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Morphological Observation of Antler Regeneration in Red Deer (*Cervus elaphus*)

Chunyi Li,* James M. Suttie, and Dawn E. Clark

AgResearch, Invermay Agricultural Centre, Mosgiel, New Zealand

ABSTRACT Deer antler offers a unique opportunity to explore how nature solves the problem of mammalian appendage regeneration. Annual antler renewal is an example of epimorphic regeneration, which is known to take place through initial blastema formation. Detailed examination of the early process of antler regeneration, however, has thus far been lacking. Therefore, we conducted morphological observations on antler regeneration from naturally cast and artificially created pedicle/antler stumps. On the naturally cast pedicle stumps, early antler regeneration underwent four distinguishable stages (with the Chinese equivalent names): casting of previous hard antlers (*oil lamp bowl*), early wound healing (*tiger eye*), late wound healing and early regeneration (*millstone*), and formation of main beam and brown tine (*small saddle*). Overall, no cone-shaped regenerate, a common feature to blastema-based regeneration, was observed. Taken together with the examination on the sagittal plane of each regenerating stage sample, we found that there are considerable overlaps between late-stage wound healing and the establishment of posterior and anterior growth centers. Observation of antler regeneration from the artificially created stumps showed that the regeneration potential of antler remnants was significantly reduced compared with that of pedicle tissue. Interestingly, the distal portion of a pedicle stump had greater regeneration potential than the proximal region, although this differential potential may not be constitutive, but rather caused by whether or not pedicle antlerogenic tissue becomes closely associated with the enveloping skin at the cut plane. Antler formation could take place from the distal peripheral tissues of an antler/pedicle stump, without the obvious participation of the entire central bony portion. Overall, our morphological results do not support the notion that antler regeneration takes place through the initial formation of a blastema; rather, it may be a stem cell-based process. J. Morphol. 262:731–740, 2004.

KEY WORDS: deer antler; pedicle stump; blastema; epimorphic regeneration; appendage regeneration; wound healing

During the course of evolution, vertebrates gradually lost the ability to regenerate missing appendages. In defiance of this general rule, deer antlers, as complex mammalian appendages, are cast and then fully regenerate from the permanent pedicles each year (Fennessy, 1985). Therefore, deer antler offers a unique opportunity to explore how nature solves the problem of appendage regeneration in mammals. Detailed examination of the early antler regeneration process, however, has thus far been lacking.

Antlers are male secondary sexual characters and their development is under the control of androgens. Previous bony antlers are cast from the pedicles in the early spring when testosterone drops below a certain threshold. Regeneration of new antlers immediately follows (in most deer species) from the pedicle stumps. The newly regenerating antlers are enveloped with soft skin, which is called velvet. The period of rapid antler growth occurs during the late spring and summer when androgen hormones are at barely detectable levels. Full antler calcification and velvet shedding take place in autumn in response to rising levels of testosterone. The exposed hard bony antlers are, thereafter, retained on the deer's head until the next spring, when they are cast and a new antler growth cycle is initiated.

Regeneration in animals can be classified into four categories: physiological regeneration, which happens to counteract every day's wear and tear; wound healing, also referred to as tissue regeneration, which is a stopgap measure to restore continuity of the interrupted tissue; compensatory growth, which occurs in some organs as a response to increase in functional load; and epimorphic regeneration, which is the phenomenon of de novo development of appendages distal to the level of amputation (Goss, 1980). Virtually all animals possess the ability to undertake the first three categories of regeneration. Epimorphic regeneration, however, is not universal.

By definition, annual antler renewal is an example of epimorphic regeneration. A fundamental step in the process of epimorphic regeneration in lower vertebrates, such as newts and lizards, is the formation of a blastema at the initial stage (Goss, 1980).

*Correspondence to: Chunyi Li, AgResearch, Invermay Agricultural Centre, Private Bag 50034, Mosgiel, New Zealand.
E-mail: chunyi.li@agresearch.co.nz

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Therefore, epimorphic regeneration has been called blastema-based regeneration. A blastema is a cone-shaped mass of cells, which is formed from the dedifferentiation of all tissue lineages on the immediate amputation plane. A blastema is endowed with the capacity to develop into a replacement structure (Goss, 1969). Blastema-based regeneration in lower vertebrates goes through distinct morphological stages that have been characterized based on model animals, such as amputated newt limb regeneration. These stages consist of cone (a protrusion regenerated from a stump), palette (the flattened cone), and notch (2–3 digits) (Wallace, 1981). Since antler regeneration is an example of epimorphic regeneration, previous studies either claimed that renewal of deer antlers was initiated through blastema formation (Goss, 1980; Allen et al., 2002), or called the early regenerating antler bud a blastema (Goss, 1995; Li and Suttie, 2003). However, this claim has not been confirmed experimentally. Thus far, a comprehensive morphological description of early antler regeneration, which permits a direct comparison with early stages of a blastema-based regeneration in model animals, has been lacking. In order to establish a deer antler model for the study of epimorphic regeneration, we carried out a series of investigations on antler regeneration. In this article we report the morphological observation of early antler regeneration from both naturally and artificially created stumps.

It is necessary to include artificially created antler/pedicle stumps because these stumps are more comparable to amputated limb stumps of model animals than those formed naturally. In the latter, a wound is created after velvet skin shedding, an event that occurs around 5 to 6 months earlier than the time when antler regeneration takes place (Kierdorf and Kierdorf, 1992; Li, 2003). In contrast, regeneration in both amputated limb stumps of model animals and artificially created antler/pedicle stumps takes place immediately after the creation of a wound.

MATERIALS AND METHODS

Natural Casting

Forty-five pedicle/antler tissue samples of red deer (*Cervus elaphus*) covering the period of precasting of hard antlers and early antler regeneration were collected in a commercial abattoir. These samples were allocated into one of five groups according to the stage of regeneration: precasting (10 samples), casting (8 samples), early wound healing (10 samples), late wound healing and early regeneration (10 samples), and formation of the main beam and brow tine (7 samples).

Following morphological observation, these tissue samples were divided sagittally into two halves using a disposable microtome blade and a saw for macroscopic examination.

In order to confirm the nature of different tissue types on a sagittally cut plane of early regenerating antler bud, one of the antler buds at the late wound healing and early regeneration stage was histologically processed and stained with counterstaining of hematoxylin and eosin, and alcian blue.

Artificial Removal

In order to make more comprehensive morphological comparisons with amputated limb stumps of model animals, we artificially created stumps by removal of antler/pedicle tissues at four different levels (see below) along the axis of this cranial appendage. At the same time, the regenerative potentials at each level were carefully recorded. Experiments 2 and 3 were approved by the Invermay Animal Ethics Committee.

Through antler base. In the commercial deer industry, antlers are generally harvested midway through their growth (50–55 days) in order to retain the maximum medicinal value. The standard technique includes injection of local anesthetic drugs and removal of velvet antlers 1–2 cm above the antler/pedicle junction. By so doing, it is believed that the species-specific pattern of the next set of antlers will be retained. This practice offered an opportunity for us to observe how antlers morphologically regenerate from the leftover antler remnants.

Forty 3-year-old red deer stags were selected for this observation. Antlers at 50–55 days of growth were removed at a site 2 cm above the antler/pedicle junction using a saw, in accordance with the regulation of New Zealand Velveting Standard Board. The regeneration pattern and potential from these antler remnants were carefully recorded.

Through casting plane (antler/pedicle junction). One way to effectively examine antler morphogenesis and the true regeneration potential from the artificially generated stumps through antler/pedicle junction (natural casting plane) or from antler remnants is to create perennial growing antlers. Perennial antlers are the antlers that remain in velvet skin permanently and are not subject to routine antler growth cycles. Creation of perennial antlers can be achieved by the manipulation of androgen hormone levels in deer. In this experiment, three adult female red deer (two of them were freemartins) were used. Under natural conditions, while possessing antlerogenic tissue, female red deer do not grow antlers, as they do not have a sufficient level of androgen hormones. However, female red deer can be induced to grow pedicles and antlers by the administration of exogenous androgen hormones. These three animals had been induced to grow pedicles and first antlers in another experiment by testosterone treatment and then withdrawal (Li et al., 2003).

The detailed procedure for inducing these female red deer to grow pedicles and antlers has been reported elsewhere (Li et al., 2003). Briefly, in June (Southern Hemisphere), a month before the time of natural pedicle initiation, each female red deer was implanted with testosterone and the implant was removed 6 months later (December). Three females successfully formed pedicles and first antlers during this period of testosterone treatment. Thereafter, an antler removal experiment was carried out based on the following design. Right-side antlers were removed at a level 2 cm above the antler/pedicle junction, and left-side antlers were removed through the antler/pedicle junction. The removal date of each set of antlers was determined by when these antlers ceased further growth. The experiment was terminated when no significant difference in length could be detected between the last two sets of antlers. The data were analyzed using ANOVA.

Through different levels of pedicle. In the examination of natural antler regeneration from pedicle stumps, it was observed that pedicle skin at the proximal end (about two-thirds of total pedicle length) was loosely attached to the underlying pedicle bone. In contrast, the rest of the pedicle skin at the distal end (about one-third of total pedicle length) was firmly associated with the underlying bone (Li and Suttie, 2003). Although pedicle tissue along the whole shaft possesses the full potential to regenerate entire antlers, the region where the pedicle bone and skin are closely associated may have moved one step further toward antler regeneration than the area where pedicle skin and bone are loosely apposed. Therefore, in that study the distal closely associated portion was termed “potentiated,” whereas the proximal loosely attached portion was termed “dormant.” This experiment was designed to observe how antler regeneration took place morphologically from the stumps created at these two different regions.

Eight 1-year-old red deer stags were selected and allocated into either of two groups. The pedicles of one side were randomly selected as the treated side, and the remainder was left intact as controls. In Group I, the pedicle and first antler of the treated side were removed at a level 0.5 cm below the antler/pedicle junction (potentiated region). In Group II, the pedicle and the first antler of the treated side were removed at 2.5 cm below the antler/pedicle junction (dormant region). Antler regeneration status was subsequently recorded.

RESULTS

Natural Casting

Although the general description of the antler growth cycle can be readily found in the English literature (Goss, 1983; Lincoln, 1992), the names that vividly describe the morphological stages of initial antler regeneration can only be found in Chinese textbooks (Zhao, 1986). Therefore, these Chinese names were also used in this article alongside the general description from the English literature.

Precasting. Before casting, hard, bony antlers were firmly attached to the living pedicles. At the time, immediately prior to hard antler casting, the distal end of the pedicle skin was swollen and shiny (Fig. 1A).

In the sagittally cut antler/pedicle, the color and density clearly delineated the living pedicle (pink–red, less dense) from dead antler (white, denser) just before antler casting (Fig. 1B). The rim of skin immediately surrounding the distal part of a pedicle was very closely attached to the pedicle bone without a visible intervening layer of subcutaneous loose connective tissue. As the time of antler casting approached, a peripheral circumferential cleft was eroded proximal to the antler bone, which created a space for distal pedicle skin to grow in.

Casting (oil bowl stage). Immediately after the antler dropped off, bleeding took place from the surface of the pedicle stump. The extent of blood lost for each antler casting varied among individuals. In some deer, bleeding was profuse, although it very rarely overflowed the distal pedicle skin rim (Fig. 1C). On the sagittally cut plane in these animals, the frontier of the distal end of the pedicle skin had not visibly migrated centrally (Fig. 1D). In other deer, bleeding was very limited (Fig. 1E). On the sagittally cut plane, the distal pedicle skin had substantial ingrowth and a layer of fresh tissue (about 1 mm in average thickness) covered the reduced casting surface (Fig. 1F).

In all cases, examination of the apical surface revealed that a depressed central bony portion on the exposed casting surface of a pedicle was surrounded by a rim of hairless skin (Fig. 1C,E). This skin gives rise to the future velvet skin, which is readily distinguishable from typical body skin. At this stage, the top of a pedicle looks like a bowl. The lost blood was retained in the depressed central portion, resembling oil in a bowl. Thus, the Chinese call this “the stage of *oil lamp bowl*.”

Early wound healing (tiger eye stage). Shortly after antler casting (1 or 2 days, pers. obs.), the blood remaining in the “bowl” (central portion of the casting surface) dried and a scab was then formed. At this stage, the scab occupied approximately half of the entire casting surface and was surrounded by a ring of shiny skin (Fig. 1G), which resembles an eye, with an eyeball, the scab, located in the center. Thus, the Chinese call this “the stage of *tiger eye*.” In the sagittal plane, distal pedicle skin migrated further centrally above the fresh tissue layer and under the scab compared to the early stages. The layer of fresh tissue was further thickened (Fig. 1H).

Late wound healing and early regeneration (millstone stage). As the wound healing process advanced, the skin ring migrated further centrally and the scab became smaller compared to the last stage. Once the diameter of a scab became smaller than the width of the shiny skin ring, when viewed from the top, a millstone-like structure with an axle, the scab, located in the center (Fig. 1I) could be seen. Thus, the Chinese call this “the stage of *millstone*.” On the sagittally cut plane, the prominent feature at this stage was the two visually discernible crescent-shaped bulges. One was located at the anterior corner and the other at the posterior corner (Fig. 1J). These bulges had obvious cartilaginous properties.

When the peripheral healing skin met in the center of the healing top, the anterior and posterior bulges became bigger and even more clearly defined (Fig. 1J,L). No further thickening for the layer of fresh tissue on the casting surface was observed at this or subsequent stages (Fig. 1L).

In some cases, although antler regeneration was only at the early and mid *millstone* stage (based on the size of the scab), the anterior and posterior portions of a regenerating antler bud already protruded, which left the central scab portion behind due to the differential growth rate (Fig. 1M). Examination of the sagittally cut tissue showed that the posterior and anterior bulges formed in the previous stages had become the growth centers for the main beam and brow tine, respectively (Fig. 1N). Due to the differential growth rate, the central fresh tissue layer was left behind.

Histological results (Fig. 2) showed that the posterior and anterior bulges were composed of discrete and continuous cartilaginous columns. The fresh tissue layer under the scab was made of granulation tissue (mixture of fibroblasts and endothelial cells), which is the main component of scar tissue.

Overall, there was an overlap between the late wound healing stage and the formation of early growth centers for the main beam and brow tine.

Formation of main beam and brow tine (small saddle stage). The next event of the antler regeneration process was the formation of the main beam and the brow tine. Morphologically, the main beam and brow tine were formed posteriorly and an-

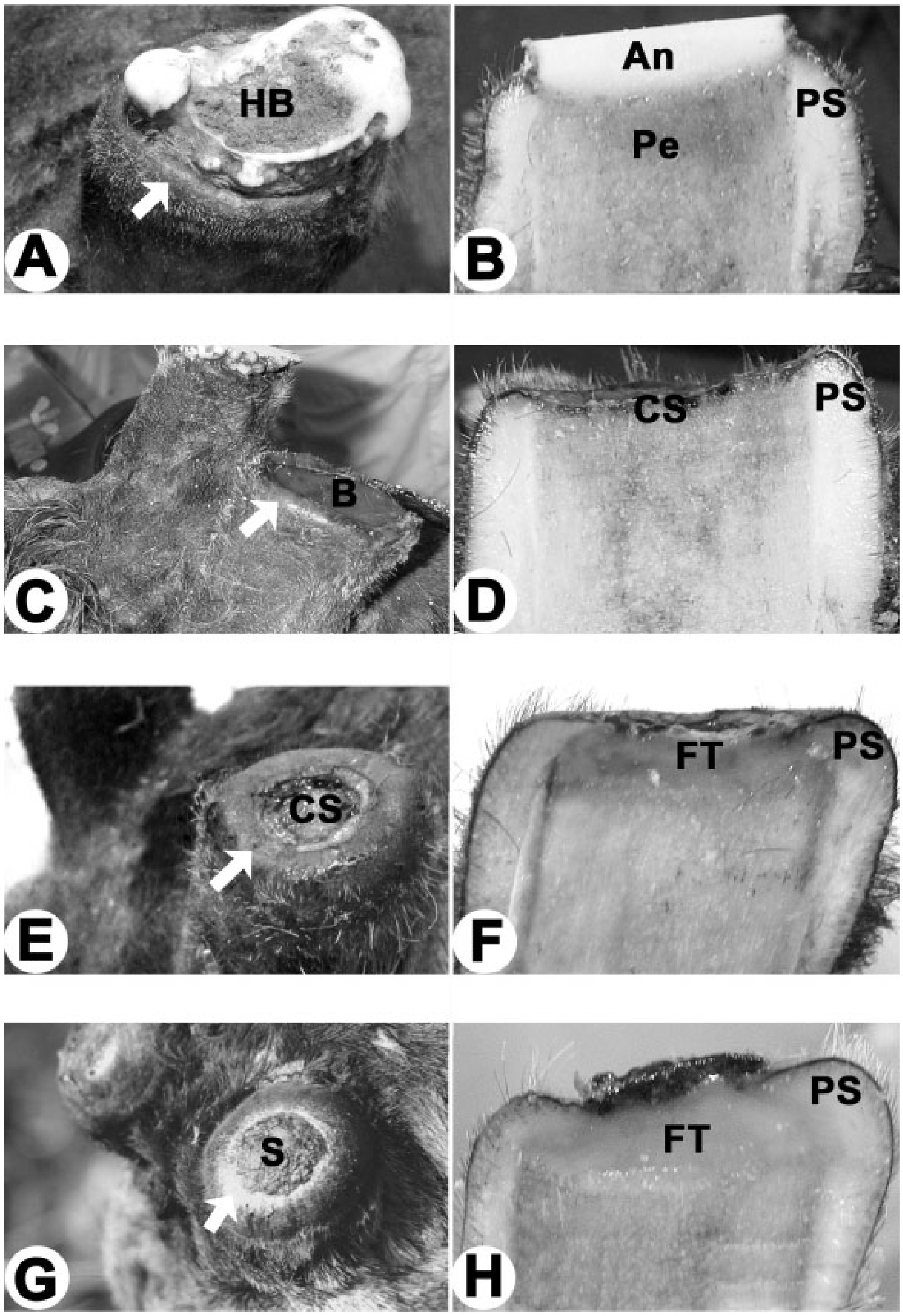

Figure 1

teriorly, respectively. At the initial stage, as the main beam and the brow tine were of a similar length, the incipient antler resembles a saddle (Fig. 1O). Thus, the Chinese call this "the stage of *small saddle*." There is no specific name in Chinese textbooks for the same regeneration stage antler with a bez tine. Here, we call it "the stage of *silver ingot*," as the shape is reminiscent of a form of ancient Chinese money (Fig. 1P).

The centrally located scab was displaced by the eccentrically formed main beam and brow tine (Fig. 1O), and eventually flaked off, and when that was to happen, the underlying scar would then be exposed (Fig. 1P).

Overall, a cone-shaped regenerate was never built up in the center of the pedicle stump surface; rather, growth centers for the main beam and brow tine emerged at the posterior and anterior corners, respectively. The general morphological descriptions during each stage of early antler regeneration are summarized in Table 1.

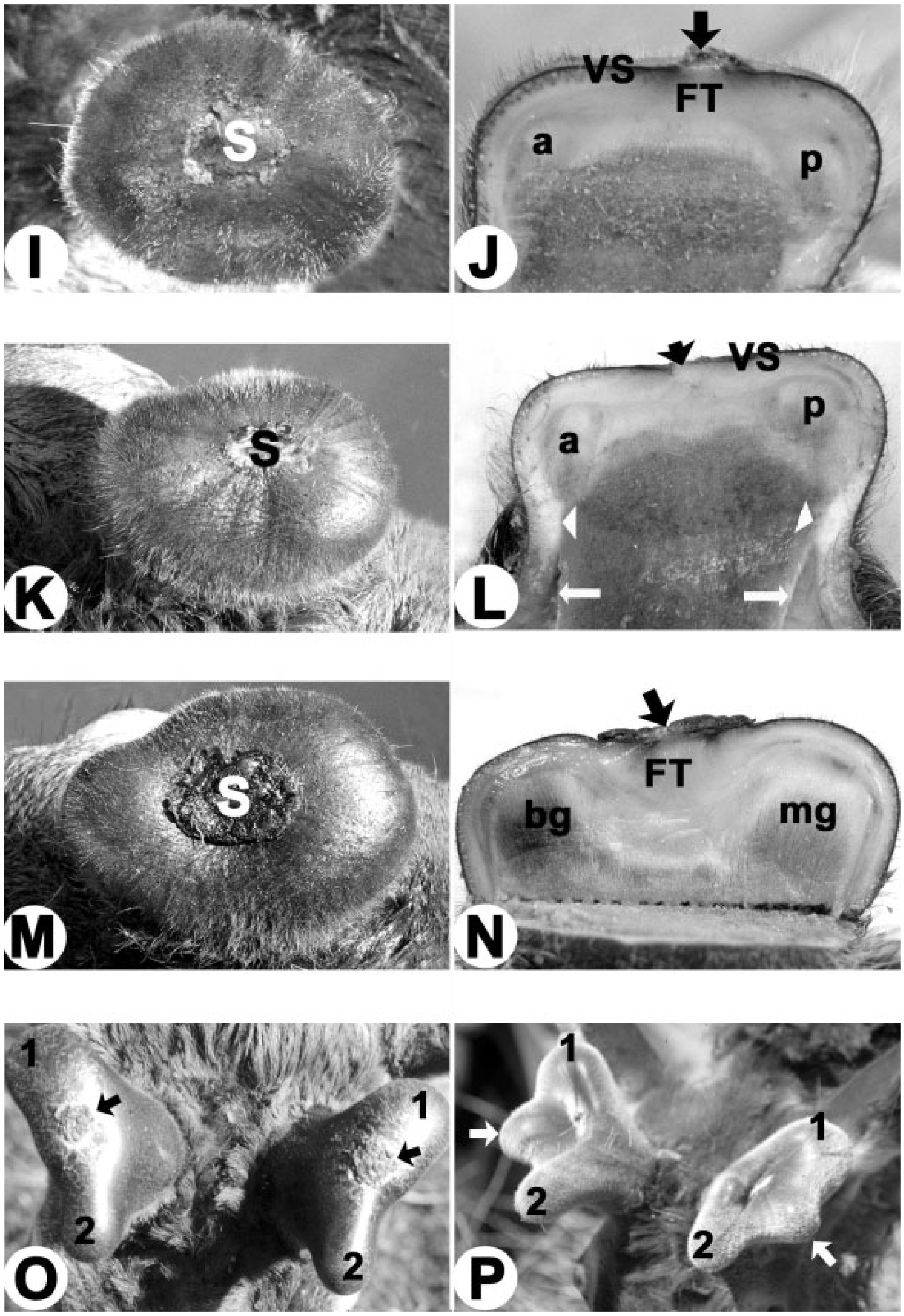

Figure 1. (Continued)

Artificial Removal

Through antler base (2 cm above antler/pedicle junction). The results showed that 33% of the stags had wounds fully healed over the antler remnants left after antler removal and had regenerated small, abnormally shaped antlers. The wound healing steps and early antler regeneration were essentially the same as those of naturally generated pedicle stumps. The remainder (67%) of the stags showed partial antler regeneration from these antler remnants. Most of these partial regenerates were small spikes (Fig. 3A) and all formed from the peripheral edge of the remnants (Fig. 3B). Morphologically, these small spikes were mainly derived from peripheral tissues of the antler remnants.

This experiment convincingly demonstrated that antler regeneration could take place without the

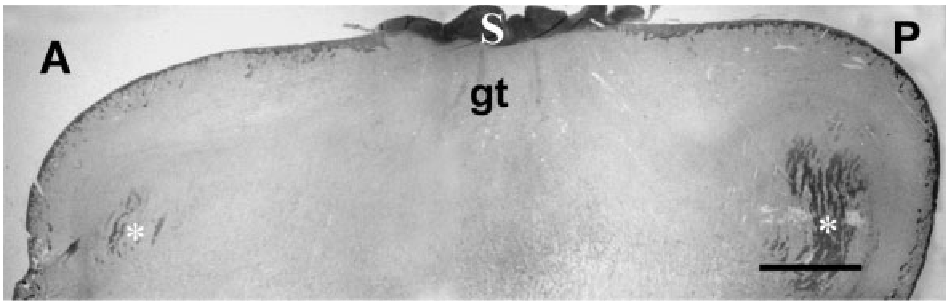

Fig. 2. Sagittaly cut section of an early regenerating antler bud over a pedicle stump (a stage similar to Fig. 1I). Note that clusters and columns of cartilage were emerged in the posterior and the anterior corners of the antler bud. These histological results confirmed the cartilaginous nature of the crescent-shaped bulges shown in Figure 1I. A, anterior; P, posterior; S, scab; gt, granulation tissue; *cartilaginous region. Counterstaining of hematoxylin/eosin and alcian blue. Scale bar = 4 mm.

obvious participation of the central bony portion of an antler remnant.

Through antler casting plane (antler/pedicle junction). No obvious morphological difference was observed in early antler regeneration between the stumps of artificially and naturally created pedicle stumps. Interestingly, cone-shaped regenerates were formed from the artificially created antler remnants (Fig. 3C,D). However, these regenerates were the terminal structures, which neither grew further nor differentiated to form branches. In the sagittal plane, the cone-shaped regenerate was developed from the posterior growth center, rather than formed from a growth center established in a pedicle stump center (data not shown). After five instances of antler removal, no significant difference ($P > 0.05$) in antler length was found between the first set and the fifth set of antlers on the pedicle stumps (Fig. 3C,D). In contrast, regeneration potential from antler remnants was decreased from the third set of antlers; antler length became significantly shorter ($P < 0.01$) than that of the first set. The fourth set of antlers was decreased in length even further (Fig. 3C,D). No further decrease in antler length was

Fig. 1. Antler regeneration from naturally casting pedicle stumps of red deer (*Cervus elaphus*). **A:** Pedicle with a hard button (HB, remnant of a hard antler following the previous season surgical velvet antler removal) on it in spring just before the hard button casting. Notice that distal end of pedicle skin was swollen and shiny (arrow). **B:** Sagittally cut surface of a pedicle at a stage similar to 1A. Note that at this stage, distal region of a pedicle becomes coarser and more vascularized, which clearly delineates the living pedicle (Pe) from dead hard antler (An). Distal pedicle skin (PS) is fused with the underlying bone without a visible intervening layer of subcutaneous loose connective tissue. **C:** Pedicle with a fresh casting surface. Notice that a rim (arrow) formed by the distal end hairless skin of the pedicle surrounds a depressed central bony portion. Some blood was retained in the depressed central portion (B). The Chinese call this stage "the stage of oil bowl." **D:** Sagittally cut surface of a pedicle stump at a stage similar to 1C. Distal end of pedicle skin (PS) has not migrated significantly toward the center and the lost blood rests on the rough casting surface (CS) of the depressed central portion. **E:** Another pedicle with a fresh casting surface. Notice that in this case, at the time of hard antler casting, the distal end of pedicle skin (arrow) has grown in and covered nearly half of the casting surface. There is no obvious blood loss in the depressed central portion (CS). **F:** Sagittally cut surface of another pedicle stump at a stage similar to 1E. Distal pedicle skin (PS) has migrated toward the center substantially, and a layer of fresh tissue (FT) has formed on the central bony surface. **G:** Apical surface of a pedicle a few days after hard antler casting. The blood in the central portion has formed a scab (S). This scab is surrounded by a ring of shiny skin (arrow), which resembles an eye. The Chinese call this stage "the stage of tiger eye." **H:** Sagittally cut surface of a pedicle stump at a wound healing stage similar to 1G. Compared to **F**, distal pedicle skin (PS) in this case has migrated further centrally above the fresh tissue layer (FT) and under the scab. **I:** Apical view of a pedicle with more advanced healing stage. At this stage, the scab (S) becomes much smaller compared to 1G due to the central migration of the healing skin. The whole apical view looks like a millstone with the axle, the scab, located in the center. The Chinese call this stage "the stage of millstone." **J:** Sagittally cut surface of a pedicle stump at a regenerating stage similar to 1I. Notice that the scab (arrow) becomes very small, and two crescent-shaped bulges can be visibly identified at anterior (a) and posterior (p) corners. No further thickening of the fresh tissue layer (FT) is detected compared to the *tiger eye* stage. **K:** An incipient antler at the late *millstone* stage. Notice that the scab (S; lost during sampling) becomes even smaller. **L:** Sagittally cut surface of an incipient antler at a regenerating stage similar to 1K. At this stage, wound healing is nearly completed (diagonal arrow) and the anterior (a) and posterior (p) bulges become clearer. Notice that at the distal end (arrowheads), pedicle skin is closely associated with the underlying pedicle bone; whereas at the proximal end (horizontal arrows), pedicle skin is only loosely attached to the bone and can be easily separated. **M:** Another incipient antler at the mid *millstone* stage (based on the scab size). However, the apical surface of the antler is no longer flat. Anterior and posterior portions protrude and leave the central scab (S) portion behind. **N:** Sagittally cut surface of the incipient antler shown in **M**. In this case, although only at the mid *millstone* stage based on the size of the scab (arrow), it becomes clear that anterior and posterior bulges are the growth centers for brow tine (bg) and main beam (mg), respectively. Notice that the fresh tissue layer (FT) is left behind due to the differential growth rate. Therefore, there is an overlap between pedicle wound healing and early antler regeneration. **O:** An incipient growing antler with similar length of the main beam (1) and the brow tine (2), which resembles a saddle-shaped structure. The Chinese call this stage "the stage of small saddle." **P:** Another incipient growing antler with three similar length branches: main beam (1), brow tine (2), and bez tine (arrowheads). This shape resembles a type of ancient Chinese money. We term it "stage of silver ingot."

TABLE 1. Morphological description of early antler regeneration on naturally casting pedicle stumps

Stage	Chinese terms	Morphological	Macroscopic
Precasting	—	Distal end of pedicle skin became swollen and acquired shinny surface.	Color and density delineate pedicle (pink-red) from antler (white). Distal pedicle skin fused with the underlying pedicle periosteum
Casting	Oil lamp bowl	A rim of hairless skin surrounded a depressed central bony portion, which contained lost blood.	For the early casting antlers, skin migration was not obvious and fresh blood directly rested on the rough surface of pedicle trabecular bone. Whereas for the late casting antlers, the margin of the casting surface had healed due to the migration of distal pedicle skin and a layer of fresh tissue formed on the exiting pedicle trabecular bone
Early healing	Tiger eye	A scab located in the center and was surrounded by a ring of shinny skin.	Distal pedicle skin migrated further centrally above the fresh tissue layer and under the scab. The layer of fresh tissue was further thickened
Late healing and early regeneration	Millstone	Diameter of the scab became smaller than the width of the skin ring, that resembles a millstone with the axle, the scab, in the center.	On a sagittally cut plane, two growth centers could be identified at the posterior and anterior sites. Once the two ends of healing skin met in the center of the healing top, the anterior and posterior growth centers could be readily discerned.
Formation of main beam and brow tine	Small saddle/ silver ingot	Main beam and brow tine possessed similar length at this stage, which resembles a saddle. For an antler that possesses bez tine, looks like ancient Chinese money, ingot.	The posterior and anterior growth centers were further developed into main beam and brow tine, the central portion was left behind.

detected at the fifth set of antlers when compared to the fourth set. Therefore, the pedicle stumps created by cutting through the antler/pedicle junction seem to have maintained their original antler regeneration potential, at least within a year, whereas regeneration potential on the antler remnants was gradually impaired (Fig. 4).

There was no significant difference ($P = 0.07$) in antler length at initial removal (day 0) between right and left antlers. Thereafter, a highly significant difference ($P < 0.01$) in length was detected between the left and right of each corresponding set of antlers (Figs. 3C,D, 4).

The results from this experiment clearly show that, although antler regeneration from both the natural casting plane and the antler remnant took place in a similar way morphologically, the former had much greater antler regeneration potential than the latter. It is reasonable to believe that after transformation from pedicle to antler, the regeneration potential of the antlerogenic tissue has been reduced significantly.

Through different levels of pedicle. Four out of four pedicle stumps created by cutting through the "potentiated region" (Group I) fully healed (Fig. 3E) and regenerated antlers with brow tines. It took about 20 days to complete the wound-healing process, which was similar to that of naturally formed pedicle stumps. In contrast, four out of four pedicle stumps created by cutting though the "dormant region" (Group II) only partially healed at the anterior and posterior sides, and formed a main beam and abortive brow tine (Fig. 3F). It took around 35 days to form these structures. In contrast to the regeneration from the antler remnants, where antler could regenerate anywhere around the distal margin of the artificially created antler stumps; partial antler regeneration from the pedicle stumps created by cutting through the "dormant region" always took place anteriorly and posteriorly. Antler regeneration potential varied along a pedicle shaft with the strongest residing in the "potentiated region" and weaker in the "dormant region."

Overall, these experiments convincingly demonstrated that antler regeneration from the stumps created artificially at the different levels along the pedicle and antler axis took place in the similar way to that of a naturally created pedicle. Together, these results support the notion that the central bony portion of a pedicle/antler stump is not essential for antler regeneration.

DISCUSSION

Annual renewal of deer antlers is the only known example of mammalian appendage regeneration. By definition, antler regeneration belongs to the same class known as epimorphic regeneration that occurs in lower vertebrates. As epimorphic regeneration can only be made possible by the initial formation of a blastema, antler regeneration has been thought to take place through a blastema stage (Goss, 1983, 1995; Allen et al., 2002; Li and Suttie, 2003). Our morphological observations in this study, however, do not seem to support this claim.

First, if blastema formation were involved in antler regeneration, a large cone-shaped regenerate,

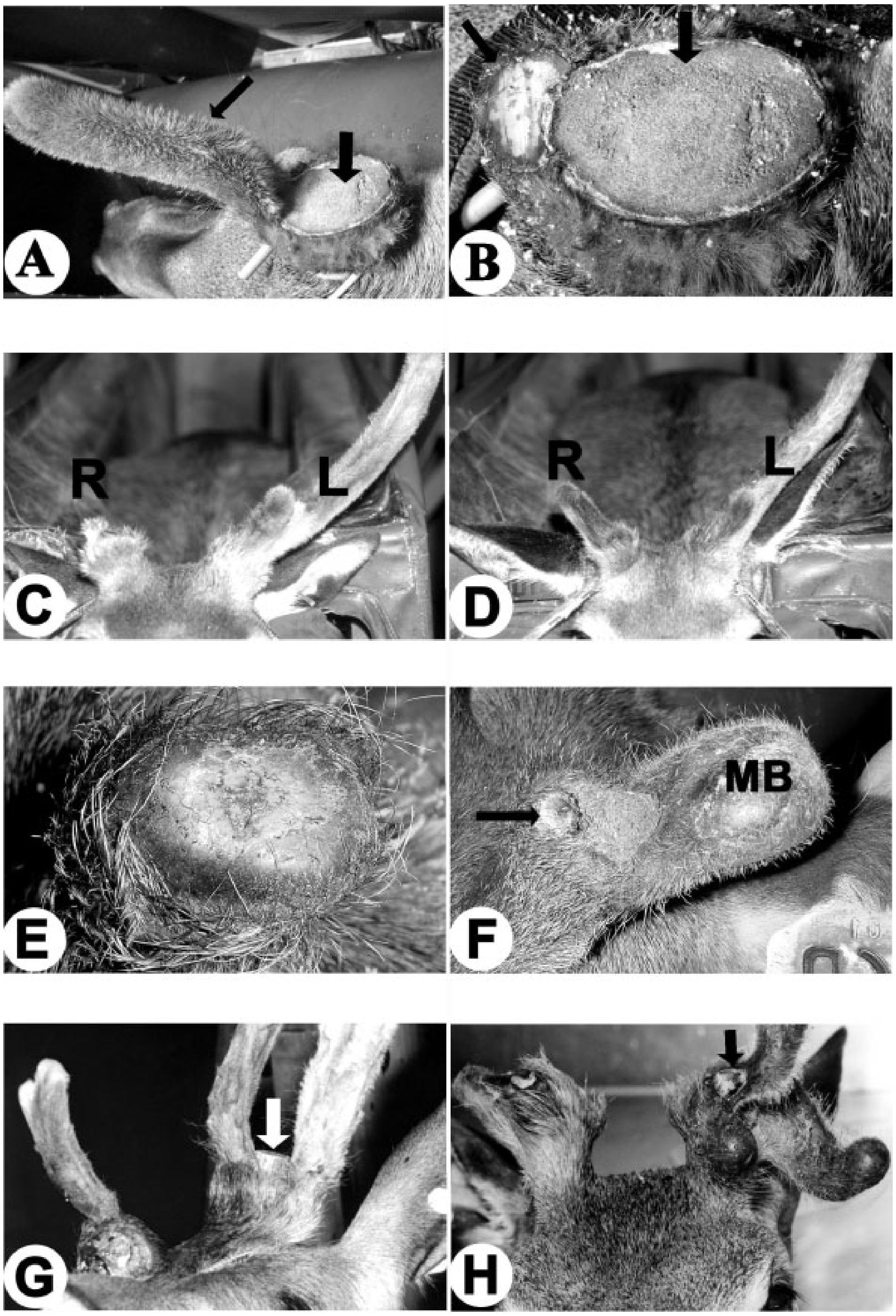

Fig. 3. Antler regeneration of red deer *(Cervus elaphus)* from artificially created stumps along the axis of a pedicle and an antler. **A:** Partial antler regeneration (thin arrow) from an antler remnant (thick arrow), which was created after removal of the 55-day antler at the level 2 cm above antler/pedicle junction. In this case, only a small spike antler regenerated from the anterior margin of the antler remnant. **B:** Top view after removal of the regenerated spike antler in **A**. Notice that the regrowth antler (thin arrow) is only formed from the periphery tissue (may include periosteum and skin) of antler remnant (thick arrow). **C,D:** Antler regeneration from either antler remnants created by cutting through the level 2 cm above antler/pedicle junction (right side) or antler casting plane (left side) from three female red deer (refer to the text for the creation of these perennial antlers in female deer). Notice that the significant difference in antler size was detected between left and right sides. **E:** Pedicle stump created by cutting through the distal pedicle region (0.5 cm below the antler/pedicle junction), which is termed "potentiated region." Notice that the cutting surface is fully healed. **F:** Partial regeneration from a pedicle stump created by cutting through the proximal pedicle region (2.5 cm below the antler/pedicle junction), which is termed "dormant region." Notice that this regeneration only formed a main beam (MB) and abortive brow tine (arrow). **G,H:** Double-head formation. Notice that the previous hard antler buttons (arrows) in these cases were not cast when the new antler regeneration took place. These new regenerates could only form from peripheral pedicle tissue in this case.

the hallmark of blastema formation (Wallace, 1981), would have been observed due to massive dedifferentiation and subsequent extensive proliferation from the dedifferentiated cells. To rebuild a large-size appendage, like deer antler, arguably the biggest regenerative animal organ in nature, a rather big blastema would have been crucial. However, our morphological observations showed that tissue mass during antler regeneration is never built up in the central region of an antler casting surface to form such a structure. The top surface of an early regenerating antler bud was flat all the way to the stage when main beam and brow tine become visible. The distinctive patterns formed during antler regeneration (*oil lamp bowl, tiger eye, millstone,* and *small saddle*) are not compatible morphologically with a blastema formation.

In addition, a scar remains after the scab has flaked off until velvet skin shedding (pers. obs.). Therefore, wound healing during antler regenera-

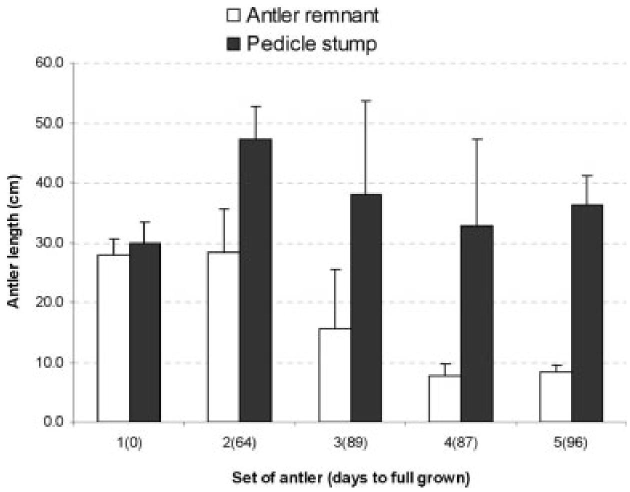

Fig. 4. Effects of antler removal level on subsequent antler regeneration.

tion produces a scar, which is also not compatible with blastema-based regeneration.

Second, if antler regeneration had gone through an initial blastema formation, antler main beam and brow tine would have been formed through the redifferentiation of blastema cells. However, our results showed that the two growth centers for the main beam and brow tine are already established even before the completion of wound healing on a pedicle stump. It is highly unlikely that both dedifferentiation and redifferentiation processes can be achieved before the completion of wound healing.

Third, if a blastema were formed at early antler regeneration stage, all tissue lineages on the immediate antler casting or cutting plane would have contributed to its formation through dedifferentiation and proliferation. However, our present results together with our immunohistochemical studies (to be published separately) showed that antler growth centers were derived from the proliferation and differentiation of distal periosteal cells of a pedicle or an antler stump. In their histological study on early antler regeneration, Kierdorf et al. (2003) also suggested that pedicle periosteum provides a cell source for regenerating antlers. Because formation of cartilage and bone from periosteal cells is the classical differentiation pathway, the two growth centers are not formed through the processes of dedifferentiation and subsequent redifferentiation, as is the case in the blastema-based regeneration, which occurs in lower vertebrates.

A natural phenomenon, so-called double-head (Fig. 3G,H) formation, further supports the claim that antler or pedicle periosteum and the enveloping skin only can give rise to antlers without participation of the entire central bony portion of a pedicle. Double-head formation is caused by the regeneration of a subsequent antler without casting of the previous hard antler (Kierdorf, 1992). In this particular case, there is no casting surface onto which the different types of cells in the distal end of the pedicle can migrate, proliferate, and dedifferentiate. Therefore, the whole bony portion on the distal plane cannot participate in new antler regeneration. Consequently, a regenerating antler can only be derived from the distal ends of the pedicle periosteum and the enveloping skin. Based on the phenomenon of double-head antler formation, Kierdorf et al. (1994) argued whether antler regeneration can be regarded as a true epimorphic response, including generation of a blastema as well as cartilage and bone formation by cells of other than periosteal origin, or would be the result of exaggerated tissue repair. This clearly indicates that these authors also questioned whether there exists a stage of blastema formation during antler regeneration, although we believe antler regeneration presents a true epimorphic response, not an exaggerated tissue repair, because true epimorphic regeneration (see the definition in the Introduction) does not necessarily have to go through a blastema stage.

The phenomenon that regeneration potential varies along a pedicle shaft with the maximum potential residing in the distal part as noted in this study is also supported by the report (Goss, 1961) that a pedicle stump, which was generated by sawing through the distal part of the pedicle, formed an antler identical to the control side both in size and shape. However, when a pedicle was removed from the proximal level, the regeneration from the resultant stump was not only significantly delayed, but also only achieved a length equal to half that of the control. However, the phenomenon has never been explained. From the present observation, we found that this differential regeneration potential may not be a constitutive one, but rather caused by whether or not pedicle antlerogenic tissue becomes closely associated with the enveloping skin at the cut plane, because close association between antlerogenic tissue and the enveloping skin is the prerequisite for both antler generation and regeneration (Goss, 1990; Li and Suttie, 2000; Li et al., 2001). It is known that pedicles of a deer shorten year after year and eventually disappear, leaving antlers directly forming from deer heads. However, at the time when a pedicle shortens to the dormant region, the distal pedicle skin has become intimately associated with the pedicle bone (pers. obs.). That means that the residual tissues on the distal end of a pedicle always become potentiated prior to antler regeneration.

Overall, our morphological observations do not support the claim that antler regeneration takes place through initial blastema formation. These and our immunohistochemistry results (to be published separately) provide convincing evidence for the notion that antler regeneration is through a stem cell (distal pedicle periosteal cells)-based process. Further, pedicle antlerogenic tissue holds much greater regeneration potential than its antler counterpart.

However, the differential regeneration potential along a pedicle shaft may not be constitutive, but may be caused by whether or not pedicle antlerogenic tissue becomes closely associated with the enveloping skin at the cut plane.

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