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Effects of Insulin-Like Growth Factor 1 and Testosterone on the Proliferation of Antlerogenic Cells In Vitro

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ABSTRACT Androgen hormones and growth factors are implicated in pedicle formation and antler transformation in deer. The potential to form a pedicle and an antler is only found in the antlerogenic periosteum (AP) overlying the presumptive antler growth region. Histological studies (Li and Suttie, '94) showed that AP consists of an inner cellular layer and an outer fibrous layer. Pedicle and antler are mainly derived from the cellular layer cells of the AP. Ossification takes place in four stages: intramembranous (IMO), transitional (OPC), pedicle endochondral (pECO) and antler endochondral (aECO). However, the precise mechanism whereby androgen hormones and growth factors control pedicle and antler formation is unknown. The aim of this study was to use cell culture techniques to investigate how testosterone and IGF1 affects the proliferation of antlerogenic cells from the four ossification stages of pedicle/antler in vitro. The results showed that in serum-free medium IGF1 stimulated the proliferation of antlerogenic cells from all four ossification stages in a dose-dependent manner. In contrast, testosterone alone did not show any mitogenic effects on these antlerogenic cells. However, in the presence of IGF1, testosterone increased proliferation of the antlerogenic cells from the IMO and the OPC stages (pedicle tissue), and reduced proliferation of the antlerogenic cells from transformation point (TP) and aECO stages (antler tissue). Therefore, the results from the present in vitro study support the in vivo findings that androgen hormones stimulate pedicle formation but inhibit antler growth. The change in the mitogenic effects of testosterone on antlerogenic cells from positive to negative occurs approximately at the change in ossification type from OPC to pECO. Therefore, these results reinforce the hypothesis that the transformation from a pedicle to an antler takes place at the time when the ossification type changes from OPC to pECO rather than at the time when the pedicle grows to its full species-specific height. *J. Exp. Zool. 284:82–90, 1999.*

Antlerogenic periosteum (AP) has the full potential to form a pedicle and an antler (Hartwig and Schrudde, '74; Goss and Powel, '85). It has been reported that AP, like the periosteum elsewhere on the bone surfaces, consists of two layers, an outer fibrous layer and an inner cellular layer, although both layers are much thicker than those of non-antlerogenic periosteum (Li and Suttie, '94). It has been suggested that the cellular layer cells (C cells) and fibrous layer cells (F cells) of somatic bone are different in lineage (Ham and Harris, '71; Sissons, '71). Our previous studies showed that the histogenesis of pedicles and first antlers, which was mainly dependent on the cellular layer cells of AP, covered four ossification stages, namely, intramembranous (IMO), transitional (OPC), pedicle endochondral (pECO) and antler endochondral (aECO) (Li and Suttie, '94).

It is well established that androgen hormones stimulate pedicle initiation (Jaczewski, '82; Goss, '83) but inhibit antler growth (Goss, '68), and have no effects on the transformation from pedicle to antler (Suttie et al., '98). However, the way in which androgen hormones function on antlerogenic cells (C and F cells) is controversial (Bubenik, '82; Fennessy and Suttie, '85). Because Li et al. ('97) found specific binding for testosterone in the AP cells, they considered that testosterone could play a role in directly stimulating deer pedicle initiation, either alone or with other factors.

Apart from androgen hormones, pedicle initiation is also dependent on body size and nutritional

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status, since deer initiate pedicle growth only when they reach a species-specific body weight (Suttie and Kay, '82). It is postulated that nutrition may control pedicle growth through the insulin-like growth factor 1 (IGF1) pathway (Li and Suttie, '96), as the peripheral IGF1 level was significantly and positively correlated with live weight gain during the period of pedicle and first antler formation (Suttie et al., '89). In addition, IGF1 increased proliferation of antler mesenchymal cells (antler C cells) as measured by thymidine incorporation in vitro in a dose-dependent manner (Sadighi et al., '94). Whether IGF1 could also influence proliferation of C and F cells from different pedicle developmental stages in vitro in a manner similar to antler mesenchymal cells is not known. Likewise, the relative roles of testosterone and IGF1 at different pedicle forming stages are not known.

The aim of this study was to use cell culture techniques to culture C and F cells from four stages of pedicle and antler development and to investigate (1) whether IGF1 could stimulate thymidine incorporation in these cells in serum-free medium in a dose-dependent manner; (2) whether testosterone could stimulate thymidine incorporation in these cells in serum-free medium; and (3) the relationship between IGF1 and testosterone in the proliferation of antlerogenic cells.

MATERIALS AND METHODS

Animals and tissue sampling

Four red deer stag calves were selected from a herd grazing on pasture at Invermay. Each calf was slaughtered when its pedicles or antlers had reached a predetermined developmental stage (Table 1). Apical antlerogenic perichondrium was taken at the transformation point (TP) stage from pedicle to antler, because it was hypothesised that the reaction of antlerogenic tissue at the TP stage to androgen hormone would be pivotal in the transition from positive to negative (Suttie et al., '98). Pedicle formation depends on androgen hormones, whereas high level androgen hormones arrest antler growth.

TABLE 1. Experimental animals

	At sampling			
Deer	Age (days)	Live weight (kg)	Pedicle height (mm)	Ossification pattern[1]
R313	314	55.5	Crest[2]	IMO
R325	326	57.5	20.0	OPC
R337	352	76.5	50.0	TP
R305	360	68.0	60.0[3]	aECO

[1]IMO, intramembranous ossification; OPC, ossification pattern change (transitional ossification); TP, transformation point from a pedicle to an antler (endochondral ossification); aECO, antler endochondral ossification.
[2]>5 mm in height.
[3]A full grown pedicle with a 10 mm long antler bud.

All APs were collected from the slaughtered deer heads using a technique reported elsewhere (Li and Suttie, '94). Briefly, for deer R313, after the removal of the scalp overlying the presumptive antlerogenic regions and exposure of the frontal lateral crests, a piece of periosteum (about 5×20 mm) overlying each crest was removed and placed into BGJb medium (BGJb 45%, F12 nutrient 45%, foetal bovine serum (FBS) 10%, penicillin 100 U/ml, and streptomycin 100 μg/ml (Sigma, St. Louis, MO). A small piece of periosteum (about 2×3 mm) with a layer of underlying bone was then taken from each crest for histology, to confirm stage of pedicle/antler development. For the remaining animals, a piece of apical periosteum/perichondrium (about 5×20 mm) without underlying tissue was removed from each pedicle/antler and placed into BGJb medium, and a small piece of apical periosteum/perichondrium (2×3 mm) with underlying tissue was taken for histology.

Separation of cellular layer and fibrous layer

The tissue samples were rinsed in the rinse medium (BGJb medium with higher doses of penicillin (500 U/ml) and streptomycin (500 μg/ml)) for 3×2 min and then cut with scissors into small pieces (about 1×5 mm). The cellular and fibrous layers of each small piece were then separated under the dissecting microscope (Fig. 1A and B).

Cell culture

The separated tissues from cellular and fibrous layers were further cut into fine pieces (about 1×1 mm). After an additional rinse in the rinse medium, the fine pieces of each type of tissue were transferred into a 25 ml culture flask (Falcon, Lincoln Park, NJ) containing 10 ml digestion medium (BGJb medium with lower percentage of FBS (2.5%), plus additional 200 units/ml collagenase (Sigma). The flasks were incubated in a humidified CO_2 incubator (95% air and 5% CO_2 at 37°C) for 24 hr with occasional shaking. The digestion medium was then discarded after centrifugation at 1500 rpm. The precipitate was resuspended using BGJb medium and seeded in 25 ml culture flasks. The flasks were incubated in the CO_2 incubator and the medium was changed every 3

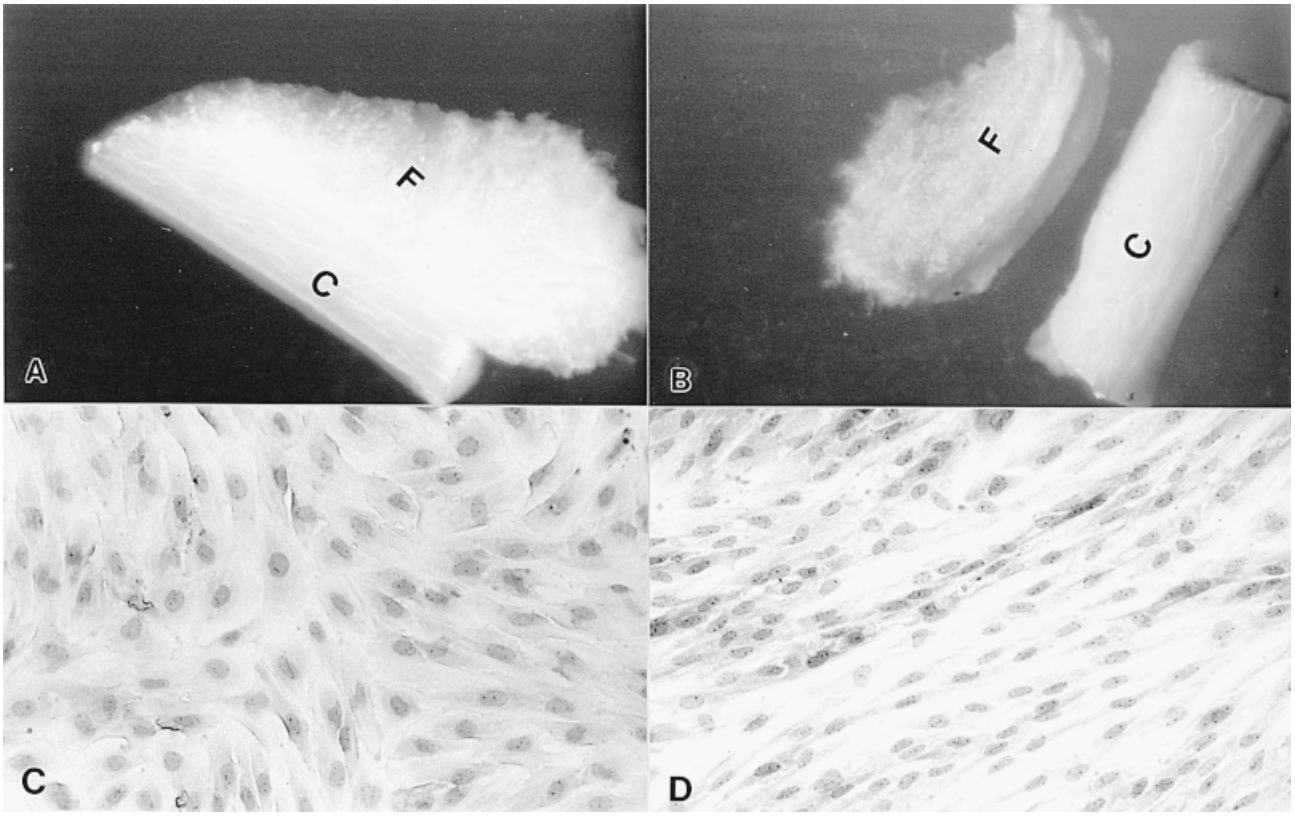

Fig. 1. (**A**) A piece of antlerogenic periosteum. (**B**) The piece of antlerogenic periosteum was separated into two parts from the junction of the cellular layer and the fibrous layer. (**C**) Cultured confluent cellular layer cells (passage 3). Note that these cells were morphologically of intermediate type between fibroblast-like and epithelial-like cells. (**D**) Cultured confluent fibrous layer cells (passage 3). Note that these cells were typical fibroblast-like cells in morphology. C, cellular layer; F, fibrous layer. (C and D: Giemsa's stain, ×175.)

days. When the cells reached confluence, they were detached using trypsin-EDTA (Sigma) and centrifuged at 1500 rpm for 10 min. The supernatant was discarded and the sedimented cells were resuspended in BGJb medium. The resuspended cells were aliquoted into 1 ml vials and frozen in a –80°C freezer in BGJb freezing medium (BGJb 40%, F12 nutrient 40%, foetal bovine serum (FBS) 10%, penicillin 100 U/ml, streptomycin 100 μg/ml and dimethyl sulphoxide 10%, Sigma). For thawing, the vials were taken out of the freezer and put directly into a 37°C water bath. The thawed cells were then cultured in 25 ml flasks in the CO_2 incubator. Once the cells reached about 80–90% confluence, they were trypsinized and seeded in 24-well plates at a density of 2×10^4 cells/well. Each treatment was performed in triplicate wells randomized within developmental stage. Cells used in this study had been through two passages. Cell viability was always above 85%, measured using Trypan blue.

Treatment

The study was carried out with cells from all four histologically distinguished developmental stages (Table 1). IGF1 concentrations used in the study covered a wide range from 0.05 to 100 nM, whereas testosterone was used at a concentration (10 nM) equivalent to plasma levels during pedicle and first antler formation (Suttie et al., '91).

Following 48 hr initial culture after seeding cells into 24 well-plates, the medium was replaced with serum-free medium (SFM) (BGJb medium without serum, but supplemented with 0.1% bovine serum albumin). The cells were cultured for a further 24 hr before the SFM was replaced by either SFM, BGJb medium, SFM with graded doses (0.05–100 nM) of IGF1, SFM + 1% ethanol, SFM + 10 nM testosterone (T) or SFM + graded doses (1.0, 10.0, and 50.0 nM) of IGF1 + 10 nM T (T dissolved in 100% ethanol with the final concentration of ethanol in the medium being 1%). The cells were then cultured for a further 24 hr. Two hours before the termination of the incubation,

^{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 scintillant (LKB product, SciTech, Dunedin NZ). The proliferation rate is expressed as incorporation of ^{3}H-thymidine (dpm).

Statistical analysis

The data (dpm/well on $\log_{10}$ scale) were analysed by ANOVA with treatment terms for developmental stage, cell type, treatment, and their interactions. Contrasts for the differences between the following treatments were assessed: FBS and the IGF1 treatment giving the maximum response for each cell line (optimum IGF1); SFM and T; the mean of 1, 10, and 50 nM IGF1 with T (denoted T + IGF1) and the corresponding mean IGF1 without T. The statistical significance was assessed at the 5%, 1%, and 0.1% levels. Geometric means are significantly different ($P < 0.05$) when the ratio of one to another is greater than the square of the standard error of ratio.

RESULTS

Histology and cell morphology

The histological data confirmed that the antlerogenic tissues taken from deer R305, R313, R325, and R337 were forming pedicles or antlers through the following ossification types, respectively: IMO, OPC, TP (transformation point, pECO), and aECO (Table 1). The morphology of cultured primary C and F cells were quite different upon reaching confluence (Fig. 1C and D, at the IMO stage). The F cells were morphologically typical fibroblast-like cells. They had a bipolar spindle shape and formed parallel arrays. In contrast, C cells were intermediate between fibroblast-like and epithelial-like cells. They had a multipolar shape and pavement-like appearance. C cells were larger than F cells (Fig. 1C and D).

Effects of IGF1, testosterone (T) and T + IGF1 on ^{3}H-thymidine incorporation in cellular layer (C) and fibrous layer (F) cells

^{3}H-thymidine incorporation for each cell type within each developmental stage is presented in Fig. 2. In each case, ^{3}H-thymidine incorporation increased strongly ($P < 0.001$) with dose of IGF1 in serum-free medium to a maximum level at doses which differed among the tested cell lines. The proliferative response then remained high or dropped off weakly for the highest doses of IGF1. A similar dose-dependent pattern was observed for the T+IGF1 treatments. No mitogenic effects of 10 nM T were detected in any of the tested cell lines ($P > 0.05$). However, averaged over C and F cells, T+IGF1 had positive mitogenic effects for the cells from IMO and OPC stages, and negative mitogenic effects for the cells from TP and aECO stages. The ratio of T+IGF1 and IGF1 treatments (1.09; SER 1.059) was not significant at the IMO stage, but differed significantly at the OPC (1.15; $P < 0.05$), TP (0.83; $P < 0.01$) and aECO (0.36; $P < 0.001$) stages. ^{3}H-thymidine incorporation was particularly low for aECO for the mean of T + IGF1 treatments (Fig. 3).

A comparison between the response to FBS and optimum IGF1 showed interesting differences between these treatments across developmental stages ($P < 0.001$), averaged over C and F cell types. At the IMO stage the response (^{3}H-thymidine incorporation) to FBS did not differ significantly from that for optimum IGF1. The response to FBS was then greater than that for optimum IGF1 by factors of 5.64 ($P < 0.001$) for OPC stage, 3.05 ($P < 0.001$) for the TP stage, and 1.56 ($P < 0.01$) for the aECO stage (common SER 1.105). This allowed for overall differences between these treatments (FBS to optimum IGF1 ratio of 2.20; SER 1.051; $P < 0.001$) and a developmental stage by cell type interaction ($P < 0.001$). For the IMO stage ^{3}H-thymidine incorporation for C cells was 1.30 ($P < 0.01$) times greater than for F cells. OPC was the only stage for which the response for F cells was greater by a factor of 2.43 ($P < 0.001$) than that for C cells. For the TP and aECO stages ^{3}H-thymidine incorporation for C cells averaged 1.13 ($P > 0.05$) and 1.30 ($P < 0.01$) times greater than that for F cells (Fig. 4). These ratios have a common SER of 1.105. There was no evidence of any interaction between treatments and cell types ($P > 0.05$).

DISCUSSION

The in vitro culture system described in the present study permits not only the survival and proliferation of antlerogenic cells, but the effects of hormones and growth factors on these cells to be tested. Successful separation of the cellular layer and the fibrous layer of the antlerogenic tissues allowed us to test, experimentally, whether C cells and F cells differ in responses to IGF1 and testosterone.

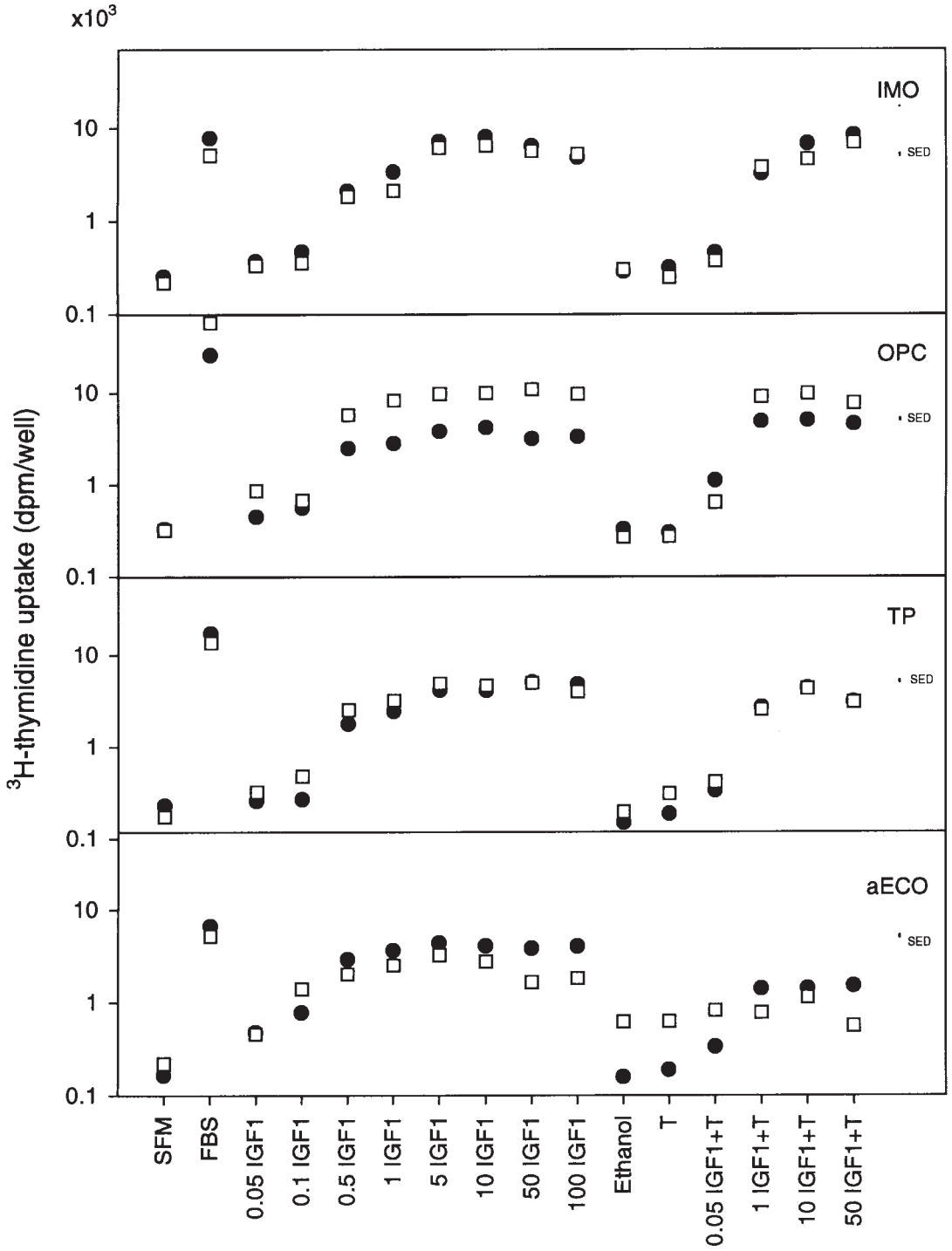

Fig. 2. Geometric mean (triplicate/treatment) ^{3}H-thymidine incorporation (note log scale) classified by IGF1 (nM) or testosterone (T, 10 nM) + IGF1 treatment for the cellular layer (C, ●) and fibrous layer (F, □) cells from the four developmental stages. These stages are IMO, intramembranous ossification; OPC, transitional ossification; TP, transformation point from a pedicle to an antler (ECO, endochondral ossification); aECO, antler endochondral ossification.

The present study clearly showed that all C and F cells from four developmental stages proliferated in response to IGF1 in a dose-dependent manner. These results agree with the reports from antler tip cells (Price et al., '94; Sadighi et al., '94). Because antler tip cells are the descendants of antlerogenic cells (Li and Suttie, '94), we conclude that IGF1 is a potent

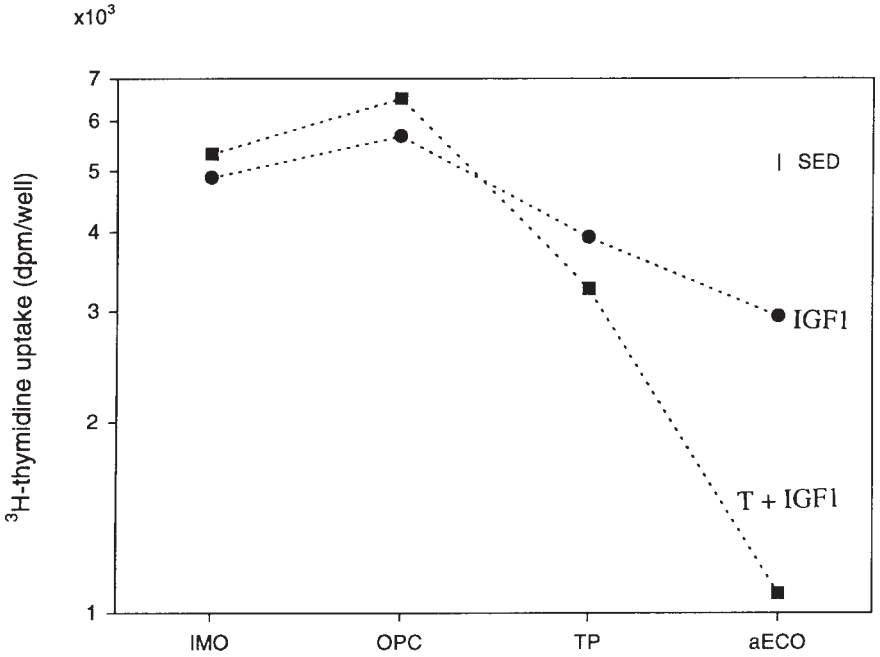

Fig. 3. Geometric mean (triplicates/treatment) ^{3}H-thymidine incorporation (note log scale) for T + IGF1 treatments (means of 1, 10, and 50 nM IGF1) (■) and corresponding concentrations of IGF1 without T (●) for the four developmental stages. IMO, OPC, TP, and aECO as shown in Fig. 2.

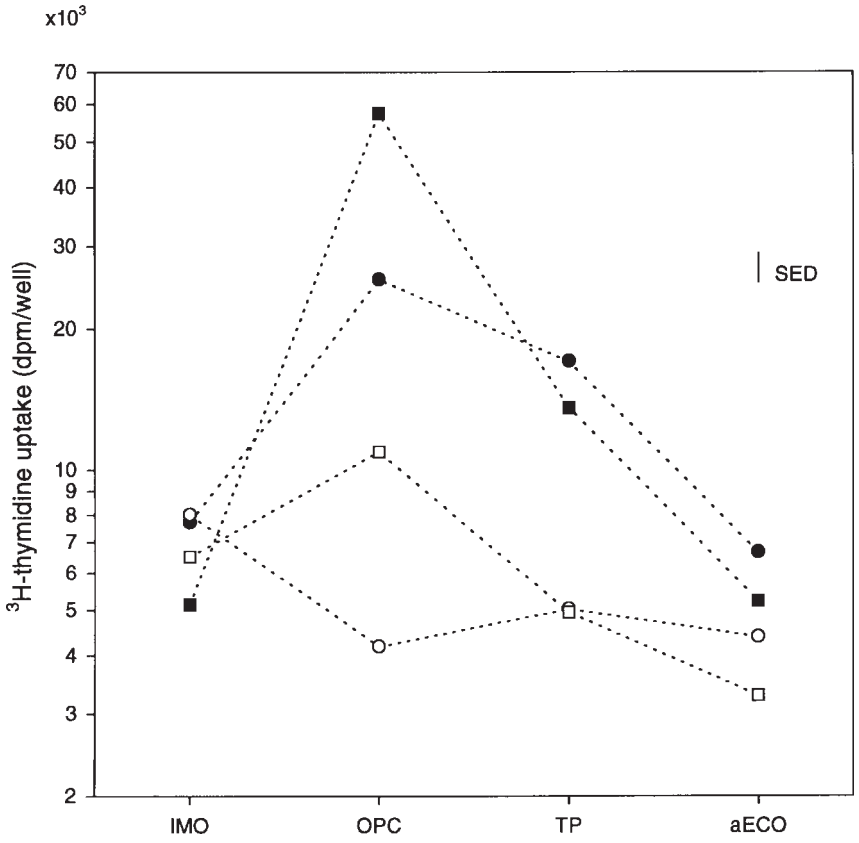

Fig. 4. Geometric mean (triplicates/treatment) ^{3}H-thymidine incorporation (note log scale) for FBS for the cellular layer (C, ●) and fibrous layer (F, ■) cells, and for the optimum IGF1 for the C (○) and F (□) cells over the four developmental stages. IMO, OPC, TP, and aECO as for Fig. 2.

mitogen for both antlerogenic cells and their progeny. It is likely that the mitogenic effects of IGF1 on these cells are via direct ligand-receptor interactions. This conclusion is based on the findings that the cultured antlerogenic cells can react to IGF1 in serum-free medium and antlerogenic tissue possesses IGF receptors (Elliott et al., '92).

It is suggested that cells from the cellular layer and fibrous layer in somatic bone are different in lineage, since the thickness of the fibrous layer remains unchanged when the cellular layer reaches its maximum thickness during bone formation (Sissons, '71). Also the broken fibrous layer is lifted away from the underlying fast forming callus by vigorous proliferation of the cellular layer cells during bone fracture repair (Ham and Harris, '71). Li and Suttie ('94) found that pedicle and antler tissues are mainly derived from the C cells of AP. However, the F cells of AP seem to be different from their somatic periosteum counterparts. The fibrous layer of AP not only becomes hyperplastic, but also forms regular waves during OPC stage (Li and Suttie, '94), although the amplitude of the waves decreases gradually as pedicle formation proceeds to the antler. Li and Suttie ('94) thought that the F cells at the early developmental stage might proliferate faster than the C cells. The rate of proliferation of the C cells, however, would catch up and eventually surpass that of the F cells. The results from the present study clearly demonstrated that the proliferative response of F cells to FBS and IGF1 was greater than that of C cells at OPC stage, although as pedicle development proceeded to later stages, the response of the C cells to FBS and IGF1 became stronger than F cells. Therefore, the present study supports the hypothesis advanced by Li and Suttie ('94).

The results from the present study showed that the dependence of the antlerogenic cells on IGF1 for proliferation altered with the change of ossification type during pedicle and early antler formation. When proliferation (^{3}H-thymidine incorporation) is expressed as a percentage of the effects of the optimum IGF1 over FBS (Fig. 5), we can see that at the IMO stage, IGF1 and FBS are equally effective in stimulating proliferation of the antlerogenic cells (C and F cells). In contrast, at the OPC stage, IGF1 is only 20% as effective as FBS, and at the TP and aECO stages the corresponding reliances on IGF1 are 80% and 60% respectively. The lesser dependence of the antlerogenic cells

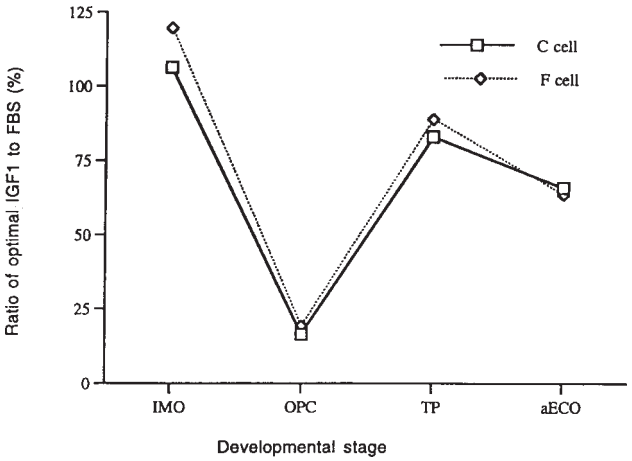

Fig. 5. Percentage of the effects of optimum IGF1 versus fetal bovine serum (FBS).

on IGF1 at the OPC stage may imply that other growth factors are also important for proliferation at this stage.

It is unexpected that testosterone alone in the present study did not have mitogenic effects on the antlerogenic cells in vitro, unless sufficient IGF1 was present. As secondary sexual characters, the formation of a pedicle and an antler depend on androgen hormones (Jaczewski, '82). However, the mechanism whereby androgen hormones stimulate pedicle initiation has been elusive. Fennessy and Suttie ('85) hypothesised that pedicle formation resulted from the direct stimulation of AP by androgen hormones. The finding that AP possesses specific binding sites for testosterone (Li et al., '90, '98) supports this hypothesis. However, this hypothesis cannot explain two phenomena: first, that deer can only start to grow pedicles when they reach their species-specific threshold body weight (Suttie and Kay, '82); and second, that hummels (antlerless red deer stags) do not grow pedicles and antlers although they have normal androgen status (Lincoln et al., '76). Therefore, Li and Suttie ('96) considered that pedicle initiation and antler formation may depend on both appropriate androgen hormone and nutritional status. Further they suggested that nutrition may control pedicle growth through the IGF1 pathway. This is because (1) nutrition promotes body growth mainly through the IGF1 signal transduction pathway (Thissen et al., '94); and (2) IGF1 can exert mitogenic effects on antler tip cells in vitro in serum-free medium (Price et al., '94; Sadighi et al., '94). The present in vitro study seems to support the notion that the regulation of antlerogenic cell proliferation during pedicle ini-

tiation relies on growth factors and androgen hormones acting in concert. However, exactly how this happens is unknown.

Westley and May ('94) reported that steroid hormones can stimulate cell proliferation by interacting with and modulating the activity of growth factor signal transduction pathways. One mechanism is by acting on the production of IGF1 either in an autocrine, paracrine or endocrine pathway. However, the inability to demonstrate a mitogenic effect of testosterone in the present study (even over a wide range of 10 different doses of testosterone, from 0.01 nM to 100 nM, data not shown) on antlerogenic cells from the IMO stage has ruled out an autocrine pathway. Further, although C and F cells do not directly react to sex hormones in vitro, the fact that AP from which C and F cells are derived can form ectopic pedicles and antlers if transplanted elsewhere on the deer's body without adjacent tissues (Goss and Powel, '85) has eliminated the possibility of paracrine regulation. In addition, if IGF1 mediates androgen-induced antlerogenic cell proliferation through the endocrine pathway, pedicle initiation from well nourished deer should abrogate the requirement for androgen hormone, a situation which has not been observed. Another mechanism is by up-regulating type I IGF receptors (van derBurg, '91) or IGF binding proteins (IGFBPs) (Clemmons et al., '90), then in turn altering the sensitivity of the cells or tissues to IGF1. It is reported that antler tip cells possess type 1 IGF receptors (Elliott et al., '92) and testosterone treatment significantly affects IGF1 binding to the mesenchymal cells within the tip of growing antlers (Elliott et al., '96). Unfortunately, so far it is still not known whether antlerogenic cells synthesise IGFBPs. If the second mechanism applies to pedicle and antler formation, then pedicle initiation can take place only when both IGF1 concentration and the sensitivity of antlerogenic cells to IGF1 reach certain thresholds. The IGF1 concentration may be controlled by nutritional status, whereas the sensitivity of antlerogenic cells to IGF1 may depend on androgen hormones.

It is well known that androgen hormone triggers pedicle initiation as castration of prepubertal stag calves will abolish future pedicle formation and administration of exogenous androgen hormones can overcome this abnormality (Jaczewski, '82; Goss, '83). In contrast, high levels of androgen hormones arrest antler growth (Goss, '68). Interestingly, transformation from a pedicle to an antler seems to be independent of androgen hormones, as testosterone in circulating blood during this transformation is hardly detectable (Suttie et al., '91), and after pedicle growth proceeds to pECO stage androgen hormone is no longer required for this transformation (Suttie et al., '98). In the present study, the mitogenic effects of testosterone on the antlerogenic cells were shown to be dependent on developmental stage. If these mitogenic effects (^{3}H-thymidine incorporation) of testosterone are expressed as the differences between the T + IGF1 and the corresponding concentration of IGF1 (Fig. 6), we can see that testosterone has negligible or positive effects on the pedicle cells (from IMO and OPC stages), and negative effects on the antler cells (from TP and aECO stages). Therefore, the in vitro results from the present study support the in vivo findings described previously. However, the change in mitogenic effects of testosterone on antlerogenic cells from positive to negative occurs much earlier than TP stage, approximately at the change in ossification type from OPC to pECO stage. This finding supports the hypothesis advanced by Li ('97) that antler transformation from a pedicle starts from the change in ossification type from OPC to pECO, rather than from the stage when a pedicle grows to the visible species-specific length. In addition, this probably explains why, once a pedicle reaches the pECO stage, androgen is no longer needed for further pedicle growth and the transformation to antler (Suttie et al., '98).

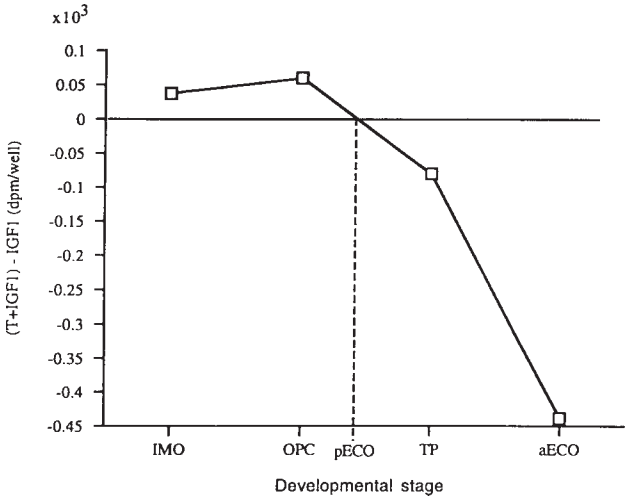

Fig. 6. Differences between the effects of T + IGF1 and the corresponding concentration of IGF1. pECO, pedicle endochondral ossification; IMO, OPC, TP, and aECO as for Fig. 2.

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