Cell proliferation during fibre growth initiation in ferret hair follicles

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ABSTRACT

The commencement and course of cell proliferation is described in proanagen hair follicles of ferrets. Initiation of autumn fur growth was synchronised in 32 animals using melatonin implants. Skin samples containing S-phase cells labelled in vivo with bromodeoxyuridine were collected from groups of four animals up until the time of development of metanagen follicles at 14 days post-implant. Cells were visualised by an indirect immunocytochemical method and counts made of proliferating cells in the hair germ, epithelial strand/outer root sheath, connective tissue sheath and dermal papilla. Telogen follicles showed no labelling in all four tissues until 4 days after melatonin implant. The largest number of proliferating cells were in the hair germ, which formed the new fibre and inner root sheath. The cell labelling index for this tissue remained high at 14 days post-implant. In other tissues, cell proliferation reached peaks during mid to late proanagen, then declined as the metanagen state was reached. These results indicate that some key signalling events controlling ferret hair growth occur between 0 and 4 days after the melatonin implant, and illustrate the separate processes of follicular regeneration and fibre growth occurring in proanagen follicles.

Keywords Bromodeoxyuridine, cell proliferation, ferret, fibre growth, hair follicle, melatonin, proanagen.

INTRODUCTION

Fibre growth cycles in ferrets (*Mustela putorius furo*), like those of all mammals, involve periods of active fibre growth (anagen) and quiescence (telogen). These cycles occur seasonally and are cued by photoperiod via a hormonal mechanism (Hammond 1952, Martinet *et al.*, 1984). The facility to manipulate the timing of the rapid transition into fibre growth (Pearson *et al.*, 1989) makes hair growth in ferrets a suitable model for seasonal wool growth. We have compared follicles at key points in the ferret hair growth cycle, as a means of identifying biochemical mechanisms that might also regulate wool growth.

One such event is the start of cell division in the follicle bulb during fibre growth initiation (proanagen). Previous mitotic studies have focused on proliferative activity of germinal matrix cells relating to wool growth (e.g. Fraser 1965, Hynd and Everett 1990). However, since we are dealing with regenerating organs, we have considered cell proliferation in several tissue compartments of activated follicles so as to distinguish different aspects of growth occurring simultaneously.

DNA labelling with the thymidine analogue 5-bromo-2'-deoxyuridine (BrdU) and detection using a commercially available monoclonal antibody has recently been demonstrated in wool follicles (Holle and Birtles 1990, Hynd and Everret 1990, Adelson *et al.*, 1991). In this study we have used melatonin to synchronise autumn initiation of fibre growth in ferret skin, and the BrdU method to determine the time and location of changes in cell proliferation in the newly activated hair follicle.

MATERIALS AND METHODS

Early onset of autumn fur growth was induced in 28 female ferrets at 16 weeks of age on 26 February 1991, using two subcutaneous 18 mg melatonin implants (Regulin). Groups of four animals were sacrificed at two day intervals between 2 and 14 days post-implant. Two additional groups of four animals sampled on days 0 and 14 served as untreated controls. A 30 mg/kg intracardiac injection of BrdU labelling reagent (Amersham) was given one hour prior to sacrifice and skin sampling. Snip biopsies of skin were taken from all animals while anaesthetised with 1.2 ml/kg alphaxalone / alphadolone acetate (Saffan, Glaxovet) at day 0 to establish that follicles were in telogen.

Skin samples were fixed in Bouins fluid for 4-6 hours, and processed to wax. Six µm longitudinal sections of hair follicles were cut and mounted onto slides precoated with poly-L-lysine (Sigma). Prepared sections were then stained using incubation times and conditions as recommended for Amersham immunoreagents. BrdU incorporated into cellular DNA was detected using monoclonal mouse anti-BrdU IgG. Detection of bound antibody was achieved using peroxidase conjugated sheep anti-mouse IgG. Peroxidase activity was localised by precipitation of diaminobenzidine in the presence of cobalt and nickel ions giving blue-black staining at sites of BrdU incorporation. Counterstaining was with 0.1% Nuclear Fast Red (C.I. 60760) in a 5% aqueous solution of aluminium sulphate.

Negative controls on each slide were sections not exposed to the primary antibody. Positive controls for resting follicles were epidermal cells in S-phase within the same section.

Counts of labelled cells of original primary hair follicles were made from median longitudinal sections in four tissue types of the developing follicles: (i) hair germ, (ii) epithelial strand/outer root sheath, (iii) connective tissue sheath and (iv) dermal papilla. As there was considerable variation in follicle size, labelled cell count for each follicle section was divided by follicle bulb width, measured using a Visilog image analysis system (Noesis). The mean of cell counts adjusted for follicle size in this manner was used as a labelled cell index. Each follicle was assessed for follicle development according to the classification of Chase *et al.*, (1951).

RESULTS

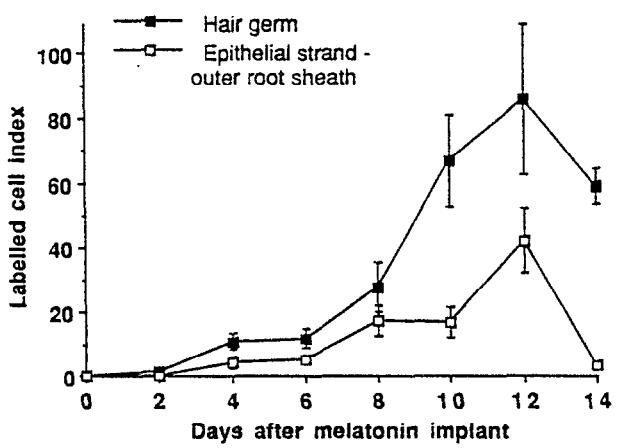
All original primary follicles from day 0 skin samples of all animals were in telogen. Three of the four control animals

sampled at day 14 post-treatment possessed spontaneously initiated proanagen follicles. However, all treated animals showed a close relationship between stage of follicle development and time after implant. Anagen I was indicated at day 6 by the first mitoses in the follicle bulb, anagen II was predominant at days 8 and 10, anagen III and IV at day 12, and anagen VI was achieved in most follicles by day 14. BrdU labelled S-phase cells were absent from the lower follicle at days 0 and 2, but were detected in all four tissue types of day 4 samples, preceding morphological changes to the follicle bulb by approximately two days. Cell proliferation and follicle structure at representative transitional stages between dormancy and full activity are shown in Fig. 1.

The hair germ began in the telogen follicle as a disk of epithelial cells overlying the dermal papilla (Fig. 1a). At day 0, no BrdU labelled cells were counted in hair germs of original primary follicles, although a small number were observed in the derived follicle type. In the anagen follicle, the hair germ increased in cell number to become the germinal matrix for inner root sheath and fibre at the distal end of the bulb. There was an increase in cell proliferation rate over the initiation period (Fig. 2) as fibre production was established.

The epithelial strand (Fig. 1b) connecting the hair germ proper with the old outer root sheath developed into the new outer root sheath in the active follicle (Fig. 1d). A burst of cell proliferation from day 8 to day 12 was associated with follicle remodelling (Fig. 2). Cell proliferation subsequently declined, but some labelled cells remained in the fully active follicle.

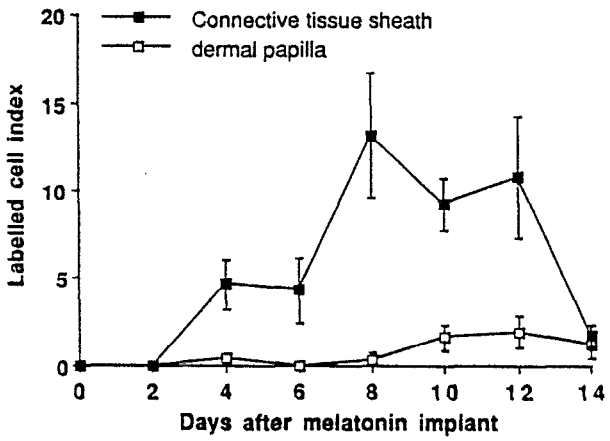
FIG 2 Cell proliferation in epithelial tissues of the ferret hair follicle: hair germ / germinal matrix, epithelial strand / new outer root sheath. Labelled cell index is the mean of the number of BrdU labelled cells per 6 µm section relative to follicle bulb width. Vertical bars represent SEM.



Like the epithelial strand, the connective tissue sheath enveloping the follicle increased in size in active follicles. The labelling index of fibrocytes within the connective tissue sheath was highest as the follicle elongated during anagen II to III between days 8 and 12, These cells then ceased dividing when the fully developed follicle was established (Fig. 3).

DNA replication was evident in the dermal papilla during fibre growth initiation (days 10 and 12 in Fig. 3). This was accompanied by changes in cell shape, cytoplasmic volume, and papilla volume and shape.

FIG 3 Cell proliferation in dermal tissues of the ferret hair follicle: connective tissue sheath, dermal papilla. Labelled cell index is the mean of the number of BrdU labelled cells per 6 µm section relative to follicle bulb width. Vertical bars represent SEM.



DISCUSSION

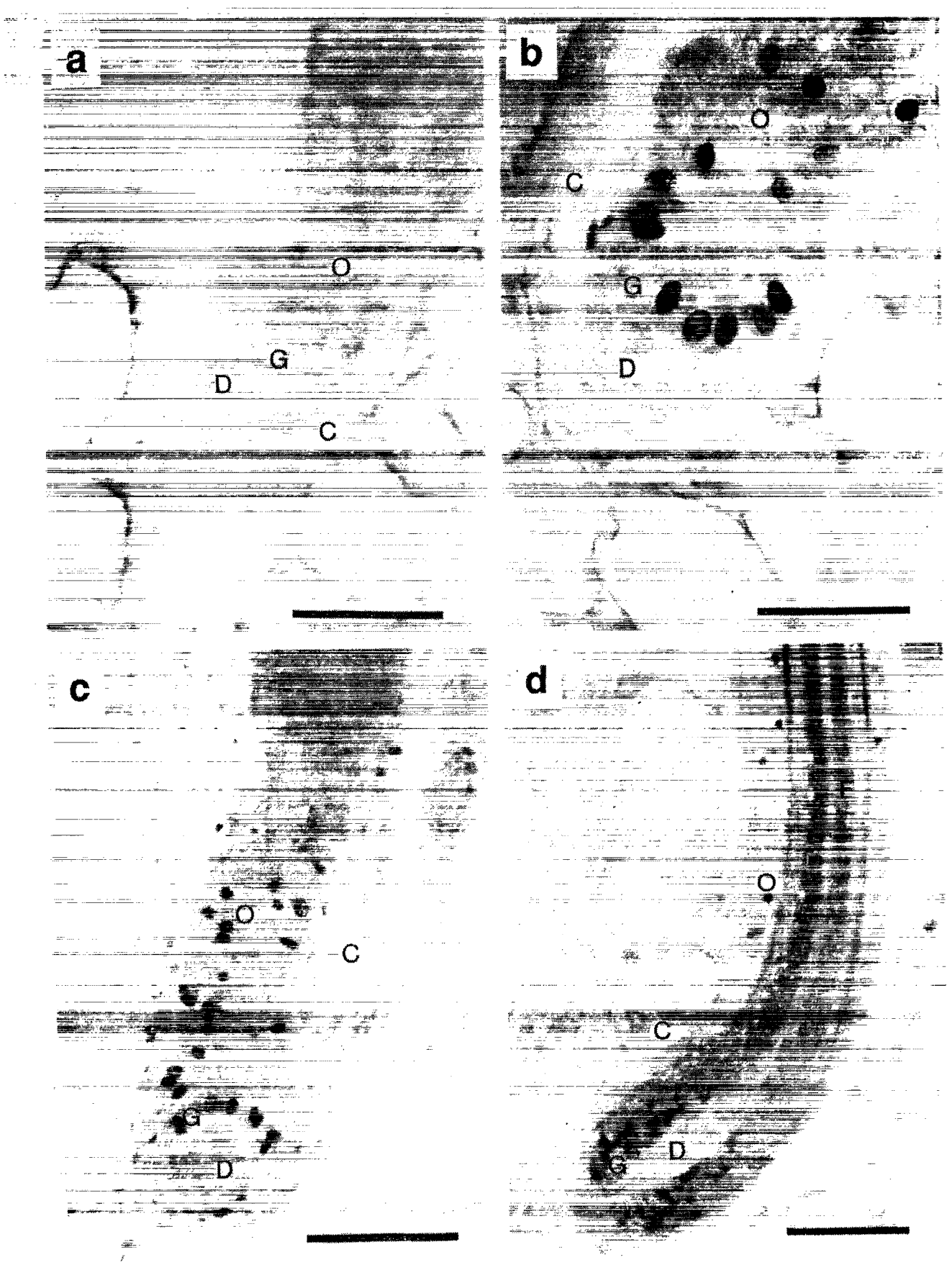
The BrdU labelling method and a crude cell labelling index was successful in determining the time of cell activation in hair follicles synchronised with melatonin. Patterns of proliferation could also be traced within separate cell populations from telogen to anagen VI stages of the hair cycle. The contrasting states provided by the model system reduced the need to ascertain cell cycle times and cell numbers that are sometimes required in experiments involving more subtle effects in continuously growing wool follicles (e.g. Hynd and Everett 1990).

Very few labelled cells were present in the bulb region of telogen follicles. Their absence indicates that most telogen follicle bulb cells were in G1 or G0 phase, as demonstrated by Silver and Chase (1970) using [3H]-thymidine treated mice. DNA synthesis started in all four tissues between days 2 and 4 after the melatonin stimulus, and about two days prior to observable structural change. In the mouse by comparison, thymidine incorporation was detected between 6 and 12 hours after plucking (Silver and Chase 1970). However, fibre plucking may invoke a different local mechanism, as indicated by trauma produced throughout the follicle (Silver *et al.*, 1969). Cell cycle times of about 12 hours in murine hair follicles are also comparatively short (Fraser 1965). Operation of endocrine or paracrine factors involved in transmitting the stimulus might therefore be elucidated in the ferret by comparing telogen follicles with day 2 to 4 proanagen follicles.

These results also illustrate the need to distinguish between closely associated cell populations when using the induced proanagen model. The hair germ region consists of some cells that form the new inner root sheath and fibre, and others that differentiate into outer root sheath. In the latter case, proliferation is rapid during follicle regeneration, but subsequently declines once the stable metanagen follicle is formed. Fibrocytes of the connective tissue sheath similarly divide to accommodate the new longer follicle. Proliferative behaviour described in proanagen follicles therefore represents follicle metamorphosis as well as initiation of keratinocyte production.

The dermal papilla, usually thought of as mitotically inactive, also showed a rise in S-phase cells during follicle regeneration. These cells have been shown to stimulate fibre growth in epidermal tissue (Oliver and Jahoda 1989) and cellular activity

FIG 1 S-phase cells labelled with BrdU in primary original hair follicles during fibre growth initiation. Nuclei of S-phase cells are stained black. (a) Telogen hair follicle showing absence of labelling. Bar = 30 µm. (b) Cell proliferation begins in early proanagen. Bar = 30 µm. (c) Follicle regeneration proceeding at eight days post-implant. Bar = 60 µm. (d) Fibre growth continues in fully active or metanagen ferret hair follicle. Bar = 300 µm. C: connective tissue sheath, D: dermal fibrocytes (papilla), F: new fibre and inner root sheath G: hair germ, O: epithelial strand - new outer root sheath.



during proanagen could be related to this regulatory function. However, it is noteworthy that the rise in cell proliferation occurred after the hair germ had begun to divide and invaginate. Silver and Chase (1977) similarly found that RNA synthesis in the hair germ preceded that in the dermal papilla during proanagen.

In conclusion, the BrdU labelling method has provided markers for early stages of follicle development during proanagen. Identification of these events is essential to ongoing work to determine significant factors controlling fibre growth.

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