Exploring the mechanisms regulating regeneration of deer antlers

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Deer antlers are the only mammalian appendages capable of repeated rounds of regeneration; every year they are shed and regrow from a blastema into large branched structures of cartilage and bone that are used for fighting and display. Longitudinal growth is by a process of modified endochondral ossification and in some species this can exceed 2 cm per day, representing the fastest rate of organ growth in the animal kingdom. However, despite their value as a unique model of mammalian regeneration the underlying mechanisms remain poorly understood. We review what is currently known about the local and systemic regulation of antler regeneration and some of the many unsolved questions of antler physiology are discussed. Molecules that we have identified as having potentially important local roles in antlers include parathyroid hormone-related peptide and retinoic acid (RA). Both are present in the blastema and in the rapidly growing antler where they regulate the differentiation of chondrocytes, osteoblasts and osteoclasts *in vitro*. Recent studies have shown that blockade of RA signalling can alter cellular differentiation in the blastema *in vivo*. The trigger that regulates the expression of these local signals is likely to be changing levels of sex steroids because the process of antler regeneration is linked to the reproductive cycle. The natural assumption has been that the most important hormone is testosterone, however, at a cellular level oestrogen may be a more significant regulator. Our data suggest that exogenous oestrogen acts as a 'brake', inhibiting the proliferation of progenitor cells in the antler tip while stimulating their differentiation, thus inhibiting continued growth. Deciphering the mechanism(s) by which sex steroids regulate cell-cycle progression and cellular differentiation in antlers may help to address why regeneration is limited in other mammalian tissues.

Keywords: deer; antler; regeneration; parathyroid hormone-related peptide; retinoic acid; oestrogen

1. INTRODUCTION

Deer antlers have fascinated zoologists for centuries, as they are undoubtedly one of nature's most dramatic and beautiful symbols of male strength and dominance. Over two thousand years ago Aristotle observed that male deer were unable to defend themselves without antlers and noted that castrated deer did not cast off their antlers. In this review, we present the case that the study of antlers remains relevant in the twenty-first century because of their importance as a unique mammalian model of epimorphic regeneration. In the late 1950s, this feature was recognized by Richard Goss, who was a great champion of antler biology and wrote that 'antlers provide an unprecedented opportunity to observe how nature solved this problem (regeneration) which has frustrated experimental zoologists for so many years' (Goss 1983). In his last review he wrote on the subject (Goss 1995), he discussed many of the unsolved problems of antler biology, most of which remain unanswered. As he pointed out, the fact that antler research remains a neglected area does not reflect its relevance, rather the fact that research on wild animals presents formidable problems of management. Patience is

another quality required of an antler biologist because the seasonal nature of antler growth limits the number of experiments that can be undertaken each year. This, understandably, can be a disincentive for young scientists hungry for publications working in an environment where research funding does not generally support long-term projects. Notwithstanding these difficulties, the study of antler regeneration remains of fundamental importance as it will help to address an important biological question; 'If deer retain the capacity to regenerate these large bony structures why is it that other mammalian organs, bones amongst them can, at best, only institute repair?' Although the complete regeneration of a human limb remains an unlikely prospect, at least in the short-term, the consequences for human health of inducing and extending regeneration are enormous.

2. ANTLER DEVELOPMENT

Antlers are cranial appendages that develop after birth in male deer, and in the females of a few species such as reindeer. They are not attached directly to the skull but develop from pedicles, permanent extensions of the frontal bone, which develop at puberty under the influence of testosterone (Lincoln 1973). The age at which the pedicles develop depends on nutrition and on an animal's body weight (Suttie *et al.* 1984). Apart from blood vessels, nerves and connective tissue the antler pedicle consists

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only of skin and bone and it is from this that antlers regenerate. Pedicles and antlers are derived from a region of the periosteum overlying the frontal bone that is known as 'antlerogenic periosteum' (AP). AP was first identified after a series of transplantation experiments (Hartwig & Schrudde 1974). These authors found that when the periosteum overlying a fawn's pedicle was transplanted onto the foreleg a pedicle and an antler developed at that site. The origin of AP remains unclear. In common with other skull bones it is likely to be neural-crest-derived, although this needs to be established experimentally. AP and the development of the first antler have been studied extensively in recent years by Li & Suttie (1994, 1998, 2000, 2001). In a recent review, they proposed that because AP appears to have a remarkable capacity for self-differentiation it can be considered as a piece of post-natally retained embryonic tissue (Li & Suttie 2001), although as yet there is no direct evidence that this is the case.

A deer's first antlers are not branched (they are described as 'spikes') and develop by a combination of intramembranous and endochondral ossification from the tip of the fully formed pedicle when it has grown to its species-specific height. When testosterone levels rise in the autumn as the breeding season approaches (the regulation of antler growth is reviewed in § 7), these first antlers mineralize, the velvet skin covering them is shed and this leaves a solid bone attached to the pedicle. It had always been a natural presumption that these hard antlers are 'dead'; however, there is evidence that some regions may contain a vascular system and viable bone cells (Rolf & Enderle 1999).

3. ANTLER CASTING AND BLASTEMA FORMATION

The first set of antlers are shed in the spring when testosterone levels fall, a process known as 'casting'. A blastema then forms on the exposed surface of the pedicle bone and from this the first set of 'mature' branched antlers regenerate (figure 1). Thus, casting of the first antlers marks the onset of repeated annual rounds of regeneration that continue throughout life. The cellular mechanisms controlling antler casting have been little studied despite this process being one of nature's most dramatic examples of site-specific osteoclastic bone resorption. Understanding this process could provide insight into why OCs target certain skeletal sites in human diseases associated with bone loss, for example, osteoporosis and metastatic bone disease. What has been established is that the event is associated with a fall in circulating levels of testosterone. Deer administered high levels of testosterone or oestrogen do not cast antlers, whereas castration will result in casting within 2–3 weeks (Goss 1968; Fletcher 1978). Antlers are normally cast within a day or so of each other, although this is not preceded by a gradual 'loosening' at the interface between the antler and pedicle bone. In fact, recently we noted an antler to be firmly attached to the pedicle at 08.00 only to find that it was cast 30 min later. Clearly there is local production of factor(s) at the pedicle–antler bone interface that stimulate the recruitment of large numbers of OC progenitors from the circulation and their subsequent differentiation. A likely candidate for regulating OC differentiation is RANKL and we have

recently identified its synthesis by mesenchymal cells in the blastema (unpublished observations). The trigger for increased osteoclastogenesis by RANKL could be the fall in circulating testosterone as recently it has been demonstrated that sex hormones can inhibit RANKL signalling in bone cells (Shevde *et al.* 2000; Huber *et al.* 2001). OCs must then be activated to resorb large amounts of matrix at the pedicle–antler interface in a coordinated manner. Almost certainly, this must involve some form of hormonal regulation. A possible candidate would be PTH as we have identified many cells expressing the PPR in the antler blastema. This process of OC recruitment, differentiation and activation must be highly site specific, otherwise a generalized increase in OC activity would lead to pathological fracture at other skeletal sites.

In some species of deer, antler casting and regrowth of the new set of antlers are not closely linked; regeneration can be delayed for several months and during that time wound healing is held in abeyance. However, in red deer (the species that we use as a model) casting is coincident with regeneration. Immediately after the old antlers have been cast a swollen ring of skin surrounds the pedicle; this has a 'shiny' surface, which is a characteristic of antler velvet skin (figure 1*a*). Within hours, a scab forms beneath which the epidermis migrates and below this mesenchymal cells are interposed between the epidermis and the exposed surface of the pedicle bone (figure 1*b*, 2*a,b*). One of the most important questions in antler biology relates to the origin of cells that form the blastema. Is there reversal of the differentiated state in local populations of cells as is known to take place in regenerating urodele limb (Lo *et al.* 1993; Simon *et al.* 1995)? Alternatively, are antler tissues derived from a population of stem cells retained in the region of the pedicle that proliferate when the environment is appropriate? In recent years evidence has accumulated that many adult tissues contain stem cell populations capable of expansion under certain circumstances (for reviews see Korbaling & Estrov 2003; Rosenthal 2003). Another possibility is, could there be a contribution from a circulating pool of stem cells? Clearly these questions cannot be addressed before cells in the antler blastema are characterized in far more detail; currently very little is known about their phenotype. Establishing the origin of antler progenitors will also require experiments to be undertaken that introduce a cell marker (e.g. green fluorescent protein) into cells of the pedicle periosteum and related tissues enabling their fate to be followed. Based on current evidence our view is that the blastema cells are likely to be derived from stem cells of the periosteum of the pedicle because it has been shown that there are multipotential stem cells in the periosteum of other bones (Nakase *et al.* 1993). The recent observation that the periosteum of the distal pedicle is markedly enlarged shortly after antler casting (Kierdorf *et al.* 2003) supports the hypothesis that there may be expansion of a progenitor pool in this tissue. Our finding that PTHrP can be immunolocalized in periosteum and in mesenchymal cells in the blastema is further evidence that the latter may be derived from the former.

4. ANTLER REGENERATION AND WOUND REPAIR

An important relationship exists between antler growth and wound healing, another neglected area of investigation.

Figure 1. Deer antlers at different stages of development. (a) An antler blastema 24 h after the previous antler has been shed (cast) (day 0). (b) The blastema after *ca.* 8 days of growth when epithelialization is almost complete. (c) A rapidly growing antler at *ca.* 21 days of development. (d) A fully formed antler that is still covered in velvet skin (*ca.* 100 days). (e) Fully mineralized 'hard' antlers after shedding of the velvet skin (*ca.* 140 days). Scale bars: (a,b) 1 cm; (c) 5 cm; (d,e) 10 cm.

It is an almost universal truism that damage to mammalian tissues results in a fibroproliferative response that produces a fibrotic scar. However, wound healing can be delayed on the antler pedicle for several months because in some species of deer old antlers are cast several months before new ones regrow. Experimental studies have also shown that if the distal end of the pedicle is amputated during the winter months in these species the cut surface will not heal and regeneration will not commence until spring, which is the normal time for casting and regeneration (Goss 1961). However, like virtually all examples of epimorphic regeneration, formation of the antler blastema requires a wound repair response. When Richard Goss (1972) covered the pedicle stump with a full-thickness skin graft antler regeneration was prevented as there was no wound. More recently it was shown that when AP was transplanted into nude mice in xenograph studies a pedicle-shaped protuberance developed, however, wounding was required to generate antler tissue (Li *et al.* 2001). These observations raise important questions: why does a blastema form on the pedicle instead of a scar? What are the local factors in the blastema that prevent the normal wound repair response? If a 'blastema' could be induced to form instead of a scar, this would improve the prospect of regenerating complex skeletal structures in humans. Furthermore, identification of the local factor(s) in antler that are capable of inhibiting scarring could have a practical application as they could be used to inhibit exaggerated fibrosis that is a complication of many injuries.

Harty *et al.* (2003) recently proposed that an ability to regenerate might relate to an organism's immune competence. They present evidence that urodeles may be 'immunodeficient' and that changes in immune competence may lead to a loss of regenerative ability that occurs during auran metamorphosis. It is intriguing to speculate that the local environment of the antler pedicle

is also 'immunodeficient' and thus the normal inflammatory responses to injury are in some way modified so that scar tissue does not form. Investigation of local cytokine-signalling pathways in the antler blastema is clearly relevant to understanding why regeneration rather than scarring takes place on the antler pedicle. Another remarkable feature of the pedicle is that despite the large area of tissue exposed after casting, bacterial infections are rare at this site. If an area of bone several centimetres in diameter were exposed to the environment at another skeletal site (e.g. femur) a severe infection would occur very rapidly. We suggest that this could be further evidence that local immune responses in antler may be unique.

5. THE ANATOMY OF ANTLER GROWTH

After 7–9 days epithelialization of the blastema is complete, future branches of the antler are visible as raised swellings (figure 1b) and a number of zones can be distinguished histologically (figure 2). Below the epithelium is a region that resembles the future perichondrium, beneath this a zone of proliferating mesenchymal cells (figure 2c), and more proximally, chondrogenesis takes place. The antler then elongates with a typical S-shape growth curve; during the first month to six weeks longitudinal growth at the distal end of each branch is relatively slow (figure 1c). Throughout the next 60–80 days there follows a period of rapid exponential growth that slows as autumn approaches (figure 1d). During this period of rapid growth, the antlers of large species of deer (e.g. wapiti or moose) will elongate by more than 2 cm per day. This represents the fastest rate of tissue growth in the animal kingdom involving the coordinated regeneration of multiple tissue types including skin, nerves, blood vessels, cartilage and bone. Although our research has focused on

Figure 2. Histology of the antler blastema. (a) Low power cross-section through a blastema at *ca.* 4 days of development. The boxed areas 1–3 correspond to the higher power views shown in (b)–(d). (b) Section taken from the centre of the blastema showing the regenerating epithelium beneath the scab and subjacent mesenchymal cells (WE, wound epithelium). (c) Section from the outer region of the blastema (box 2) (also corresponds to the region covered by skin in figure 1b) showing mesenchymal cells. (d) Section from the inner region of the blastema showing mesenchymal cells in a more 'columnar' arrangement between which are vascular channels (arrows). All sections are stained with haematoxylin and eosin. Scale bars: (a) 0.5 cm; (b) 100 µm; (c,d) 50 µm.

skeletal tissues, the antler is also a relevant model for investigating skin and peripheral nerve regeneration.

Longitudinal growth of antlers is by a process of modified endochondral ossification at the distal tip of each branch. In section, the distal antler tip can be divided into several distinct regions representing the whole spectrum of cellular differentiation and bone development (figure 3a). Below the dermis of the velvet skin is the perichondrium, which contains cells that are not highly differentiated (figure 3b). They express type I collagen (a phenotypic marker of cells of the osteoblast and fibroblast lineages) but only express low levels of ALP, a marker of more differentiated chondrocytes or osteoblasts (Price *et al.*

al. 1994). *In vitro* studies have shown that PER cells are able to differentiate into cells of more than one lineage, indicating that they are multipotential progenitors. For example, in the presence of rabbit serum PER cells will differentiate into adipocyte-like cells whereas in the presence of dexamethasone ALP expression increases indicating differentiation into osteoblast-like cells (Price & Faucheux 2001). The perichondrium is continuous proximally with the periosteum that surrounds the shaft of the antler, which is the site of intramembranous bone formation. Our preliminary studies have shown that cells cultured from periosteum share many of the characteristics of PER cells and *in vivo* they express a similar range of molecules including PTHrP, RANKL and the RA-synthesizing enzyme (RALDH2).

Below the perichondrium is a densely cellular zone (figure 3c) that has been variously described as the zone of proliferation (Goss 1983), or reserve mesenchyme (Banks & Newbrey 1983), although our preferred descriptor is 'mesenchyme'. The cells in this region are also not highly differentiated; they do not express ALP (Price *et al.* 1994), there is a low level of type I collagen mRNA expression and type IIA mRNA, a marker of chondroprogenitor cells in developing cartilage is not expressed (Price *et al.* 1996). In our view, this represents a 'growth zone'; proliferating cell nuclear antigen staining shows that mesenchyme cells have a high rate of proliferation (unpublished observations) and a significant proportion of them (*ca.* 60%) are apoptotic (M. Colitti, unpublished observations). This high rate of apoptosis may provide a defence against mutagenesis in these rapidly dividing cells, as discussed by Jeremy Brockes in the context of amphibian limb regeneration (Brockes 1998). The local factors that regulate cell growth and apoptosis at this site need to be identified. Potential candidates include components of the Wnt-signalling pathway and BMPs which are known to regulate these processes during development (Yokouchi *et al.* 1996; Zou & Niswander 1996; Yamaguchi *et al.* 1999; Ellies *et al.* 2000; Chen *et al.* 2001; Grotewold & Ruther 2002; Yang *et al.* 2003).

In longitudinal sections (figure 3a) chondrogenic differentiation occurs in a region that can be distinguished histologically as one where cells start to become aligned in vertical columns, between which are small vascular channels (figure 3d). In this 'transition zone' type I, IIA, IIB and type X collagen mRNAs are all expressed (Price *et al.* 1996), consistent with this region being composed of cells at different maturational stages in the chondrocyte lineage. Such temporal co-expression of matrix proteins may be a feature of tissues in which there is rapid differentiation from prechondrogenic mesenchyme to cartilage and in this regard, the antler is different to the epiphyseal growth plate. Antler cartilage is also structurally different from embryonic or growth plate cartilage in that it is highly vascularized. Between the columns of chondrocytes is a region of perivascular tissue and at this site both osteoblast and OC progenitors can be localized (figure 3e; Price *et al.* 1994, 1996; Faucheux *et al.* 2001). Like other hyaline cartilages, antler cartilage contains types II and X collagen, although the region expressing type X collagen is far more extensive than that seen in the growth plate.

The extremely rapid growth of antlers requires extensive resorption of mineralized cartilage matrix by OCs, which

Figure 3. Histology of the growing antler. (a) Longitudinal section through the tip of the main antler branch after approximately four weeks of growth. PC, perichondrium; Mes, mesenchyme; CP, chondroprogenitors; CART, cartilage; PO, periosteum. Higher magnification views showing: (b) perichondrium; (c) mesenchymal zone (Mes) and the distal zone of chondroprogenitors (Cp) between which are vascular channels (arrows); (d) chondroprogenitors; (e) columns of chondrocytes (Ch) bordered by vascular channels and perivascular cells (arrowheads); (f) bone from the centre of the section in (a) showing osteoblasts (arrows) aligned along the bone (B) surface; (g) periosteum showing the outer more fibrous zone (Fp) and inner cellular layer (Cper). Scale bars: (a) 1 cm; (b-g) 100 µm.

Figure 4. Antler OC development *in vivo* and *in vitro*. (a) Cryostat section of antler cartilage stained for the enzyme TRAP, a marker of the OC phenotype. Numerous TRAP-positive cells (arrows) are present within the perivascular tissue. C, cartilage; V, vascular channel. (b) Numerous TRAP-positive OCs differentiate in micromass cultures of cells derived from antler cartilage. (c) Confocal image of an OC cultured on a mineralized substrate showing localization of cathepsin K (green) (another marker of the OC phenotype) and an actin ring (red) which indicates active resorption. The outline of the resorption pit is indicated by the dashed line. Scale bars: (a) 100 µm; (b) 40 µm; (c) 20 µm.

is then replaced by bone. Degradation of extracellular matrix by OCs also facilitates invasion of antler cartilage by blood vessels and the abundant blood supply of the antler is the source of haematopoietically derived OC progenitors. OC-like cells, identified by the expression of TRAP and VNRs are first seen to differentiate in perivascular tissues in non-mineralized cartilage. The number of TRAP-positive multinucleated cells increases proximally in mineralized cartilage (Fauchaux *et al.* 2001; Szuwart *et al.* 2002) and large numbers of OCs can be extracted from antler cartilage and bone (Gray *et al.* 1992; Gutierrez *et al.* 1993). The regulation of OC formation by chondrocytes/mesenchymal cells has also been shown to occur in foetal bones (Thesingh & Burger 1983; Van De Wijngaert *et al.* 1989). To investigate antler OC biology more closely we have developed an *in vitro* model in which cells from non-mineralized cartilage are cultured as high-density micromasses. OC-like cells differentiate in these cultures and these cells have the phenotype of 'classical' mammalian OCs (figure 4); they express TRAP, VNRs, MMP-9, cathepsin K and calcitonin receptors and when cultured on a mineralized substrate form F-actin rings and large resorption pits (Fauchaux *et al.* 2001). Candidate molecules that support OC differentiation from circulating progenitors are discussed in § 6.

6. LOCAL FACTORS THAT REGULATE ANTLER REGENERATION

The nature of the cellular interactions that control antler regeneration remain poorly understood, not least because the number of scientists working in this field remains small and thus progress has inevitably been slow. For many years, antler research has focused on anatomy and endocrinology because the tools were not available for investigating the molecular mechanisms involved. However, advances in knowledge and technology from cell and molecular biology, genetics, developmental biology and the study of regeneration in lower organisms can now be applied to antler regeneration. There has also been an increased demand for antler velvet in recent years, not only for use in oriental medicine, but also in the West where it is now being marketed as a health food or nutraceutical. Because Western consumers demand safe and effective products, this has led to renewal of interest in the identification of components of antler tissues and their function. As New Zealand is the main exporter of antler velvet, it has led the field in research of antler growth mechanisms. More recently, these scientists have combined this work on basic antler biology with research on the efficacy of deer antler products (Suttie & Haines

2001). Nevertheless, one of the main difficulties facing those of us who work on antlers is the paucity of genetic information available. However, in the near future, gene chips will be available for microarray analysis based on bovine sequences and there is generally considerable identity between bovine and cervid sequences. The application of this, and related techniques, holds considerable promise for identifying genes that regulate antler regeneration. In the longer term, it is likely that the genome of important deer species will also be mapped.

The extraordinary features of antler growth have led some scientists to propose the existence of unique regulatory factors in antler tissues. However, evidence is accumulating that developmental programmes can be recapitulated in the adult under specific circumstances. The hypothesis that we have explored in recent years is that the molecular signals that regulate skeletal development in the embryo and regeneration in lower vertebrates have been highly conserved throughout evolution and are recapitulated during the repeated cycles of antler regeneration. The number of molecules that control skeletal development is large and ever expanding, and to investigate more than a few of these has been beyond the scope of our laboratory. We review studies relating to the role of PTHrP and RA in the context of antler regeneration.

(a) *Parathyroid hormone-related peptide*

PTHrP was first identified as the hypercalcaemic factor associated with malignancy (Suva *et al.* 1987). However, its paracrine/autocrine functions are most important in the context of development where it has an important role in a number of systems. Acting through the type 1 receptor (PPR), it regulates chondrocyte differentiation in the developing skeleton (Kronenberg 2003). Mice with a null mutation in the PTHrP and PPR genes have severe skeletal malformations in the growth plate resulting from accelerated chondrocyte differentiation and premature bone formation (Amizuka *et al.* 1994; Karaplis *et al.* 1994; Lanske *et al.* 1996). The first evidence that PTHrP may have a similar effect on chondrocyte differentiation in the antler came from *in vitro* studies, which showed that PTHrP increased antler chondrocyte proliferation but inhibited differentiation (Fauchaux & Price 1999). More recently, we have used immunocytochemistry to show that the distribution of PTHrP in the distal tip of the growing antler is consistent with it playing a role in the regulation of cell differentiation at sites of chondrogenesis (Fauchaux *et al.* 2004). There is a high level of PTHrP expression in the antler perichondrium, in mesenchyme, in chondroprogenitors and in perivascular cells in antler cartilage; however, it is not synthesized by differentiated chondrocytes. Thus, although the patterns of expression of PTHrP in the developing limb and the growing antler are similar (e.g. expression in perichondrium), some distinct differences can be observed (Kartsogiannis *et al.* 1997; Farquharson *et al.* 2001; Medill *et al.* 2001; Nakase *et al.* 2001; Kindblom *et al.* 2002). The most obvious difference is the lack of PTHrP expression in differentiated antler chondrocytes. We also have evidence that there may be an interaction between PTHrP and the morphogen IHH, similar to that existing in embryonic bone where PTHrP and IHH control growth plate chondrocyte differentiation at multiple steps (Lanske *et al.* 1996; Vortkamp *et al.* 1996;

Chung *et al.* 2001; Kobayashi *et al.* 2002). IHH can be immunolocalized in recently differentiated antler chondrocytes; a similar pattern of expression to that seen in the growth plate where it is specifically expressed by prehypertrophic and early hypertrophic chondrocytes (Lanske *et al.* 1996; Vortkamp *et al.* 1996, 1998; St Jacques *et al.* 1999; Nakase *et al.* 2001; Kindblom *et al.* 2002). IHH is also localized in osteoblasts in the mid-shaft of the antler; however, unlike PTHrP it is not expressed in the periosteum suggesting that it only has a function during endochondral bone formation. This finding is consistent with the observation that osteoblast differentiation is impaired in the IHH null-mutant mouse, but only during endochondral bone formation (St Jacques *et al.* 1999). PTHrP and the PPR are also expressed by antler osteoblasts, which suggests an additional role in the regulation of osteoblast differentiation. PTHrP has been shown to regulate osteoblast differentiation in several *in vitro* models (Motomura *et al.* 1996; Du *et al.* 2000; Carpio *et al.* 2001; Miao *et al.* 2001). More recent *in vivo* studies have confirmed that PTHrP is required for the development of normal bone mass because mice heterozygous for the knockout of the PTHrP gene display signs of osteopaenia in adulthood (Amizuka *et al.* 2000). Mice lacking the PPR have a more abnormal bone phenotype; there are few osteoblasts in primary spongiosa whereas cortical bone is thicker than normal (Lanske *et al.* 1999).

Another function for PTHrP in antlers is to stimulate antler OC formation. As described previously, OCs differentiate in perivascular tissues in antler cartilage and PTHrP can be immunolocalized in cells at this site (Fauchaux *et al.* 2001, 2002). Out of the factors tested in our micromass model, PTHrP is the most potent stimulator of OC differentiation (Fauchaux *et al.* 2001). Although RANKL and macrophage colony stimulating factor are also expressed in antler and stimulate OC formation *in vitro*, the magnitude of this increase is less than that observed after PTHrP treatment. Furthermore, when cells are treated with PTHrP and RANKL in combination, the increase in OC-like cells formed is not significantly different from the effect of PTHrP alone. Of particular interest is the observation that OPG, the soluble 'decoy' receptor for RANKL, does not completely abrogate the effects of PTHrP on OC formation. This means that in antlers PTHrP has effects on OC formation that are RANKL independent. By contrast, in other species most studies have shown that the effects of PTHrP on osteoclastogenesis are entirely mediated by RANKL (Morony *et al.* 1999). These RANKL-independent effects could be direct as antler OCs express the PPR at a mRNA and protein level (Fauchaux *et al.* 2002). There are only two other reports describing the expression of PPRs in mammalian OCs (Langub *et al.* 2001; Nakashima *et al.* 2003). Interestingly, the PPR has a predominantly nuclear localization in antler OCs, which suggests that the receptor may directly regulate gene transcription.

These results show that during the phase of rapid longitudinal growth PTHrP may regulate the differentiation of several cell types in the antler including chondrocytes, osteoblasts and OCs. However, there is evidence that it could also play a particularly important role during blastema formation as PTHrP and PPRs are both expressed in regenerating epithelium, as well as in a significant

proportion of uncharacterized mesenchymal cells in the blastema (figure 5). As referred to earlier, the localization of PTHrP in the blastema provides further evidence that these cells may be derived from anterogenic periosteum because PTHrP is also highly expressed in periosteum covering the antler shaft and in the perichondrium (which is continuous with the periosteum). The widespread distribution of PTHrP in these tissues leads us to consider PTHrP to be a phenotypic marker of antler progenitor cells. A particularly intriguing observation is that PTHrP can be immunolocalized in a population of cells that appear to be 'squeezing' out of the vascular spaces and into the perivascular tissue in cartilage. This suggests that some progenitor cells in the antler may be derived from circulating stem cells, a possibility that deserves further investigation. The observation that PTHrP is also expressed during fracture repair highlights the relevance of the antler model to the study of bone repair and provides further support that developmental signalling pathways are recapitulated during repair and regeneration (Vortkamp *et al.* 1998; Okazaki *et al.* 2003).

(b) Retinoic acids

RAs are a class of molecules that regulate skeletal development and regeneration in urodeles (Tassava 1992; Schilthuis *et al.* 1993; Lohnes *et al.* 1994; Scadding & Maden 1994; Pecorino *et al.* 1994, 1996; Cash *et al.* 1997; Yamaguchi *et al.* 1998; Koyama *et al.* 1999). Before we began our series of studies, there was *in vivo* evidence that exogenous RA can influence the development of the first antler. In a single case study, Bubenik (1990) found that application of RA into an early antler led to accelerated growth and a change in the direction of growth. Injection of RA into the pedicle anlage of a male fallow deer also resulted in accelerated growth and alteration in the shape of the pedicle and first antler (Kierdorf & Kierdorf 1998). In a larger study, it was shown that RA applied to the growing pedicle led to an increase in the volume of the first antler (Kierdorf & Bartos 1999). Our work provides several lines of evidence to confirm that RA plays a potentially vital role in the regulation of cellular differentiation in growing antlers (Allen *et al.* 2002). We cloned by RT-PCR the members of two families of receptors, RAR α , β and γ and RXR α , β and γ , and analysed their expression pattern. When compared with the expression patterns seen in the developing mouse limb some similarities and several differences were observed. The major difference is the low level of RAR γ -receptor expression in antlers. In chick and mouse limbs RAR γ is upregulated as mesenchymal cells differentiate into chondrocytes (Dollé *et al.* 1989; Mendelsohn *et al.* 1992; Koyama *et al.* 1999), but no such increased expression is seen in antler cartilage. This raises the possibility that a receptor other than RAR γ may regulate chondrocyte differentiation. The most likely candidate is RXR β whose expression increases as cells differentiate from chondroprogenitors into mature chondrocytes.

We used HPLC analysis to show that significant amounts of vitamin A (retinol) are present in antler tissues at all stages of differentiation. The levels of all-*trans*-RA measured were similar to those found in developing limb buds (Thaller & Eichele 1987; Horton & Maden 1995) and regenerating amphibian limbs (Scadding & Maden

1994). Of particular interest was the identification of 9-*cis*-RA in perichondrium, mineralized cartilage and in bone; 9-*cis*-RA has not been detected in the developing limb, although it is synthesized and released in the wound epidermis of the regenerating urodele limb (Viviano *et al.* 1995). This suggests that the RXR ligand may have a specific function during bone regeneration. RAs are locally synthesized in antler because the RA-synthesizing enzyme RALDH2 is present in several tissues including skin, perichondrium, mesenchyme, perivascular cartilage tissue and bone. Furthermore, sites of RALDH2 expression correlate well with the presence of endogenous retinoids and the localization of cells that respond to RA; chondrocytes and osteoblast progenitors. It is noteworthy that in embryonic chick limb mesoderm there is little RALDH2, it is absent in cartilage although it is highly expressed in the perichondrium, as it is in antler (Berggren *et al.* 2001). In the antler, as in the developing limb, chondrocytes are an important RA target; *in vitro* RA treatment leads to loss of the differentiated chondrocyte phenotype. However, RA also appears to play a role in controlling the differentiation of cells of the osteoblast lineage because mature osteoblasts in the antler express significant amounts of RALDH2 and RA induces ALP expression in progenitor cells *in vitro*.

RA also regulates OC differentiation in the antler. In micromass cultures formation of OCs can be induced by the addition of 10–100 nM of all-*trans*-RA, which is similar to the levels found in developing limbs (20–50 nM; Thaller & Eichele 1987; Horton & Maden 1995). When grown on dentine slices RA-treated osteoclasts resorb a larger area than control cultures. In contrast to the situation with PTHrP, the effect of RA on osteoclastogenesis is entirely indirect, through the induction of RANKL synthesis (S. P. Allen, M. Maden and J. S. Price, unpublished data). In RA-treated cultures, the levels of RANKL mRNA are significantly increased before OC differentiation and addition of OPG, an inactive soluble receptor for RANKL (Simonet *et al.* 1997; Tsuda *et al.* 1997; Yasuda *et al.* 1998) will abolish the RA-induced increase in OC differentiation.

In common with PTHrP, RA may also regulate the early stage of antler regeneration since RALDH2 and the receptors RAR α , RAR β and RXR β are expressed in the blastema (figure 6). Also, HPLC analysis has detected several RAs, including what appear to be 4-*oxo*, 9-*cis* and all-*trans*, in blastema tissues (S. Allen, unpublished observations). To explore the potential role of RA in the blastema *in vivo*, we recently undertook a study designed to inhibit RA signalling by transfection of a dominant negative form of RAR, RAR-E. The RAR-E contains a point mutation originally identified as the homologous position of the thyroid hormone receptor of thyroid hormone-resistant patients; the dominant-negative phenotype is transferable to RAR by a single amino acid substitution (Saitou *et al.* 1995). The mutated RAR suppresses wild-type RAR function and targeted expression in skin or chondrogenic cells inhibits skin development and retards skeletal development (Saitou *et al.* 1995; Yamaguchi *et al.* 1998). The antler blastema was transfected using a biolistic particle-delivery system 24–48 h after the old set of antlers had been cast (figure 7). One side was transfected with RAR-E, the other with a control, beta galactosidase-expressing plasmid. The transfection efficiency was high;

Figure 5. Localization of PTHrP in the antler blastema. (a) PTHrP is present in the regenerating wound epithelium (WE) and the underlying mesenchymal cells (Mes). (b) Higher-power view of the boxed area in (a) showing PTHrP expression in mesenchymal cells and in cells lining blood vessels (arrowheads). Scale bars: (a) 100 μ m; (b) 25 μ m.

Figure 6. Localization of the retinoic acid synthesizing enzyme RALDH2 and RA receptors in the antler blastema (ca. 4 days). (a) RALDH2 immunolocalization (brown stain) in mesenchymal cells of blastema. (b–d) Bright field images showing expression of RA receptor mRNAs by *in situ* hybridization. (b) RAR α has a distinct pattern of expression in a population of vertically aligned mesenchymal cells alongside vascular channels. (c) RAR β is expressed at lower levels and has a more generalized pattern of expression. (d) RXR has a similar pattern of expression to RAR α . Scale bars, 50 μ m.

after 48 h we observed that in the centre of the blastema ca. 70–80% of cells were transfected. This demonstrates that the biolistic particle-delivery system has significant potential for manipulating gene expression in the antler blastema *in vivo*. Analysis of the cellular effects is ongoing but preliminary results indicate that blockade of RA signalling has an effect on OC differentiation.

Several other molecules have been identified in antler tissues including growth factors, cytokines and oncogenes. IGFs play a particularly important role in regulating antler cell growth. However, the relative importance of circulating compared to locally produced IGF-I remains unclear. IGF-I and IGF-II have been extracted from antler tissues and show close homology to other mammalian forms (Mundy *et al.* 2001). Ligand-binding studies have revealed

Figure 7. Transfection of an antler blastema using biolistic particle delivery 30 h after the old antler had been cast.

the presence of IGF-I and IGF-II receptors in the antler tip (Elliott *et al.* 1992, 1993), and *in vitro* both stimulate proliferation of antler cells (Price *et al.* 1994; Sadighi *et al.* 1994). In a search for anabolic agents that could be used to treat diseases such as osteoporosis, Mundy *et al.* (2001) examined antler extracts for potential osteoblast stimulating factors. This revealed the presence of other known factors including BMP-4 (Feng *et al.* 1995), BMP-2 (Feng *et al.* 1997), FGFs and IGF-I and IGF-II. Novel peptides identified included OT-2 that has 70% homology to IGF-I and in tissue sections was localized in OCs (Guitierrez *et al.* 1993). Three other novel factors were identified which had activity in cell proliferation assays. Using a PCR approach, Francis & Suttie (1998) identified several mRNAs including TGF β 1, TGF β 2, c-fos, c-myc and IGFs I and II. More recently, the same group used a subtraction technique to identify a growth factor, dermatopontin and an anti-oxidant agent, phospholipid hydroperoxide glutathione peroxidase (GPX4), which are not expressed in adult tissues (Lord *et al.* 2001). In their recent review, Li & Suttie (2001) propose that the ontogeny of antlers and limbs is comparable and report that some factors that control limb development are also expressed in antlers, including FGF8 and Shh, although these findings are currently only published in a thesis (de Alwis *et al.* 1996). Interestingly, we have failed to detect the expression of Shh in regenerating antlers, although this may well be a technical issue. However, we have detected the expression of other developmentally important molecules including Wnt 3a and the active form of β -catenin, leptin, the beta isoform of the leptin receptor, Msx-1 and Msx-2, FGF-4, cbfa1 and osterix (unpublished data). The potential role of components of the Wnt-signalling pathway is of particular interest because this pathway has recently been revealed to play an important role in regulating normal bone physiology (Gong *et al.* 2001; Little *et al.* 2002). Our recent studies suggest that this pathway may have multiple functions in antlers because its

Figure 8. Changes in concentrations of plasma testosterone and IGF-1 during the growth cycle of red deer antlers in the Northern Hemisphere. Solid line, IGF-1; dashed line, testosterone; dot-dash line, antler growth.

activation (by lithium chloride) inhibits PER cell differentiation (as determined by ALP activity) and OC formation (T. Althnaian, unpublished observations).

7. THE CONTROL OF ANTLER DEVELOPMENT

Antlers are unique among regenerating structures in that their primary function is to serve as secondary sexual characteristics and so their seasonal pattern of development is tightly coordinated to the animal's reproductive cycle (Goss 1983; Bubenik 2001). In temperate zones, the mating season must be timed so that the young are born in the spring, and it is changing day length that regulates both antler growth and the reproductive cycle. As described earlier, antlers are cast in the spring, at a time when testosterone levels are low and day length is increasing (Lincoln & Kay 1979; figure 8) and then grow rapidly during the spring and early summer. As the days shorten in late summer, elongation slows, testosterone levels rise, the antlers become heavily mineralized and velvet is shed in preparation for the autumn mating season (Muir *et al.* 1988). It is in this state that the antlers are used for the fighting, intimidation and display necessary to assemble and protect a harem of females. The first experiment to demonstrate the link between photoperiod, antler development and the breeding season was undertaken 50 years ago (Jaczewski 1954) and has been confirmed by several later studies (reviewed by Goss 1983). The mechanism whereby the photoperiod is linked to antler growth and reproductive activity is not clear but is likely to involve changes in melatonin secretion from the pineal gland, which then acts on the hypothalamo-pituitary axis (Bubenik *et al.* 1986).

Of the intrinsic factors that control first antler development and the timing of subsequent cycles of regeneration, sex steroids are the most important. The development of pedicles is induced by increasing and elevated plasma testosterone concentrations whereas the first antler develops when testosterone levels are decreasing (Suttie *et al.* 1984, 1991). Casting is triggered by low levels of circulating testosterone, and most evidence suggests that blastema

formation and the rapid phase of antler growth do not require the presence of sex steroids *in vivo*, at least not in red deer. This is confirmed by *in vitro* studies that have been unable to demonstrate a direct effect of testosterone on the proliferation of mesenchymal or cartilage cells, despite the presence of testosterone binding sites. The same group found that testosterone did not sensitize antler cells to the effects of IGF-I (Li *et al.* 1999; Sadighi *et al.* 2001), which has been proposed to be the elusive AGS (Suttie *et al.* 1985). AGS is the non-gonadal trophic factor whose existence was first suggested 60 years ago (Wislocki 1943). The rationale for concluding that IGF-I could be AGS comes from the observation that seasonal peaks in circulating IGF-I coincide with the period of rapid antler growth (Suttie *et al.* 1985), the presence of IGF receptors in the antler tip (Elliott *et al.* 1992) and the fact that IGFs increase the proliferation of cells from perichondrium, mesenchyme and cartilage (Price *et al.* 1994; Sadighi *et al.* 1994). There is evidence for an association between IGF-I concentrations and serum testosterone (Ditchkoff *et al.* 2001; Li *et al.* 2003), although whether previously elevated plasma testosterone levels are directly responsible for the subsequent IGF-I peak remains unclear.

The obvious association between antler development, male reproductive behaviour and high testosterone levels led to the natural assumption that antler regeneration was controlled by testosterone. However, there is increasing evidence from other species that in males as well as females the effects of testosterone on the skeleton are indirect, occurring after its local conversion to oestrogens by aromatase (reviewed by Riggs *et al.* 2002). Recent reports of two men with aromatase deficiency showed that they had unfused epiphyses implying continued bone growth. In common with male mice lacking the aromatase gene they also had low bone mass (Morishima *et al.* 1995; Carani *et al.* 1997). A man lacking the ER has a similar phenotype to male mice lacking its alpha isoform (Smith *et al.* 1994). Over 30 years ago, it was shown that growing antlers were more sensitive to the effects of injected oestrogen than testosterone (Goss 1968). Treatment with 100 mg of oestrogen, but not testosterone, stopped antler growth and induced velvet shedding. At the time, a mechanism for this apparent anomaly was not suggested. More recently, antler perichondrium has been shown by immunocytochemistry to contain ERs (Barrell *et al.* 1999), although the specific isoforms of the ER were not identified. High-affinity binding of oestrogen has also been shown to occur in periosteum, cartilaginous tissue and calcified cartilage, but not in bone (Lewis & Barrell 1994).

Taken together, these findings led us to propose that the effects of androgens on antler development in antlers might also be indirect, following their local conversion to oestrogen by aromatase. We have explored this hypothesis in several ways. The ER was blocked using a physiological dose of a long-acting preparation of the pure ER blocker ICI 182,780 (Zeneca Pharmaceuticals) that binds both ER-alpha and ER-beta. This treatment was associated with a substantial increase in the length of time for which the antlers grew as measured from the time of first growth from the pedicle to shedding of the velvet (Price *et al.* 2000). The means for local conversion of testosterone to oestrogen is available because aromatase mRNA is expressed in the antler tip and in bone. Semi-quantitative

Figure 9. The effect of oestrogen administration *in vivo* on mineralization of the distal antler. (a) Section through the distal antler tip of a 2-year-old red deer stag treated with vehicle control. (Mineralization was revealed by Von Kossa staining (shows black).) There is an extensive zone of chondroprogenitors (CP), and cartilage mineralization (MC) is observed only in proximal regions of the section. (b) Six days after a single injection of oestradiol there is a significant reduction in the extent of the region of chondroprogenitors and there is now extensive mineralization of the distal antler. This is associated with bone formation (not shown). Scale bar, 1 cm.

PCR and immunocytochemistry has shown that the predominant isoform of the ER in antlers is ER α .

To examine in more detail the cellular effects of exogenous oestrogen on antler growth we injected red deer stags subcutaneously on a single occasion with 17- β oestradiol during the early phase of antler growth. The effect was dramatic; longitudinal growth stopped and 14 days after the injection the antler's growing tip was transformed into almost solid bone (Price *et al.* 2002). This response was similar to that described by Richard Goss in sika deer over 30 years ago (Goss 1968). Oestrogen treatment was associated with an inhibition of cell proliferation in the mesenchymal zone, a reduction in the normally extensive region of chondroprogenitors and non-mineralized cartilage and a significant increase in mineralization (figure 9). Oestrogen appears to induce the differentiation of progenitor cells in the perichondrium into osteoblasts instead of along their normal 'default' pathway into chondrocytes. These observations lead us to propose that oestrogen operates by inhibiting the growth of multi-potential progenitor cells in the antler tip (and thus inhibits continuous longitudinal growth of the antler) but stimulates their differentiation. Observations relating to the antlers of castrated animals support this suggestion and raise several further questions about the potential role of sex steroids as natural inhibitors of regeneration. Aristotle first described the effects of castration on antler development: 'if stags be mutilated when, by reason of his age, they have as yet no horns, they never grow horns at all; if they be mutilated when they have horns, the animal does not lose them'. These observations have been confirmed in several studies (reviewed by Goss 1983); if an animal is orchidectomized during the 'hard antler' stage in autumn or winter there is premature casting of the antler as a result of a decline in testosterone levels; essentially this accelerates the natural process of casting. The antlers grown by the castrated deer are often grossly normal in appearance except that they do not completely mineralize and the

velvet skin covering them is not shed. Freezing conditions during the winter will often lead to necrosis and loss of tissue and then these antlers can assume a variety of forms, depending on the species. They have been reported to increase in length and diameter, develop extra branches or, in some species, to develop into large masses on the animal's head (Goss 1983). In roe deer, the effects of castration are particularly dramatic; the antler tissue (known as 'perukes') grows extravagantly to cover the top of the animal's head. Unfortunately very little is known about these 'antleromas' of castrated deer beyond a few small-scale histological studies. There are no reports to suggest that they metastasize and they have generally been assumed to be local growths consisting of transformed fibroblast-like cells (Goss 1983). The administration of testosterone or oestrogen leads to their ossification, shedding of velvet and, as the hormone levels fall, the abnormal antlers will be cast (Fletcher & Short 1974; Lincoln 1975).

It has been proposed that the absence of replicative senescence may deprive an animal's ability to sustain multiple rounds of regeneration (Brookes 1998). However, in mammalian cells replicative senescence serves a purpose; it reduces the possibility of mutagenesis and tumour formation (Campisi 2001; Schmitt *et al.* 2002). Do castrate antlers form bizarre growths in some species because the natural inhibitor (testosterone/oestrogen), which is responsible for controlling the lifespan of progenitor cells with an enhanced capacity for proliferation, is no longer present at the appropriate level? The mechanisms by which sex steroids (and oestrogen in particular) regulate cell-cycle progression and cell differentiation in antlers now need to be identified because this could provide insight into why regeneration is prevented in other mammalian organs. It is worth noting in this context that newt limb blastema cells in culture do not undergo crisis or senescence even after more than 200 generations (Ferretti & Brookes 1988). Furthermore, the incidence of tumours in the regenerating tissues of urodeles is rare (for a review see Tsonis 1983) and this may be because they use different methods of cell-cycle regulation to mammals (Brookes 1998). This is why it is so important that we continue to compare the process of regeneration in lower vertebrates with that in mammalian models, such as the deer antler, if rational strategies are to be developed that will enable complex structures to be regenerated in humans.

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GLOSSARY

- AGS: antler growth stimulus
 ALP: alkaline phosphatase
 AP: antlerogenic periosteum
 BMP: bone morphogenetic protein
 ER: oestrogen receptor
 IGF: insulin-like growth factor
 IHH: Indian hedgehog
 OC: osteoclast
 OPG: osteoprotegerin
 PER: perichondrium-derived
 PPR: parathyroid/parathyroid hormone-related peptide (PTH/PTHrP) receptor
 PTH: parathyroid hormone
 PTHrP: parathyroid hormone-related peptide
 RA: retinoic acid
 RANKL: receptor activator of NF- κ B ligand
 Shh: sonic hedgehog
 TRAP: tartrate-resistant acid phosphatase
 VNR: vitronectin receptor