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No Direct Mitogenic Effect of Sex Hormones on Antlerogenic Cells Detected *in Vitro*

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Deer pedicles, antecedents of antlers, develop from a specialized periosteum (antlerogenic periosteum) which overlies the lateral crest of the deer frontal bone. The initiation of pedicle growth is triggered by androgen hormones. Thus far, it is not known whether pedicle initiation is caused by direct stimulation of androgen hormones on the antlerogenic periosteum or whether some intermediate mechanisms are necessary. The present study took an *in vitro* approach to investigate whether sex hormones have direct mitogenic effects on primary cultured antlerogenic periosteal cells (antlerogenic cells). Antlerogenic cells were obtained from two 5-month-old red deer calves. The cells were passaged twice and then treated with testosterone, dihydrotestosterone, and estradiol. The proliferation assays showed that no direct mitogenic effects on the second passage antlerogenic cells could be detected with any of the sex hormone treatments ($P > 0.05$). Testosterone-binding studies showed that at the second passage, specific testosterone-binding sites were present in the antlerogenic cells. Therefore, we conclude that androgens do not have mitogenic effects on antlerogenic cells *in vitro*. Our results suggest that pedicle formation may not be the result of direct stimulation of androgen hormones on antlerogenic tissue. Instead, androgen hormones may only allow the process to proceed by increasing the sensitivity of antlerogenic cells to mitogens, e.g., some growth factors.

Key Words: antlerogenic periosteum; antler; pedicle; *in vitro*; sex hormone; IGF1.

Deer antlers are cranial appendages which are cast and which fully regenerate each year. Antlers do not form directly from the heads of deer; instead, they form from the permanent protuberances called pedicles (Li and Suttie, 1994). Deer are not born with pedicles; these develop from the frontal bone when deer approach puberty. When a pedicle reaches its species-specific length (5–6 cm long in red deer), a first antler generates spontaneously from its apex. After initiation, first antlers enter a rapid growing period and then become calcified during the rutting season. The first hard antlers drop off from the pedicles in the next spring and growth of new soft antlers immediately follows. From then on, antler development enters a well-defined annual cycle.

Pedicles and antlers are male secondary sexual characters. As such, pedicle formation and the antler growth cycle are under the control of androgen hormones (Jaczewski, 1982). However, the mechanism underlying this control is not known. Unlike cattle or sheep horns, which are skin derivatives, deer antlers are organs of bone (Goss, 1983). It has been shown that histogenesis of a pedicle and an antler depends solely on the periosteum overlying the lateral crest of deer frontal bone (Hartwig and Schrudde, 1974). This specialized periosteum is called antlerogenic periosteum (AP) (Goss and Powel, 1985). The discovery of the AP offers an opportunity to investigate whether pedicle

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initiation results from direct stimulation by androgen hormones or whether some intermediate mechanisms are necessary.

Li *et al.* (1999) reported the mitogenic effects of IGF1 on antlerogenic cells (the cells released from antlerogenic periosteum) *in vitro* in serum-free medium, and also tested the mitogenic effects of a physiological concentration (10 nM) of testosterone either alone or in the presence of IGF1. The results showed that no direct mitogenic effects were detected at a physiological concentration of testosterone, although some additive effects were detected in combination with IGF1. However, it is possible that antlerogenic cells *in vitro* might react to a different level of testosterone from those *in vivo*. Testing a wide range of concentrations of testosterone would be necessary to evaluate this possible activity. Besides, testosterone also functions on the target tissues through the following two metabolic pathways: reduction to dihydrotestosterone (DHT) or aromatization to estradiol (Bonsall *et al.*, 1989; Cooper *et al.*, 2000). Therefore, these two pathways also need to be investigated in addition to the direct effect. In addition, it is not known whether the primary cultured antlerogenic cells still retain specific androgen-binding sites. Also, in the previous studies the mitogenic effects of testosterone might have been masked by the cell culture medium which contained fetal bovine serum (FBS) prior to the step of incubating with serum-free medium for the addition of special growth factors. It is known that FBS normally contains some level of endogenous steroids (Makin *et al.*, 1995). Finally, D-MEM culture medium used in that experiment may not be appropriate for the purpose as the medium contains phenol red, which has lipophilic impurities that can act as estrogens (Bindal *et al.*, 1988).

The aim of this study was to determine whether sex hormones have direct mitogenic effects on antlerogenic cells.

MATERIALS AND METHODS

Tissue Biopsy and Cell Culture

Antlerogenic periosteum (AP, about 5 × 20 mm) was biopsied from two 5-month-old red deer stag calves, G657 and B627 (for detailed procedure refer to Li and Suttie, 1994). The biopsied AP was placed in culture medium (D-MEM, 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mmol/ml GlutamaxII, Gibco BRL), and then transfered to the tissue culture laboratory.

Cell culture was carried out as previously reported (Li *et al.*, 1999). Briefly, the cells were released from the tissues by incubating fine pieces (about 1 × 1 mm/piece) of AP in digestion medium (D-MEM, 2.5% FBS and 200 U/ml collagenase, Sigma, St. Louis, MO) at 37° for 24 h with occasional shaking. After release, the cells were cultured in medium in a humidified CO_2 incubator (95% air and 5% CO_2 at 37°). To carry out the tests under the same culture condition for these antlerogenic cells, which were obtained from two deer at different times, all the cells were kept frozen until the start of sex hormone treatments. The procedure for this was as follows. Upon reaching confluence, the cells were detached and then frozen in the freezing medium (D-MEM, 15% FBS, and a 15% mixture (4:6) of dimethyl sulfoxide and glycerol) in a −80° freezer. Before hormone treatments started, the frozen cells were thawed and cultured in 25-ml flasks. Once the cells reached 80–90% confluence, they were trypsinized and seeded in 24-well plates at a density of 1.5×10^4 cells/well. Each treatment was performed in triplicate wells. Cells used in this study had been through two passages. Cell viability was measured using trypan blue and was always found to be above 95%.

Proliferation Assay

The mitogenic effects of three sex hormones, testosterone, DHT, and estradiol, on antlerogenic cells were tested individually. These tests were assigned to Experiments 1, 2, and 3. These hormones were dissolved in 100% ethanol, with the final concentration of ethanol in the culture medium being 1%. The culture medium used in each treatment was phenol red-free D-MEM (Gibco BRL), and the FBS used was charcoal-treated to remove endogenous steroid hormones. Endogenous steroid hormones were removed from FBS by adding activated charcoal to FBS (100 mg/ml serum) and mixing overnight on a magnetic stirrer in a cold room (4°). The serum was then centrifuged for 1 h at 6200 rpm using a wind-shielded rotor on a DPR 7000 centrifuge to remove most of the charcoal. The centrifuged serum was filtered through a glass wool plug to remove suspended charcoal, and then further filtered through a 0.25-μm filter.

Following a 48-h initial culture after seeding of cells into 24-well plates, the D-MEM medium (10% FBS) was replaced with serum-free medium (SFM, phenol red-free D-MEM without serum, but supplemented with 0.1% bovine serum albumin). The cells were cultured for a further 24 h before the SFM was replaced with SFM, SFM + 10% serum, SFM + graded doses (0.05, 1.0, 10.0 nM) of IGF1, SFM + 1% ethanol, SFM + graded doses (0.5–50 nM) of each sex hormone or SFM with graded doses (0.5–50 nM) of each sex hormone + 0.05 nM IGF1. The cells were then cultured for a further 24 h. Two hours before the termination of the incubation, [*methyl*-^{3}H]thymidine (85 Ci/mmol, Amersham) at 2.5 μCi/ml was added into each well. When the incubation was finished, the radioactive medium was removed and the cells were washed three times with 10% trichloroacetic acid (TCA, w/v, BDH). The cells were then dissolved in 0.1 M NaOH (BDH). The solution was counted in HiSafe3 scintillatant (LKB product, SciTech, Dunedin, NZ). The proliferation rate is expressed as incorporation of [^{3}H]thymidine (dpm).

Testosterone-Binding Study

Testosterone-binding studies were carried out following the protocols described by Cullen *et al.* (1990) with some modifications. Briefly, following a 48-h initial culture after seeding of cells into 24-well plates and a further 24-h incubation with the serum-free medium, the antlerogenic cells (only from deer G657) were washed with PBS and then incubated at 0° for 1 h in binding buffer (0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 8 mM glucose, 0.1% BSA, and 0.2% azide) which contained graded doses (8, 10, 12, and 14 nM) of ^{3}H-T (95.0 Ci/mmol and 322 mCi/mg, Amersham, NZ) with or without 200 times cold testosterone. Then the cells were gently washed with cold PBS and dissolved in 200 μl 0.1 N NaOH and 100 μl was counted. The binding capacity is expressed as dpm.

Statistical Analysis

Data from the proliferation study for each sex hormone were pooled from two deer, log transformed, and analyzed by analysis of variance (ANOVA), fitting treatment, and inspecting contrast for (a) SFM versus 1% ethanol; (b) 1% ethanol versus the mean of the hormone treatments; and (c) 0.05% nM IGF1 versus the mean of the hormone treatments in combination with 0.05 nM IGF1. Data from the testosterone-binding assays were analyzed by ANOVA on the natural scale. In the analysis, a separate term for control was included in the model.

RESULTS

Proliferation Assay

Incorporation of [^{3}H]thymidine (dpm/well) by antlerogenic cells for each sex hormone is presented in Fig 1. In each case, 10% FBS culture medium showed the strongest mitogenic effects on antlerogenic cells. The effects of 0.05 nM IGF1 were significantly greater than those of SFM ($P < 0.001$) in each treatment, and then [^{3}H]thymidine incorporation increased strongly ($P < 0.001$) with increasing doses of IGF1 to a maximum level of either 1.0 nM in Experiment 1 or 10.0 nM in Experiments 2 and 3. There was no significant difference in mitogenic effects between the treatments of 1% ethanol and SFM in any of the hormone treatments ($P > 0.05$). No mitogenic effects from any of the sex hormone treatments (testosterone, DHT, or estradiol) in SFM were detected ($P = 0.38$, 0.90 and 0.09, respectively; compared to 1% ethanol) on the antlerogenic cells. The mean [^{3}H]thymidine incorporation in the 0.05 nM IGF1 + testosterone or 0.05 IGF1 + DHT treatments was not significantly different from that of 0.05 nM IGF1 ($P = 0.27$ and 0.63, respectively). The mean [^{3}H]thymidine incorporation of 0.05 nM IGF1 + estradiol was significantly higher ($P < 0.001$) than that of 0.05 nM IGF1.

Testosterone-Binding Study

There was a highly significant increase in [^{3}H]testosterone uptake for each dose of [^{3}H]testosterone

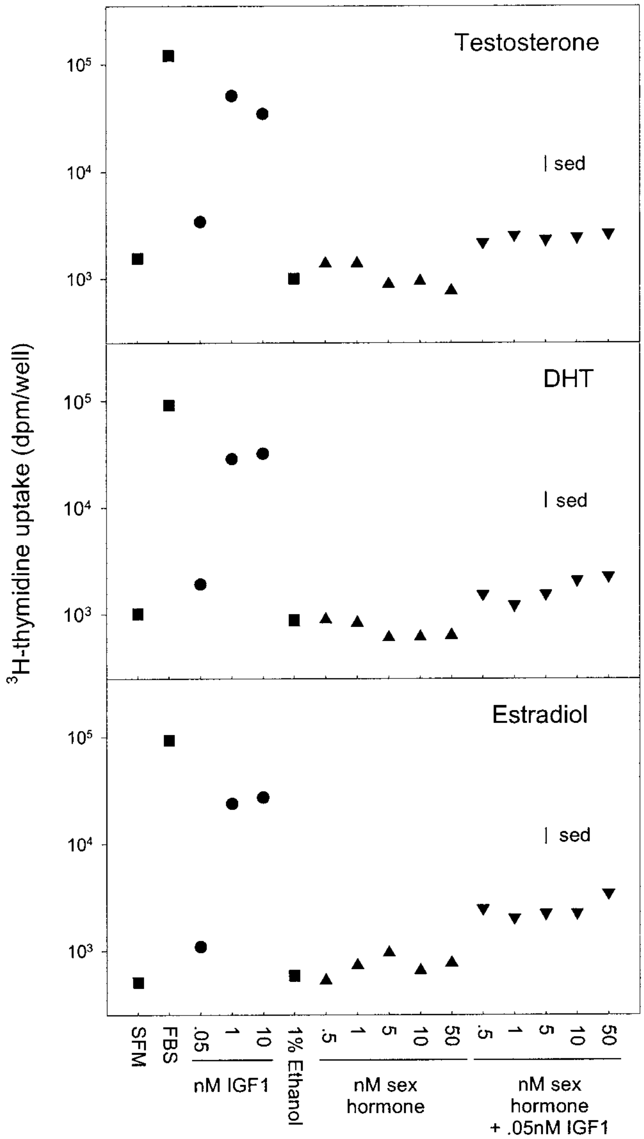

FIG. 1. Mitogenic effects of testosterone, DHT, and estradiol either alone or with IGF1 on antlerogenic cells, which were from two 5-month-old male red deer calves. After biopsy, the antlerogenic cells were enzymatically released from the fine pieces of antlerogenic periosteum and cultured in D-MEM medium with 10% FBS. The cells were detached and frozen upon reaching confluence. Each hormone treatment was carried out using the thawed cells at passage 2. Following 48-h initial culture and 24-h serum starvation, the hormone treatments were carried out. The result from each treatment is the mean of triplicates. DHT, dihydrotestosterone; SFM, serum-free medium; FBS, fetal bovine serum; IGF1, insulin-like growth factor 1; sed, standard error of difference.

treatment compared to the control ($P < 0.001$). As [^{3}H]testosterone concentration increased (8–14 nM), the [^{3}H]testosterone uptake increased significantly ($P < 0.001$) in a dose-dependent manner. A 200-fold dose of cold testosterone over [^{3}H]testosterone in each treatment was found to significantly displace [^{3}H]testosterone from binding sites in the antlerogenic cells ($P < 0.001$) (Fig. 2).

DISCUSSION

The hypothesis that pedicle initiation results from stimulation by androgen hormones on antlerogenic periosteum was advanced by Fennessy and Suttie (1985) based on two findings. One is that deer pedicle initiation and formation are under the control of androgen hormones, because prepubertal castration prevents pedicle growth in young stags. Administration of exogenous androgen hormones can readily reverse this abnormality (Jacezwski, 1982). The second is that pedicles develop from antlerogenic periosteum (AP) only, because removal of AP prior to pedicle initiation

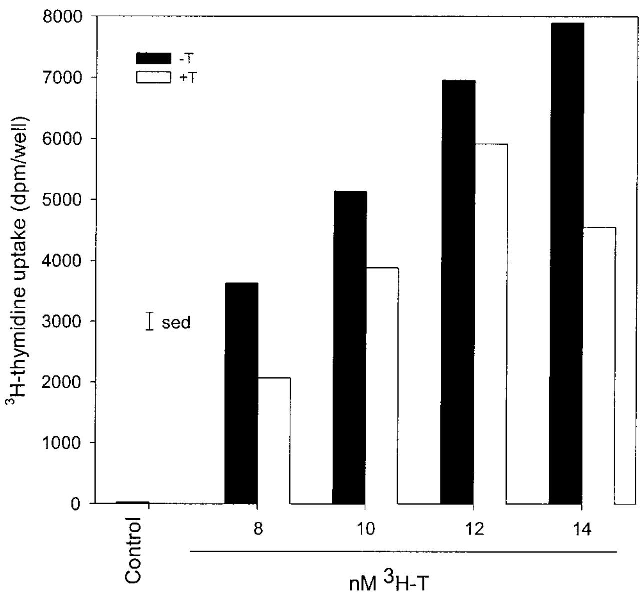

FIG. 2. Testosterone-binding assay. The process for cell culture is the same as in Fig. 1. Following 48-h initial culture and 24-h serum starvation, the cells, which were from a 5-month-old male red deer calf, were washed and incubated at 0° for 1 h in binding buffer containing different doses of [^{3}H]testosterone with or without 200 times cold testosterone. T, testosterone; −T, without cold testosterone; +T, with 200-fold cold testosterone; sed, standard error of difference.

will stop subsequent pedicle and antler formation and implantation of the AP elsewhere on the deer body will create an ectopic pedicle and antler (Hartwig and Schrudde, 1974). In addition, the fact that AP possesses specific binding sites for testosterone (Li *et al.*, 1990, 1998) strongly supports this hypothesis. However, the results from the present study showed that no direct mitogenic effects on antlerogenic cells could be detected *in vitro* from any of the sex hormone treatments. Therefore, these results are consistent with our previous findings (Li *et al.*, 1999), although the previous study showed a trend of an additive mitogenic effect of testosterone + IGF1 over IGF1 only on antlerogenic cells (statistically not significant, $P > 0.05$) and the present study did not.

The lack of mitogenic effect by testosterone is not due to the use of alternative steroid metabolic pathways, because DHT and estradiol were tested in addition to testosterone in the present study. Neither is it due to an unsuitable concentration of these hormones used *in vitro*, as a wide range of doses (0.5–50 nM) was investigated. Therefore, something else should be taken into account.

One possibility that changes in structure and function of these primary cultured antlerogenic cells took place after removal from their extracellular matrix. One possible change may be the loss of specific androgen-binding sites if the *in vitro* selection favors androgen-independent cells, as shown in some cases of primary cultured prostatic epithelial cells (Berthon *et al.*, 1997). However, our testosterone-binding studies showed that the specific binding sites for testosterone are well preserved in the present study. To retain the continuous expression of androgen hormone receptors, Peehl and Stamey (1986) cultured just released prostatic epithelial cells in a medium supplemented with DHT, as the expression of androgen receptor is autoregulated by its own ligand. In the same way we added testosterone or DHT to the medium for culturing newly released antlerogenic cells, but failed to produce cells that responded to androgens (data not shown). Czerwiec *et al.* (1997) concluded, based on their findings, that the presence of androgen hormone receptors does not guarantee that some osteoblastic cell lines will directly respond to androgen stimulation. Therefore, it is unlikely that the failure to demonstrate a dependency on androgens for growth *in vitro* of antlerogenic cells is due to the loss of specific androgen-binding sites.

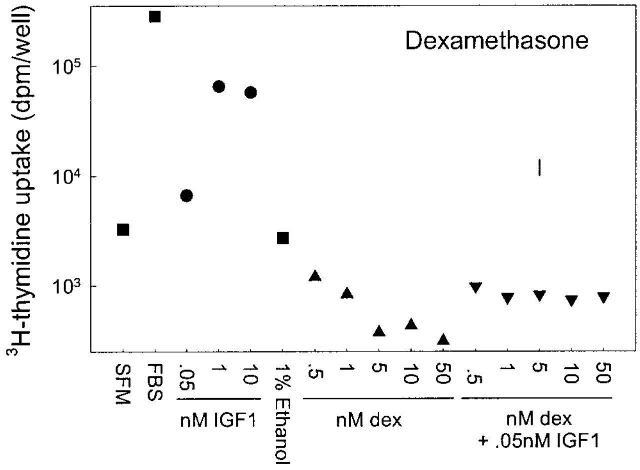

FIG. 3. Mitogenic effects of dex either alone or with IGF1 on antlerogenic cells, which were from a 5-month-old male red deer calf. The process for cell culture is the same as in Fig. 1. The result from this treatment is the mean of triplicates. Dex, dexamethasone; SFM, serum-free medium; FBS, fetal bovine serum; IGF1, insulin-like growth factor 1; sed, standard error of difference.

Another possible change may be that the sensitivity of antlerogenic cells to the stimulation of mitogens decreased after culture *in vitro*. However, the cells used in this experiment reacted not only to IGF1 in a dose-dependent manner, but also to IGF1 at doses as low as 0.05 nM ($P < 0.001$). This concentration is about 200 times lower than that *in vivo* at the time of pedicle initiation (Suttie *et al.*, 1989). Therefore, these cells should be sensitive enough to respond to any mitogenic stimulation from the sex hormones if these hormones do have mitogenic effects on these cells.

Finally, these cultured antlerogenic cells may have lost sensitivity only to the stimulation of steroid hormones, although the response to the stimulation of growth factors was well retained. However, the results from the following experiment do not support this claim. Following the same cell culture procedure for the sex hormones in the present study, we tested mitogenic effects of dexamethasone (dex), another type of steroid hormone, on these cells. The results showed that dex had a significant suppressive effect on the proliferation of the antlerogenic cells ($P < 0.001$) (Fig. 3). Beresford *et al.* (1994) reported that dex could markedly increase alkaline phosphatase expression of bony colonies that formed from the primary culture of human marrow fibroblasts, but decrease the average colony size. These results imply that dex stimulates differentiation of bone progenitor cells but inhibits proliferation of these cells. Indeed, Sadighi *et al.* (1996) found that dex could cause a threefold increase in alkaline phosphatase expression of primary cultured antler cells compared to controls. Therefore, the results from the antlerogenic cells are consistent with those from the bone projector or the antler cells. Consequently, the antlerogenic cells in the present study still retained the sensitivity to react in proliferation to at least one steroid hormone, dex.

These *in vitro* results from the present study may, nevertheless, represent the true *in vivo* situation. That is, deer pedicle formation is not the result of direct stimulation of androgen hormones on antlerogenic cells. If that is the case, what could be the real role played by androgen hormones in pedicle initiation? Instead of direct stimulation to antlerogenic cells, androgens may only allow the process to proceed by increasing the sensitivity of these cells to mitogens, e.g., potent growth factors. If this is true, anything that can sensitize antlerogenic cells should be able to trigger pedicle initiation. Indeed, pedicle formation in reindeer does not need the stimulation from androgen hormones (Lincoln and Tyler, 1994). Chemical (Robbins and Koger, 1981) or mechanical (Jaczewski, 1982) injuries to antlerogenic periosteum can readily initiate pedicle formation from castrated male deer or from normal female deer; in some cases injury is more potent than androgen hormones (Lincoln and Fletcher, 1976). However, if this explanation were conceivable, one would ask why the cultured antlerogenic cells in the present study responded to IGF1 in a dose-dependent manner without androgen hormone presensitization? One possibility would be that these antlerogenic cells had gone through mechanical stimulation due to tissue sampling and cell disaggregation, and this mechanical stimulation might have sensitized these cells. Alternatively, unknown potent growth factor(s), rather than the currently tested IGF1, may be the candidate(s) for stimulating pedicle and antler growth. These putative candidates, unlike IGF1, which does not require androgen presensitization to exert its mitogenic effects on antlerogenic cells, may only promote proliferation of androgen-sensitized androgenic cells.

In conclusion, whether pedicle growth can take place or not depends on whether antlerogenic cells are sufficiently sensitized to receive the stimulation of some potent growth factors. These cells can be sensitized by androgen hormones in most deer species of Cervinae, by something else in reindeer (Rangifer), or by injuries in any deer species that can grow antlers, although the mechanisms underlying the sensitization are thus far unknown. However, that this sensitization is linked to reproductive hormones in most deer species may have the advantage of natural selection.

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