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Vascular Localization and Proliferation in the Growing Tip of the Deer Antler

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ABSTRACT

The process of angiogenesis is of interest because of the significant clinical benefits associated with controlling vascular growth. Within the antler, chondrogenesis and antler elongation are occurring at the rate of 1–2 cm per day and thus blood vessels are growing at this same rapid pace. We demonstrate that the process of angiogenesis in the antler is controlled at various tissue locations. The findings clearly differentiate the spatial location of the stem cells that drive chondrogenesis from the proliferation process driving the angiogenesis. Vessels within the lateral dermis contained BrdU-positive cells, suggesting that these vessels were elongating. Within the precartilage region, proliferating vessels were detected in bundles of complex structure evenly distributed throughout this tissue layer. The support cells within these bundles of vessels were detected by staining with α-smooth muscle actin, while the endothelial cells were negative. Additionally, the α-smooth muscle actin staining was found in association with the cartilage cells of the antler. The marked proliferation of the vascular associated cells in the precartilage region identified this area as a major region of vascular growth in the antler. We propose that within the precartilage region, the most likely mechanisms to explain the observed vascular morphology are that of vascular extension of the existing vessels and intussusceptive angiogenesis or sprouting to generate the small bundles of vessels. Anat Rec Part A, 288A: 973–981, 2006.

Key words: angiogenesis; cervine; velvet; cartilage; endothelial

Blood vessel formation occurs either via vasculogenesis, which is the de novo formation of vessels from angioblastic precursors, or via angiogenesis, where vessels form from an existing framework (Risau, 1997). The proposed mechanisms by which angiogenesis can occur vary and include angiogenic sprouting, lymphangiogenesis, intussusceptive growth, and growth via endothelial precursors from bone marrow (Carmeliet and Jain, 2000b; Burri and Djonov, 2002; Djonov et al., 2002).

Relatively little is known about the vasculature of deer antler. Angiography of red deer stags has revealed that the arterial vessels of the antler originate from branches of the superficial temporal artery. Blood returns via the core of the antler (Suttie et al., 1985). Rates of antler growth vary between the deer species with measurements in red deer estimating a growth rate of 10 mm/day (Fennessy et al., 1991). Cartilage, bone, nerves, support tissues, and blood vessels must also therefore grow at this rate. We have investigated the vascular proliferation response occurring during angiogenesis in the antler. Growth in the red deer antler is driven from the tip of the antler. The antler tip is made up of several tissue layers. Distal to proximal, these include the dermis, reserve mesenchyme, precartilage, transition zone, and cartilage (Li et al., 2002). The antlerogenic stem cells are located within the mesenchymal layer, which measures less than 3 mm in depth and lies as a cap at the tip between the dermis and precartilage

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regions (Fig. 3). Within this region, the mesenchymal cells proliferate and drive the chondrogenesis processes and thus the upward growth of the antler. Therefore, blood vessels that lie above this region are pushed upward and the vessels below within the less calcified precartilage region are extended.

BrdU labeling and Ki67 immunostaining are markers of proliferating cells (MacCallum and Hall, 1999). We have also investigated the expression of smooth muscle actin (α-SMA) as a means of labeling the antler blood vessels. α-SMA filaments are important for cell mobility, shape, and contractility (Janmey and Chaponnier, 1995; Spector, 2001).

The aim of this research was to localize the vessels and tissue regions in the antler associated with angiogenesis. The rapid growth of the vasculature in the antler and the uniqueness of a vascularized cartilage make this an ideal model for the discovery of novel angiogenic growth factors. The results presented here provided important information about localization of vessels in the tip of the growing deer antler and where proliferation of the vasculature is occurring. These studies are thus an important reference point for work focused on the isolation of known and novel angiogenic growth factors from antler for therapeutic use.

MATERIALS AND METHODS

Animals and Ethics

For all histology, antler tissue was collected from red deer 55 to 60 days after casting of the previous year's antler (n = 3 animals). Local anesthetic (bromacaine) was injected around the junction of the pedicle and the skull before the whole antler was removed above the pedicle junction. The antler removal procedure was done in accordance with regulations set by the New Zealand National Velveting Standards Board. The distal 5 cm of each antler main beam was used for this study. All deer were supplied and maintained by AgResearch Invermay Farm, Mosgiel, New Zealand. The BrdU study was conducted under approval from the Invermay Animal Ethics Committee and in compliance with the Code of Ethical Conduct for Animal Experimentation of the NZ Pastoral Agriculture Research Institute.

Angiography

Angiography was performed on the first antler of a red deer stag as previously described (Suttie et al., 1985). Animals were anaesthetized and the carotid artery was cannulated and maintained patent with a saline infusion. A radio-opaque contrast media (Urograffin, Schering, Berlin, Germany) was injected and the image was recorded on Kodak Min-R film 12 sec postperfusion.

BrdU Localization

BrdU (5-bromo-2′-deoxyuridine) incorporation was conducted on 3-year-old red deer. The BrdU was injected intravenously at a dose of 25 mg/kg of body weight. The label was left for 2–4 hr and the tissue sampled. Antler tissue was either frozen in liquid nitrogen-cooled isopentane and embedded in OCT compound or fixed in 10% formalin and embedded in paraffin wax. The paraffin blocks were sectioned at 5 μm, dewaxed in xylene, rehydrated through alcohol, then washed in PBS for 10 min (× 2). The sections were incubated in 2 M HCl for 20 min at 37°C and then washed twice in PBS for a total of 20 min. A monoclonal BrdU antibody was diluted 1:50 with PBS/1% BSA and incubated for 2 hr at room temperature (RT). The slides were then incubated with a biotinylated antimouse secondary antibody at 1:100 dilution for 0.5 hr and washed in PBS (2 × 10 min). Incubation for 10 min in 3% H_2O_2 diluted in methanol was followed by washing in PBS (2 × 10 min). A streptavidin-HRP conjugate was added at a dilution of 1:100 for 15 min at RT. Washing in PBS (2 × 5 min) was followed by incubation in DAB (3,3′-diaminobenzidine) for 1 min. PBS washes (2 × 5 min) were then followed by dehydration through ethanol, xylene washes, and coverslipping.

Immunohistochemistry for Ki67 and α-SMA

Paraffin-embedded tissue was sectioned at 5 μm onto APES (3-aminopropyltriethoxy-silane)-coated slides. The sections were then dewaxed in xylene and rehydrated through ethanol to water. Antigen unmasking of Ki67 slides was conducted by placing in 800 ml of boiling 10 mM citrate buffer (pH 6.0) for 10 min and allowing to cool slowly to RT for 30 min before washing in PBS. To block endogenous peroxidases, all Ki67 slides were treated with Peroxo block (Zymed) for 5 min while the α-SMA slides were treated with 0.3% H_2O_2 diluted in methanol for 10 min. Nonspecific binding was blocked with 20% goat serum/1% BSA in PBS for 30 min at RT.

Ki67 antibody (Lab Vision) at 1:100 dilution in 1% BSA/PBS or control rabbit IgG at 5 μg/ml (Dako, Denmark) were incubated for 1 hr at 37°C. Slides were washed in 2 × PBS/0.1% Tween-20/1% nonfat milk powder for 25 min before addition of the biotinylated goat antirabbit antibody (ZyMax grade, Zymed) at 1:100 dilution in PBS/1% BSA for 30 min.

For α-SMA immunostaining, sections were incubated overnight at 4°C with either α-SMA antibody (Zymed) or mouse IgG (Zymed) at 2 μg/ml. The sections were washed in 0.1% Tween-20/1% nonfat milk powder in PBS (3 × 5 min), followed by incubation with a biotinylated goat antimouse secondary antibody (ZyMax grade, Zymed) at 2.5 μg/ml for 20 min and rinsed again in 0.1% Tween-20/PBS (5 min × 3).

All sections were incubated with a HRP-streptavidin conjugate at 1.7 μg/ml (ZyMax grade, Zymed) for 20 min. The sections were washed in PBS (5 min × 3) and developed with DAB (Zymed). Counterstaining with Gills II hematoxylin was conducted on some sections. All sections were washed in water, dehydrated in ethanol and xylene before being coverslipped using DEPEX.

RESULTS

Velvet antler was collected about halfway through its annual period of growth. The results of the angiogram conducted on the growing tip of the antler demonstrated that the arterial blood supply was situated at the junction of the dermis and the perichondrium, which we call the vascular layer (Fig. 1). At locations along the antler shaft, but predominantly at the distal end of the antler, arteries branch into the central core of the antler. At the tip of the antler, the branches develop into parallel vessels, which then from flow distally to proximally through the core of the antler. Histology confirms that the

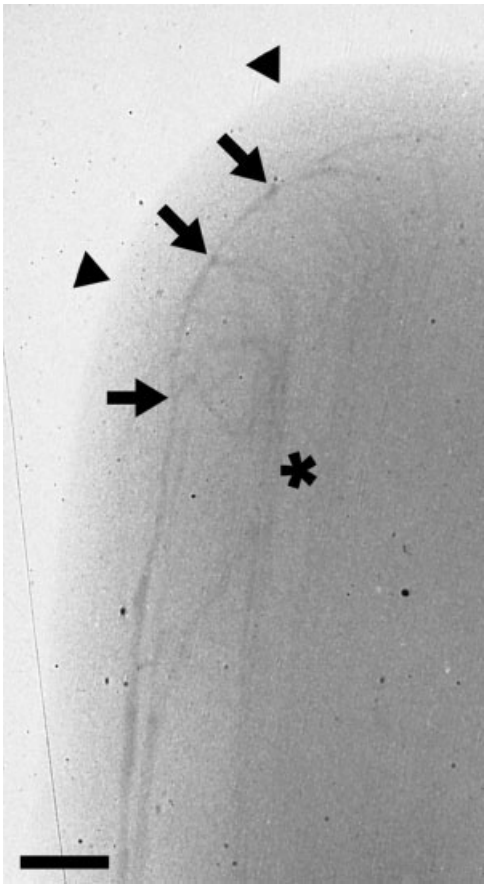

Fig. 1. Arterial blood vessels in the antler are concentrated at the base of the dermis (apical dermis, arrowheads). The vessels branch into the core of the antler when nearing the antler tip (arrows). The venous vessels run in parallel arrays through the core of the antler (asterisk). Reproduced with permission from the Royal Society of New Zealand Bulletin (Suttie et al., 1985). Scale bar = 1 cm.

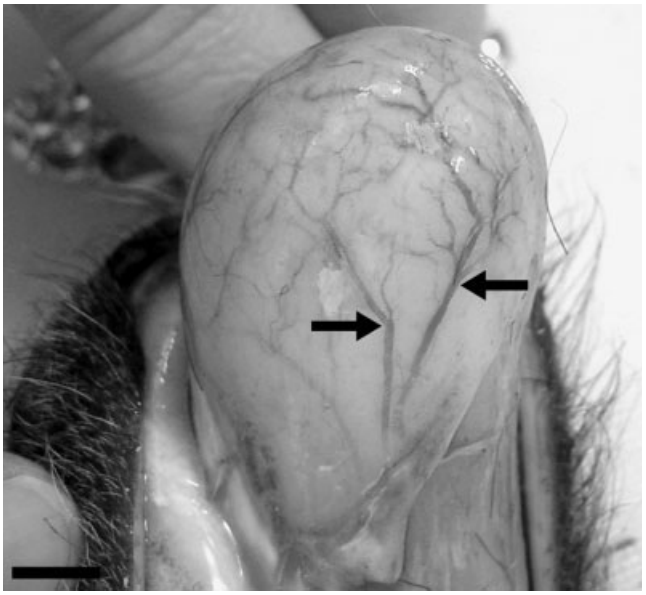

Fig. 2. The skin retracted from a first-year antler. Major arteries running up the antler are visible, which then branch (arrows) to form a network covering segments of the tip region. Scale bar = 1 cm.

vessels at the tip radiate with a regular pattern. Elongation and branching of vessels must occur to support rapid antler growth.

In the first-year antler, known as the spiker, the skin can be retracted and the vessels are clearly visible (Fig. 2). The vessels confirm the findings from the angiogram in that major arteries run along the antler shaft in the vascular layer and it is only in the region near the tip that they branch significantly. The branching vessels give rise to the parallel array of venous vessels running distal to proximal in the antler core and which are first seen within the precartilage region (Fig. 3).

BrdU incorporation and immunostaining in the antler revealed that within the lateral dermis, positive endothelial cells were clearly visible (Fig. 4A and B). Positive cells were also visible within the lateral dermis and in association with hair follicles and sebaceous glands (Fig. 4C). At the tip of the antler, fewer positive cells were observed within the dermis and vascular layer (Fig. 4D and E). The inner mesenchyme contains chondrogenic progenitor cells and stained intensely for BrdU (Fig. 4E and F). This is the main growth center for the antler cartilage. The vasculature was, however, labeled within the precartilage region, where both endothelial and support cells were labeled but no chondrogenic cells were labeled (Fig. 4F and G). This indicates that the growth centers for chondrogenesis and angiogenesis are distinct. Within the cartilage, an occasional positive cell was observed and this was always associated with vasculature (Fig. 4G and H).

The Ki67 immunohistochemistry identified proliferating cells within the vascular layer of the antler (Fig. 5A and B). The inner mesenchyme was clearly positive, indicating the proliferating chondrogenic cells (Fig. 5). Within the precartilage layer, only cells associated with vessels were labeled (Fig. 6A and B). This was very similar in manner to the BrdU incorporation. Within the cartilage, more extensive staining of proliferating cells was observed within cells associated with the vasculature as compared to BrdU (Fig. 6C and D). The cartilage cells in this region showed no staining for Ki67.

To define which cells were associated with the vasculature, the smooth muscle was stained with α-SMA. The stain was specific for smooth muscle and not endothelium (Figs. 7E, 8C, and 9C). The chondrogenic cells were, however, found to contain α-SMA (Figs. 8 and 9). The results clearly revealed a close network of vessels associated with the hair follicles and sebaceous glands of the dermis (Fig. 7A and B). Small vessels were also distributed throughout the dermis. The vascular layer at the base of the dermis contains large vessels with a complex muscle wall (Fig. 7C–F). These vessels gave rise to the vascular bundles distributed throughout the mesenchyme and precartilage regions. Within the precartilage, the α-SMA-positive chondroblasts become evident. The positive vascular support cells are more darkly stained while the endothelial cells are negative (Fig. 8). The vascular support cells are 2–3 layers thick and unevenly distributed around the vessels. Vessels were oval or irregular in shape and occurred in small bundles throughout the precartilage layer. Within the more mature carti-

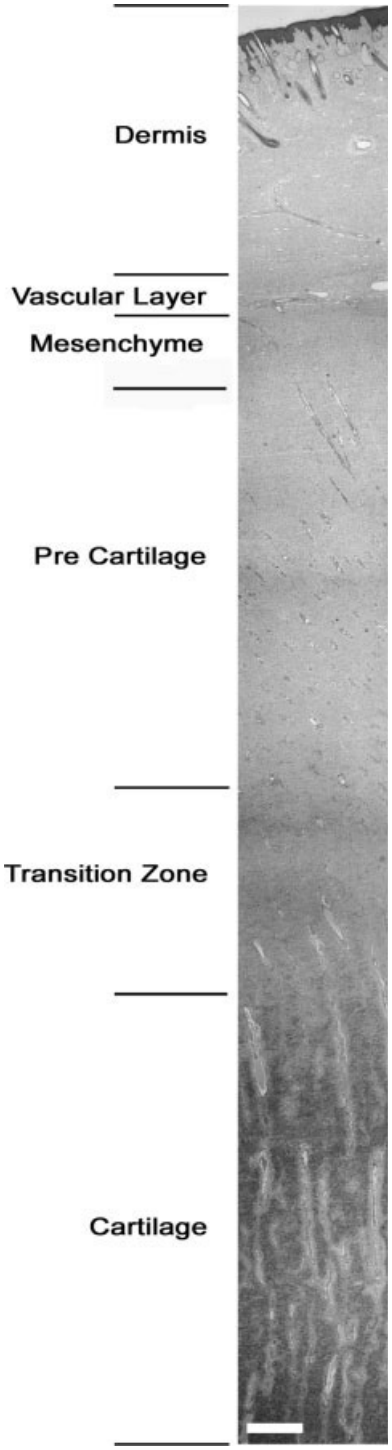

Fig. 3. Overview of tissue layers at the tip of the antler. Tissue stained with hematoxylin/eosin and alcian blue. Dermis, vascular layer, mesenchyme, precartilage, transition zone, cartilage. Scale bar = 1 mm.

lage, the vascular channels continued to be lined by α-SMA-positive cells, which were 2–3 layers in thickness (Fig. 9). The endothelial cells were evident and did not stain. The cartilage within this region had variable staining for α-SMA (Fig. 9).

DISCUSSION

The vascularization of antler cartilage is fascinating and provides an informative model with which to examine the regulation of chondrogenesis, angiogenesis, and calcification processes.

In other chondrogenic systems, angiogenesis appears to be associated with calcification. During embryonic development of the chondroepiphysis of the long bone, the cartilaginous matrix is invaded by the vasculature before forming the secondary ossification center (Doschak et al., 2003). This is not unlike the epiphyseal plate of the long bone, where angiogenesis is associated with calcification. An exception to this may be in transplanted regenerating perichondrium, where marked vascularization occurs that regresses as the cartilage matures (Ljung et al., 1999). The antler is unique and informative in that the angiogenesis is not directly associated with calcification. The discovery that during endochondral ossification endothelial cells can regulate the process of chondrogenesis, in particular the progression of chondrocytes into a hypertrophic phenotype (Bittner et al., 1998), raises questions of whether endothelial cell signaling is substantively different in the antler or whether the regulation of chondrogenesis is markedly different.

Angiograms and gross observations have indicated that the antler vasculature is fed via arteries, which are found within the dermis but typically at the base of the dermis, which we have called the vascular layer (Figs. 1 and 2). These arterial vessels grow up from the base of the antler within the subdermal region (also called the vascular layer) with minimal branching. They begin to branch as the vessels begin to curve around the tip of the antler (Fig. 1). The antler that results from the first year's growth is less complex, with only a main beam and no tines; these animals are referred to as spiker deer. The antler in Figure 2 is short, having just initiated its growth, as it is not easy to retract the skin from the more mature stages of development. However, the angiogram and the vasculature of the spiker are remarkably similar. The extensive vascular branching remains in the tip region of the antler and the vessels give rise to the parallel arrays of venous vessels within the precartilage, cartilage, and bone. The balance of angiogenic and antiangiogenic factors within the antler tip is undoubtedly crucial for this process. In the spiker antler, it appears that the straight vessels below the arrows in Figure 2 must extend to keep the branching at the tip. The angiogram in Figure 1 is also suggestive of this process. This extension of arteries within the vascular layer was confirmed by the BrdU labeling, where the arteries along the antler shaft are labeled while those at the tip have very little label. Growth factors as well as mechanical stretch caused by the antlers elongation may play a role in this process (Iwasaki et al., 2000; Li and Suttie, 2000). There are several possible mechanisms that could contribute to this phenomenon. One possibility is that of vascular extension, whereby the main artery undergoes intrinsic growth with proliferation of the endothelium and support cells occurring along the length of the vessel or at specific sites along the vessel. Other possibilities include the process of sprouting of new vessels or of intussusceptive remodeling, whereby the lower branches fuse with, or are removed from, the main artery (Carmeliet and Jain, 2000b). Intussusceptive remodeling has been described by others but is often associated with

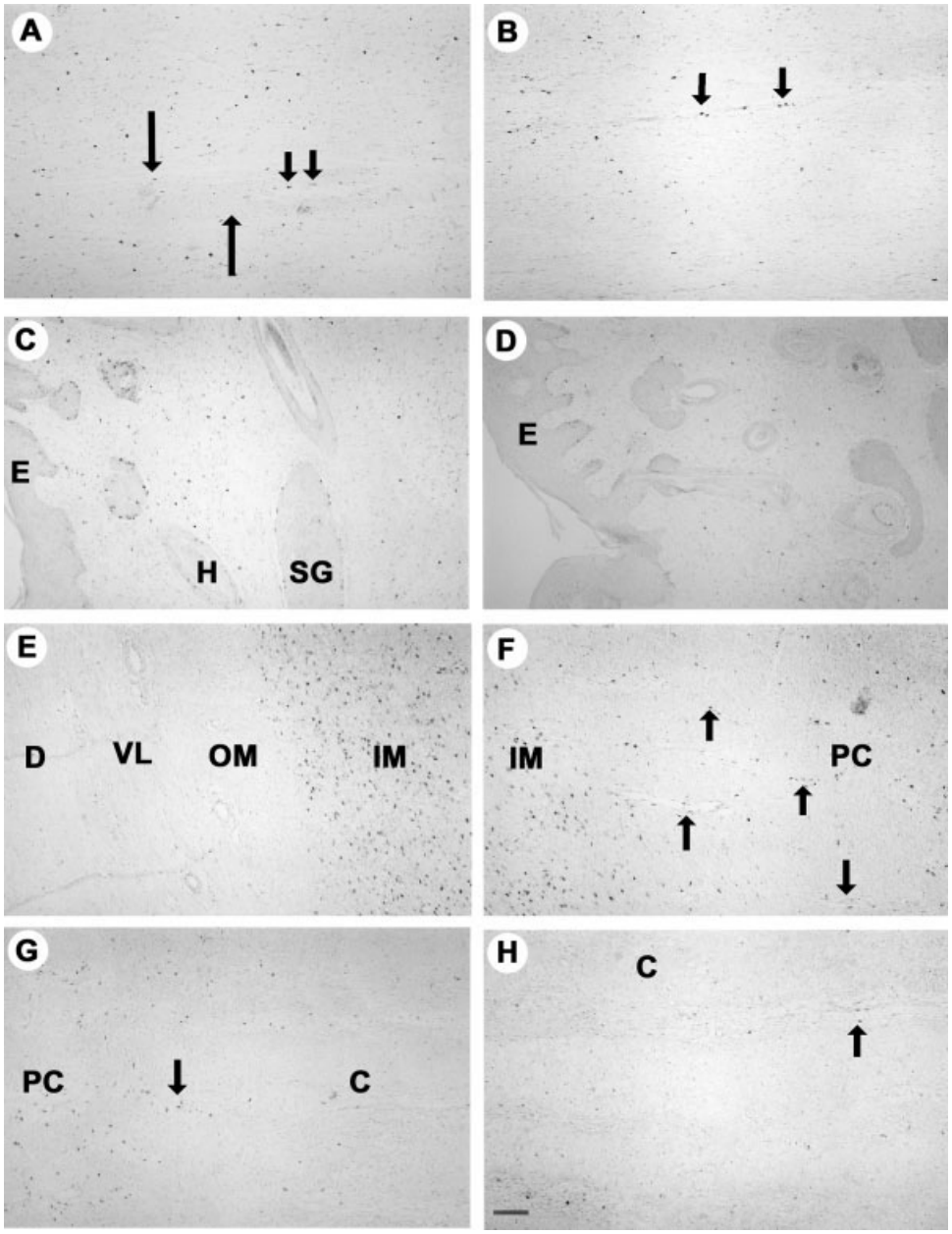

Fig. 4. BrdU staining of the antler tip. **A:** Lateral antler dermis containing a large vessel (long arrow) with BrdU-positive endothelial cells (short arrow), some dermal fibroblasts are also positive. **B:** Vessel within the lateral dermis containing positive endothelial cells (short arrow). **C:** Lateral dermis and epidermis (E). Some positive fibroblasts observed. Hair follicles (H) and sebaceous glands (SG) have some positive cells. **D:** Antler tip dermis and epidermis with only scattered positive cells. **E:** Antler tip dermis (D), vascular layer (VL), outer mesenchyme (OM), and inner mesenchyme (IM). **F:** Inner mesenchyme (IM) with positive chondroblasts and the precartilage region (PC) with positive vessels (arrows). **G:** Precartilage (PC) with positive vessels (arrow) and the cartilage (C) region. **H:** Cartilage region with only the occasional positive endothelial cells. Scale bar = 100 μm.

the formation or proximal movement of arterial branches (Carmeliet and Jain, 2000b; Burri and Djonov, 2002; Djonov et al., 2002; Burri et al., 2004).

Within the dermis and reserve mesenchyme of the antler, α-SMA-positive immunostaining was only found in association with the smooth muscle cells and pericytes. The outer dermis contained multiple blood vessels particularly in association with the hair follicles and sebaceous glands (Fig. 7A and B). The vascular layer at the base of the dermis was found to contain large vessels with a highly developed layer of α-SMA positive cells. These vessels branched proximally into the mesenchyme to give the ordered array of vessels in the precartilage and which then become the sinusoidal vessels within the cartilage layer as the antler elongated. The precartilage zone, just below the mesenchymal stem cells, is where vascular growth appears to be concentrated. This is supported by the marked BrdU labeling of these vessels

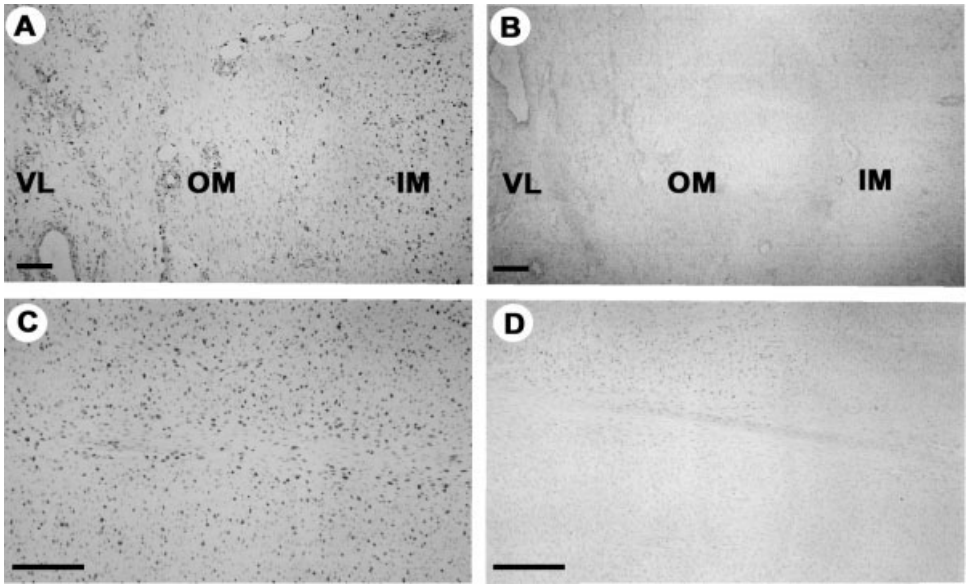

Fig. 5. Ki67 immunohistochemistry of the antler tip. **A:** Some Ki67-positive cells are seen in the vascular layer (VL), few positive cells in the outer mesenchyme (OM), and many positive chondroblasts in the inner mesenchyme (IM). **B:** IgG control of A. **C:** Inner mesenchyme with positively staining chondroblasts. **D:** IgG control of C. Scale bars = 100 μm.

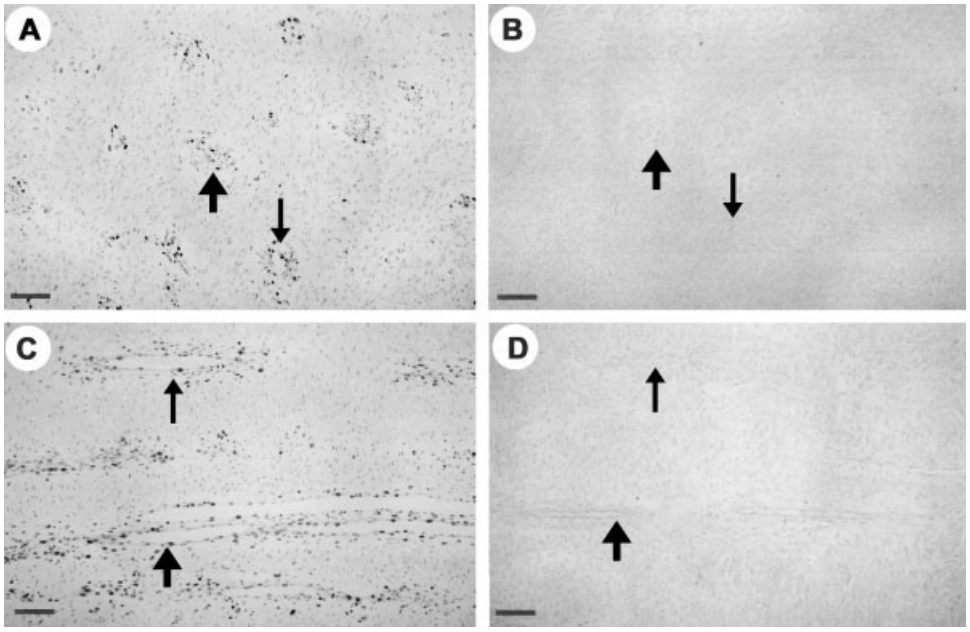

Fig. 6. Ki67 immunohistochemistry of antler precartilage and cartilage. **A:** Precartilage region with positive blood vessels (arrows). **B:** Precartilage IgG control. **C:** Cartilage region with positive cells only in association with the blood vessels (arrows). **D:** Cartilage region IgG control. Scale bars = 100 μm.

(Fig. 4F and G). The unique vascularization of the antler cartilage has been proposed as a solution to the intense metabolic demands of this tissue (Li and Suttie, 1994). Vascularization within the precartilage zone was found in distinct bundles of vessels separated from each other by the precartilage tissue. The vascular bundles had 1–2 layers of cells staining positively for α-SMA associated with them (Fig. 8A–D). The processes by which the vascular bundles form in the precartilage are unknown. Intussusceptive growth could possibly contribute to this type of morphology (Burri and Djonov, 2002), as could sprouting (Carmeliet and Jain, 2000a). The rate of proliferation suggests that significant angiogenic pressure must be exerted on these vessels. It is possible that a

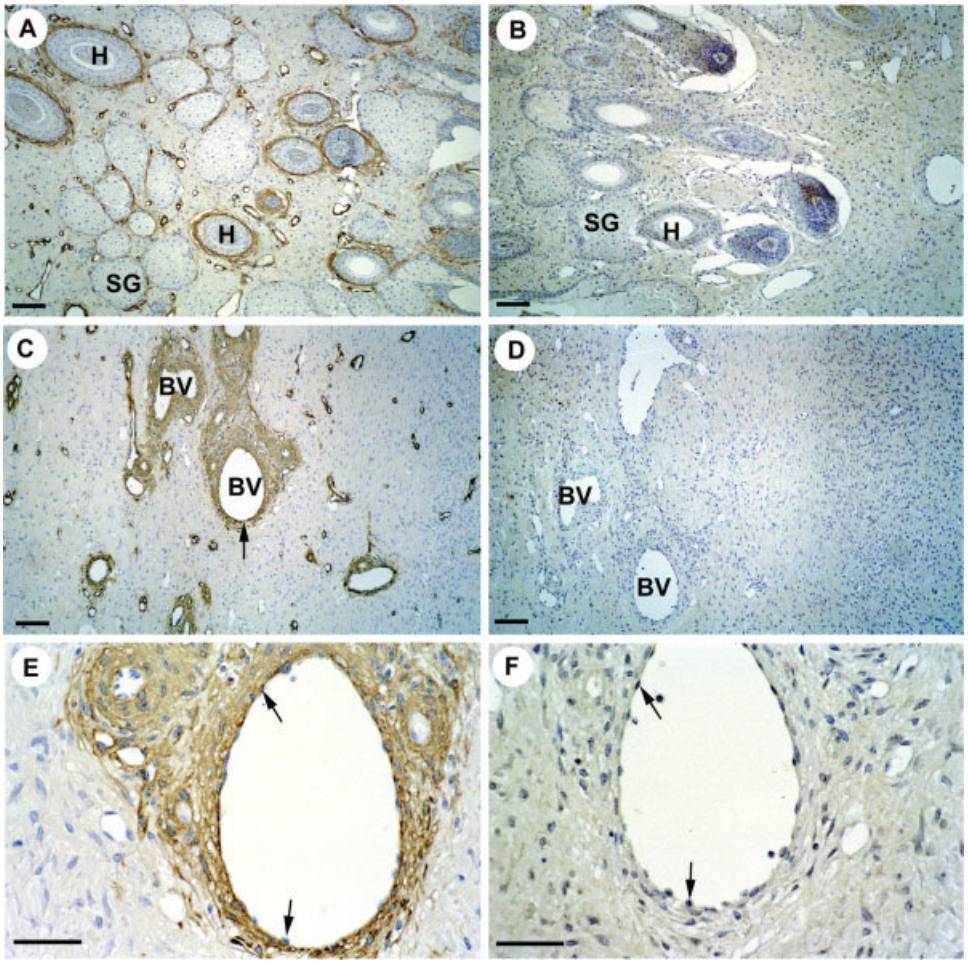

Fig. 7. Antler α-SMA immunohistochemistry of dermis. **A:** Hair follicles (H) and sebaceous glands (SG) in the apical dermis. Positive vessels are stained brown and typically surround these structure as well as being located within dermal tissues. **B:** IgG control. **C:** Vascular layer between the dermis and the mesenchyme. Blood vessels (BV) dominate this region. Arrow indicates vessel used in E. **D:** IgG control. **E:** High magnification of C with unstained endothelial cells (arrows). **F:** IgG control. Scale bars = 100 μm.

consequence of this is not only the elongation of the vessels but intussusception or sprouting of the vessels, resulting in small bundles of vessels.

At the transition between precartilage and cartilage, the immunohistochemistry reveals that vessels join to become the sinusoids of the mature antler. The endothelium is detected as one or two layers thick and is not a flat sheet as seen in the dermis or in most tissues. The vessels eventually transform into open sinusoids at the base of the cartilage region. The α-SMA staining of the cartilage region confirms the structure of these vessels with an endothelial cell layer and the α-SMA reveals that they are likely to have 1–3 layers of smooth muscle/pericyte supporting them. This suggests that by the middle of the cartilage zone, much of the vascular proliferation, which occurs in response to antler growth, has occurred.

The α-SMA immunostaining was also found within the precartilage and cartilage cells themselves (Figs. 8 and 9). These cells are chondroblasts and chondrocytes existing within an immature and mature cartilaginous extracellular matrix. α-SMA is usually associated with smooth muscle cells; however, others have reported it in association with a percentage of normal articular cartilage cells, in articular cartilage explants and in healing articular cartilage (Qiu et al., 2000; Wang et al., 2000). In vitro α-SMA has been found in association with micromass cultures of chick limb mesenchymal cells as they undergo chondrogenesis (Yoo et al., 2001). Other nonmuscle expression has been reported in tendon, ligament, meniscus, intervertebral disk, and in some trabecular bone (Spector, 2001). The expression of α-SMA does not appear to be a feature of endochondrial bone formation and thus there are features of antler chondrogenesis that distinguish it from endochondrial cartilage. The role of a contractile form of actin in the chondrocytes of antler appears to be related to the process of chondrogenesis. In soft tissues, the production of α-SMA is related to the wound-healing process and, in particular, in conjunction with wound contraction as well as with pathological processes (Spector, 2001; Gabbiani, 2003). It thus appears probable that the expression of α-SMA provides the cells with the motility and contrac-

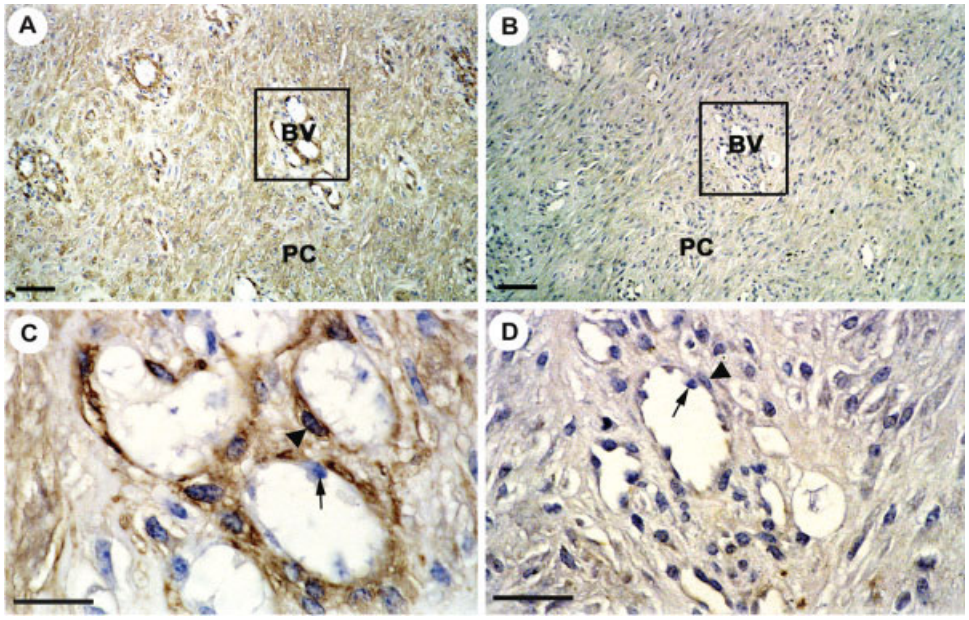

Fig. 8. Antler α-smooth muscle actin immunohistochemistry of precartilage region (PC). **A:** Groups of vessels staining positively in the PC region as well as positive staining in the chondroblasts. **B:** IgG control in PC. **C:** High power of boxed area in A. Darkly stained smooth muscle evident (arrowhead) and unstained endothelial cells (arrow). **D:** IgG control. Scale bars = 100 μm.

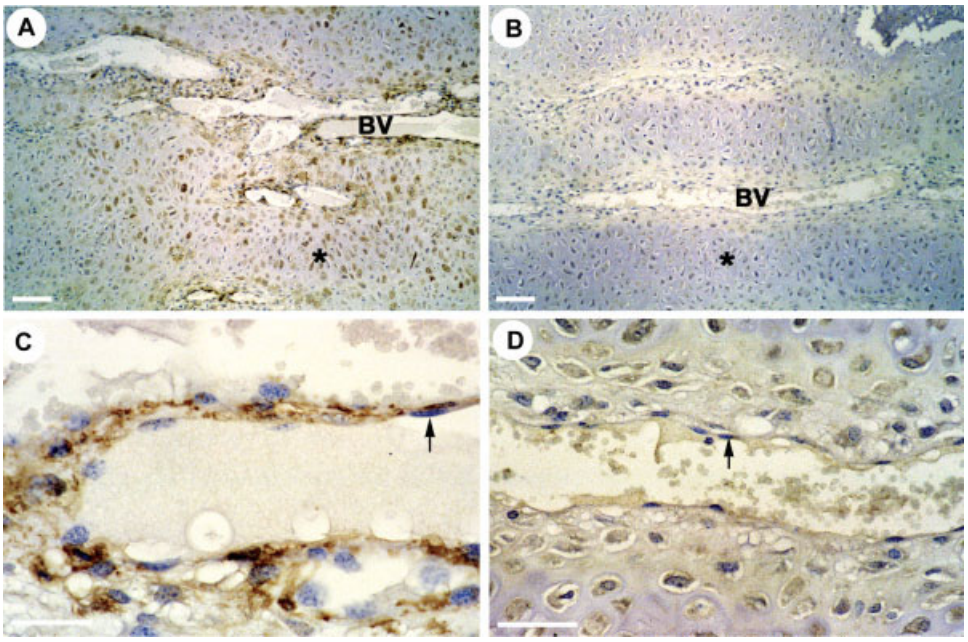

Fig. 9. Antler α-SMA immunohistochemistry of cartilage region. **A:** Cells associated with the vascular channels (BV) label positively as do the maturing cartilage cells (asterisk). **B:** IgG control. **C:** High power of vessel from A. Vascular support cells are positive (arrow) while endothelial cells do no stain. Vascular support cells can be 2–3 layers deep. **D:** IgG control in cartilage. Scale bars = 100 μm.

tility required for the cartilage to grow at a rapid rate and still maintain a defined structure.

These results show that the cellular proliferation associated with chondrogenesis and angiogenesis is spatially separate in the growing antler. There is now evidence to support the idea that the precartilage region is likely to contain factors that specifically regulate the growth of blood vessels. Likewise within the dermis and particularly in the dermis along the shaft of the antler, vessels are being induced to proliferate. The findings also sup-

port a role for α-SMA not only in association with blood vessels but also as part of the orchestrated differentiation processes associated with chondrogenesis in the tip of the antler.

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