

Histogenetic aspects of deer antler development

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1. ABSTRACT

Deer antlers are the only mammalian organs that, once lost, can fully grow back; therefore, they offer a unique opportunity for investigating the mechanism underlying mammalian organ regeneration. This review summarizes the current knowledge of antler histogenesis. The axis of a pedicle (antecedent of antler) and a first antler consists of an internal component and an external component. Formation of the internal component commences from the proliferation of antlerogenic periosteal cells and undergoes 4 ossification stages: Formation of the external component goes through 3 distinguishable stages. Antler velvet transformation is mainly associated with alteration in the skin appendages. Subsequent antler regeneration is divided into 5 stages. The present account, together with the companion paper on antler morphogenesis in this special issue (E4, 1836-1842), provides a foundation for further mechanistic study of this fascinating model for mammalian organ regeneration.

2. INTRODUCTION

Biomedical models have played and will continue to play an indispensable role in revealing the mechanisms of developmental processes and in carrying out proof-of-concept testing for treating debilitating diseases. Deer antler regeneration holds the potential to be developed into a unique model for biomedical research (see the paper on this issue: "Morphogenetic aspects of deer antler development"; cited below as "Morphogenesis"); however, to unleash this potential, a comprehensive understanding of basic antler biology is required. A typical example is the finding that antlers can be developed as a novel stem-cell research model, which was inspired by the discovery of the stem cells for antler regeneration when carrying out an investigation on how an antler is regenerated from its pedicle (1, 2). Undoubtedly, further antler research will yield more valuable insights about this unique mammalian organ. The detailed histological description of antler development featured in this review

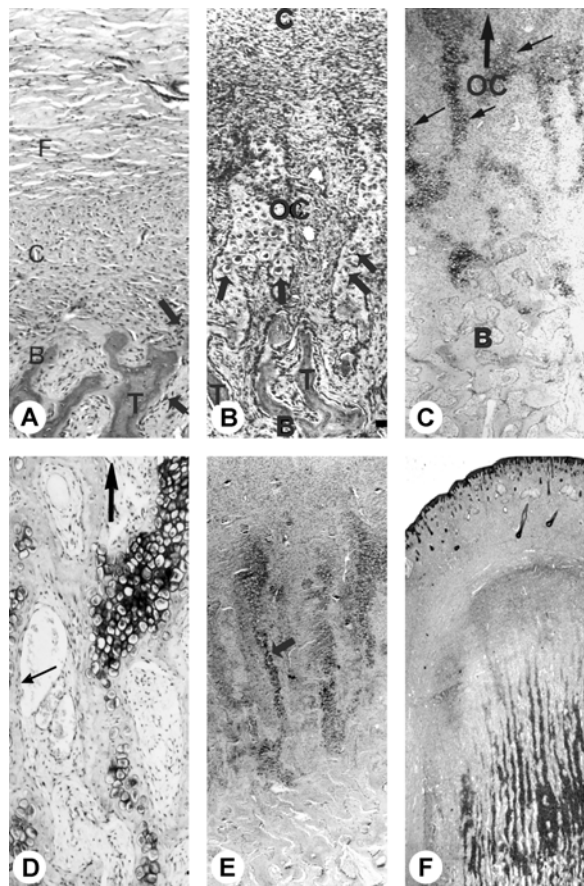


Figure 1. Histogenesis of deer antler osteocartilage (vertical section). A. Through the antlerogenic periosteum (AP) and the cancellous bone of a frontal crest. Surfaces of the trabeculae (T) and the spicules are covered with active osteoblasts (arrows), indicating the crest formation is through IMO. B. Through the AP cellular layer (C), the osseocartilage (OC), and the cancellous bone (B) of a palpable pedicle. Note that some discrete clusters of mature chondrocytes (arrows) appeared in the fast forming bony trabeculae (T), indicating the pedicle formation is through TO. C. Through the OC and the B of an incipient pedicle. Note that in the OC region, the proportion of cartilaginous tissue (arrows) increased towards the distal end (large arrow). D. Through the OC of an incipient pedicle. Note that a bony trabecula (arrow) with discrete cartilaginous cores, large arrow points the growth direction. E. pECO stage pedicle to show the continuous cartilaginous columns (arrow). F. aECO stage pedicle to show the continuous cartilaginous columns. Note that there is no distinguishable difference in histological makeup between the pECO and the aECO. IMO, intramembranous ossification; TO, transitional ossification; pECO, pedicle endochondral ossification; aECO, antler endochondral ossification; F, fibrous layer.). Reproduced with permission from (3).

together with the companion paper, "Morphogenesis," would lay a solid foundation for further antler research and

provide a comprehensive background for readers to read other papers presented in this special issue.

3. GENERATION OF PEDICLES AND THE FIRST ANTLERS

The axis of a pedicle and an antler consists of an internal component (osseocartilaginous tissue) and an external component (skin).

3.1. Internal component

3.1.1. Formation

This description of antler formation is based mainly on the report by Li and Suttie (3). The internal component of a pedicle consists of enveloping periosteum/perichondrium, cartilage, osseocartilaginous tissue (a mixture of bone and cartilage), and bone. This component is initially created from the proliferation and differentiation of the cellular layer cells of the antlerogenic periosteum (AP), the bone membrane that overlies the frontal crest in a prepubertal deer and consists of the fibrous and the cellular layers (3, 4), and proceeds through 3 ossification stages (3): first intramembranous ossification (IMO) up to 1.0 cm in height (palpable pedicle), followed by transitional ossification (TO) between 1.0 cm and 2.5 cm in pedicle height (visible pedicle), and finally pedicle endochondral ossification (pECO) to complete the rest of the pedicle tissue formation (2.5–3.0 cm in height). The internal component of a first antler is also constructed through the same type of ossification of pECO, which is termed here as antler endochondral ossification (aECO). Both the pECO and the aECO are histologically indistinguishable and belong to atypical endochondral ossification, known as "modified endochondral ossification." This term was coined by Banks and Newbery (5) to distinguish it from its classic counterpart. The former is richly vascularized, whereas the latter is relatively avascular (6). The vascular phenomenon is thought to be caused by the high metabolic demand resulting from rapid antler growth (5).

The tissue formed by AP cells during the IMO stage is solely cancellous bone. Underlying the thickened AP, bony trabeculae are rapidly under construction by the lining-active osteoblasts (Figure 1A), direct derivatives of the AP cells. At the TO stage, AP cells overlying some of the trabeculae in the apical region of a pedicle begin to differentiate into chondroblasts (Figure 1B), instead of osteoblasts, and osseocartilaginous tissue (mixture of bone and cartilage) is formed (Figures 1C to 1E). In the stages of pECO and aECO, the cellular layer cells of AP exclusively differentiate first into prechondroblasts and then into chondroblasts; therefore, only cartilage is formed. Interestingly, cartilage tissue formed in these stages is well vascularized (Figures 1E and 1F). No clear histological demarcation in the internal component between the pedicle and the first antler can be detected, although, exteriorly, these 2 structures are readily distinguishable by the change in skin-type (see "Morphogenesis"). Both periosteum/perichondrium and

cartilaginous columns are continuous between a pedicle and an antler (Figure 1F).

In prepubertal male red deer calves (age, 4 months), the subperiosteal bone of the frontal crests in presumptive pedicle growth regions is cancellous and actively formed through typical IMO. It is known that periosteal cells differentiate into osteoblasts, and then bone is directly formed in the presence of rich capillaries (6). Because the periosteum is richly supplied with blood vessels under normal conditions (6), once activated, periosteal cells begin to differentiate into osteoblasts, such as in the early stages of bone-fracture repair (7). Deer AP is also richly supplied with capillaries derived from the superficial temporal arteries (8, 9); therefore, it is conceivable that, at the initial stage, the AP cells differentiate into osteoblasts, and then cancellous bone is directly formed in the growing apex of these frontal crests.

Two questions arise here. First, should the IMO stage be defined as frontal crest formation or pedicle initiation? Although no histological study of deer frontal crests is available for the period between birth and puberty, it is likely that the IMO is an integral part of frontal crest formation. There are 2 reasons for this: (1) pedicle initiation depends on a high level of androgen hormones (10–12), and at the IMO stage, endogenous testosterone is still undetectable in male red deer calves (13); (2) frontal crests in both female and castrated male deer develop to the IMO stage (3). Second, how is the IMO stage initiated or sustained? Sissons (14) reported that proliferating bone tissue has considerable autonomous powers of growth. In the course of normal development, the tissue undergoes important modifications under the influence of local and general factors. These include nutritional, genetic, and other non-specified factors that play their part in sustaining or modifying bone growth. It seems quite possible that the IMO stage is related to dietary factors because it does not occur in malnourished deer of either sex (3).

The reason that the change in ossification-type from IMO to ECO takes place during pedicle and first antler formation is subject to speculation. Banks and Newbrey (5) considered that the metabolic demands of rapid proliferation, differentiation, and growth of antlers might require the additional nutrient supply provided by the unique vascularized cartilage. Stockwell (15) reported that, although a cartilage matrix is optimal for diffusion (the normal way in which cartilaginous cells obtain their nutrients), there is a limit beyond which the chondrocytes cannot be adequately nourished by the blood vessels in the perichondrium; therefore, if pedicle and antler development had adopted typical mammalian ECO, growth would have been limited or cartilage cells in the central part of the tissue block would have to die before they become calcified because they cannot obtain adequate nourishment. Conversely, if pedicle and antler development had proceeded continuously through IMO, that is, no change in ossification-type, antlers would not have existed at all because the bone tissue that is formed through IMO grows exceedingly slow. The weekly growth rate of the cortical bone through IMO is about 10–15 μm (14). The antler

growth period is about 15 weeks (16); therefore, the final length of an antler would have reached only approximately 0.2 mm! The modified mammalian ECO has probably evolved for late-stage pedicle and antler formation/regeneration to permit rapid growth over a restricted time period (about 2 months).

3.1.2. Remodeling

When a pedicle develops to the visible stage (>20 mm in height), chondroclasia first occurs in the proximal region of the osseocartilaginous portion and is mainly associated with the earliest formed chondrocytes. With advancement of pedicle development, the chondroclasia extends progressively upwards, towards the distal region. At the pECO stage, chondroclasts can be observed in the entire osseocartilaginous portion. The spaces created at this stage by removing chondrocytic clusters and destroying the peripheral bony wall of osseocartilaginous columns become continuous. In some cases, chondroclastic activity is found in the proximal region of the cartilage portion. The activities of chondroclasts and osteoclasts convert the smooth-surfaced osseocartilaginous trabeculae into irregular and broken columns. At the beginning of the aECO stage, the cartilaginous portion consists of 2 zones—forming and remodeling—from the distal region to the proximal region. The progression of cartilage formation occurs distally, whereas cartilage remodeling takes place from the proximal region upwards (Figure 2A); however, remodeling is more pronounced in the proximal region (Figure 2B) than in the distal region (Figure 2C). The proximal region of the osseocartilaginous portion contains osseous trabeculae with cartilaginous cores; however, nearly all the nuclei of these cartilaginous cells in the core regions are pyknotic (Figure 2D). On the surface of some trabeculae, osteogenesis is prominent but still accompanied by osteoclasia and chondroclasia (Figure 2E).

3.2. Exploration of the mechanism underlying the change in ossification-type

It is understandable that to grow antlers to the size that is proportional to a deer's body within a limited period, ossification-type has to be changed from intramembranous to modified endochondral; however, the factors that drive this change can only be speculated at this stage. Li *et al.* (3) thought that one of the main factors is mechanical compression exerted by the stretched skin covering and the fibrous layer of periosteum/perichondrium (see the following section).

To test this hypothesis, Li *et al.* (17) cultivated the apical periosteum/perichondrium from the different developmental stage pedicles/antlers *in vivo* by using diffusion chambers. By so doing, the mechanical pressure that is normally imposed onto these antlerogenic tissues was effectively lifted. The results showed that all tissues cultivated in the diffusion chambers, without exception, formed trabecular bone *de novo* during the 42- to 68-day period, regardless of whether they were forming osseous, osseocartilaginous, or cartilaginous tissue at the time of the initial biopsies. In contrast, all the apical periosteum/perichondrium at the control sides (intact) were

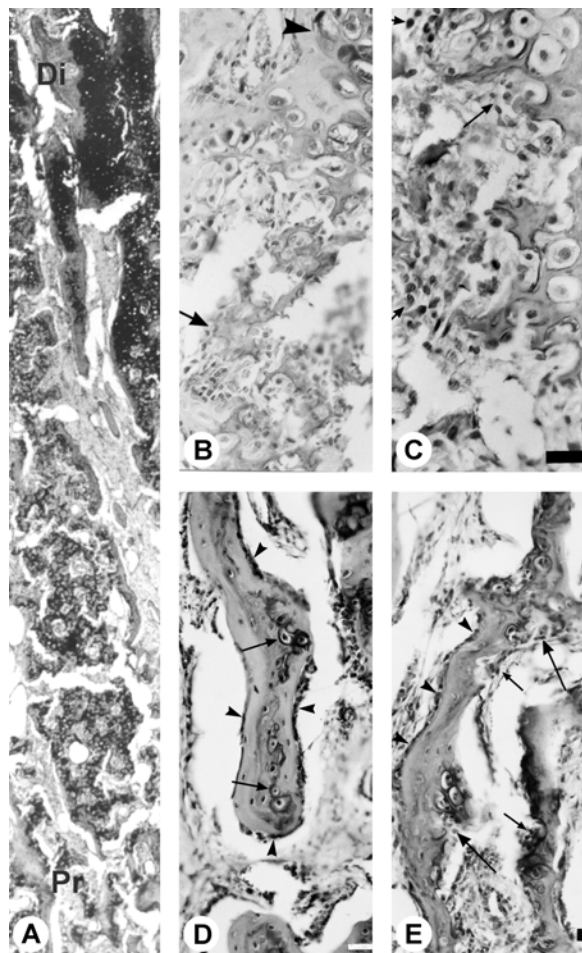


Figure 2. Remodelling of deer antler osteocartilagere (vertical section). In the OC region of a late stage pedicle. Note that cartilage remodelling has taken place intensively in the proximal part (Pr), whereas the cartilage is still intact in the distal part (Di). A. The arrowhead points to the chondrocytes and the arrow points to the remodelling area. B. Proximal part of the OC in an incipient pedicle to show the space created by chondroclasia. C. Distal part of the OC in an incipient pedicle to show the intensive chondroclasia. D. Part of the OC region in a full-grown pedicle with an incipient antler to show that a bony trabecula was covered by osteoblasts (arrowheads) and had cartilaginous cores (arrows). Note that the nuclei of the cartilaginous cells are pyknotic. E. An active area of chondroclasia (arrows) in the proximal part of the OC region, still with osteoblasts (arrowheads) that lined the surface of the trabecula. OC is the same as shown for Figure 1A.

exclusively forming cartilage; therefore, antlerogenic cells have a tendency to differentiate into osteoblasts and then form bone, and the change in ossification-type during pedicle and early first antler formation would be caused by extrinsic factors, such as mechanical pressure.

3.3. External component

Formation of the external skin of a pedicle and an antler also proceeds through 3 histologically

distinguishable stages: compression of the apical subcutaneous loose connective tissue (SLCT) when pedicles are at the TO stage (>1.5 cm in height), stretching of the apical undulated epidermis when pedicles are at the early pECO stage (~2.5 cm in height), and neogenesis of the overlying skin and its associated appendages when pedicles are at the mid-pECO stage (>3.0 cm in height). Antler velvet transformation from pedicle skin does not occur until the apical pedicle skin becomes intimately attached to the underlying antlerogenic tissue when pedicles are at the late pECO stage (>3.5 cm in height). This description is based mainly on the report for red deer by Li and Suttie (18).

At the onset of pedicle formation (IMO stage), there is a very loose and thick layer of SLCT overlying the frontal crest (Figure 3A), within the layer the major vascular and nerve systems are located. As pedicle growth proceeds, the layer becomes thinner and denser. At the aECO stage, the layer is significantly compressed and stretched and has become a strip that is 2–3 cells in thickness (Figure 3B). The change in configuration of the overlying epidermis begins at the late TO stage, when the SLCT layer is substantially compressed and stretched. At the IMO stage, the epidermis is a greatly undulated layer (Figure 3C). At the pECO stage, undulation of the epidermis is barely detectable (Figure 3D), and at the aECO stage, the epidermis has become totally flat (Figure 3E). The change in epidermal thickness commences at the mid-pECO stage, when the epidermis becomes more or less flat. At the IMO stage, the epidermis is very thin (2–3 cells in thickness; Figure 3F). As pedicle growth proceeds, the thickness of the epidermis gradually increases (Figure 3G). At the aECO stage, the epidermis has become about 10 times thicker than that at the IMO stage (Figure 3H). Antler velvet transformation, which occurs at the late pECO stage, is associated mainly with alteration in skin appendages. This alteration includes the loss of arrector pili and sweat glands and the gain of the large bilobed or multilobed sebaceous glands (Figures 3F and 3G); therefore, histological transformation from pedicle skin to antler velvet is a change mainly in the epidermis and its associated appendages that initiates from the apical center of a growing pedicle in the very first stage. Thereafter, velvet skin increases in area as the pedicle/antler continuously elongates until the boundary of the pedicle skin and velvet skin moves to the shoulder of the pedicle, which marks the termination of pedicle formation and the initiation of antler growth (Figure 4).

3.4. Exploration of the mechanism underlying pedicle skin formation and transformation to antler velvet

The factors that drive pedicle skin and antler velvet to expand so quickly and commensurate with the elongation of the internal component is open to speculation. On the basis of the findings of their histological examination, Li and Suttie (18) suggested that mechanical tension/stretch that originates from the fast expansion of the internal antlerogenic tissue is the main cause of this rapid skin formation. When a pedicle begins to grow under androgen hormone stimulation (11, 19), the first sign of change in the overlying skin is the compression of the

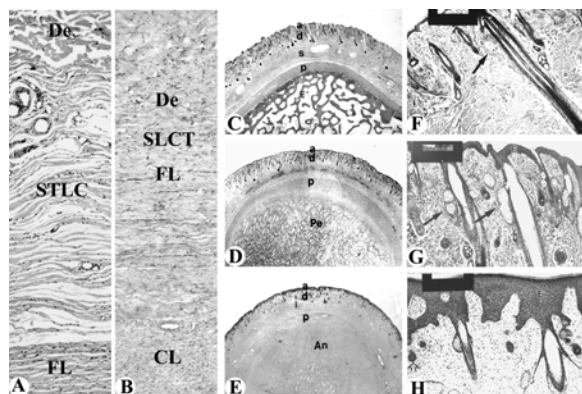


Figure 3. Histogenesis of antler velvet skin (Vertical sections) A and B. Subcutaneous loose connective tissue (SLCT). A. At the IMO stage, the SLCT is a very loose and thick layer. B. At the early aECO stage, the SLCT layer has become a thin strip. C-E. Changes in configuration of the apical epidermis. C. Scalp skin overlying a frontal crest (F) to show the undulated nature. D. Skin overlying a pECO stage pedicle to show the epidermis with the greatly reduced undulation. E. Velvet skin overlying an early aECO stage antler to show the flat epidermis. F-H. Changes in thickness of the apical epidermis and in the associated appendages. F. At the IMO stage, the epidermis is very thin; the sebaceous gland is small and monolobar with an obvious arrector pili muscle (arrow) attached. G. At the pECO stage, the epidermis is much thicker than the previous one, and sebaceous glands become bigger than those at the earlier stage but still with associated arrector pili muscles (arrows). H. At the early aECO stage, the epidermis becomes very thick, and the sebaceous glands become even bigger than those of the pECO stage but without arrector pili muscles attached. E/a, apical epidermis; De/d, dermis; FL, fibrous layer; CL, cellular layer; SLCT/s, subcutaneous loose connective tissue; p, periosteum/perichondrium; Pe, pedicle; An, antler; Star, sweat gland. IMO, pECO and aECO are the same as shown for Figure 1A. Reproduced with permission from (18).

SLCT layer, followed by stretching of the overlying undulated epidermis. Formation of new pedicle skin and its associated appendages is not commenced until the overlying epidermis is fully stretched and begins to increase in thickness. *In vivo* BrdU labeling experiments functionally confirmed this finding (18).

It is a common practice in the medical field to produce an extra piece of new skin by subcutaneous insertion of an inflation-controlled tissue expander. Austad *et al.* (20) concluded that skin expansion is a physiological process for accommodating an enlarged mass beneath it by increasing the surface area. Moreover, the newly formed pedicle skin is histologically comparable to artificially induced skin. Both newly formed apical pedicle skin (18) and artificially induced skin have thicker epidermis (21, 22) and thinner SLCT (23) than the skin from which they are originally derived. In this regard, the fast-forming antlerogenic tissue could be considered as a special kind of “tissue expander.” It is likely, then, that at the initial stage,

pedicle skin stretches to accommodate this expanding “expander” and that pedicle skin formation at the later stage releases the tension resulting from the continuously expanding “expander.”

The factors that cause the transformation from pedicle skin to antler velvet are not yet known. Mechanical tension, which may drive pedicle and/or antler skin growth, cannot, in itself, accomplish this transformation because mechanical tension can stimulate only skin neogenesis and cannot alter skin-type (21, 22). Putative inductive molecules from the underlying antlerogenic tissue must play a pivotal role in this skin transformation (2, 24, 25).

Nonetheless, chemical induction alone, without mechanical stimulation, may not be sufficient to complete the entire process of antler velvet transformation. Chemical induction may be effective only to alter the type of growing skin but not the type of existing skin, as demonstrated by Li *et al.* (17), again by using the diffusion-chamber approach. In their study, the diffusion chambers loaded with the minced AP were subcutaneously implanted into deer forehead regions for more than 2 months. No visible change in the overlying skin-type was observed, although the tissue derived from the AP had filled the chamber and formed close contact with the overlying skin, with interposition by a thin permeable membrane. Because the chambers were sealed using a membrane with a pore-size of 0.45 μm , passage of molecules was allowed but antlerogenic tissue outgrowth was limited; thus, it would not create any mechanical tension beyond the chamber to the overlying skin and, hence, would not promote new skin formation. It was therefore suggested that antler-velvet transformation could be accomplished using a combination of mechanical tension and chemical induction. It is likely that mechanical stimulation drives skin formation, whereas chemical induction determines the type of neoforming skin.

The mechanically stretched apical pedicle skin would inevitably exert mechanical pressure on the underlying internal component; therefore, there might be reciprocal mechanical interactions between the apical pedicle skin and the underlying growing antlerogenic tissue. Rapidly growing AP-derived tissue may drive pedicle skin expansion and formation and, in turn, the stretched pedicle skin might exert mechanical pressure on the underlying antlerogenic tissue, causing the change in ossification-type. Histogenesis of a pedicle and first antler is diagrammatically presented in Figure 4.

3.5. Elongation, calcification, velvet skin shedding, and antler casting

Elongation, calcification, velvet skin shedding, and antler casting of the first antlers bear great resemblance to those of the second and subsequent antlers at the histological level; therefore, they are described in the following sections on antler regeneration.

4. REGENERATION OF THE SECOND AND SUBSEQUENT ANTLERS

To simplify the description of antler regeneration, the process of regeneration of the second and

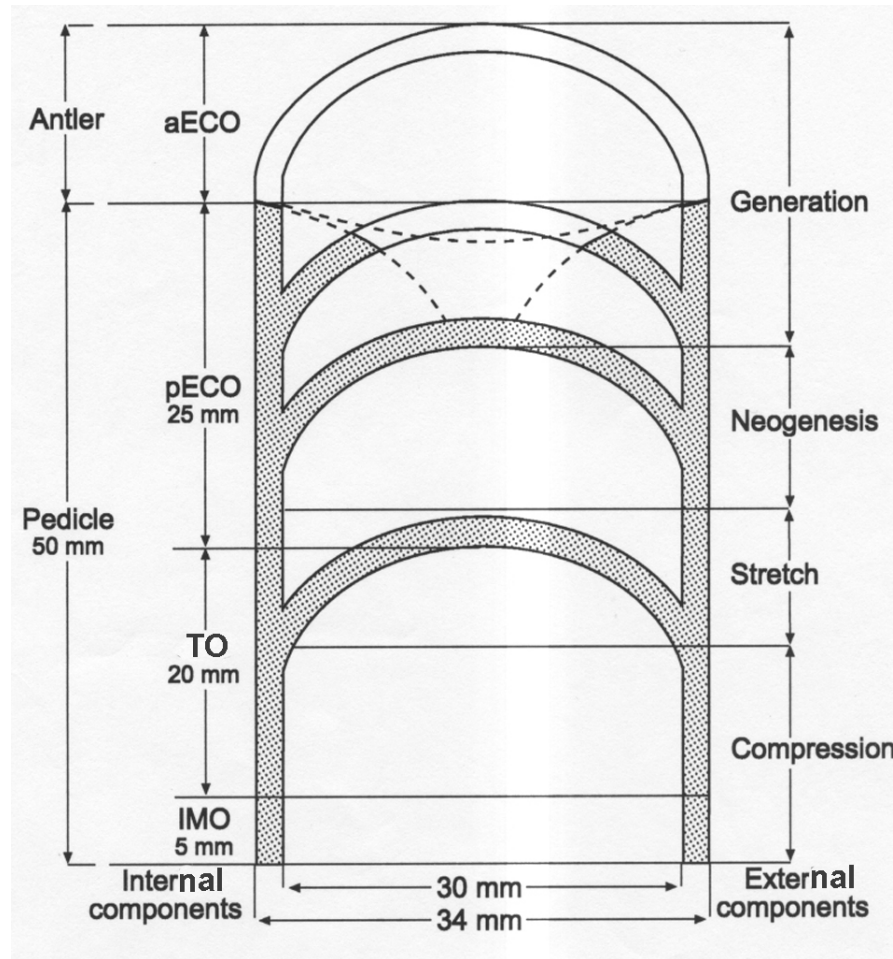


Figure 4. Illustration of formation of the internal and the external components of a pedicle and a first antler. For the description of the figure, refer to the text. IMO, TO, pECO and aECO are the same as shown for Figure 1A. Reproduced with permission from (18).

subsequent antlers was divided into 5 stages: precasting, casting, early wound healing, late wound healing, and early antler regeneration. The descriptions are based mainly on the report for red deer by Li *et al.* (26).

4.1. Precasting

Before a hard antler drops off, the future casting line is not detectable. The distal pedicle epidermis bends inward over the distal end of the dermis and directly attaches to the underlying pedicle periosteum without intervening with loose connective tissue.

When the antler casting date (indicated by the cast of a hard antler from the contralateral side) approaches (within 24 hr), enlarged cavities along the junction between a pedicle and an antler develop into an abscission line, which is a narrow dark-red zone sharply delineating the plane of future separation (Figure 5). Both sides of trabecular bone along the abscission line are densely studded with active osteoclasts (Figure 5A). Erosion at the periphery before antler casting leads to the excavation of a circumferential cleft into which healing pedicle skin has

begun to migrate (Figure 5B). The distal pedicle periosteum from both the anterior and posterior sides is tightly bound to the subperiosteal trabecular bone by Sharpey's fibers (Figures 5C and 5D). These periosteal are different from those ordinarily observed, in that no clear demarcation between the cellular layer and the fibrous layer can be readily detected.

4.2. Casting

Immediately after antler casting, the rim of the skin and periosteum tissue surrounding the distal end of a pedicle stump covers the margin of the cast plane and encroaches on the space formerly occupied by the periphery of the bony antler base (Figure 6). The epidermis at the distal end is thicker than that at the more proximal end down the pedicle shaft and has already acquired some velvet skin features (i.e., formation of new hair follicles) (Figure 6A). The osseous trabeculae on the immediate cast surface of the pedicle are denuded of connective tissue, which detaches when the antler is shed. The surface of spiky bone trabeculae of the pedicle stump is densely populated with osteoclasts, which help smoothen the rough

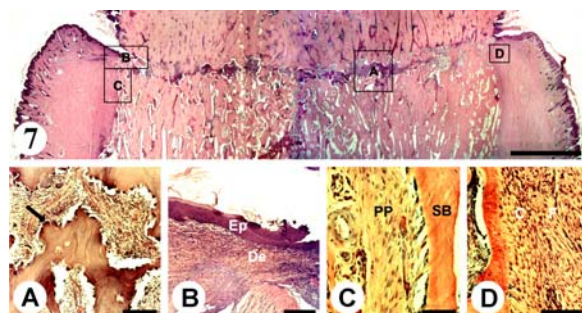


Figure 5. Junction between a pedicle and an antler just before antler casting (sagittally cut section). A narrow dark-reddish abscission line sharply delineates the plane of future separation. A. Higher magnification of an area similar to “A” in Figure 5 to show densely studded osteoclasts (arrow) on the bone trabeculae alongside the abscission line. B. Higher magnification of an area similar to “B” in Figure 5 to show the healing epidermis (Ep) and dermis (De) of pedicle skin. C. Higher magnification of an area similar to “C” in Figure 5 to show the distal pedicle periosteum (PP) and subperiosteal bone (SB). D. Higher magnification of an area similar to “D” in Figure 5 to show a peculiar phenomenon that there is no clear demarcation between the cellular (C) and the fibrous (F) layers of the pedicle periosteum. . Reproduced with permission from (Li and Suttie, 2000. *Anat Rec.* 260:62-71). Reproduced with permission from (26).

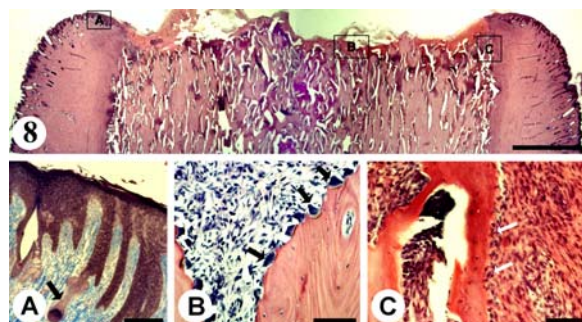


Figure 6. Pedicle stump immediately after antler casting (sagittally cut section). Fresh cast plane on the pedicle stump shows a very rough surface. A. Higher magnification of an area similar to “A” in Figure 6 to show that epidermis of the pedicle skin rim has acquired some features of velvet skin, e.g. de novo hair follicle formation (arrow). B. Higher magnification of an area similar to “B” in Figure 6 to show the active osteoclasts (arrows) lined along the surfaces of bone trabeculae on the cast plane. C. Higher magnification of an area similar to “C” of Figure 6 to show that the surface of the subperiosteal bone of distal pedicle is densely populated by active osteoblasts (arrows). Reproduced with permission from (26).

cast surface (Figure 6B). The lateral surface of the existing subperiosteal pedicle bony trabeculae at both the anterior and posterior ends is densely lined with active osteoblasts (Figure 6C).

4.3. Early wound healing

Within a day or two following antler casting, the newly formed hairless skin has made substantial ingrowth

from the periphery to seal nearly all of the cast plane, although the very central area is still denuded. A layer of granulated tissue, possibly of dermal origin, is observed overlying this denuded central area. Beneath the granulation are newly formed short, slender osseous trabeculae, which extend from the much thicker existing ones of a pedicle stump. These slender trabeculae appear to be directly formed from the osteogenic cells located in and above the eroded bony spicules of the cast surface. This observation is consistent with the finding of Kierdorf *et al.* (27), who reported that in addition to the osteoclasia, osteoblastic activities led to a partial restoration of the distal pedicle portion that was lost along with the cast antler; however, given that a substantial decrease in pedicle height (5.5 mm/year) occurs after each hard-antler casting (2), restoration of the distal pedicle portion has to be minimal if it were to happen. Otherwise, it would be hard to reconcile with the phenomenon of yearly reduction in pedicle height. Interestingly, the newly formed slender trabeculae at the anterior and posterior corners are inclined toward the center, as if there is an external force imposed on them. The undersurface of the migrating epidermis forms “tongue-like” structures in the tumescent skin. Most of these tongue-like structures have specific angles, as if working as pegs to clip the leading end of the healing epidermis tightly onto the underlying connective tissue. Pedicle periosteal cells at both the anterior and posterior ends begin to form slender bony trabeculae laterally and distally on the existing thick ones.

4.4. Late wound healing and early antler regeneration

Histologically, this stage could be subdivided into 3 phases: (1) initiation of the anterior and posterior growth centers, (2) formation of the continuous cartilaginous columns in each growth center, and (3) commencement of tissue remodeling in the earliest formed cartilaginous region. These phases are established to correspond to the appearance of new features rather than to rigidly defined length of time.

4.4.1. Initiation of the anterior and posterior growth centers

The prominent feature at this stage is that discrete clusters of chondrocytes that emerge in the posterior and anterior corners of a late wound healing–stage pedicle/early regenerating antler. These cartilaginous clusters are formed from rapidly proliferating and differentiating cells of the thickened distal pedicle periosteum. Formation of these cartilaginous clusters indicates that initiation of the posterior and anterior growth centers has started. Tips of both the anterior and posterior growth centers are capped by a layer of hyperplastic periosteum/perichondrium, which is formed from the distal end of the thickened pedicle periosteum. This periosteum/perichondrium consists of an outer fibrous layer and a very thick inner cell-rich layer. Within this thick periosteum/perichondrium, mesenchymal cells in the cellular layer differentiate into osseocartilaginous tissue; therefore, discrete clusters of chondrocytes are formed. The much thinner, newly formed slender trabeculae over the central region of a pedicle stump are conspicuously continuous with the existing thick osseous trabeculae.

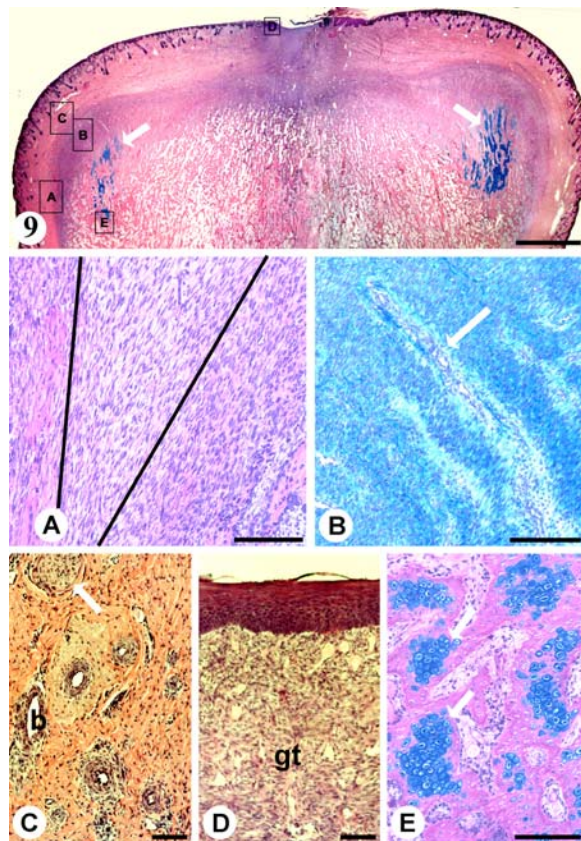


Figure 7. Early regenerating antler bud over a pedicle stump (sagittally cut section). Substantial amount of cartilaginous tissue and continuous cartilaginous columns have formed in the anterior and the posterior growth centres (arrows). A. Higher magnification of an area similar to “A” of Figure 7 to show the thickened hyperplastic perichondrium formed by the distal pedicle periosteal cells. B. Higher magnification of an area similar to “B” of Figure 7 to show that the continuous precartilaginous columns formed in a particular angle toward the growth direction of each growth centre (arrow). C. Higher magnification of an area similar to “C” of Figure 7 to show the richly distributed blood vessels (b) and nerves (arrow) directly overlying each growth centre. D. Higher magnification of an area similar to “D” of Figure 7 to show the granulation tissue (gt) underneath the healing epidermis. E. Higher magnification of an area similar to “E” of Figure 7 to show the cartilaginous clusters (arrows) formed during the TO stage. TO is the same as shown for Figure 1A. Reproduced with permission from (26).

Distally, the zone of newly formed slender osseous trabeculae merges into vascularized tissue.

4.4.2. Formation of the continuous cartilaginous columns in the growth centers

The distinguishing feature at this stage is the formation of the continuous cartilaginous columns under the perichondrium cap in each growth center (Figure 7) and the thickened distal pedicle periosteum (Figure 7A). The newly formed tissues (bone, osseocartilage, and cartilage)

of periosteal origin are clearly building on the bony trabeculae of pedicle bone origin. Interestingly, the continuous periosteum-derived precartilaginous columns in each growth center are oriented in a way that is directly against the external force imposed from both anterior and posterior corners (Figure 7B), indicative of the growth direction of each center. Directly overlying the hyperplastic cap of each growth center is a vascular layer densely populated with blood vessels and nerves (Figure 7C). The re-epithelialization of the entire antler cast plane is nearly complete at this stage. At the healing ends, the epidermis is thin and devoid of both hair follicles and sebaceous glands (Figure 7D), beneath which granulated tissue (a mixture of fibroblasts, immune cells, and endothelial cells) is found. Formation of each growth center by the hyperplastic periosteum/perichondrium cap also proceeds through the same 3 ossification stages that occur in the first antler generation (3) (i.e., IMO to form trabecular bone, TO to form osseocartilaginous tissue [Figure 7E], and aECO to form solely cartilage).

4.4.3. Commencement of remodeling in the region of the earliest formed cartilage

Convergence of healing epidermis at the central point over the top of a regenerating antler bud (Figures 8 and 8A) marks the completion of the wound-healing stage. Internally, chondroclasia (Figure 8B) has begun in the cartilaginous region formed during the TO stage (Figure 8C). In the central region (between the 2 growth centers), a limited number of cartilaginous clusters are discernible (Figure 8D). As the cartilaginous tissue in both the anterior and posterior growth centers continuously builds up, both corners of a pedicle stump begin to bulge outward (Figure 8).

4.5. Formation of the main beam and brow tine

The contour of the distal end of a regenerating antler bud has changed from slightly concave to deeply concave at this stage as a result of the rapid growth of tissue mass in the anterior and posterior growth centers, which push up from both corners. Now, it becomes clear that the posterior and anterior growth centers, the derivatives of the pedicle periosteal cells, are the centers for the formation of the antler main beam and brow tine (first branch), respectively. Mesenchymal, precartilaginous, and transitional layers in the growth center of a main beam are much thicker than the corresponding layers of the growth center of a brow tine. Granulated tissue and pedicle bone-derived bony trabeculae still exist beneath the scar in the area between the 2 growth centers. Once these growth centers are established, antler regeneration at the histological level takes place in the same way as in the first antler generation (3). Histogenesis of the second and subsequent antler regeneration is schematically presented in Figure 9.

4.6. Elongation and ramification

Antlers elongate by addition of the new tissue mass at the tips of the main beam and each tine as they arise. A growing antler tip has previously been divided into 6 zones: proliferation, maturation, hypertrophy, calcification, primary spongiosa, and secondary spongiosa

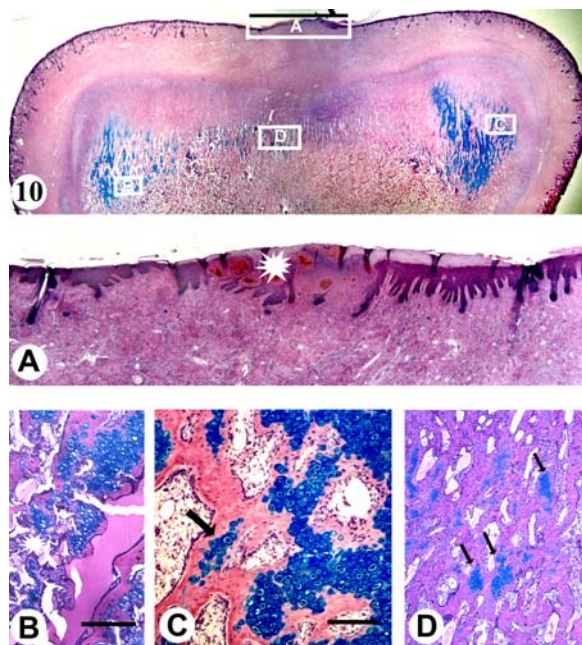


Figure 8. Early regenerating antler bud (sagittally cut section). Completion of the wound healing has achieved at this stage, and the growth centres in the anterior and the posterior portions have further extended. A. Higher magnification of an area similar to “A” in Figure 8 to show the convergence of the healing epidermis at the central point over the top of an antler bud (asterisk). B. Higher magnification of an area similar to “B” in Figure 8 to show that chondroclasia occurred in the region that is formed during the TO stage (asterisk). C. Higher magnification of an area similar to “C” in Figure 8 to show the osseocartilaginous tissue (arrow) formed during the TO stage. D. Higher magnification of an area similar to “D” in Figure 8 to show the limited amount of cartilage formed in the central region, where lies between the two growth centres (arrows). Reproduced with permission from (26).

(28). These zones represent successive differentiation stages from the reserve mesenchymal cells.

On the basis of existing and their own histological results, BrdU incorporation findings, and morphologically identifiable markers, Li *et al.* (29) further refined the classification for the proliferation zone by Banks and Newbery (28): distoproximally, a layer of mesenchyme including outer (essentially devoid of dividing cells) and inner (intensively labeled by BrdU) sublayers, a layer of precartilage (discrete columns), a layer of transition (between discrete and continuous columns), and a layer of cartilage (continuous columns). The cells in the proliferation zone are, on the one hand, dividing rapidly and on the other, progressively differentiating into various tissue components of the antler; therefore, antler elongation continues as proliferation rate keeps pace with that of differentiation.

The zone of cartilage hypertrophy in antlers is far more diffuse and extensive than that in the embryonic

growth plate, with type X collagen being expressed by majority of the chondrocytes (30). Because the hypertrophic chondrocytes are scattered among the vertical cartilage columns, the antler ossification center does not possess a distinct hypertrophic zone. Histological examination of the process of antler branching as well as the investigation of the mechanism underlying the branching process has thus far been lacking.

4.7. Ossification and hard antlers

The process of antler ossification has been well studied by Banks and Newbery (28). The results showed that the bone nearest the antler base is the most mature, whereas that at the tips is still forming. The cortical part is converted into a dense, compact bone with primary Haversian systems, while the cancellous interior is composed of fewer and coarser spicules of spongy bone enclosing relatively wide marrow spaces.

The histology of hard antlers is a relatively neglected field compared to that of growing antlers for obvious reasons. The hard antler shaft in fallow deer has been divided into 4 zones histologically from the periphery to the center by Rolf and Enderle (31): subvelvet, osteonic bone, transition, and spongiosa. The subvelvet zone is the outermost thin spongy layer, which encompasses the antler shaft and contains extensive osteoid seams. The osteonic bone zone is the layer of compact lamellar bone surrounding the substantial spongiosa of a hard antler. Only small osteoid seams can be found within this zone. The transition zone is the area between the osteonic bone zone and spongiosa. The spongiosa zone is the central-most region resembling bone marrow and occupies the main part of the antler shaft. This zone consists of spongy trabeculae and spacious lacunae.

The hard antler base is initially connected to the distal pedicle tissue by blood vessels, which are closed just days before antler casting (31). When examining hard antler specimens from different species, the axial channels and sinus located in the antler core prompted Acharjyo and Bubenik (32) to speculate that antlers from some deer species might remain alive through these vascular systems after velvet shedding. Bubenik *et al.* (33) also hypothesized that androgen blockade can cause long-term survival of the antler core when the internal parts of the antlers are not completely calcified as a result of a low level of androgen hormones. To test these hypotheses experimentally, Rolf and Enderle employed 2 techniques: histological analysis (31) and fluorescent-dye tracing (34). Histologically, a polished hard antler of fallow deer contains a well-intact system of capillaries that are connected to the vascular system of the deer body through pedicle tissue. The main parts of these hard antlers are still alive, at least until 3 weeks before antler casting. These histological results are further supported by the fluorescent-dye tracing study. When the fluorescent dye “calcein” was injected into the jugular vein of a deer in mid-winter, it was found to not only be transported into the polished antlers through the blood vessels inside the pedicles but also incorporated into the bony tissue of the antler, up to a height of 62 cm.

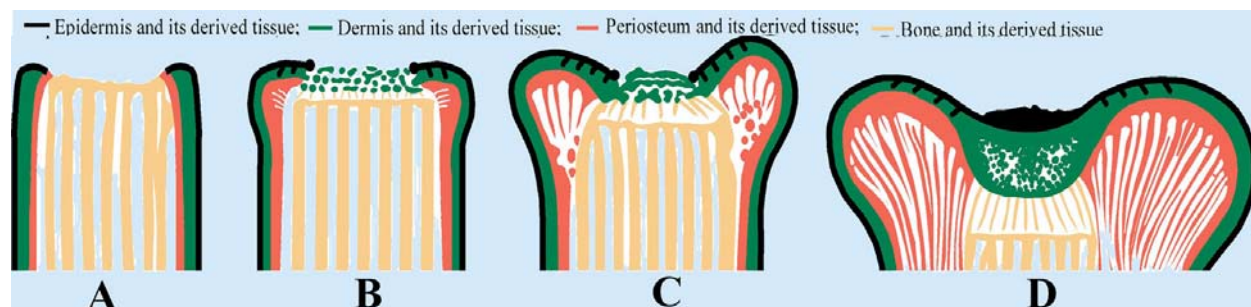


Figure 9. Schematic drawings of histogenesis of antler regeneration. A. Casting. B. Early wound healing. C. Late wound healing and growth center formation. D. Formation of main beam and brow tine. Note that it is the distal pedicle periosteum that gives rise to the growth centres for antler regeneration. Reproduced with permission from (26).

Belanger *et al.* (35) described that, in the bone, there is a deep-seated, osteocyte-governed type of resorption (osteocytic osteolysis), independent of osteoclasts and apparently related to calcium homeostasis. In their study, the authors found that osteolytic resorption was predominant in antlers from both male and female reindeer in July, when the antler is young and grows very fast. In December, although antler growth seems to have practically ceased in both males and females, osteolytic resorption is still occurring to some extent, indicating that the antlers may well be a calcium bank during winter for reindeer, at least in the female, who retains these appendages until spring and sheds them just before or after parturition.

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