THE ANATOMICAL RECORD PART A 282A:163–174 (2005)

Histological Examination of Antler Regeneration in Red Deer (*Cervus elaphus*)

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ABSTRACT

Annual antler renewal presents the only case of epimorphic regeneration (de novo formation of a lost appendage distal to the level of amputation) in mammals. Epimorphic regeneration is also referred to as a blastema-based process, as blastema formation at an initial stage is the prerequisite for this type of regeneration. Therefore, antler regeneration has been claimed to take place through initial blastema formation. However, this claim has never been confirmed experimentally. The present study set out to describe systematically the progression of antler regeneration in order to make a direct histological comparison with blastema formation. The results showed that wound healing over a pedicle stump was achieved by ingrowth of full-thickness pedicle skin and resulted in formation of a scar. The growth centers for the antler main beam and brow tine were formed independently at the posterior and anterior corners of the pedicle stump, respectively. The hyperplastic perichondrium surmounting each growth center was directly formed in situ by a single type of tissue: the thickening distal pedicle periosteum, which is the derivative of initial antlerogenic periosteum. Therefore, the cells residing in the pedicle periosteum can be called antler stem cells. Antler stem cells formed each growth center by initially forming bone through intramembranous ossification, then osseocartilage through transitional ossification, and finally cartilage through endochondral ossification. There was an overlap between the establishment of antler growth centers and the completion of wound healing over the pedicle stump. Overall, our results demonstrate that antler regeneration is achieved through general wound healing- and stem cell-based process, rather than through initial blastema formation. Pedicle periosteal cells directly give rise to antlers. Histogenesis of antler regeneration may recapitulate the process of initial antler generation.

Key words: deer antler; pedicle stump; epimorphic regeneration; blastema; stem cells

Annual renewal of deer antlers represents true epimorphic regeneration and the only case of mammalian appendage regeneration (Li, 2003). Epimorphic regeneration is the phenomenon of de novo development of appendages distal to the level of amputation (Goss, 1969, 1980). As formation of a blastema during the initial stages of regeneration is a prerequisite for all the known cases of epimorphic regeneration in lower vertebrates, e.g., newts and lizards, epimorphic regeneration is also referred to as blastema-based regeneration (Wallace, 1981). A blastema is the cone-shaped mass of dedifferentiated cells from diverse origins on the immediate amputation plane of an appendage stump (Mescher, 1996).

Deer antler regeneration, as the only example of epimorphic regeneration in mammals, has been previously consid-

Grant sponsor: Foundation for Research, Science and Technology of New Zealand.

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Received 25 June 2004; Accepted 16 September 2004
DOI 10.1002/ar.a.20148
Published online 7 January 2005 in Wiley InterScience (www.interscience.wiley.com).

ered to be a blastema-based process (Goss and Holt, 1992; Goss, 1995; Allen et al., 2002; Li, 2003). Goss (1992) stated that "very much the same mechanism is utilized in the epimorphic regeneration of all appendages. In each case, be it the fin of a fish, the limb of an amphibian, the tail of a lizard, or the antler of a deer, regeneration is made possible by the development of a blastema. The existence of a blastema is indicative of epimorphic regeneration." However, this claim in the case of deer antler has never been tested experimentally. In a recent study (Li et al., 2004), we described that the early stages of antler regeneration were not morphologically compatible with blastema formation. Hence, this has raised a question as to whether the histogenesis of regenerating antlers resembles that of blastema formation? Unfortunately, currently available histological data do not permit a direct comparative analysis between the early stages of antler regeneration and blastema formation in classic models of epimorphic regeneration.

Whether regenerating antlers are of dermal or periosteal origin has been an issue of controversy. Wislocki and Waldo (1942, 1953) and Goss (1969, 1992, 1995) stated that after the previous hard antler is cast, the cells in the dermal layer of the pedicle skin give rise to the antler blastema. Thus, the dermis, which elsewhere in the body is responsible for producing the regeneration-inhibiting scar, is the very tissue that makes antler regeneration possible. However, other authors suggested that pedicle periosteum (Kierdorf and Kierdorf, 1992), or pedicle periosteum and the marrow spaces on the immediate pedicle casting plane [Gruber (1937), cited in Kierdorf et al. (2003)], provides the main cell source for antler regeneration. These conflicting conclusions, consequently, merit further investigation.

Deer antler regeneration and initial antler generation (in yearling stags) have been considered to be similar, although antler generation takes place in the absence of wound healing and wound healing is believed to be the prerequisite for normal antler regeneration (Goss, 1983). Thus far, it is not clear whether histogenesis of regenerating antlers recapitulates that of antler generation. In their recent study, Kierdorf et al. (2003) stated that although the change in ossification type takes place during early antler regeneration, the factor causing this change may not be the mechanical pressure, which has been hypothesized to be the case in first antler generation (Li and Suttie, 2000). However, an alternative factor was not suggested in their report. A detailed histological analysis of antler regeneration during the change in ossification type may yield some clues.

The aim of this study was to take a light microscopy approach to describe systematically the progression of antler regeneration from precasting to full establishment of the antler growth centers. While examining the histological sections, special attention was placed on determining whether formation of an early regenerating antler bud is a blastema-based process; which tissue type, skin or periosteum, gives rise to a regenerating antler; and whether histogenesis of regenerating antlers recapitulates that of initial antler generation or through an alternative pathway.

MATERIALS AND METHODS

Tissue Samples

Tissue samples were collected from 3-year-old red deer (*Cervus elaphus*) stags in a commercial abattoir and allocated into one of the five groups based on the stage of regeneration advancement. These stages include precasting (three samples), casting (four samples), early wound healing (five samples), late wound healing and early regeneration (six samples), and formation of main beam and brow tine (three samples). The samples were cut sagittally to produce a center slice up to several millimeters wide. These sampled tissue blocks were then immediately fixed in 10% neutral buffered formalin.

Histology

Detailed histological processing procedures were reported elsewhere (Li and Suttie, 1994). Briefly, after 7 days of fixation, the tissue samples were decalcified in commercial decalcification solution (BDH Chemical) for a period of 5 days (late regenerating tissue samples) to 2 months (precasting to early wound healing tissue samples) based on the radiographic results. Subsequently, the decalcified tissues were embedded in paraffin wax and sectioned at 5 μm. Three different types of staining were used: Gill's hematoxylin and alcoholic eosin (H&E), H&E and Alcian Blue (AB) counterstaining, and Mallory trichrome (MT) staining. For MT staining, tissue sections were saturated in alcoholic picric acid for 5 min, stained in hematoxylin for 10 min, in 1% acid fuchsin for 10 min, and washed and rinsed in 1% acetic acid, stained with 2.5% Aniline Blue, and finally rinsed in 1% acetic acid for 1 min. Sections were then passed through graded alcohol for differentiation of Aniline Blue stainings.

RESULTS

Precasting

Well before hard antler casting, the future casting line was not detectable (Fig. 1). However, the living bone of the pedicle and dead bone of the antler could still be distinguished by histological staining. In the proximal region of dead antler bone, the contents of bone cavities and lacunae stained much darker than those of distal living pedicle bone (Fig. 1A, arrows). The transition zone between the differential staining was the region where the future separation line would develop. The distal pedicle epidermis bent inwardly over the distal end of the dermis and directly attached to the underlying pedicle periosteum without intervention of loose connective tissue (Fig. 1B, e and p). The skin surrounding the antler/pedicle junction contained rather thick-walled arteries (Fig. 1C, arrow) and bundles of nerves (Fig. 1D, arrow). These nerve fascicles were peculiar in that they were enclosed by a thick sheath or capsule. The stumps of the blood vessels and nerves were preserved in the rim of dermal tissue at the distal margins of the pedicle after the velvet was shed.

As the time of antler casting approached (the contralateral side hard antler had cast), enlarged cavities within the transitional zone became evident. This region subsequently developed into the abscission line, which was a narrow dark-reddish zone sharply delineating the plane of future separation (Fig. 2A, B). Both sides of trabecular bone alongside the abscission line were densely studded with active osteoclasts (Fig. 2A, arrows). Erosion at the periphery before the antler casting led to the excavation of a circumferential cleft into which healing pedicle skin had begun to migrate (Fig. 2B, ep and de). The distal pedicle periosteum from both anterior and posterior sides was

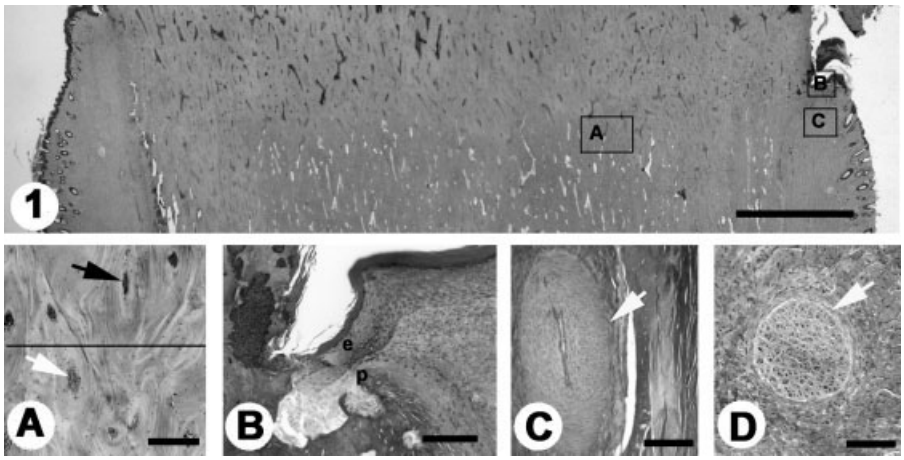

Fig. 1. Sagittally cut histological section of junction between a pedicle and an antler well before casting. Notice that although no future casting line was detectable at this stage, the living pedicle bone and dead antler bone could be distinguished readily by the differential staining of bone cavities. Heamatoxylin and eosin (HE) staining. Bar = 4.0 mm. **A**: Higher magnification of an area similar to A in Figure 1 to show differentially stained bone cavities: darker in antler (black arrow) and lighter (white arrow) in pedicle. Black line: separating the pedicle tissue from antler tissue. HE. Bar = 0.2 mm. **B**: Higher magnification of an area similar to B in Figure 1 to show that distal pedicle epidermis (e) was bent inwardly over the distal end of the dermis and directly attached to the pedicle periosteum (p). Mallory trichrome (MT) staining. Bar = 0.1 mm. **C** and **D**: Higher magnification of an area similar to C in Figure 1 to show thick-walled artery (arrow; bar = 0.2 mm) and heavy sheathed nerve fascicles (arrow; bar = 0.1 mm) located in the dermis of distal pedicle skin. MT.

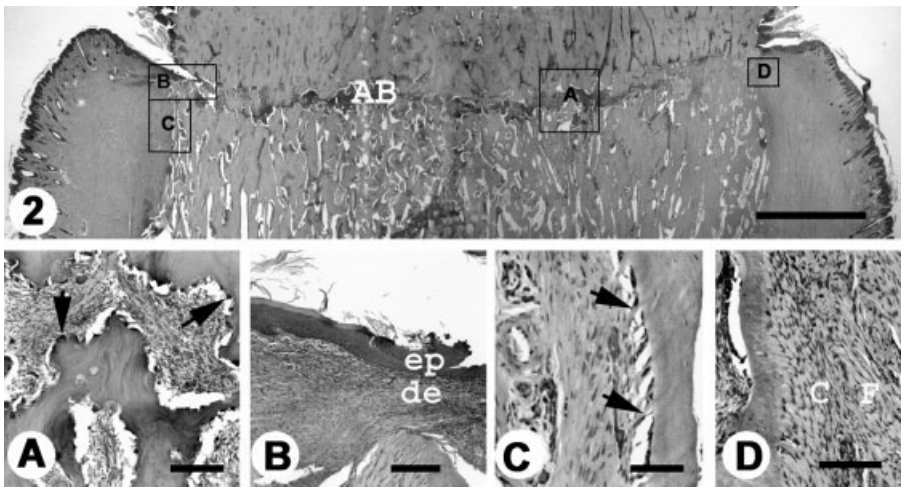

Fig. 2. Sagittally cut section of junction between a pedicle and an antler just before hard antler casting (on the contralateral side, hard antler had cast). Notice that an abscission line (AB), a narrow dark-reddish zone, sharply delineated the plane of future separation. HE. Bar = 4.0 mm. **A**: Higher magnification of an area similar to A in Figure 2 to show densely studded osteoclasts (arrows) on the bone trabeculae alongside the abscission line. HE. Bar = 0.1 mm. **B**: Higher magnification of an area similar to B in Figure 2 to show the healing epidermis (ep) and dermis (de) of pedicle skin had begun to migrate into the circumferential cleft eroded by osteoclasts. HE. Bar = 0.1 mm. **C**: Higher magnification of an area similar to C in Figure 2 to show the distal pedicle periosteum and subperiosteal bone. Notice that periosteum was bound to the subperiosteal bone via Sharpey's fibres (arrows). Bar = 89 μm. **D**: Higher magnification of an area similar to D in Figure 2. Notice that there is no clear demarcation between the cellular (C) and fibrous (F) layers of the periosteum. HE. Bar = 41 μm.

tightly bound to the subperiosteal trabecular bone via Sharpey's fibers (arrows in Fig. 2C; Fig. 2D), C and F. These periostea were different from those of ordinarily observed ones in that there was not a clear demarcation between the cellular layer and fibrous layer. The rim of skin immediately surrounding the pedicle periosteum at anterior and posterior sites contained several large arteries, veins, and nerves in the inner reticular layer of the dermis (Fig. 2C and D).

Casting

Immediately after antler casting, the rim of skin and periosteum tissue surrounding the top of the pedicle overlapped the margin of the bone and encroached on space formerly occupied by the periphery of the antler base (Fig. 3). The epithelium was thicker than that of the more proximal skin down the pedicle shaft and had already acquired some velvet skin features, i.e., new hair follicle

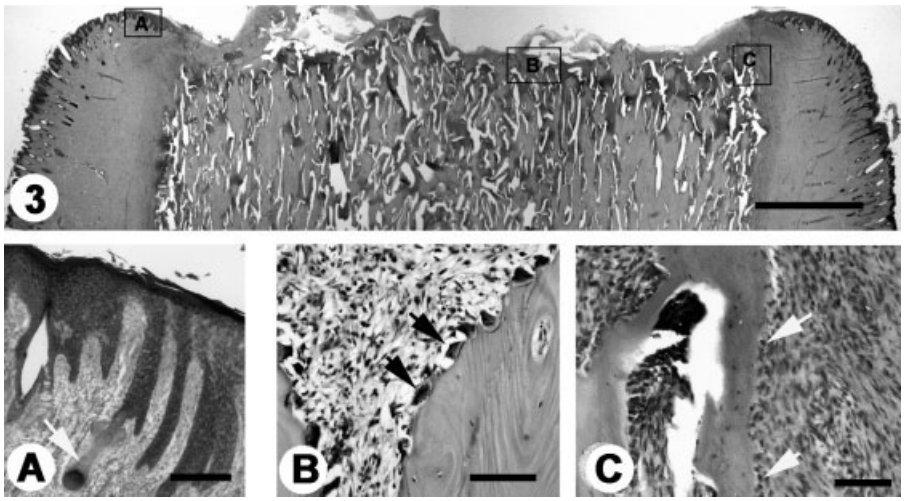

Fig. 3. Sagittally cut histological section of a pedicle stump immediately after antler casting. Notice that the rough surface of the casting plane of the pedicle stump. Epithelium of the rim formed by distal pedicle skin became thicker and acquired some velvet skin features. HE. Bar = 4 mm. **A**: Higher magnification of an area similar to A in Figure 2 to show the rim epidermis had acquired some features of velvet skin, e.g. forming new hair follicles (arrow). MT. Bar = 0.2 mm. **B**: Higher magnification of an area similar to B in Figure 2 to show the active osteoclasts (arrows) lined along the surfaces of bone trabeculae on the casting plane. HE. Bar = 0.1 mm. **C**: Higher magnification of an area similar to C of Figure 2 to show that the surface of the subperiosteal bone of distal pedicle was densely populated by active osteoblasts (arrows). HE. Bar = 40 μm.

formation (Fig. 3A, arrow). The osseous trabeculae on the immediate casting surface were denuded of connective tissue that was detached when the antler was shed. In some cases, a layer of fibrocellular tissue was found covering the denuded casting surface. The surface of spiky bone trabeculae of a pedicle stump was densely populated with osteoclasts, which were actively smoothening the rough casting surface (Fig. 3B, arrows). The lateral surface of the existing subperiosteal pedicle trabeculae, at the distal end, was densely lined by active osteoblasts (Fig. 3C, arrows).

Early Wound Healing

Within 1 or 2 days following antler casting (judged by the fresh color of newly formed scab), the newly formed hairless epidermis and associated dermal tissue made substantial ingrowth from the periphery to cover the surface of the pedicle, except for the central depressed area (Fig. 4). A layer of granulation tissue (Fig. 4A), possibly of dermal origin, was observed overlying the bony pedicle. Beneath the granulation were many newly formed slender osseous trabeculae, which extended from the much thicker existing trabeculae of the pedicle stump, including the central region where the wound was still exposed. These slender trabeculae were directly formed from the osteogenic cells located in and above the eroded pedicle bone trabeculae. Interestingly, the newly formed slender trabeculae at the periphery were oriented toward the center (Fig. 4B, arrow and PS), as if there were mechanical pressure imposed from anterior and posterior corners. The undersurface of the migrating epidermis began to form tongue-like structures in the tumescent skin. Most of these tongue-like structures had special angles (Fig. 4C, arrow) and worked as pegs to clip the leading end of healing epidermis tightly onto the underlying connective tissue. Pedicle periosteal cells, at the posterior end, formed slender bony trabeculae laterally and distally to the existing pedicle bone, which in turn was covered by the newly formed bone (Fig. 4).

Late Wound Healing and Early Antler Regeneration

Histologically, this stage could be divided into three substages. These substages were initiation of the anterior and posterior growth centers; formation of the continuous cartilaginous columns in the growth centers; and commencement of the remodeling in the earliest formed cartilaginous region. These substages were established to correspond to the appearance of new features rather than to rigidly defined time periods.

Initiation of anterior and posterior growth centers. The prominent feature was that discrete clusters of chondrocytes emerged at the posterior and anterior corners of a regenerate (Fig. 5). These cartilaginous clusters were formed from rapidly proliferating and differentiating cells of the thickening distal pedicle periosteum. Formation of these cartilaginous clusters indicated the initiation of the posterior and anterior growth centers. Tips of both anterior and posterior growth centers were capped by a layer of hyperplastic periosteum/perichondrium, which was formed from the distal portion of the thickening pedicle periosteum (Fig. 5A, D and P). The periosteum/perichondrium consisted of an outer fibrous layer and a much thicker inner cell-rich layer (Fig. 5B). Within this thick periosteum/perichondrium, mesenchymal cells differentiated into osseocartilaginous tissue. Therefore, discrete clusters of chondrocytes were formed (Fig. 5B and C, arrows). The much thinner, newly formed slender trabeculae over the central region of a pedicle stump were conspicuously continuous with the existing thick osseous trabeculae (Fig. 5). Distally, the zone of newly formed slender osseous trabeculae merged into vascularized tissue.

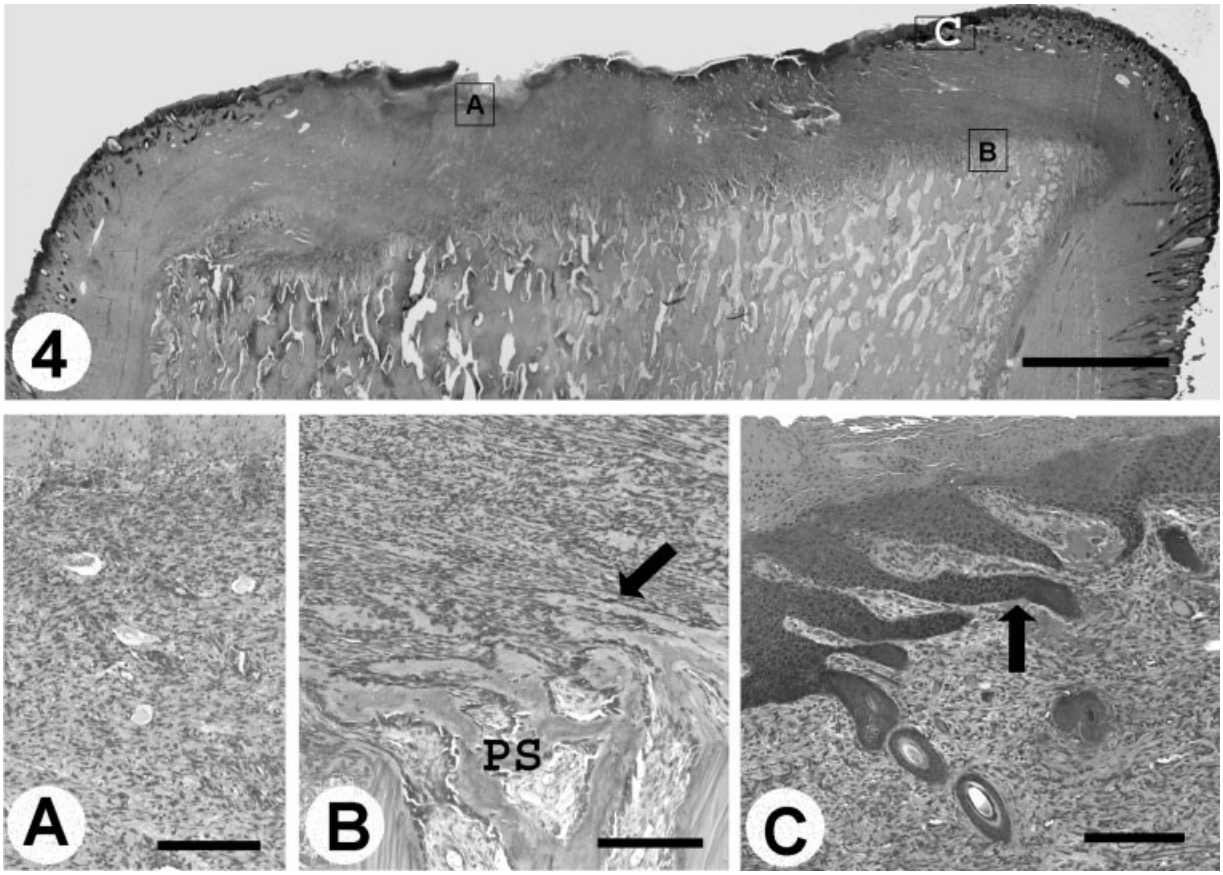

Fig. 4. Sagittally cut section of a pedicle stump at early wound healing stage. Notice that full thickness of distal pedicle skin had made substantial ingrowth. However, the central depressed region was still devoid of skin. HE. Bar = 4.0 mm. **A**: Higher magnification of an area similar to A in Figure 4 to show the granulation tissue located in the central depressed region. HE. Bar = 0.2 mm. **B**: Higher magnification of an area similar to B in Figure 4 to show the newly formed slender bony trabeculae at the periphery of the pedicle stump (PS). Notice that these slender trabeculae (arrow) had an orientation which was inclined to the centre, as if there was a mechanical pressure imposed from posterior corner. HE. Bar = 0.2 mm. **C**: Higher magnification of an area similar to C in Figure 4 to show the "tongue-like structures" formed along the undersurface of healing epidermis. Notice that these structures (arrow) had special angles and worked as pegs to clip the leading end of healing epidermis tightly onto the underlying connective tissue. HE. Bar = 0.2 mm.

Formation of continuous cartilaginous columns in growth centers. The distinguishing feature at this stage was the formation of the continuous cartilaginous columns under the perichondrium cap in both posterior and anterior growth centers (Fig. 6, arrow) and the thickened distal pedicle periosteum (Fig. 6A, P). The newly formed tissues (bone, osseocartilage, and cartilage) of periosteal origin were clearly built up on the slender bony trabeculae of pedicle bone origin. Interestingly, the continuous precartilaginous columns formed a particular angle (Fig. 6B, arrow) toward the anterior (in anterior growth center) or posterior corner (in posterior growth center), indicative of the growth direction of each center. Directly overlying the hyperplastic cap of each growth center was a vascular layer, within which blood vessels and nerves were densely populated (Fig. 6C, b and arrow). The reepithelialization of the entire pedicle was almost completed at this stage. At the healing ends, the epidermis was thin and devoid of both hair follicles and sebaceous glands (Fig. 6D), underneath which granulation tissue (gt, mixture of fibroblasts and endothelial cells) was found. Formation of each growth center by the hyperplastic periosteum/perichondrium cap went through three ossification stages. These stages sequentially were intramembranous ossification to form trabecular bone (Figs. 4 and 5), transitional ossification to form osseocartilaginous tissue (Fig. 6E, arrows), and modified endochondral ossification to form continuous cartilaginous columns (Fig. 6B).

Commencement of remodeling in the earliest formed cartilaginous region. Convergence of healing epidermis at the central point over the top of a regenerating antler bud (Fig. 7 and A) marks the completion of the wound healing stage. Interiorly, chondroclasia (Fig. 7B, arrow) started to occur in the cartilaginous region formed during the transitional ossification stage (Fig. 7C, arrow). In the central region and between the two growth centers, a limited number of cartilaginous clusters were discernible at this stage (Fig. 7D, arrows). As the cartilaginous tissue in the growth centers continuously built up, anterior and posterior portions of a new regenerating antler bud started to bulge out (Fig. 7).

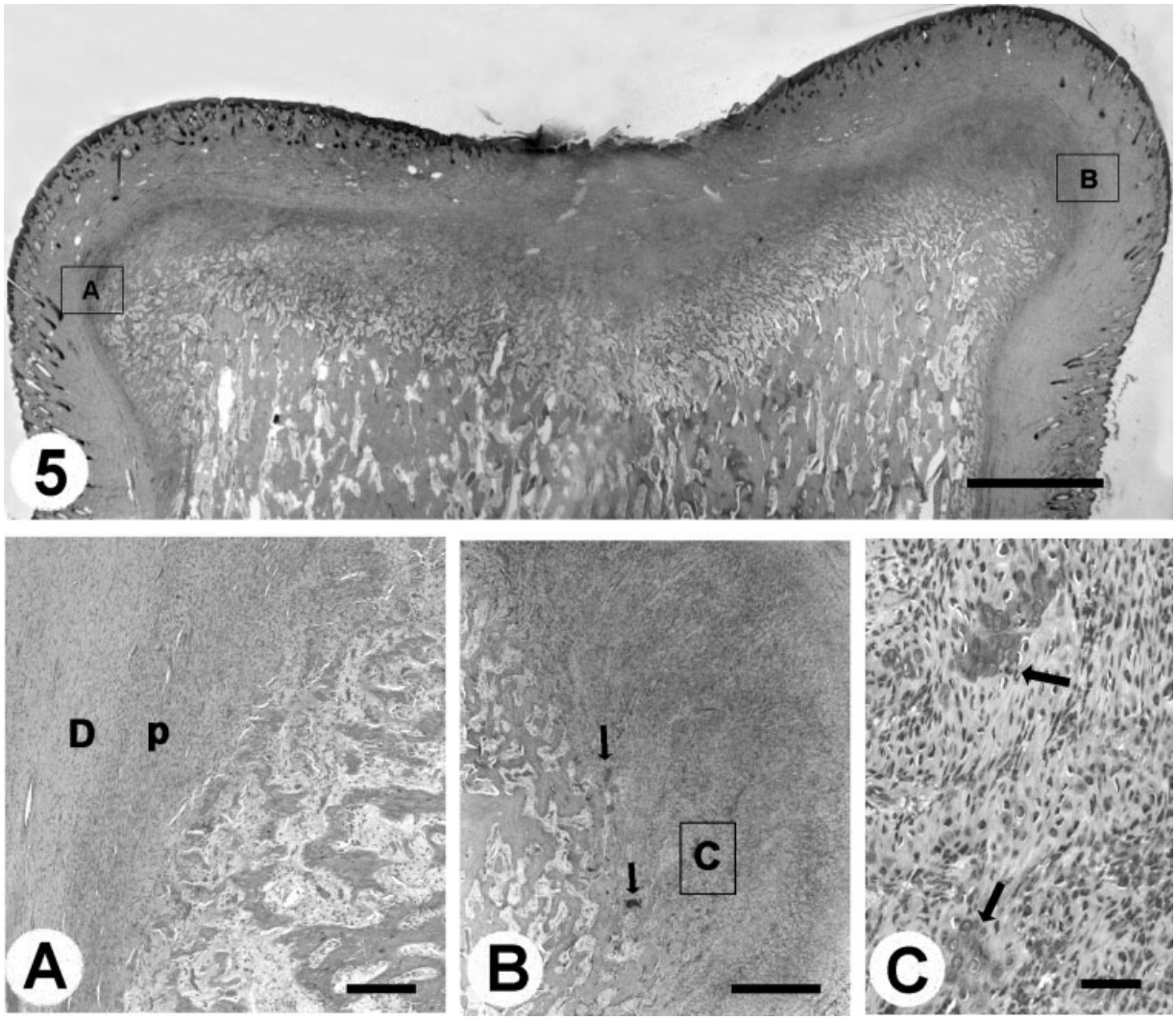

Fig. 5. Sagittally cut section of a pedicle stump at late wound healing stage. Notice that wound healing over the stump was nearly completed. Counter-staining of HE and alcian blue (AB). Bar = 4.0 mm. **A**: Higher magnification of an area similar to A in Figure 5 to show the thickening distal pedicle periosteum. D, dermis; P, periosteum. HE/AB. Bar = 0.2 mm. **B**: Higher magnification of an area of B in Figure 5 to show the cartilaginous clusters formed in the posterior corner. Notice that the distal pedicle periosteum was building a new growth centre via initial formation of trabecular bone through intramembranous ossification to form osseocartilaginous tissue (mixture of bone and cartilage (arrows)) through transitional ossification. HE/AB. Bar = 1.0 mm. **C**: Higher magnification of the area of C of Figure 5B to show the cartilaginous clusters (arrows). HE/AB. Bar = 0.1 mm.

Fig. 6. Sagittally cut section of an early regenerating antler bud over a pedicle stump. Notice that a substantial amount of cartilaginous tissue had formed in the anterior and posterior growth centres, and continuous cartilaginous columns had formed in the posterior growth centre (arrow). There was a considerable overlap between the completion of wound healing and the establishment of anterior and posterior growth centres. HE/AB. Bar = 4.0 mm. **A**: Higher magnification of an area similar to A of Figure 6 to show the thickening hyperplastic perichondrium formed by distal pedicle periosteum in situ. HE. Bar = 0.2 mm. **B**: Higher magnification of an area similar to B of Figure 6 to show that the continuous pre-cartilaginous columns formed a particular angle toward the growth direction of each growth centre (arrow). MT. Bar = 0.2 mm. **C**: Higher magnification of an area similar to C of Figure 6 to show the richly distributed blood vessels (b) and nerves (arrow) directly overlying each growth centre. HE. Bar = 0.1 mm. **D**: Higher magnification of an area similar to D of Figure 6 to show the granulation tissue (gt) underneath the leading ends of healing epidermis. Notice that the epidermis was thin and devoid of both hair follicles and sebaceous glands. HE. Bar = 0.1 mm. **E**: Higher magnification of an area similar to E of Figure 6 to show the cartilaginous clusters (arrows) formed during the stage of transitional ossification. HE/AB. Bar = 0.2 mm.

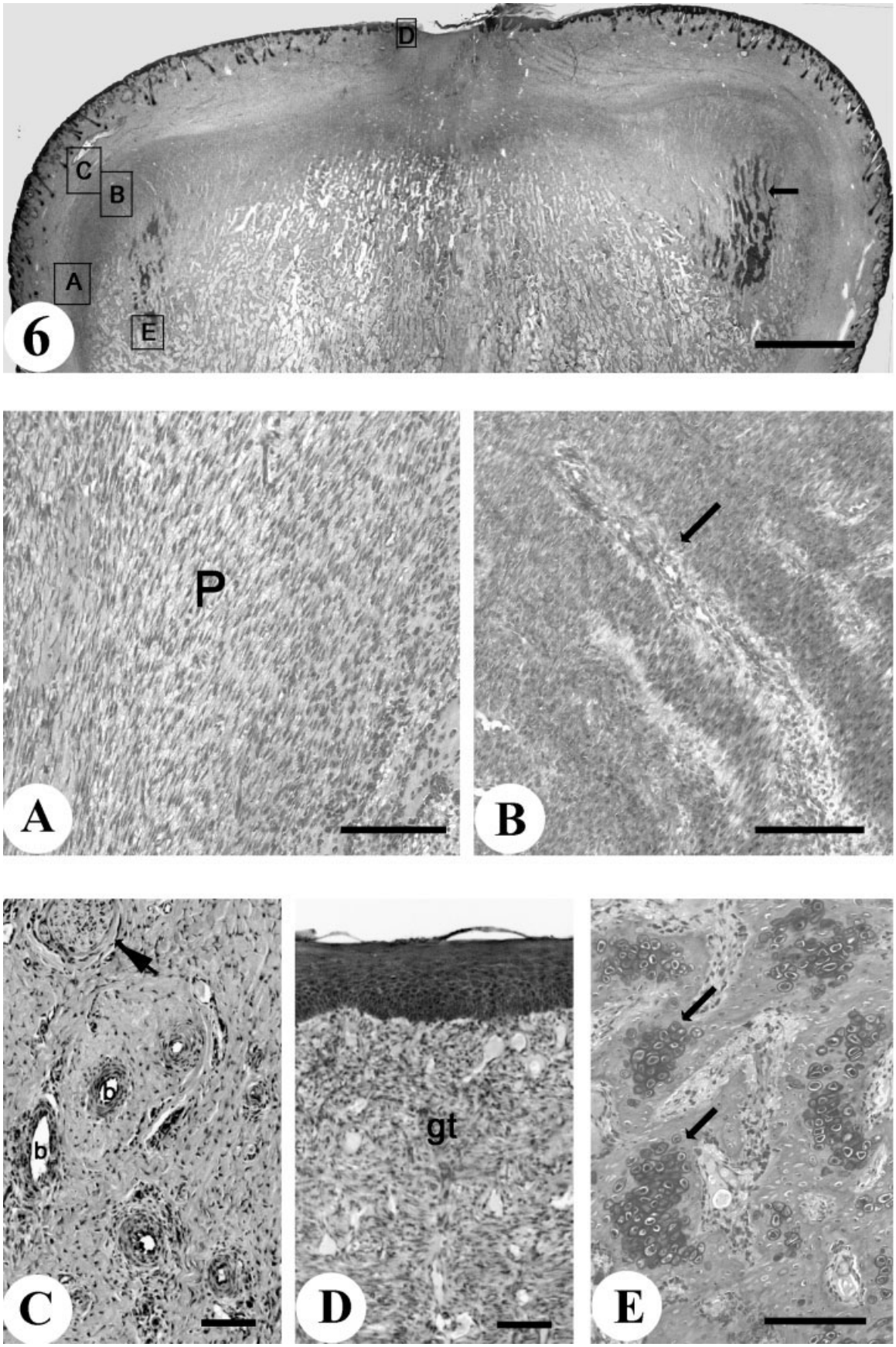

Figure 6.

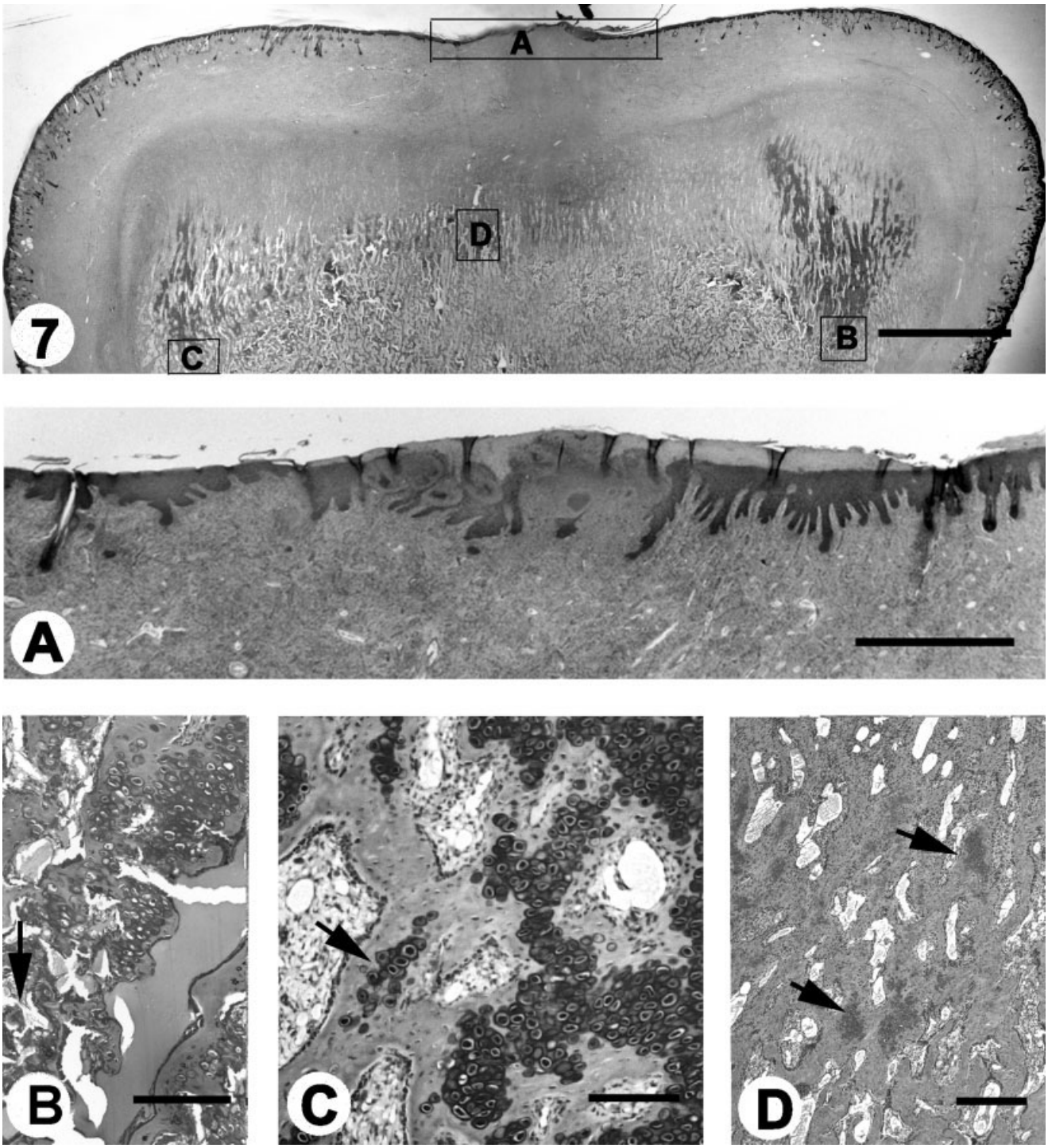

Fig. 7. Sagittally cut section of an early regenerating antler bud. Notice that wound healing process had completed. Anterior and posterior growth centres had been gradually built up. HE/AB. Bar = 4.0 mm. **A**: Higher magnification of an area similar to A in Figure 7 to show the convergence of healing epidermis at the central point over the top of an antler bud, which marks the completion of wound healing stage. HE. Bar = 1.0 mm. **B**: Higher magnification of an area similar to B in Figure 7 to show that chondroclasia occurred in the region formed during transitional ossification (arrow). HE/AB. Bar = 0.2 mm. **C**: Higher magnification of an area similar to C in Figure 7 to show the osseocartilaginous tissue (arrow) formed during transitional ossification. HE/AB. Bar = 0.2 mm. **D**: Higher magnification of an area similar to D in Figure 7 to show the limited amount of cartilaginous tissue formed in the central region and between the two growth centres (arrows). HE/AB. Bar = 0.5 mm.

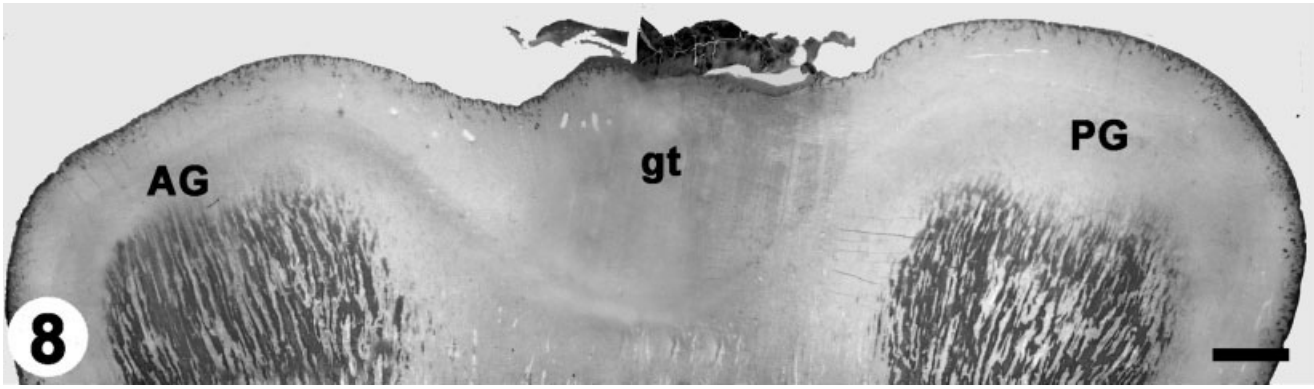

Fig. 8. Sagittally cut section of a regenerating antler bud. Notice that rapidly built tissue mass in each growth centre had pushed anterior and posterior corners up. It became clear that posterior (PG) and anterior (AG) growth centres were the centres for the main beam and brow tine formation. Granulation tissue (gt) was located underneath the scab. HE/AB. Bar = 4.0 mm.

Formation of Main Beam and Brow Tine

The contour of the distal end of a regenerating antler bud changed from flat to deep concave due to the rapid built-up of tissue mass in the growth centers, which pushed up from anterior and posterior corners (Fig. 8, AG, gt, and PG). At this stage, it became clear that the posterior and anterior growth centers, periosteal derivatives, were the centers for the formation of the main beam and brow tine. Mesenchymal, precartilaginous, and transitional layers in the main beam growth center are much thicker than those of the brow tine growth center respectively. Both granulation tissue and pedicle bone-derived bony trabeculae still existed beneath the scab between the two growth centers.

DISCUSSION

The main findings of the present histological examination are that, one, antler growth centers for the main beam and brow tine are formed independently at the posterior and anterior corners of a pedicle stump, respectively, and approximately at the same time. Two, the hyperplastic perichondrium surmounting each growth center is directly formed in situ by thickening of the distal pedicle periosteum. Three, the main beam and brow tine growth centers are established well before the completion of the wound healing over a pedicle stump. Four, wound healing over a pedicle stump is achieved by the ingrowth of full thickness pedicle skin and results in formation of a scar. These findings are summarized in Figure 9.

Is Early Antler Regeneration a Blastema- or Stem Cell-Based Process?

As an example of epimorphic regeneration, annual antler renewal has been considered to take place through initial blastema formation (Goss, 1992). This claim has never been experimentally tested. In recent morphological studies on antler regeneration (Li et al., 2004), we noted that the early antler regeneration process is not morphologically compatible with blastema formation, as no cone-shaped regenerating antler bud was formed. A histological comparison of early antler regeneration with blastema formation would provide crucial data to determine whether antler regeneration is a blastema-based process. However, currently available results on antler regeneration do not allow us to do so. Our detailed histological examination of early antler regeneration has made this comparison possible.

In the case of blastema formation, wound healing over the stump of an amputated appendage is achieved solely by the migration of epithelial cells within 24 hr. Cells from all mesenchymal lineages of the immediate amputation plane undergo a process called dedifferentiation (a loss of their specialized characteristics). These dedifferentiated cells start to migrate, proliferate, and accumulate beneath the thickened wound epidermis. Once a critical mass has built up, these cells begin to redifferentiate to form the replacement structure (Mescher, 1996). However, in antler regeneration, wound healing over a pedicle stump takes up to a week to complete and more importantly is achieved by the ingrowth of full thickness skin. Underlying the healing skin in the central region was granulation tissue [Goss (1983), Goss et al. (1992), Kierdorf et al. (2003), and the present study], which is known to be the main constituent of scar tissue.

Beneath the granulation tissue is the newly formed slender trabecular bone. These new trabeculae are clearly formed from the osteogenic cells lining the distal ends of the existing pedicle trabeculae, rather than differentiated from the overlying granulation tissue. Formation of these slender trabeculae has started in the central region by the time when the skin healing process starts at the periphery of the pedicle stump. Therefore, no obvious dedifferentiation and redifferentiation processes are detected histologically during the course of pedicle wound healing.

In blastema formation, no clear distinctive growth center can be identified in the whole cone-shaped regenerate (blastema). Cell proliferation takes place evenly in the blastema including the redifferentiating tissues to provide the cells destined to become the replacement structures in the regenerate (Wallace, 1981: p. 145). However, in antler regeneration, growth centers for main beam and brow tine formation are established at the posterior and anterior corners of a pedicle stump even before the completion of wound healing.

Interestingly, the process of early antler regeneration is histologically more comparable to that of postamputational healing of nonregenerative mammalian append-

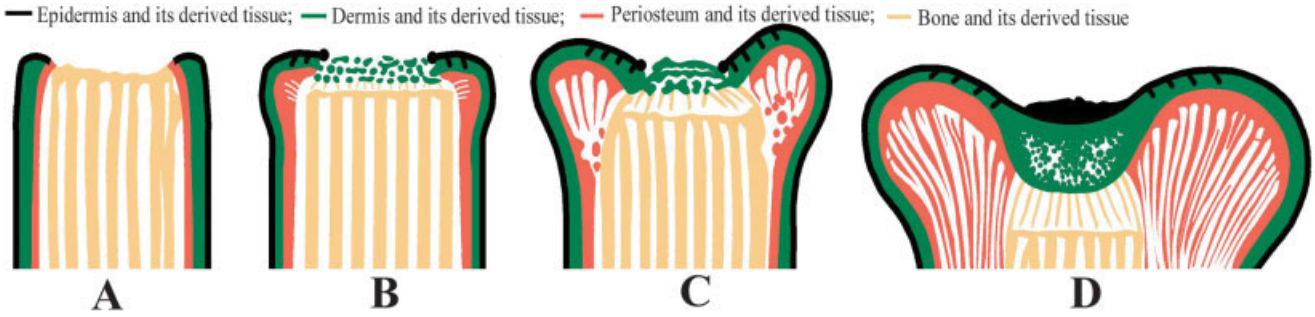

Fig. 9. Schematic drawings of histogenesis of antler regeneration. **A:** Casting. **B:** Early wound healing. **C:** Late wound healing and growth center formation. **D:** Formation of main beam and brow tine.

ages, such as limbs of rats or mice (Neufeld, 1985). Wound healing over both a pedicle and a limb stump is achieved by formation of full thickness of skin and results in formation of a scar. Scar formation over a pedicle stump after wound healing was also reported by Waldo and Wislocki (1951). Both types of scar are composed of granulation tissue. Internally, a very limited amount of cartilage is formed distal to the pedicle casting and limb amputating planes, but a substantial amount of cartilage is formed laterally surrounding the distal ends of these stumps. However, the cells in the peripheral periosteum of a pedicle stump have a much greater proliferation potential than those of a limb stump, because these pedicle cells will eventually form the replacement structure, whereas limb cells cannot.

The ability to delay the formation of basal lamina (a layer located between epidermis and dermis) until after a blastema has formed is the feature that distinguishes regenerative from nonregenerative appendages (Neufeld et al., 1986). So if antler regeneration takes place through initial blastema formation, the basal lamina should be absent in the healing skin over a pedicle stump. However, our results using laminin, one of the main components of basal lamina, antibody staining showed that all the healing skin has a well-formed basal lamina layer between the epidermis and dermis (Li et al., 2004). Although we cannot exclude the possibility that the well-formed basal lamina may be broken down when proceeding to the more advanced antler regenerating stage, as we only examined the stages of late wound healing and early regeneration. The incidence that the initially well-formed basal lamina is broken down in later regeneration stage does happen during the regeneration of a mouse ear hole (Gourevitch et al., 2003). However, any antler bud beyond the early regenerating stage should no longer be considered as a proper blastema, even if the incidence of basal lamina breaking down happens.

It is known that early regenerating blastema is an avascular tissue, as blastema formation is not compatible with angiogenesis (Mescher, 1996). However, the early regenerating antler bud is a richly vascularized tissue. Further, early-stage blastema formation is a nerve-dependent process (Singer, 1978). However, denervation to a future antler growth region cannot inhibit antler generation or regeneration (Li et al., 1993). Consequently, we conclude that deer antler regeneration is not a blastema-based but rather a conventional wound healing- and stem cell-based process.

The stem cell-based epimorphic regeneration does not seem unique to deer antlers. Recently, Gargioli and Slack (2004) reported that the regeneration of the Xenopus tadpole tail operates through mechanism that are completely different from those found in the appendage regeneration of urodeles, the classic epimorphic regeneration model animal. Because regeneration of Xenopus tail does not involve in dedifferentiation or metaplasia (conversion of one differentiated cell type to another), and each compartment (spinal cord, notochord, and muscle) regenerates from its own undifferentiated reserve cells. These authors predicated that the regeneration that might be stimulated in mammals will be closer to the anuran amphibians (stem cell-based) than to the urodeles (blastema-based). Deer antler regeneration, a case of naturally occurring mammalian appendage regeneration, therefore strongly supports their prediction.

Here one wonders why any other epimorphic regeneration has to be realized through initial blastema formation, but antler (Xenopus tadpole tail?) epimorphic regeneration can be an exception? When reasoning why epimorphic regeneration has to take place through blastema formation, Goss (1983) stated that the blastema concept was invented in the first place for epimorphic regeneration, because the segmental nature of most appendages seems to militate against their regeneration by means of exaggerated versions of tissue regeneration alone. However, deer antler, as a mammalian appendage, does not have segmentation. Consequently, antler regeneration, as an example of epimorphic regeneration, may be achieved by an exaggerated version of tissue regeneration, rather than going through the process of blastema formation.

Is a Regenerating Antler the Derivative of Pedicle Skin or Pedicle Periosteum?

If antler regeneration is achieved through an exaggerated version of tissue regeneration, rather than by dedifferentiation and redifferentiation, what tissue type from a pedicle stump gives rise to antlers? Pedicle stumps are composed of two main tissue types: bone and skin. Whether antler is a bone (periosteum) or skin derivative has been a matter of controversy. Wislocki (1942) found that it was the proliferating deeper portion of the corium, rather than adjacent periosteal tissue, which restored the surface of the pedicle and gave rise to the osteogenic germinal bed. Goss (1972, 1984, 1995) stated that following previous hard antler casting, the skin around the upper margin of the pedicle thickened and gave rise to

cells that healed over the exposed pedicle bone and provided a potential source of cells for the formation of antler buds. Both researchers concluded that it was the migrating pedicle dermal cells that formed regenerating antlers. This conclusion makes the process of early antler regeneration well aligned with the blastema formation in the classic models of epimorphic regeneration.

Recently, Kierdorf et al. (2003) reported that distal pedicle periosteum increased in thickness during the period of early antler regeneration. They suggested that antlers are formed mainly from pedicle periosteal cells. This suggestion is consistent with the discovery that deer frontal crest periosteum, from which pedicle periosteum derives, exclusively possesses the potential to form pedicles and first antlers (Hartwig and Schrudde, 1974).

Although each group has its own plausible theory to back up the argument, none of them had the tissue samples covering the period when the antler growth center forms. Our present histological study has for the first time provided the sufficient number of tissue samples covering the period of antler growth center formation. Our results showed that after the previous hard antler drops off, the margin of the exposed wound is rapidly healed by the ingrowth of the distal pedicle skin, and the exposed bony surface in the central region is subsequently covered with migrating dermal cells. At the same time, distal pedicle periosteum at anterior and posterior sides becomes thickened. Therefore, our results confirmed all the previous findings from Wislocki, Goss, and Kierdorf et al. However, our results further showed that the cellular layer cells of the thickening distal pedicle periosteum begin to form antler growth centers in situ, rather than migrate to the center, at the anterior and posterior corners by laying down first osseous tissue, then osseocartilaginous tissue, and finally cartilage. In contrast, the migrating dermal cells, which healed the central region of a pedicle stump, only formed granulation tissue under the scab and did not participate in antler growth center formation. Consequently, regenerating antlers are the derivatives of pedicle periosteum, but not pedicle skin.

Does Antler Regeneration Recapitulate Antler Generation or Reinvent the Wheel?

The present study has clearly demonstrated that antler regeneration takes place initially by intramembranous ossification, then proceeds to modified endochondral ossification (vascularized cartilage) through the transitional ossification (formation of a mixture of bone and cartilage). This result is consistent with the findings from initial antler generation (Li and Suttie, 1994) and the recent report by Kierdorf et al. (2003). However, Kierdorf et al. (2003) argued that the ossification type change during early antler regeneration might not be brought about by the mechanical pressure, which has been suggested to be the case in first antler generation (Li and Suttie, 2000). Because in first antler generation, the formation of interior osseocartilaginous tissue is under substantial pressure created by the overlying mechanically stretched skin. In contrast, in antler regeneration, the formation of both interior (bone) and exterior components (skin) occur simultaneously, so the newly formed velvet skin will be unlikely to exert any significant pressure on the underlying tissue. Hence, an alternative stimulus rather than mechanical pressure would play a major role in antler regeneration. That is, deer has to "reinvent the wheel" in order to accomplish the ossification type change during antler regeneration. However, our present histological study seems to demonstrate that ossification type change in antler regeneration may also be caused by mechanical pressure.

First, in contrary to the currently held view that the antler growth center over a pedicle stump forms in the central region that is not covered by healing skin at the time, our results showed that the growth centers for main beam and brow tine are established at the posterior and anterior corners of a pedicle stump. At this time, these corners have been well covered by the healing pedicle skin. Second, the undersurface of healing pedicle skin epidermis formed specific angled tongue-like structures, which appear to act as pegs to anchor the leading end of migrating epidermis to the underlying connective tissue (Fig. 3C). Third, the newly formed slender trabeculae located in the central region were vertically straight, but at the periphery were inclined centrally, as if some mechanic pressure is imposed onto them from anterior and posterior corners (Fig. 3B). The rapidly forming tissue mass at the anterior and posterior corners gradually pushes up into round buds. During this period, the newly formed velvet-like skin over the areas of anterior and posterior corners becomes substantially stretched (Figs. 4 and 5). Interestingly, the distal part of the healing skin, particularly epidermis, not only withstands the mechanical push from underlying rapidly expanding tissue, but continuously extends its leading edge to heal the rest of wound. Undoubtedly, the mechanically stretched skin would impose some mechanical pressure on the underlying expanding tissue. Consequently, we conclude that histogenesis of antler regeneration may recapitulate the events of first antler generation including skin stretch and mechanical pressure to drive chondrogenesis.

Overall, our present study is the first to provide direct histological evidence demonstrating that antler regeneration is realized initially by a conventional wound healing-based process, rather than through blastema formation. Regenerating antlers are of direct distal pedicle periosteum origin, rather than derived from pedicle skin. Histogenesis of regenerating antlers recapitulates the process of initial antler generation.

ACKNOWLEDGMENTS

The authors thank Mrs. Marion Labes for help with histology preparation.

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