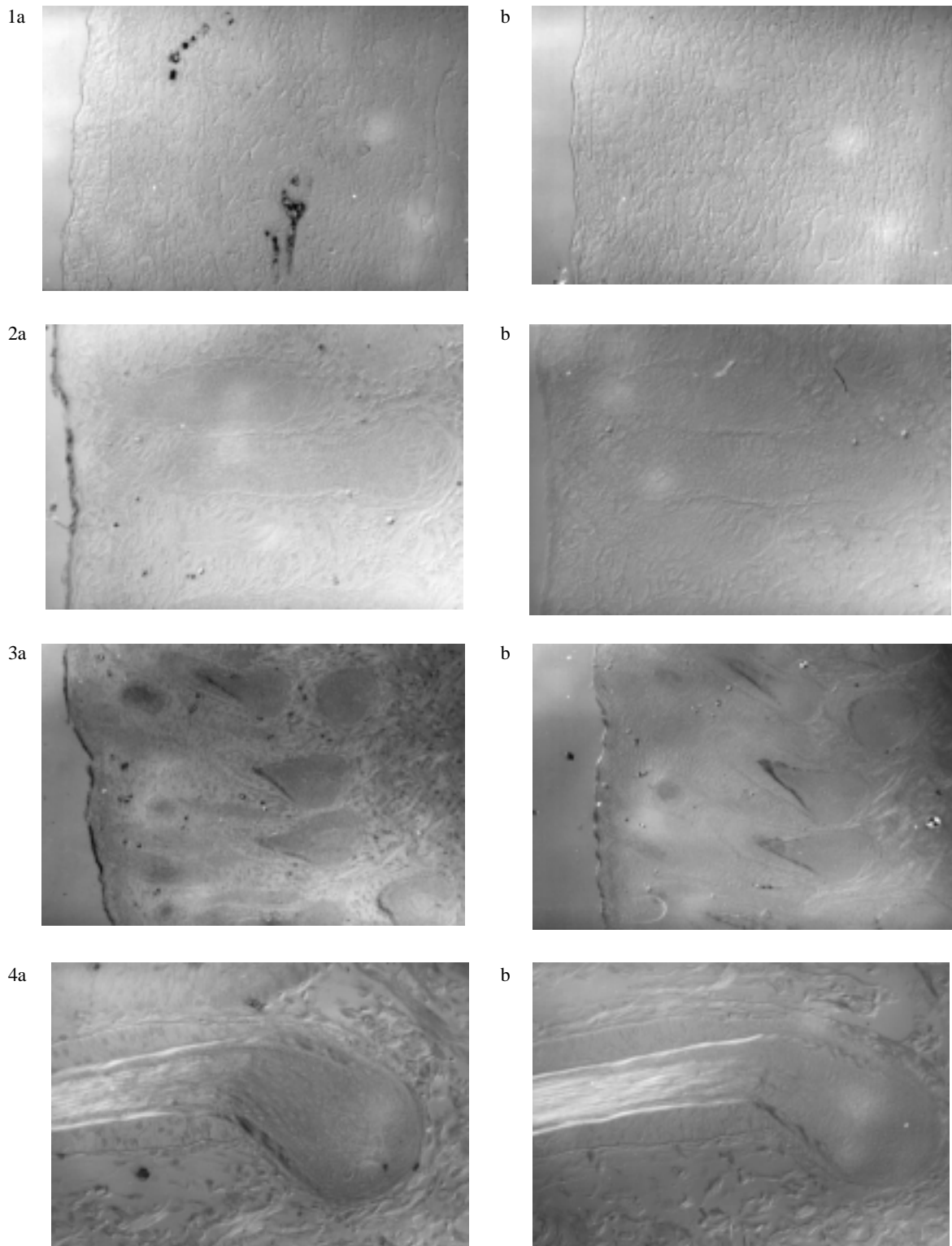
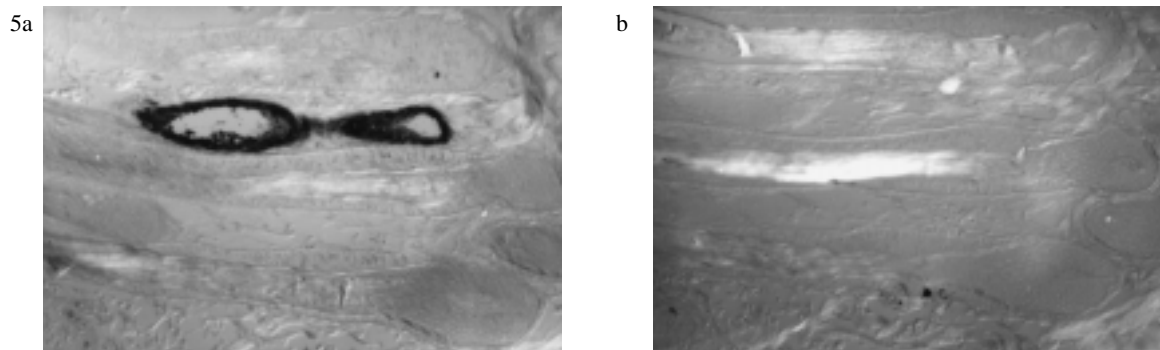


FIGURE 5A: At day 118 post-conception, the follicular pattern of staining is unchanged, although epitrichial sweat glands now show intense immunoreactivity. Magnification 60x.

All sections were incubated with anti-TGF β_1 (mc), streptavidin-biotin.

Control sections (Figs 1b, 2b, & 5b) were negative for any immunoreactivity, while Figure 4b shows no immunoreactivity in endothelial and ORS cells, but slight background staining in the IRS.



follicles were negative for TGF β_1 -like immunoreactivity. Negative control sections (Figs. 1b, 2b & 5b) show no immunoreactivity, while Figure 4b shows no immunoreactivity in endothelial and ORS cells, but slight background staining in the IRS.

DISCUSSION

There is a high degree of conservation of the TGF β_1 gene between the species examined to date including human (Derynck *et al.*, 1985), mouse (Derynck *et al.*, 1988) and sheep (Woodall *et al.*, 1994). All reported mature processed TGF β_1 peptides are identical (Massague, 1990), therefore ovine TGF β_1 can be detected by an antibody raised against human TGF β_1 .

The present study has shown that a TGF β_1 -like molecule was present in both ectodermal and mesodermal-derived tissues within sheep skin during wool follicle development (see Table 1). Previous studies have implicated members of the TGF β family, including TGF β_1 , in mouse skin morphogenesis (Heine *et al.*, 1987; Pelton *et al.*, 1991). However, there were discrepancies between the results from the present and the two previous immunocytochemical studies. The first (Heine *et al.*, 1987) showed TGF β_1 -like immunoreactivity in the dermal tissue associated with the developing vibrissa follicles, with the most intense staining reported when critical interactions with the adjacent epithelium were occurring. In the present work, intense TGF β_1 -like immunoreactivity was not shown in dermal tissue, although discrete staining was observed in blood vessels within the dermis closely associated with developing follicles. The second study (Pelton *et al.*, 1991) in contrast showed TGF β_1 -like immunoreactivity confined to epidermal tissue prior to follicle initiation and further localisation within the cytoplasm of pelage follicle cells prior to fibre formation. Neither published study reported immunoreactivity associated with sweat glands.

One explanation for the discrepancies between these results is that the different antibodies used in each study were raised against different epitopes of the TGF β mol-

ecule and recognise a possible conformational change which is present between extracellular and intracellular forms of TGF β (Flanders *et al.*, 1989). The 'extracellular' TGF β_1 antibody used in the study by Heine *et al.* (1987) showed a TGF β distribution confined to dermal tissues during follicle development and also did not stain normal adult skin keratinocytes *in vivo* (Kane *et al.*, 1990). In contrast, the 'intracellular' antibody used by Pelton *et al.* (1991) showed TGF β intracellular immunoreactivity in epidermal tissue and within follicles during development, and in non-proliferating, differentiated suprabasal keratinocytes in normal adult skin (Kane *et al.*, 1990). These results suggest that the TGF β_1 antibody used in the present study is recognising a different epitope to those used previously. In particular the antibody was capable of reacting with TGF β_1 in both ectodermal- and mesodermal-derived tissue with an extra- and intracellular distribution. However, the discrepancies between TGF β_1 distribution in the different studies could also be due to non-specific cross reactivity with related or unrelated molecules.

TGF β_1 is generally released from cells in a biologically inactive form which is unable to bind to cellular receptors and is not recognised by antibodies to the active form of TGF β (Miyazono *et al.*, 1988). However, because the tissue fixation process (Heine *et al.*, 1987; and present study), utilised a pH low enough to activate latent TGF β it is probable that the antibody in the present study detected both active TGF β_1 and activated latent TGF β_1 .

In vitro studies have indicated that TGF β_1 regulates the formation of extracellular material either by inducing synthesis of dermal proteins (such as collagen, fibronectin, glycosaminoglycans and cell adhesion receptors) or by controlling proteolytic degradation of extracellular matrix proteins (reviewed by Roberts & Sporn, 1991). Our results suggest that the *in vivo* synthesis of extracellular material is not necessarily related to the presence of TGF β_1 in developing skin. Extracellular tissue formation begins prior to follicle initiation in human (Smith *et al.*, 1986) and rat (Gibson *et al.*, 1983) skin whereas TGF β_1 was present in the epidermis only after the appearance of hair germ

cells.

TGF β_1 -like immunoreactivity was consistently associated with endothelial cells from 56 days of gestation, the first age at which vascularisation was observed in the skin. TGF β has been shown to induce angiogenesis when injected into new born mice (Roberts *et al.*, 1986) possibly by attracting and activating monocytes (Wahl *et al.*, 1987).

Weak TGF β_1 -like immunoreactivity was present throughout the skin between 83 and 104 days of gestation. Towards the end of this period, specific staining was observed first in the developing sebaceous gland and then in the sweat gland and arrector pili muscle. Original and derived secondary follicles begin to form in the skin between 100-125 days development (Hardy & Lyne, 1956; Hocking Edwards *et al.*, 1996). However, no association was found between TGF β_1 -like immunoreactivity in the original or branching secondary derived follicles in the present study even though TGF β_1 was shown to accumulate in mesenchymal cells during branching of lung alveoli in the mouse (Heine *et al.*, 1990).

In the mature follicle the TGF β_1 peptide was present in the distal region of the ORS, the cells of both the sebaceous gland and sweat gland, and the proximal region of the IRS. The most intense immunoreactivity was present in the secretory lower portion of the sweat gland. Recent work has shown the expression of mRNA from activin beta (B), a member of the TGF β family, in sweat glands of the rat (Fann & Patterson, 1995). Previous studies also revealed TGF β_1 immunostaining localised in the proximal region of the IRS of vibrissae follicles (Pelton *et al.*, 1991). Close examination of the wool follicle bulb in the present study showed immunostaining of cells about to differentiate into the Henle layer. This suggests that TGF β_1 may have a role in directing the paths of differentiation of the cells as they move up the follicle. This view is supported by results from a recent study which emphasised the inhibitory role of the TGF β family of growth factors on cell division in the epidermis, hair follicles and other tissues in mice (Lange *et al.*, 1996).

Several conclusions can be drawn from these results. First, there is no difference in the distribution of TGF β_1 in developing skin between Romney and Merino sheep. However, TGF β_1 does not appear necessary for the initiation of follicles in either breed, since little immunoreactivity is associated with developing primary and secondary follicles. Second, the presence of TGF β_1 in cells of the sebaceous and sweat glands during development suggests a role in this process. However, the more intense immunoreactivity in the sweat glands of mature follicles indicates a osmoregulatory role for TGF β_1 in the skin. These findings may have important practical implications to the sheep industry since the problem of wool yellowing has been linked to the production of sweat in Indian wool breeds (Jatkar *et al.*, 1979).

ACKNOWLEDGEMENTS

We thank Mrs. S. Khalaf for skilled technical assistance. We also gratefully acknowledge Wools of New Zealand and PGSF (Contract number C10606) for their

financial support of this work.

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