Androgen receptors are only present in mesenchyme-derived dermal papilla cells of red deer (*Cervus elaphus*) neck follicles when raised androgens induce a mane in the breeding season

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Abstract

Red deer stags produce an androgen-dependent mane of long hairs only in the breeding season; in the non-breeding season, when circulating androgen levels are low, the neck hair resembles the rest of the coat. This study was designed to determine whether androgen receptors are present in deer follicles throughout the year or only in the mane (neck) follicles when circulating testosterone levels are high in the breeding season. Although androgens regulate much human hair growth the mechanisms are not well understood; they are believed to act on the hair follicle epithelium via the mesenchyme-derived dermal papilla. The location of androgen receptors in the follicle was investigated by immunohistochemistry and androgen binding was measured biochemically in cultured dermal papilla cells derived from mane and flank follicles during the breeding season and from neck follicles during the non-breeding season.

Immunohistochemistry of frozen skin sections using a polyclonal antibody to the androgen receptor localised nuclear staining only in the dermal papilla cells of mane follicles. Saturation analysis assays of 14 primary dermal papilla cell lines using [^{3}H]-mibolerone demonstrated high-affinity, low-capacity androgen receptors were present only in mane (breeding season neck) cells; competition studies with other steroids confirmed the specificity of the receptors. Androgen receptors were not detectable in cells from either the breeding season flank nor the non-breeding season neck follicles.

The unusual biological model offered by red deer of androgen-dependent hair being produced on the neck in the breeding, but not the non-breeding season, has allowed confirmation that androgen receptors are required in follicle dermal papilla cells for an androgen response; this concurs with previous human studies. In addition, the absence of receptors in the non-breeding season follicles demonstrates that receptors are not expressed unless the follicle is responding to androgens. Androgen receptors may be induced in mane follicles by seasonal changes in circulating hormone(s).

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Introduction

Clearly visible long, pigmented hair near the face is a secondary sexual characteristic which distinguishes the adult male of several species including man, the lion and the red deer. These follicles are known to respond to androgens in man (reviewed Randall 1994, Randall 2000*a*) and the red deer (Lincoln 1971*a*); this is inferred for the lion. Although hormones are known to regulate the type of hair produced by many mammalian hair follicles (reviewed Ebling *et al.* 1992), the precise mechanisms of how alterations occur in the type of hair produced by a hair follicle are unclear. The red deer stag offers an exciting model for studying androgen action in hair follicles because the annual seasonal rises in testosterone secretion associated with the breeding season are directly involved in altering various secondary sexual characteristics, including the conspicuous neck mane (Lincoln 1971*b*, Lincoln *et al.* 1972).

Seasonal coat changes occur twice a year in the red deer (Ryder & Kay 1973, Curlewis *et al.* 1988) and many other mammals (Ebling *et al.* 1992) to accommodate alterations in the environment due to the insulative and camouflage functions of their hair. In addition, many follicles produce different hair types in adults as part of social and sexual communication, such as human beard and pubic hair

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follicles (reviewed Randall 1994, Randall 2000*a*). All hair follicles pass through regular cycles of regression (telogen) and regeneration (anagen) to form new hairs enabling these changes to occur. This is frequently under hormonal control (reviewed Randall 1994). The hair produced may be similar to the previous one or markedly different, such as the neck mane hairs of the adult male red deer which are only produced during the breeding season (winter coat). Hormones link the hair growth cycle in many mammals to climatic variation, particularly the length of daylight and temperature (Ebling *et al.* 1992), and the moulting and reproductive cycles are closely harmonised in seasonal breeders like the red deer (Loudon *et al.* 1989). The details of how this occurs are not yet known. The long hairs of the red deer neck mane are at least twice the length of the other winter coat hairs and develop from August until December (Lincoln 1971*b*), coinciding with the increase in concentrations of plasma testosterone. Testosterone concentrations remain low throughout the spring and early summer (Suttie *et al.* 1984) when the winter coat is replaced by the summer coat and the mane is replaced with short neck hairs, similar to the rest of the summer coat. The red deer stag, therefore, offers an interesting model system in which large androgen-dependent hair follicles are readily available unlike human material.

Although the mechanisms involved in the hair cycle are not fully understood and are the subject of much current research (Paus *et al.* 2000), the mesenchyme-derived dermal papilla found at the base of the hair follicle is known to play a major regulatory role inducing new follicular development and strongly influencing the epithelial components of the hair follicle to determine the type of follicle and fibre produced (Jahoda & Reynolds 1996). The dermal papilla is generally believed to be the site of androgen action in the hair follicle (discussed Randall *et al.* 1991, Randall 1994, Randall 2000*a*), particularly since rat follicle dermal papillae, but not follicular epithelial cells, take up [^{3}H]-testosterone *in vivo* (Stumpf & Sar 1976) and monoclonal antibodies have located androgen receptors only in the dermal papilla of human follicles (Choudhry *et al.* 1992, Itami *et al.* 1995*a*).

This study was designed to determine the location of androgen receptors in red deer follicles and whether the presence of androgen receptors in dermal papilla cells from red deer stag follicles varied depending on their ability to respond to androgens *in vivo* and their exposure to circulating hormones. Frozen sections of mane skin were immunostained with a polyclonal antibody to the androgen receptor to localise receptor expression. To confirm that immunostaining reflected androgen receptor activity, androgen-binding activity was assessed in cultured dermal papilla cells by saturation analysis. Cultured dermal papilla cells are an important model system in hair follicle research as cultured cells retain their ability to induce follicular growth when re-implanted *in vivo* (Jahoda *et al.* 1984, Jahoda & Reynolds 1996) and human cells retain androgen-related characteristics reflecting their sites of origin (Randall *et al.* 1992, Thornton *et al.* 1993, Itami *et al.* 1995*b*, Hamada *et al.* 1996, Hibberts *et al.* 1998, Thornton *et al.* 1998). Markedly androgen-sensitive and apparently androgen-independent follicles were compared by establishing primary lines of dermal papilla cells from hair follicles from the androgen-dependent mane and from the control flank skin of 5 stags during the breeding season. To determine whether androgen receptor activity was still present in the non-breeding season, parallel lines of dermal papilla cells established from neck skin in the non-breeding season, when the mane is no longer produced, were also assayed.

Materials and Methods

Animals

Adult sexually mature male red deer, body weights 130–180 kg, were maintained at the Institute of Zoology, Whipsnade Park. They were housed in outside paddocks for the duration of the study. Biopsies were taken using a 6 mm biopsy punch (Stiefel Laboratories (UK) Ltd, Woodburn Green, Bucks, UK) under general anaesthetic, (large animal immobilon; Intervet, Bury St Edmunds, UK); animals were intubated for the duration of the sampling (5–10 min). The biopsy wound was sprayed with aerosol antibiotic (Alamycin; Norbrook Laboratories, London, UK) and each animal received an intramuscular dose of long-acting antibiotic (Duphacen LA; Solvay Duphar Veterinary Ltd, Hedge Hill, Southampton, UK) at 1 ml/20 kg bodyweight. The anaesthetic was reversed and the animals regained their feet within 3 min. Animals were under veterinary supervision throughout the procedures and were monitored for any sign of ill effect; none was observed. The biopsy sites were healed within 2 days and no post-sampling inflammation or infection was observed.

Biopsies were taken from 5 stags during the breeding season (September and October) from the androgen-dependent mane area and the mid-rib areas of the flank as a non-androgen dependent control. Skin biopsies were removed from both sites of the animals at the same time, to enable a direct comparison of receptor levels from the two regions. During the non-breeding season (May/June) when the summer coat was growing, corresponding biopsies were taken from similar areas from 5 stags and processed in the same manner. All procedures were undertaken in accord with appropriate project and personal licences under the Animal (Scientific Procedures) Act of 1986, UK.

Immunohistochemistry

Full depth skin samples were frozen in OCT (Raymond A Lamb, London, UK) and maintained at –20°C until

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required. Vertical frozen sections (7 μm) of deer skin were cut carefully in parallel to the direction of hair growth and mounted on poly-L-lysine coated slides. After fixing immediately in Zamboni's fixative (Steffanni *et al.* 1967) for 10 min at room temperature, sections were processed for immunostaining. Each incubation step was carried out at room temperature and separated by washing with phosphate-buffered saline unless specified otherwise.

Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in 1:1 methanol: water for 30 min and non-specific binding reduced by 20 min with 3% bovine serum albumin. Sections were incubated overnight at 4°C with a rabbit polyclonal antibody (Novocastra Laboratories Ltd, Peterborough, UK) to the human androgen receptor at a 1:600 dilution in 2% normal horse serum. After 30 min with the secondary anti-rabbit Ig biotinylated antibody (Vector Labs Ltd, Peterborough, UK), the sections were incubated with an avidin-biotin complex, Vectastain ABC reagent, (Vector) for 30 min, followed by the chromagen solution VIP peroxidase substrate (Vector) for 15 min. Positive staining was purple. Before mounting in DPX (Merck Ltd, Leicester, UK), sections were dehydrated in 95% alcohol and absolute alcohol and cleared in 50:50 (v:v) histoclear:ethanol (National Diagnostics, Hessle, UK) and histoclear, each step taking 2 min.

Positive (rat prostate) and negative controls (phosphate buffered saline (PBS) and a monoclonal antibody to *Asperigillus niger* (plant protein; Dako Ltd, High Wycombe, UK)), were carried out with each set of incubations. Histochemical staining with SACPIC (Nixon 1993, Nutbrown & Randall 1996), which clearly defines many layers of the follicle, was also carried out in parallel to confirm the structure of red deer follicles.

Cell culture

Red deer dermal papilla and dermal fibroblast cell cultures were established and grown as described previously (Thornton *et al.* 1996). Briefly, biopsies were wrapped in sterile gauze soaked in PBS and kept at 4 °C until the following day. Individual anagen follicles were dissected out from the surrounding skin under the microscope and the intact, membrane-bound dermal papilla removed from the surrounding epithelium at the base of each follicle using 21 gauge syringe needles. Five to six isolated papillae were incubated in a sterile 35 mm Petri dish (Falcon Labware, Becton Dickenson, Cowley, Oxford, UK) and left undisturbed for one week, after which most had attached to the bottom of the dish and cells had begun to grow out from the explants. The cells from each biopsy were kept separate at all times. Dermal fibroblasts from the same skin samples were also established by explant culture. The culture medium was E199 supplemented with glutamine (2 mmol/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml), amphotericin B (2·5 μg/ml) (Flow Laboratories Ltd, Ivrine, Strathclyde, UK) and 20% foetal bovine serum (Globefarm Ltd, Esher, Surrey, UK). Cultures were established and maintained in a humidified atmosphere containing 5% CO_2 in air. After one week the dishes were topped up with fresh medium. After about four weeks cell outgrowths were of sufficient density for subculture and were transferred to 25 cm^2 tissue culture flasks (Flow Laboratories Ltd); thereafter, medium was supplemented with 10% foetal bovine serum. These cells, forming passage 1, were grown for 1–2 weeks in the flask before subculturing with a split ratio of 1:3 or 1:4.

Androgen receptor assay

The ability of deer dermal papilla cells to bind [^{3}H]-mibolerone was determined by saturation analysis in 14 cell lines derived from primary cultures all at the fourth passage, by the method previously described for human dermal papilla cells (Randall *et al.* 1992). Briefly, the cells were seeded at 2×10^5 cells/dish and grown in 100 mm Petri dishes in normal growth medium until almost confluent. The growth medium was removed, and the cells washed twice with PBS, before incubation for 24 h in serum-free medium. The cells were incubated for 2 h at 37 °C in fresh serum-free medium with a range of concentrations of [^{3}H]-mibolerone (0·05–10 nmol/l; 7–10 concentration points). A 100 × excess of radioinert 5α-dihydrotestosterone (Sigma Chemicals, Poole) or mibolerone (Amersham International plc, Amersham, Bucks, UK) was added to a duplicate set of cells to determine the non-specific binding. An excess (1000 ×) of triamcinolone acetonide (9-fluoro-16-hydroxyprednisolone-16α,17α-acetonide) (Sigma Chemicals) was added to all incubations to prevent any potential binding to progestin receptors. The cells were washed with cold (4 °C) PBS (× 4) and scraped from the dishes into cold PBS before centrifuging; the supernatant was discarded and the cellular radioactivity extracted with chloroform:methanol (1:1). The radioactivity in the supernatant was measured on an LKB liquid scintillation spectrophotometer for 10 min with a counting efficiency of 50%. Aliquots of the medium were also counted to obtain the amount of unbound or 'free' radioactivity remaining. The remaining cell pellets from the individual Petri dishes were retained for protein measurement (Lowry *et al.* 1951). The number of cells present at the time of the assay, about 250 000 per dish, was determined by counting the cells from a parallel Petri dish six times using a haemocytometer.

Specificity of steroid binding

The ability of various unlabelled steroids to compete for the [^{3}H]-mibolerone binding was investigated to confirm that the binding was to specific androgen receptors. Dermal papilla cells were derived from the mane of 3 animals during the breeding season and prepared as

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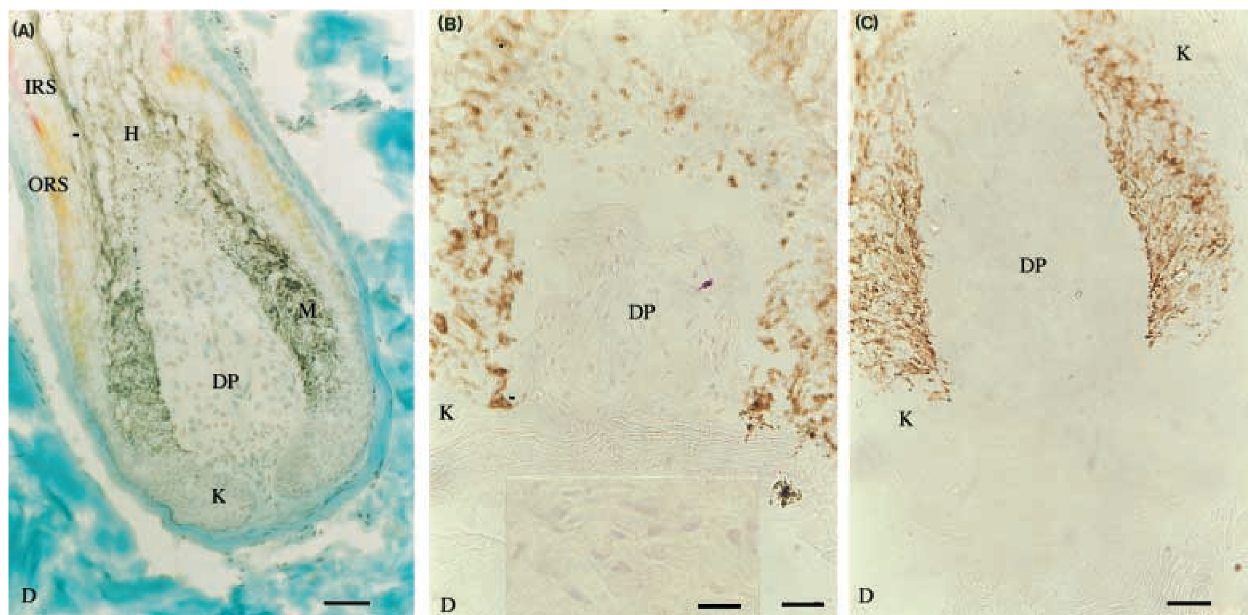

Figure 1 (A) Longitudinal section through the hair bulb of a red deer stag mane follicle stained with the histological stain, SACPIC. The membrane-bound dermal papilla (DP) is surrounded by keratinocytes (K) dividing and differentiating to form the various layers of the hair follicle. ORS: outer root sheath, IRS: inner root sheath, H: hair, D: dermis, scale bar: 9·2 µm. In the upper part of the bulb dark melanocytes (M) can be seen producing dark pigment. (B) Nuclei of dermal papilla (DP) cells but not keratinocytes (K) of red deer mane follicles were stained purple by immunostaining with a polyclonal antibody to the androgen receptor. (Note the relatively low cell numbers in the dermal papilla which contains extensive extracellular matrix as shown in Fig. 1A.) Dark pigment produced by the melanocytes (M) can be seen above and around the dermal papilla. D: dermis, scale bar: 7·3 µm. Insert at higher magnification shows the nuclear nature of staining. Scale bar: 2·9 µm. (C) Staining was not seen in neck follicles from the non-breeding season. Details as Fig. 1B.

described previously (Randall *et al.* 1992). Cells from passage 4 were incubated in triplicate with 1 nmol/l [^{3}H]-mibolerone or with 1 nmol/l [^{3}H]-mibolerone plus a range of radioinert steroids; these included the androgens, testosterone (17β-hydroxy-4-androsten-3-one), 5α-dihydrotestosterone (17β-hydroxy-5α-androstan-17-one), mibolerone (Amersham International plc), 17β-oestradiol (1,3,5(10)estratriene-3,17β-diol), triamcinolone acetonide and cortisol (11β,17,21-trihydroxy-4-pregnene-3,20-dione) (Sigma Chemicals), and the antiandrogen, cyproterone acetate (a gift from Schering), all at a concentration of 100 nmol/l for 2 h in serum-free medium at 37 °C. Binding was assessed as described previously (Randall *et al.* 1992).

Results

Immunohistochemistry

The structure of the red deer hair follicle was very similar to that of the human and other mammals with sebaceous glands attached to the upper follicle and a distinct membrane-bound dermal papilla situated at the base of the hair bulb (Fig. 1A). Anagen follicles exhibited all the normal differentiating layers to produce the outer and inner root sheaths and the hair itself. Mane hair contained a very well-developed medulla. The hair bulb around and above the dermal papilla consisted of dividing and differentiating keratinocytes and melanocytes visibly producing pigment (Fig. 1A).

Purple androgen receptor staining was seen in the control rat prostate sections; negative controls showed no colour. In deer skin staining was seen only in the nuclei of dermal papilla cells of the hair follicle and the outer cells of the associated sebaceous glands of follicles from the mane (Fig. 1B). Corresponding neck follicles from the non-breeding season did not exhibit staining in the dermal papilla (Fig. 1C).

Cell culture

After one week in culture primary dermal papilla cells began to grow out from the isolated dermal papilla explants. There was no evidence of epithelial contamination and the dermal papilla cells, although fibroblastic, displayed a different morphology to corresponding fibroblasts grown for comparison when examined under phase contrast microscopy. There was no difference in the morphology of cells isolated from mane or flank follicles. Dermal papilla cells from both sites, and from both seasons,

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assumed a polygonal shape and irregular organisation, but unlike human (Messenger *et al.* 1986) and rat (Jahoda & Oliver 1981) dermal papilla cells, did not form aggregates in culture. Dermal fibroblasts exhibited an elongated shape and formed parallel arrays (Thornton *et al.* 1996). There was no difference in the rate of growth of cells from mane or flank established at the same time, although winter cells did grow slightly faster than summer cells (Thornton *et al.* 1996). Cells from mane and flank follicles from both seasons were successfully established and passaged several times in culture.

Androgen-dependent follicles: breeding season mane

Saturation of specific [^{3}H]-mibolerone binding occurred in all 5 of the primary cell lines derived from the mane during the breeding season at about 1 nM. Scatchard plots were calculated for each cell line (e.g. Fig. 2) to enable the determination of the dissociation constant (Kd), to indicate the affinity of the receptor for the ligand, and the maximum number of binding sites available (Bmax). Cells derived from the mane cells had a mean Kd of 0·38 ± 0·12 nmol/l (n=5; ± S.E.M.) and Bmax of 0·035 ± 0·008 fmol/10^4 cells (n=5; Fig. 3).

Follicles not exhibiting an androgen response: breeding season flank and non-breeding season neck

Specific binding was undetectable in three lines of dermal papilla cells from the breeding season flank follicles of the same individuals and very low in the other two lines (Fig. 3). Similarly, little or no specific binding could be measured in dermal papilla cells from the neck region of the summer coat, i.e. the non-breeding season and low levels were only detectable in one line (Fig. 3).

Specificity of radioligand binding

The use of either unlabelled mibolerone (10 cell lines) or 5α-dihydrotestosterone (4 cell lines) to determine non-specific binding made no difference to levels of specific binding. The androgens, testosterone, 5α-dihydrotestosterone and mibolerone, and the antiandrogen, cyproterone acetate, were all potent competitors for the radioligand in mane dermal papilla cells (Fig. 4). Oestradiol also had some effect, reducing binding by about one third; triamcinolone acetonide (with progestational and corticosteroid action) and cortisol had no effect.

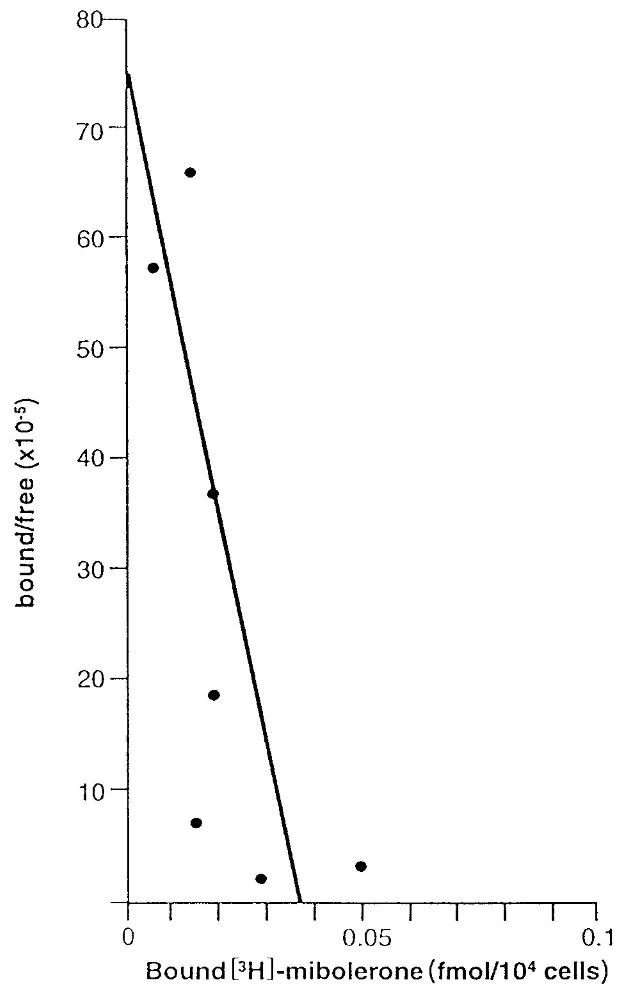

Figure 2 A typical example of a Scatchard analysis of [^{3}H]-mibolerone binding to the androgen receptor of cultured dermal papilla cells (passage 4) derived from red deer mane hair follicles.

Discussion

The polyclonal antibody to the human androgen receptor stained the nuclei of the mesenchyme-derived dermal papilla cells, but not follicular keratinocytes, in the red deer stag mane follicles (Fig. 1). Although caution is required to avoid overinterpretation when using immunohistochemistry, especially across different species, this distribution replicates that already reported in the human hair follicle using monoclonal antibodies to the human androgen receptor. (Choudhry *et al.* 1992, Itami *et al.* 1995*a*, De Oliveira & Randall 2000). It also concurs with the current hypothesis that androgens act on other components in the follicle via the dermal papilla (Randall 1994, 2000*a*). No staining was detected in neck follicles from the non-breeding season when a mane is not present.

The immunohistochemical observations are also supported by the biochemical measurement of androgen-binding activity in cultured dermal papilla cells from mane follicles (Fig. 3). The saturation analysis method using the non-metabolisable androgen mibolerone as the ligand was established for cultured human dermal papilla cells

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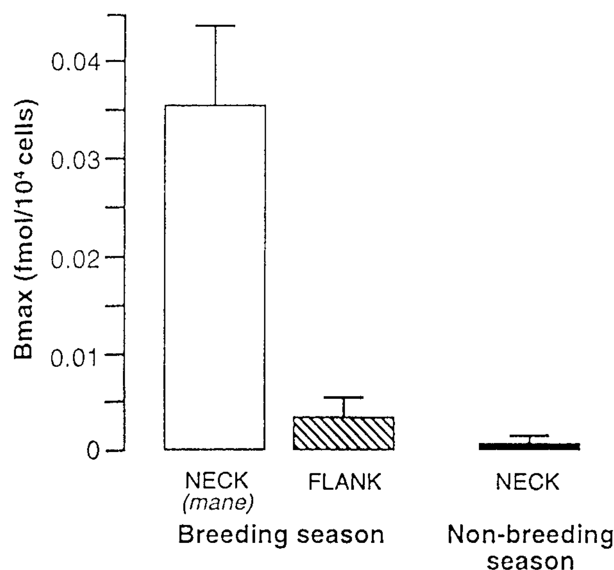

Figure 3 Androgen receptors (Bmax) were only present consistently in primary lines of cultured dermal papilla cells derived from androgen-dependent mane follicles and not those from androgen-independent flank follicles in the breeding season or neck follicles in the non-breeding season. The breeding season mane and flank cells are matched pairs from 5 stags, derived from follicles taken at the same time. The non-breeding season neck cells (n=4) were derived several months later. All cells were assayed at passage 4. Results are the mean ± standard error of the mean.

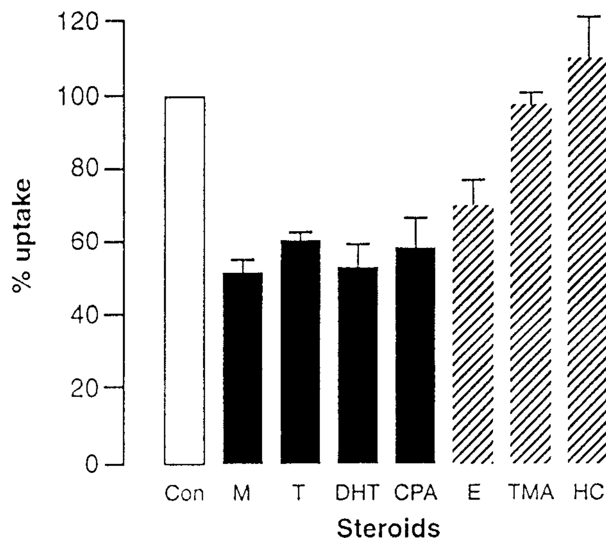

Figure 4 Effect of 100x excess (100 nM) of various natural and synthetic steroids on the uptake of 1 nM [^{3}H]-mibolerone by dermal papilla cells from breeding season mane follicles of red deer stags. Uptake was expressed as a percentage of the amount retained with [^{3}H]-mibolerone alone. Each point represents the mean of 3 measurements in each of 3 cell lines ± the standard error of the mean. ^{3}H: [^{3}H]-mibolerone alone; M: mibolerone; T: testosterone; DHT: 5α-dihydrotestosterone; CPA: cyproterone acetate; E: oestradiol; TMA: trimancinolone acetonide; HC: hydrocortisone.

(Randall *et al.* 1992, Hibberts *et al.* 1998). Since previous studies of 5α-reductase activity (Itami *et al.* 1990, 1991) and testosterone metabolism by cultured human dermal papilla cells (Thornton *et al.* 1993, Hamada *et al.* 1996) have shown that beard cells have a greater metabolic activity than scalp or pubic cells, there may be some differential metabolism of the 100-fold excess of 5α-dihydrotestosterone used to saturate non-specific binding in the various types of deer cells. However, this is unlikely to affect the results presented here as there were no differences in the amount of specific binding detected when either radioinert mibolerone or 5α-dihydrotestosterone was used to determine non-specific binding.

Saturation of binding occurred in all cell lines established from the large mane follicles during the breeding season at around 1 nM. The affinity for the ligand (Kd) was similar to that of our previous studies on human dermal papilla cells (Randall *et al.* 1992, Hibberts *et al.* 1998), and was also in the range reported for androgen receptors in classical androgen-target tissues such as the prostate (Schilling & Liao 1984) and genital skin fibroblasts (Kaufman *et al.* 1983). Since cultured dermal papilla cells produce large amounts of extracellular matrix proteins (Messenger *et al.* 1991) which may contribute to the amounts of protein measured, it is more accurate to compare the amounts of receptor bound (Bmax) in relation to cell rather than protein content. The levels of binding detected in dermal papilla cells derived from the mane follicles (Bmax 0·035 ± 0·008 fmol/10^4 cells; mean ± S.E.M.) were very similar to those reported in our previous study on human dermal papilla cells derived from androgen-dependent beard follicles (0·033 fmol/10^4 cells) (Randall *et al.* 1992). The specificity of the androgen binding was also similar to our previous studies on human dermal papilla cells (Randall *et al.* 1992), since the binding of [^{3}H]-mibolerone to mane deer cells was significantly inhibited by various androgens and an antiandrogen and to a lesser extent, by oestradiol, but not other steroids (Fig. 4).

In contrast to mane dermal papilla cells, cells simultaneously derived from the corresponding flank regions of the same animals contained little or no specific binding (Fig. 3). This is similar to previous observations in human follicles where cells from relatively androgen-insensitive non-balding scalp follicles contain significantly lower levels of receptors than those from androgen target follicles including beard, genital skin and balding scalp follicles (Randall *et al.* 1992, Hibberts *et al.* 1998). However, the difference between the deer mane and flank cells was much more pronounced than that between the androgen-sensitive human follicles and those from non-balding scalp which consistently contained measurable, although low, levels of receptors. This may be because it is difficult, if not

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impossible, to say that human scalp hair follicles are androgen-independent as eventually many follicles in most people exhibit inhibition in response to androgens (Hamilton 1951, reviewed Randall 2000*b*). Eyelash follicles would be much more suitable as androgen-insensitive human follicles, but this material is not normally available for study.

In the non-breeding season, when a mane is not distinguishable from the rest of the coat, cells derived from neck follicles contained very little androgen binding activity (Fig. 3). This suggests that the response of follicles to androgens is complex. Androgen-dependent follicles must have the genetic ability to express the androgen receptor in their dermal papilla cells; this is presumably determined by patterning mechanisms during embryonic development both in human beings (e.g. beard versus eyelashes) and deer. In addition, some other factor, probably from the circulation, appears to facilitate the expression of androgen receptors within those follicles genetically programmed to respond which enables the raised androgen levels of the breeding season to stimulate mane growth in the stag. Seasonally changing hormones, such as androgens (Lincoln 1971*a*,*b*; Lincoln *et al.* 1972), or prolactin, which is implicated in the regulation of hair growth in several species (Webster & Barrell 1985, Duncan & Goldman 1984) are potential candidates for a role in controlling receptor expression in hair follicle.

In summary, this study has identified the presence of androgen receptors in dermal papilla cells in the mane follicles of the red deer both *in vivo* and *in vitro*. The correlation of the presence of specific, high affinity, low capacity androgen receptors *in vitro* in dermal papilla cells with the androgen sensitivity of follicles *in vivo* supports the hypothesis that androgens act on androgen-dependent hair follicles via the dermal papilla. Since the mane of the red deer only grows for a short period each year, unlike the several year cycle of the human beard or scalp, it provides a good model to study the effects of androgens on hair follicle cells. Further studies taking advantage of this excellent model should provide a better understanding of the hormonal regulation of hair growth and should enable better therapeutic regimes to be developed for poorly controlled androgen-dependent disorders such as hirsutism or androgenetic alopecia.

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