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RESEARCH ARTICLE

Recapitulation of the Parathyroid Hormone-Related Peptide–Indian Hedgehog Pathway in the Regenerating Deer Antler

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Parathyroid hormone (PTH)-related peptide (PTHrP) and the PTH/PTHrP receptor (PPR) play an essential role in controlling growth plate development. The aim of the present study was to use the deer antler as a model to determine whether PTHrP and PPR may also have a function in regulating cartilage and bone regeneration in an adult mammal. Antlers are the only mammalian appendages that are able to undergo repeated cycles of regeneration, and their growth from a blastema involves a modified endochondral process. Immunohistochemistry was used to establish sites of localization of PTHrP and PPR in antlers at different stages of development. The pattern of Indian Hedgehog (IHH) and transforming growth factor-beta1 (TGFβ1) distribution was also investigated, because PTHrP expression in the developing limb is regulated by IHH and during embryonic growth plate formation TGFβ1 acts upstream of PTHrP to regulate the rate of chondrocyte differentiation. In the antler blastema (<10 days of development), PTHrP, PPR, and TGFβ1 were localized in epidermis, dermis, regenerating epithelium, and in mesenchymal cells but IHH expression was not detected. In the rapidly growing antler (weeks 4–8 of development), PTHrP, PPR, and TGF β1 were localized in skin, perichondrium, undifferentiated mesenchyme, recently differentiated chondrocytes, and in perivascular cells in cartilage but not in fully differentiated hyperytrophic chondrocytes. IHH was restricted to recently differentiated chondrocytes and to perivascular cells in cartilage. In mineralized cartilage and bone, PTHrP, PPR, IHH, and TGFβ1 were immunolocalized in perivascular cells and differentiated osteoblasts. PTHrP and PPR were also present in the periosteum. TGFβ1 in vitro stimulated PTHrP synthesis by cells from blastema, perichondrium, and cartilage. The findings of this study suggest that molecules which regulate embryonic skeletal development and postnatal epiphyseal growth may also control blastema formation, chondrogenesis, and bone formation in the regenerating deer antler. This finding is further evidence that developmental signaling pathways are recapitulated during adult mammalian bone regeneration. *Developmental Dynamics 231:88–97, 2004.*

Key words: type 1 PTH/PTHrP receptor; PTHrP; IHH; TGFβ1; immunolocalization; deer antler growth; regeneration

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INTRODUCTION

An ability to regenerate complex structures is widespread among lower organisms and is retained in some vertebrate species such as anuran amphibians (Goss, 1969; Tanaka et al., 1999). However, adult mammalian examples of epimorphic regeneration are extremely rare with the most dramatic example being the annual replacement of antlers in deer. These large complex bones function as secondary sexual characteristics used for fighting and display in males, although in some species, they also develop in females (Goss, 1983).

Each year the old set of antlers is shed (cast) in the spring and a regeneration blastema forms from which the new set of antlers develops. There then follows a period of rapid longitudinal growth, by a mod-

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ified endochondral process in the distal tip of each branch, until the final form of the mature antlers is achieved (Banks and Newbrey, 1983). In the growing antler, the whole spectrum of endochondral bone development takes place; uncommitted progenitors in the perichondrium and subjacent mesenchyme differentiate into chondrocytes arranged in vertical columns. The cartilage columns then become mineralized before being replaced with bone by the actions of osteoclasts and osteoblasts. Although the matrix composition of antler cartilage is similar to that of hyaline cartilage (Price et al., 1996), it is structurally different as it contains large numbers of vascular channels. Perivascular tissues in antler cartilage contain large numbers of osteoclasts that are required for the extensive tissue remodeling that is associated with the antlers' rapid rates of endochondral ossification (Faucheux et al., 2001).

The local cellular mechanisms that regulate antler regeneration are not well understood, although it is well established that the annual cycle of antler regeneration is regulated by sex steroids; antler casting, blastema formation, and rapid longitudinal growth are associated with low circulating levels of testosterone (Suttie et al., 1995). As the period of reproductive activity approaches in late summer circulating levels of testosterone increase and are associated with cessation of longitudinal growth and increased intramembranous bone formation and mineralization. The velvet skin covering the antlers is then eventually shed leaving solid "dead" structures that are able to fulfill their biological function (Brown et al., 1983).

Our recent studies support the hypothesis that the molecular signals that regulate antler morphogenesis are similar to those that regulate embryonic limb development. For example, retinoic acids and their receptors are expressed in antler tissues and regulate chondrocyte and osteoclast differentiation in vitro (Allen et al., 2002). We also have evidence from in vitro studies that parathyroid hormone-related peptide (PTHrP) stimulates the proliferation and inhibits differentiation of antler chondrocytes (Faucheux and Price, 1999). PTHrP also regulates the differentiation of antler osteoclasts, which express the G protein-coupled type I PTH/PTHrP receptor (PPR; Faucheux et al., 2002).

Numerous studies have shown a fundamental role for PTHrP and PPR in regulating chondrocyte differentiation during skeletal development. Mice with a null mutation in either the PTHrP or PPR gene show similar abnormalities in the growth plate; they have severe skeletal malformations resulting from an increase in bone maturation, i.e., accelerated chondrocyte differentiation, and premature ossification (Karaplis et al., 1994; Amizuka et al., 1994; Lanske et al., 1996). Similarly, in humans, inactivating mutations in the PPR that occur in Blomstrand lethal osteochondrodysplasia result in increased bone maturation (Jobert et al., 1998), whereas the activating mutation that occurs in Jansen-type metaphyseal chondrodysplasia delays chondrocyte maturation (Schipani et al., 1995).

PTHrP expression in the developing limb is regulated by the morphogen Indian Hedgehog (IHH), a vertebrate homolog of the *Drosophila* segment polarity gene Hedgehog, which is expressed in prehypertrophic chondrocytes (Lanske et al., 1996; Vortkamp et al., 1996; Chung et al., 2001). Studies in transgenic mouse models have shown that PTHrP and IHH control differentiation of growth plate chondrocytes at multiple steps (Kobayashi et al., 2002). For example, IHH controls proliferation of chondrocytes independently of PTHrP and also regulates osteoblast development while PTHrP acts by inhibiting chondrocyte differentiation (St. Jacques et al., 1999; Kobayashi et al., 2002).

The aim of the present study was to use the deer antler as a model to explore the hypothesis that the PTHrP/IHH signaling pathway is recapitulated during adult mammalian bone regeneration. Immunohistochemistry was used to establish sites of localization of PTHrP, IHH, and PPR in antlers at different stages of development. The potential role of TGFβ1 as a primary regulator of PTHrP expression in antlers was also investigated since TGFβ1 mRNA has been shown to be expressed in antler tissues (Francis and Suttie, 1998) and during mouse embryonic growth plate formation. TGFβ1 acts upstream of PTHrP to regulate the rate of hypertrophic chondrocyte differentiation (Serra et al., 1999). TGFβ has also been shown to up-regulate PTHrP expression in osteoblasts (Guenther et al., 1995) and in nonskeletal tissues such as the mammary gland (Ferrari et al., 1992).

RESULTS

Antler Morphology and Histology

Twenty-four hours after the old set of antlers are cast, there is a large open wound on the surface of the pedicle (the extension of the frontal bone to which antlers are attached) surrounded by a rim of skin (Fig. 1A). A regeneration blastema then forms, consisting of mesenchymal progenitor cells surrounded by epithelial tissue. By 7–9 days, re-epithelialization is almost complete and the position of the future branches can be identified with the naked eye (Fig. 1B). There then follows a period of rapid longitudinal growth (Fig. 1C) by a modified endochondral process until the final form of the mature antlers is achieved (Fig. 1D). Growth in diameter occurs as a result of intramembranous bone formation along the antler shaft. In sections through the growing tip of the branch of an antler, the whole spectrum of endochondral bone development can be observed (Fig. 2A). Although the histology of the growing antler has been reported previously (Banks and Newbrey, 1983; Price et al., 1996), it will be described here briefly to orientate the reader. Antler skin, or velvet, consists of an epidermis and a dermis containing numerous hair follicles and sebaceous glands (Fig. 2B). Immediately below the skin is the perichondrium, which consists of an outer, more collagenous layer containing numerous blood vessels and a subjacent layer of mesenchymal cells that are not highly differentiated (Fig. 2C). Previously, we have shown that these

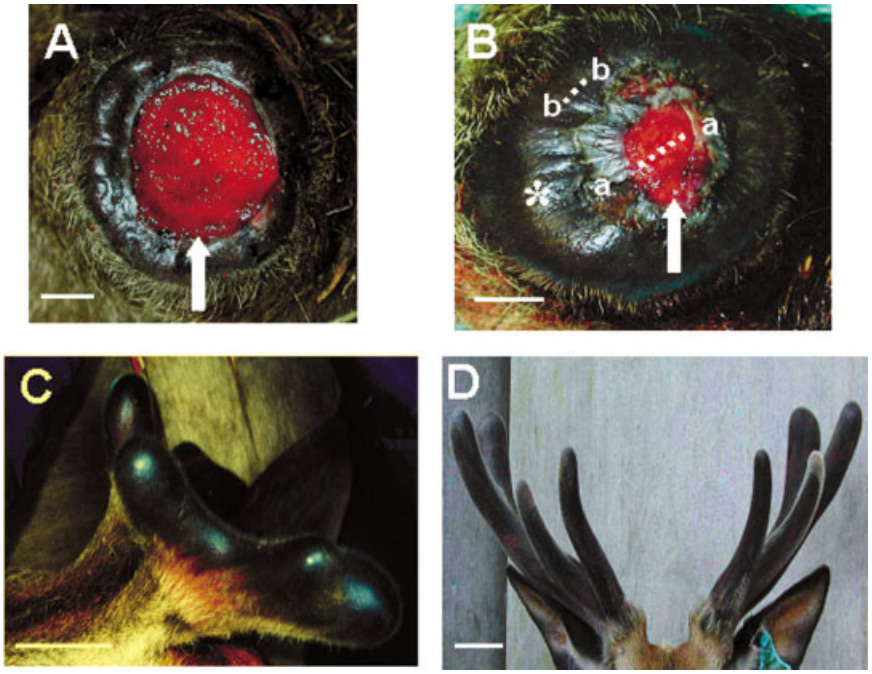

Fig. 1. Red deer antlers at different stages of development. **A:** Blastema 24 hr after the old antler has been cast. White arrow shows the leading edge of the regenerating epithelium. **B:** Blastema ~7 days after casting. The asterisk marks the position of a future branch. Dotted lines a–a and b–b show regions from which sections were cut. **C:** At 4 weeks of growth. **D:** At 8 weeks of growth. Scale bars = 1 cm in A,B, 4 cm in C,D.

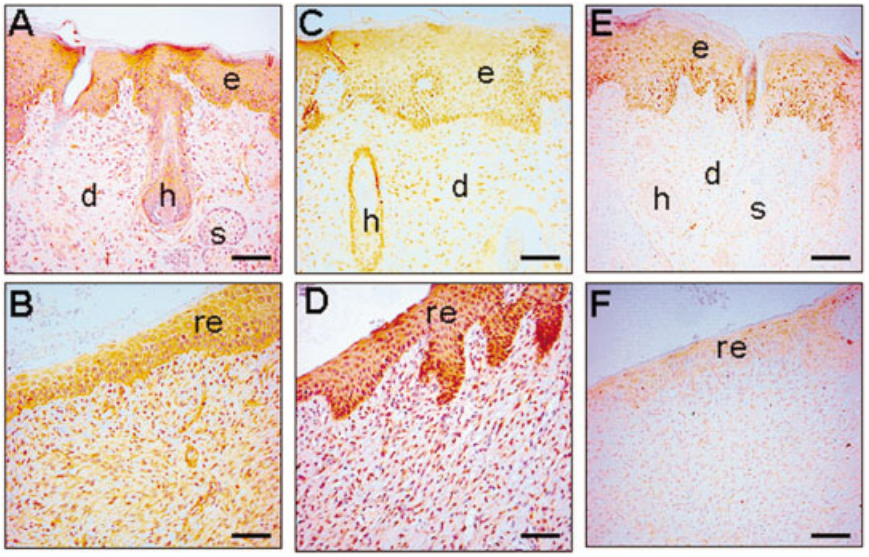

Fig. 3. Immunolocalization of parathyroid hormone (PTH) -related peptide (PTHrP), PTH/PTHrP receptor (PPR), and transforming growth factor-beta1 (TGFβ1) in the early antler blastema, 4 days after the previous hard (dead) antler had been cast. **A,C,E:** Sections through the periphery of the blastema (b–b in Fig. 1). **B,D,F:** Sections through the centre of the blastema (a–a in Fig. 1), illustrating regenerating epidermis and subjacent mesenchymal cells. Immunoreactivity is revealed as a brown stain. A,B: PTHrP. C,D: PPR. E,F: TGF-β1. e, epidermis; d, dermis; re, regenerating epithelium; h, hair follicle; s, sebaceous gland. Scale bars = 100 μm in A–F.

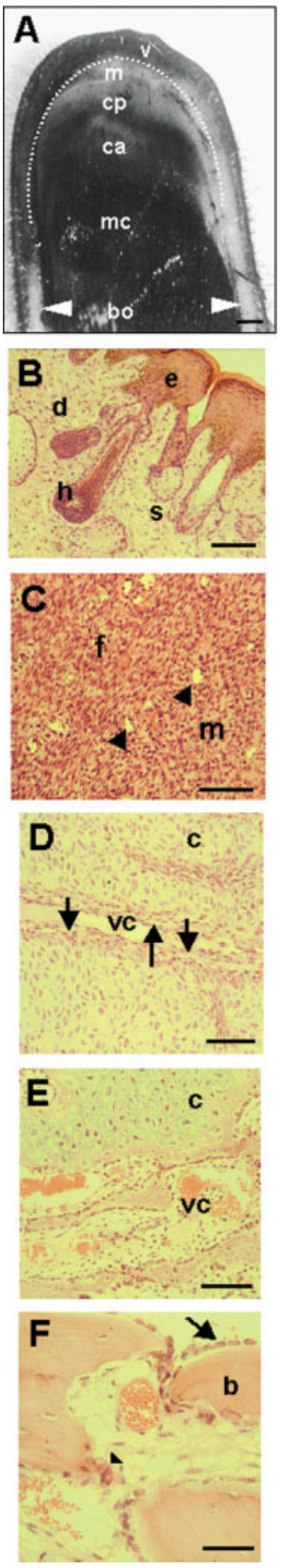

Fig. 2.

mesenchymal cells do not express alkaline phosphatase (ALP) or type II collagen (Price et al., 1994, 1996). As they differentiate into chondrocytes they become arranged into columns (Fig. 2D) separated by vascular channels and perivascular tissue, which contains cells of the osteo-

blast and osteoclast lineages (Price et al., 1996; Allen et al., 2002; Fig. 2D). There is an extensive zone of cartilage distally that is not mineralized, more proximally mineralization takes place (Fig. 2E) and cartilage is replaced with bone by the coordinated actions of osteoclasts and osteoblasts (Fig. 2F).

Immunolocalization of PTHrP, PPR, IHH, and TGFβ1 in the Antler Blastema

In the early blastema (<day 4), PTHrP and PPR were localized in the peripheral epidermis and dermis (Fig. 3A,C), in the regenerating epithelium of the healing wound and in subjacent mesenchymal cells (Fig. 3B,D). TGFβ1 was also present in these tissues, although the intensity of staining was less than that for PTHrP and PPR (Fig. 3E,F). IHH was not expressed in any of these tissues (data not shown).

Immunolocalisation of PTHrP, PPR, IHH, and TGFβ in the Growing Antler

The localization of PTHrP, PPR, IHH, and TGFβ were similar in late stage blastemas (≥9 days) where chondrogenesis has begun and at the distal end of the main branch of the growing antler (4–8 weeks). PTHrP was localized in the dermis of the skin and in hair follicles, and in the perichondrium and associated blood vessels (Fig. 4). In the underlying proliferating (proliferating cell nuclear antigen (PCNA) -positive) undifferentiated mesenchymal cells, PTHrP was also present. More proximally, PTHrP antigen was present in perivascular cells, which continue to proliferate and in recently differentiated, nonproliferating, chondrocytes (Fig. 4). In these chondrocytes, staining was mainly confined to cytoplasmic granules, although some nuclear staining was also detected. Fully differentiated chondrocytes that secrete collagen type X did not possess PTHrP, although high levels of staining were seen in the proliferating perivascular cells in cartilage (Fig. 4).

The pattern of PPR localization in the skin and the perichondrium of the growing antler was similar to that of PTHrP, although no staining was seen in blood vessels of the perichondrium (Fig. 4). Higher levels of PPR antigen were observed in recently differentiated chondrocytes where both nuclear and perinuclear staining was detected (arrow in insert). The PPR antigen was not detected in fully differentiated chondrocytes and was also low in perivascular cells (Fig. 4).

IHH was not detected in the skin, the perichondrium, or undifferentiated mesenchyme but was localized in recently differentiated chondrocytes (Fig. 4). In cartilage, IHH antigen was not detected in fully differentiated chondrocytes; however, like PTHrP, it was present in perivascular cells (Fig. 4).

TGFβ1 was found to have a similar pattern of localization to PTHrP, although the intensity of staining in some regions was lower. Immunoreactivity was identified in cells in the dermis, in perichondrium cells, in the collagenous extracellular matrix of the perichondrium but not in associated blood vessels (Fig. 4). TGFβ1 was present at low levels in mesenchyme and recently differentiated chondrocytes, and, although positive staining was only seen in a small proportion of fully differentiated chondrocytes (Fig. 4E3, arrow), it was detected in perivascular cells in cartilage (Fig. 4).

Immunolocalisation of PTHrP, PPR, IHH, and TGFβ in Mineralized Cartilage, Bone, and in Periosteum

In mineralized cartilage, the strongest staining for PTHrP was in perivascular tissue, particularly in osteoblastic cells (Fig. 5A). At more proximal sites of bone formation, positive staining was observed in fully differentiated osteoblasts (Fig. 5E). The periosteum of the antler is multilayered consisting of an outer fibrous component and an inner more cellular layer immediately adjacent to sites of intramembranous bone formation. PTHrP was localized in a proportion of cells in the fibrous periosteum and in the majority of cells in the cellular periosteum as well as in periosteal blood vessels (Fig. 5I). The pattern of localization of PPR was similar to that of PTHrP in cartilage, bone, and periosteum (Fig, 5B,F,J); however, PPR was not present in blood vessels in the periosteum (data not shown). In cartilage and bone, TGFβ-1 was detected in perivascular cells and in osteoblasts (Fig. 5C,G,K), although in periosteum TGFβ-1 was only expressed in the cellular layer and then at only low levels. IHH, like PTHrP and PPR, was localized in perivascular cells in cartilage (Fig. 5D), and in osteoblasts (Fig. 5H), IHH was not present in the periosteum (data not shown). No staining was seen in negative control sections (Fig. 5L–N).

Regulation of PTHrP Synthesis by TGFβ1 in Cultured Antler Cells

The synthesis of PTHrP by cultured antler cells was measured in the presence and absence of 2 ng/ml TGFβ1 (Fig. 6). In control cultures, cells derived from the perichondrium synthesize significantly higher concentrations of PTHrP than cells derived from either blastema or cartilage ($P < 0.0001$). When treated

Fig. 2. Histology of the antler tip. **A:** Longitudinal tissue section of antler to show macroscopic appearance of tissues. v, velvet skin; m, mesenchyme; cp, chondroprogenitor region; ca, nonmineralized cartilage; mc, mineralized cartilage; bo, bone. Arrowheads mark sites of intramembranous bone formation. Perichondrium is indicated by the dotted line. B–F: Hematoxylin and eosin–stained undecalcified paraffin sections. **B:** Velvet skin. e, epidermis; d, dermis; h, hair follicle; s, sebaceous gland. **C:** Perichondrium. Fibrous perichondrium (f) and subjacent zone of mesenchymal cells (m). Blood vessels are marked by arrowheads. **D:** Nonmineralized cartilage. Recently differentiated chondrocytes (c) are arranged in columns separated by vascular channels (vc). Perivascular tissue is marked with arrows. **E:** Mineralized cartilage. Chondrocytes (c) are more hypertrophic and the vascular channels (vc) larger. **F:** Bone. Cuboidal osteoblasts are marked with the arrow. The arrowhead shows an osteoclast. Scale bars = 0.5 cm in A, 100 μm in B–E, 50 μm in F.

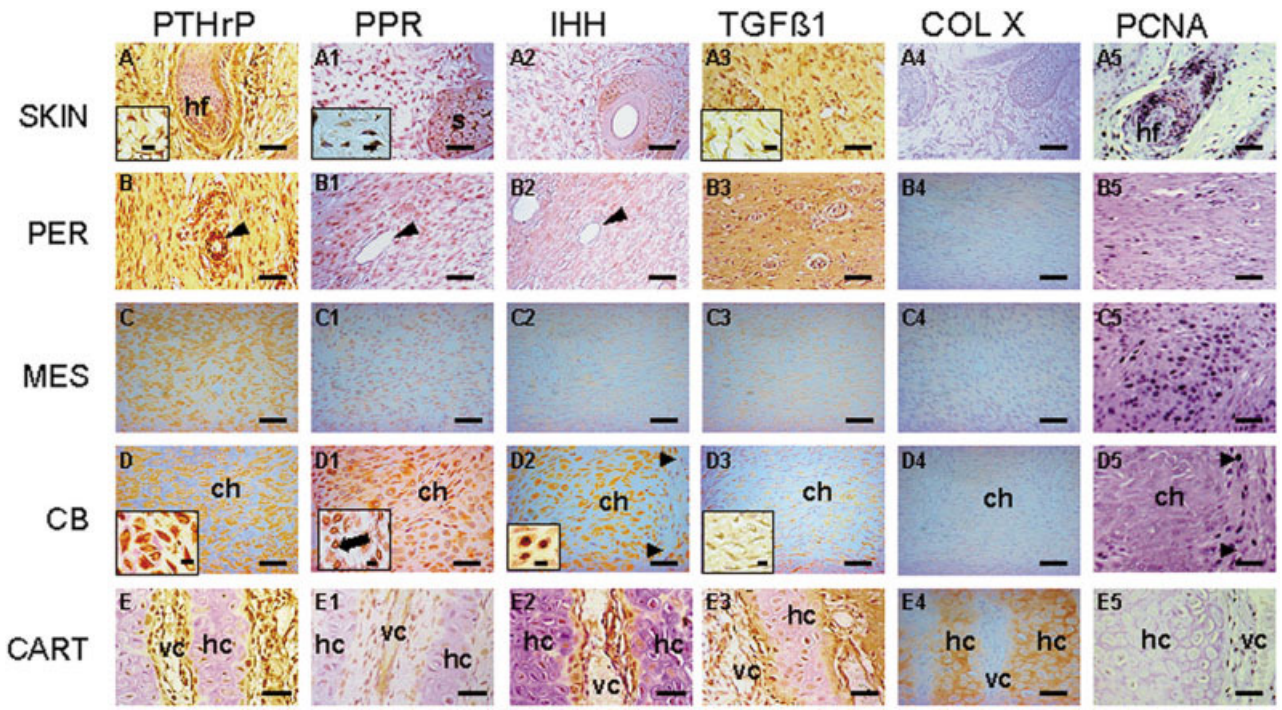

Fig. 4. Immunolocalization of parathyroid hormone (PTH) -related peptide (PTHrP), PTH/PTHrP receptor (PPR), Indian Hedgehog (IHH), and transforming growth factor-beta1 (TGFβ1), type x collagen (COLX), and proliferating cell nuclear antigen (PCNA) in tissues of the distal antler tip. Antler harvested at 4 weeks of growth. **A–A5:** Skin (row 1). **B–B5:** Perichondrium (row 2, PER). Arrowheads indicate blood vessels. **C–C5:** The region of undifferentiated mesenchyme cells (row 3, MES). **D–D5:** The region of chondroblasts/recently differentiated chondrocytes (row 4, CB), chondrocytes are aligned in columns separated by small vascular channels and perivascular cells (arrowheads). The arrow points to a positive chondrocyte. **E–E5:** Cartilage (row 5, CART). Negative control sections, skin (PTHrP) and perichondrium (PPR), are shown in the box in the upper right-hand corner. Inserts: Higher-power images. ch, chondrocyte; hc, hyalin cartilage; hf, hair follicle; s, sebaceous gland; vc, vascular channels. Scale bars = 50 μm in all panels, 12.5 μm in all inserts.

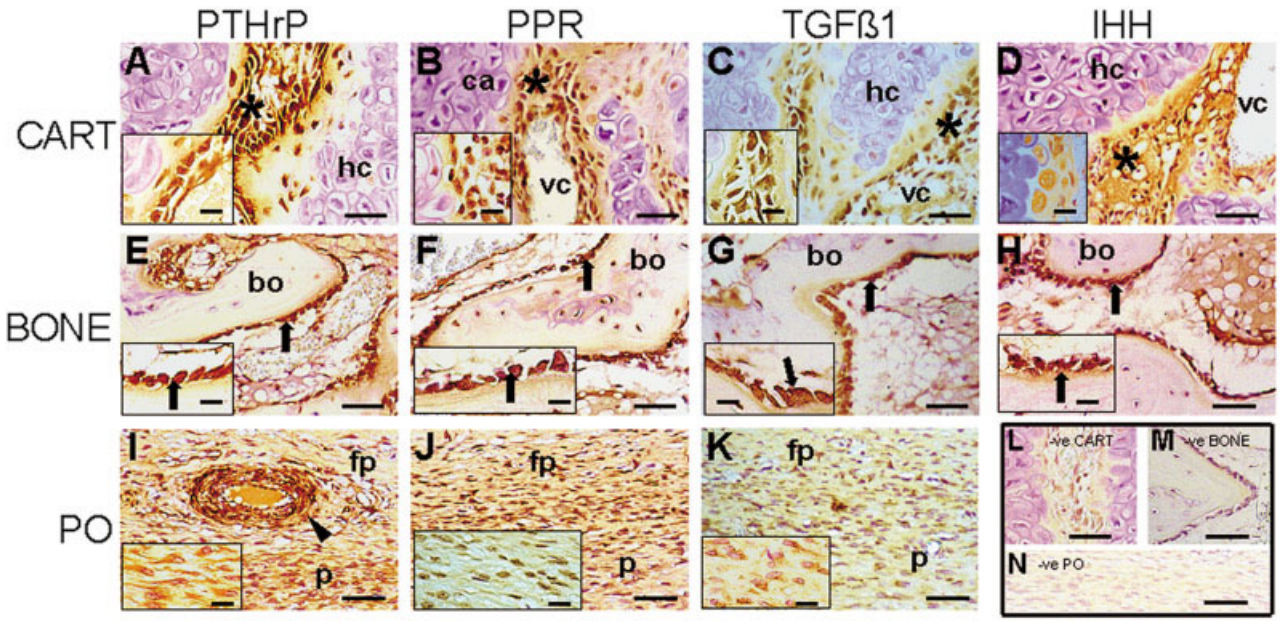

Fig. 5. Immunolocalization of parathyroid hormone (PTH) -related peptide (PTHrP), PTH/PTHrP receptor (PPR), Indian Hedgehog (IHH), and transforming growth factor-beta1 (TGFβ1) in mineralized cartilage, bone, and in periosteum of the growing the antler. Antler harvested at 8 weeks. **A–D:** Mineralized cartilage (CART): A, PTHrP; B, PPR; C, TGFβ1; D, IHH. vc, vascular channels; ca, differentiated chondrocytes; hc, hyalin cartilage. Asterisks indicate perivascular cells. The larger cuboidal cells are osteoblastic cells. Pictures in inserts are higher magnification images of these tissues. **E–H:** Bone: E, PTHrP; F, PPR; G, TBFβ1; H, IHH. bo, bone. Osteoblasts are indicated by arrows. **I–K:** Periosteum (PO): I, PTHrP; J, PPR; K, TGFβ-1. fp, fibrous periosteum; p, inner cellular periosteum. The arrowhead indicates a blood vessel. **L–N:** Negative (-ve) control sections of cartilage (L), bone, and periosteum (PO). Scale bars = 50 μm in all panels, 12.5 μm in all inserts.

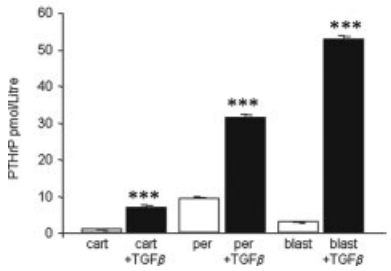

Fig. 6. Parathyroid hormone-related peptide (PTHrP) synthesis in cultured antler cells and its regulation by transforming growth factor-beta1 (TGFβ1). Primary cultures isolated from blastema (blast), perichondrium (per), and cartilage (cart) tissue were cultured with 2 ng/ml TGFβ1 or vehicle control for 48 hr. Results are expressed in picomoles PTHrP/L and represent the mean ± SEM (n = 4). Triple asterisks indicate $P < 0.0001$ compared with control conditions. PPR, the parathyroid hormone/PTHrP receptor; IHH, Indian Hedgehog.

with TGFβ1, cells derived from all tissues showed significantly increased PTHrP synthesis ($P < 0.0001$). Cells from the blastema showed the greatest increase in PTHrP synthesis after treatment with TGFβ1.

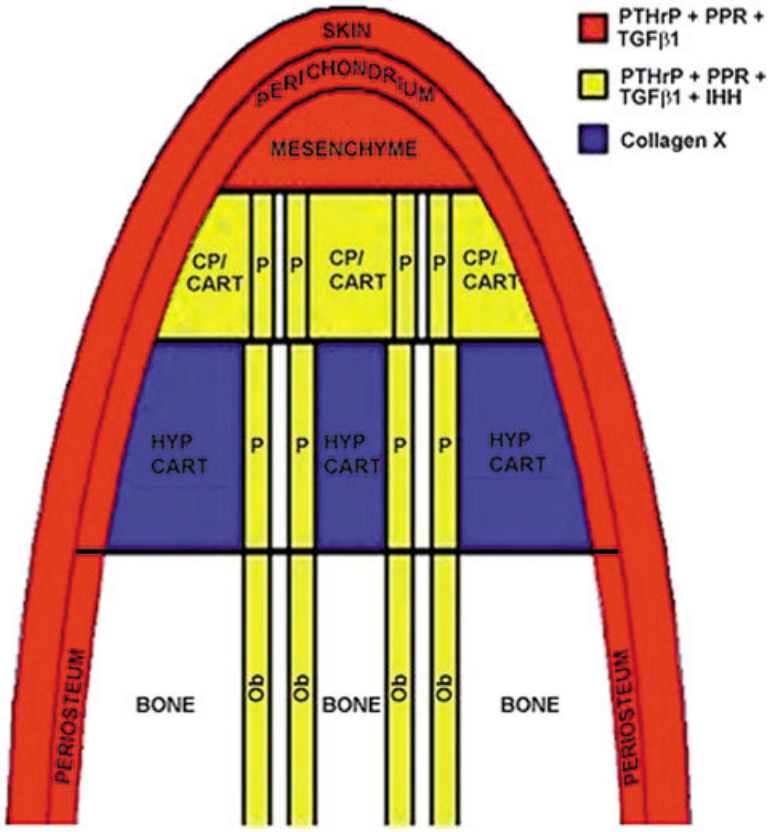

Fig. 7. Schematic diagram summarizing the pattern of localization of PTHrP, PPR, IHH, and TGFβ1 in the growing antler tip.

DISCUSSION

In this study, sites of PTHrP, PPR, IHH, and TGFβ1 localization in the regenerating deer antler at different stages of development were identified using immunohistochemistry (summarized in Fig. 7). Although numerous factors determine cellular interactions in the embryonic limb (reviewed by Capdevila and Izpisua Belmonte, 2001), we focused on these molecules, because PTHrP and IHH are "master regulators" of chondrocyte proliferation during endochondral bone formation and IHH also regulates osteoblast differentiation (reviewed by Kronenberg, 2003). Furthermore, in previous in vitro studies, we had shown that PTHrP regulates the differentiation of antler chondrocytes and osteoclasts (Faucheux and Price, 1999; Faucheux et al., 2002).

Antlers grow by a process of epimorphic regeneration; after the previous set of solid dead antlers are cast off in the spring a blastema forms, which is composed of a healing wound surface and a subjacent layer of mesenchymal cells. PTHrP and its receptor (PPR) are present in regenerating epithelium of the early blastema, which suggests that PTHrP may regulate epithelialization and/or epithelial–mesenchymal interactions. However, because PTHrP and PPRs are also localized in the majority of mesenchymal cells in the blastema, PTHrP can be considered to be a phenotypic marker of antler progenitor cells. The synthesis of PTHrP by cells cultured from the blastema was increased significantly by TGFβ-1, which is also expressed in the epithelium and in mesenchymal cells of the blastema. Localization of TGFβ1 in the blastema was unexpected as TGFβ1 promotes wound repair and scar formation (Cordeiro et al., 2003) and Goss (1983) proposed that antler regeneration represented a failure of scar formation, based on his observation that suturing the skin over the regenerating blastema inhibited antler development. However, the mechanisms regulating TGFβ's action in vivo are extremely complex and require further study in the context of antler regeneration. IHH is not present in the early blastema and, thus, does not regulate PTHrP synthesis at this stage of antler regeneration.

The function(s) of PTHrP in the regenerating antler blastema are likely to be complex and now need to be explored in greater detail. PTHrP does not appear to regulate proliferation of progenitor cells derived from perichondrium (Faucheux and Price, 1999). However, once chondrogenesis is evident, it may maintain proliferation of chondroprogenitor cells, since our earlier in vitro observations showed that PTHrP inhibited differentiation but stimulated growth in chondrocyte micromass cultures (Faucheux and Price, 1999). The observation that there is no PTHrP in terminally differentiated, nonproliferating chondrocytes in the blastema would support this suggestion.

After blastema formation, antlers grow by a process of modified endochondral ossification in the distal

tip of each branch. At these sites of chondrogenesis, PTHrP and the PTH/PTHrP receptor are present in cells at several stages of differentiation. Staining for PTHrP was strongest in the perichondrium, which is further evidence that PTHrP is a phenotypic marker for early progenitor cells in the antler. Of interest, PTHrP (but not PPR) was detected in cells lining the blood vessels of the perichondrium, raising the intriguing possibility that some antler stem cells may be derived from a circulating pool. PPR is also localized in the antler perichondrium, which suggests that PTHrP could regulate its own expression at this site. This finding represents an important difference from embryonic long bones and postnatal growth plate where PPR expression is limited to chondrocytes and PTHrP synthesis in the perichondrium is regulated by IHH acting by means of its receptor *patched* (Lee et al., 1994, 1996; Vortkamp et al., 1998; Bram et al., 2000).

In growing antler, PTHrP and PPRs are also present in a zone of undifferentiated (they do not express ALP or type I collagen) proliferating mesenchymal cells below the perichondrium. PTHrP and its receptor are then maintained in a population of cells that we have defined previously as chondroprogenitors, because they express type IIA as well as type IIB collagen mRNA (Price et al., 1996); however, there is no PTHrP or PPR expression in differentiated hypertrophic chondrocytes. This pattern of localization is consistent with PTHrP playing a role in the regulation of chondrocyte growth and differentiation in regenerating antlers, as it does in growth plate.

PTHrP mRNA and protein have been found to be expressed in perichondrium, proliferating chondrocytes, prehypertrophic and hypertrophic chondrocytes in the postnatal growth plate of the mouse (Kartsogiannis et al., 1997), chicken (Medill et al., 2001; Farquharson et al., 2001), and human (Nakase et al., 2001; Kindblom et al., 2002). The pattern of localization of PTHrP and its receptor in antler cartilage, therefore, is not entirely consistent with findings in the embryonic limb. Probably the most obvious difference between antlers and the embryonic and postnatal growth plate is that, in antlers, PTHrP antigen is not present in differentiated hypertrophic chondrocytes, although the functional significance of this observation remains unclear. It is worth noting that the zone of cartilage hypertrophy in antlers is far more extensive than that in the growth plate, with type X collagen being expressed by the majority of chondrocytes (Price et al., 1996).

This study also provides evidence that an interaction between IHH and PTHrP may take place in the regenerating antler as it does in the growth plate. IHH is present in recently differentiated antler chondrocytes but not in less differentiated cells in the perichondrium or the mesenchyme zone. This finding represents a similar pattern of localization to that described in developing long bones and the postnatal growth plate where IHH is expressed by prehypertrophic and early hypertrophic chondrocytes (Lanske et al., 1996; Vortkamp et el., 1996, 1998; St. Jacques et al., 1999; Nakase et al., 2001; Kindblom et al., 2002). Because TGFβ1 is also present in tissues that express PTHrP a feedback loop may exist whereby IHH induces PTHrP expression by means of the up-regulation of TGFβ1, as has been described in the developing limb, although in the limb, TGFβ2 was the member of the TGFβ family involved (Alvarez et al., 2002). Furthermore, in antler cells cultured from perichondrium and cartilage, TGFβ1 increases the synthesis of PTHrP. Although it would be extremely difficult to explore this interaction in vivo, ex vivo studies should now be undertaken to establish the mechanism(s) by which IHH regulates PTHrP synthesis. Clearly an autocrine/paracrine process could be involved because PTHrP, its receptor and IHH are all expressed in prehypertrophic antler chondrocytes.

Antler cartilage is unique in that it contains several large vascular spaces and perivascular cells that express ALP (Price et al., 1994), type I collagen mRNA (Price et al., 1996), and osteocalcin (Allen et al., 2002), indicating that they are of the osteoblast lineage. Although there is no PTHrP in hypertrophic chondrocytes, the adjacent perivascular tissue possesses the PTHrP, PPR, IHH, and TGFβ1 antigens. This finding is consistent with observations that have previously shown the presence of IHH (Murakami et al., 1997), PTHrP (Walsh et al., 1997), PPR (McCauley et al., 1996), and TGFβ1 (Linkhart et al., 1996) in osteoblastic cells. These factors could act independently or as part of a complex autocrine/paracrine network to regulate osteoblast differentiation. Both IHH (St. Jacques et al., 1999; Nakamura et al., 1997) and TGFβ (Reyes-Botella et al., 2002; Gurlek and Kumar, 2001) have been shown to be capable of promoting osteoblastic differentiation, while PTHrP may induce (Motomura et al., 1996; Carpio et al., 2001) or inhibit (Du et al., 2000; Miao et al., 2001) osteoblast differentiation.

Another potential target cell for PTHrP in the perivascular tissue of antler cartilage are osteoclast progenitors. TRAP staining reveals that there are numerous cells of the osteoclast lineage differentiating at this site, and in vitro studies have shown that PTHrP acts as a potent stimulator of osteoclast differentiation (Faucheux et al., 2001, 2002). In addition to RANKL-mediated effects, PTHrP also acts directly on the antler osteoclasts and these cells express PPR at an mRNA and protein level (Faucheux et al., 2002).

In conclusion, this study has demonstrated that some of the major signaling molecules that regulate embryonic long bone development and postnatal growth plate growth are also present in the regenerating deer antler. Sites of PTHrP, PPR, TGFβ1 and IHH localization indicate that these factors may regulate several biological processes in the antler, including blastema formation, chondrogenesis, and osteoblast differentiation. This finding supports our hypothesis that there is evolutionary conservation in the antler of developmental signaling pathways that occur during adult bone regeneration. A similar recapitulation of the signaling pathways has also been observed in fracture repair models (Vortkamp et al., 1998; Ferguson et al., 1999; Murakami and Noda, 2000). Establishing the molecular and cellular mechanisms involved in antler regeneration may lead to the development of strategies for im-

proving cartilage and bone regeneration in man and other animals.

EXPERIMENTAL PROCEDURES

Animals

Antlers were harvested at postmortem from red deer (*Cervus elaphus*) stags (2 years old, and weighing approximately 100 kg) at different stages of development: < 4 days after casting (early blastema stage), 9 days after casting (late blastema stage), and between 4 and 8 weeks (period of longitudinal growth; Fig. 1A–D).

Tissues

The distal tips of antlers were removed aseptically and sectioned longitudinally (Price et al., 1994, 1996). Half was placed in medium (Dulbecco's medium containing 10% fetal bovine serum (FBS), penicillin–streptomycin (PS, 100 IU–100 mg/ml), and Fungizone (F, 2.5 mg/ml, Invitrogen, Paisley, Scotland)) before tissue culture. The remaining tissue was cut into 0.5 cm^3 blocks, fixed for 1 week in 4% paraformaldehyde (pH 7.4), and embedded in paraffin wax for sectioning. Sections (10 μm) were mounted on Superfrost plus slides (BDH, Merck, Leicestershire, UK). For immunohistochemical staining, sections were deparaffinized in xylene, rehydrated through graded ethanols, and stained with 1% Harris hematoxylin for 30 sec (Sigma, Poole, UK). Sections were then cleared in xylene and mounted.

Immunohistochemistry

Deparaffinized tissue sections were placed in methanol containing 1% H_2O_2 for 30 min to block endogenous peroxidase activity. Sections were rinsed three times with phosphate-buffered saline (PBS), and nonspecific binding blocked with PBS containing 5% new born calf serum (PBS/NCS) and 10% normal swine serum (Dako, Cambridge, UK) for 30 min at room temperature. Sections were incubated overnight at 4°C in a humidified chamber with primary antibody diluted in PBS/NCS. Depending on the primary antibody used, the second antibody (Dako) was either a biotinylated multi-link swine anti-goat/rabbit/mouse (1:200 dilution), a biotinylated swine anti-rabbit (1:40 dilution), or horse anti-rabbit/mouse(1:285 dilution) and was applied for 30 min. Detection was carried out using the vector ABC method with diaminobenzidine tetrahydrochloride as the substrate (Sigma, Poole, UK). After counterstaining in a 1% hematoxylin solution for 30 sec, sections were mounted using VectaMount (Vector Labs, Peterborough, UK).

The following primary antibodies were used: (1) goat anti-IHH raised against an amino acid sequence at the amino terminus of human IHH (1:100; Santa Cruz, CA); (2) rabbit anti-PTHrP (1:50) raised against amino acids 1–16 and 1–34 of human PTHrP, which recognizes deer PTHrP (Faucheux et al., 2002); (3) rabbit anti-PPR raised against an amino acid sequence of human PPR (1:30; Babco, Berkeley, CA), which cross-reacts with deer PPR (Faucheux et al., 2002); (4) rabbit anti-TGFβ1 raised against an amino acid sequence at the carboxy-terminus of human TGFβ1 (1:200; Santa Cruz, CA); (5) mouse anti-deer collagen type X (1:100; a gift from Gary Gibson), and mouse anti-human PCNA (Dako). In control sections, the primary antibody was substituted with rabbit (Vector Labs), goat (IgG, Sigma), or mouse (Vector Labs) immunoglobulins.

Cell Culture and PTHrP Assay

All products for tissue culture were purchased from GibcoBRL (Paisley, Scotland, UK) except where specified. Cell digests were prepared from the blastema, perichondrium and cartilage using techniques described previously (Price et al., 1994; Faucheux et al., 2001).

First-passage cells were seeded into 12-well plates (10^5 cells/well) and cultured in growth medium (Fitton Jackson's modification of BGJb medium, supplemented with 10% FBS and PS antibiotics (100 IU–100 mg/ml)) until confluent. Cells were then cultured for 2 days in the presence or absence of 2 ng/ml recombinant human TGFβ1 (R&D systems, Abingdon, UK). PTHrP levels in the conditioned culture media were determined by a human IRMA (Mitsubishi Chemical Co, Tokyo, Japan). The species cross-reactivity of the assay was assessed by a parallel assay of a serial dilution of samples from antler cells with the standard curve.

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