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Fracture repair of reptilian dermal bones: can reptiles form secondary cartilage?*

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INTRODUCTION

The process of repair of fractured dermal bones of birds and mammals is well documented (Hall & Jacobsen, 1965; Girgis & Pritchard, 1958; Pritchard, 1946). Conversely, repair of fractured reptilian dermal bones has rarely been studied: the only published work is a short abstract (Nissenbaum, 1971). A detailed account is thus required. Fractured dermal bones of both birds and mammals repair by depositing a callus containing secondary cartilage. However, fractured amphibian dermal bones do not form secondary cartilage (Goss & Stagg, 1958; Goss, 1983; Hall & Hanken, 1985). A study of the repair of fractured reptilian dermal bones would provide an insight into the evolution of this repair process and the phylogenetic stage at which vertebrates first acquired the ability to form secondary cartilage as part of their fracture callus.

Three species of lizard and one snake species were used in the present study. Experimental incisions were made in the dermal parietal bone so as to create a fracture site of limited vascularity and with an increased potential for movement – both environmental conditions are known to induce secondary chondrogenesis in birds (Hall & Jacobsen, 1975) and mammals (Girgis & Pritchard, 1958). The stages of fracture repair from 0 to 26 days were studied concurrent with a search for secondary cartilage.

MATERIALS AND METHODS

A total of twenty lizards (including different species) and two garter snakes was used (Table 1). These were obtained in good condition (age unknown) from a commercial supplier (Dowds), housed in a vivarium containing heat lamps, stones, wood, sand and water *ad libitum* and fed a diet of earthworms, grubs and crickets twice daily. In each experimental animal a longitudinal fracture, approximately 0·5–1 cm in length was surgically created involving the frontal and parietal skull bones (both of dermal origin; Figs. 1, 2). Additional fractures, both parallel and perpendicular to the initial fracture site, were made to decrease the blood supply, and to increase the potential movement, of the bones involved (Table 1). The fractures were made under ether anaesthesia using a surgical scalpel which minimised the size of the fracture gap to between 0·5 mm and 1 mm. Manual pressure and a cutting motion were carefully applied to the scalpel until a spurt of blood appeared in the wound

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Table 1. *The species of lizard and snake used, the fracture type and the time interval between fracture and sampling of the tissue histologically*

Specimen number	Species name	Fracture type	Length (cm)	Day killed (after fracture)
Lizard 1	*Scincidae scincomorphus*	S	19·6	0
Lizard 2	*Scincidae scincomorphus*	S	18·2	2
Lizard 3	*Scincidae scincomorphus*	P	19·9	6
Lizard 4	*Scincidae scincomorphus*	P	22·7	9
Lizard 5	*Lacerta muralis*	T	15·8	10
Lizard 6	*Lacerta muralis*	T	18·8	11
Lizard 7	*Scincidae scincomorphus*	S	22·1	12
Lizard 8	*Lacerta muralis*	T	16·1	12
Lizard 9	*Lacerta muralis*	T	19·5	14
Lizard 10	*Scincidae scincomorphus*	S	19·0	15
Lizard 11	*Lacerta muralis*	T	17·9	16
Lizard 12	*Scincidae scincomorphus*	S	19·7	18
Lizard 13	*Lacerta muralis*	T	17·8	18
Lizard 14	*Lacerta muralis*	T	17·1	20
Lizard 15	*Lacerta muralis*	T	19·1	22
Lizard 16	*Agamidae*	P	21·8	24
Lizard 17	*Scincidae scincomorphus*	P	22·9	26
Snake 1	*Thamnophis radix*	P	49·6	15
Snake 2	*Thamnophis radix*	P	52·5	20
Lizard 18	*Scincidae scincomorphus*	—	18·7	2*
Lizard 19	*Scincidae scincomorphus*	—†	19·3	10*
Lizard 20	*Scincidae scincomorphus*	—	20·1	20*

* 'equivalent'; † Controls; S, single incision; P, parallel incisions; T, T-shaped incision.

showing that the blade had passed through the pericranium, the skull bone and the underlying dura mater. After operation, animals were returned to their cages, and continued on their normal diet. None showed any adverse reactions to the operation. Three control lizards were kept under identical conditions but were not operated upon (Table 1).

Animals were killed using chloroform vapour at varying intervals between 0 and 26 days (Table 1). They were measured, decapitated and the heads decalcified in 5% trichloroacetic acid for a period ranging from 5 to 10 days (end point determined radiographically). Specimens were washed, softened in 5% sodium hydroxide, dehydrated in ascending concentrations of alcohols, cleared in 6% celloidin in methyl benzoate, followed by ligroin (Ferguson, 1981) and embedded in Railwax, melting point 56·8 °C (Raymond A. Lamb, London). Serial sections 10 μm thick were cut in the coronal plane and alternate slides were stained with: Harris' iron haematoxylin, eosin and Alcian blue, Weigert's iron haematoxylin, Alcian blue and Van Gieson's stain (Una's variation), Weigert's iron haematoxylin and Mallory's trichrome stain.

RESULTS

These are summarised in Table 2. The principal variable affecting the chronology of the repair process was the width of the fracture (compare Figs. 6–9, 10–14, 17 and 18, 19 and 20). This in turn was related not only to the width of the initial incision but also to the amount of bone death and sequestration – itself dependent upon the degree of avascularity of the fracture site and hence the type of operation. Incisions

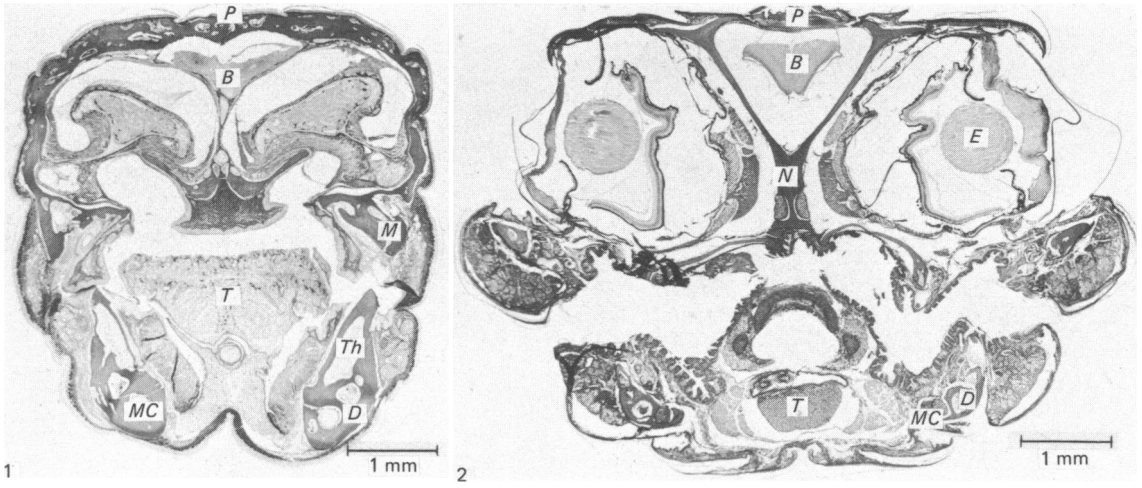

Fig. 1. Coronal section through the anterior part of the skull of the lizard *Lacerta muralis.*

Fig. 2. Coronal section through the middle part of the skull of the snake *Thamnophis radix.* ***B***, brain; ***D***, dentary; ***E***, eye; ***M***, maxilla; ***MC***, Meckel's cartilage; ***N***, nasal septum; ***P***, parietal bone and site of fracture; ***T***, tongue; ***Th***, tooth.

made parallel to the fracture site resulted in a greatly reduced blood supply, large areas of bone death, huge bony sequestra and hence wide fracture gaps (Figs. 4, 5, 7, 13, 18, 19). Incisions made at right angles to the ends of the fracture site only slightly reduced the vascularity and did not cause such large bony sequestra. Wide fracture gaps repaired more slowly than the time scale outlined in Table 2 and may only have achieved fibrous union by 26 days post fracture (Fig. 14). It seems likely that such fibrous unions eventually ossify (Fig. 14). The effect of fracture gap size on the repair process was seen most dramatically when the width of the fracture varied in the same specimen (Figs. 17 and 18, 19 and 20). Thus, the *Lacerta muralis* examined after 18 days had a narrow fracture gap anteriorly, which had achieved bony union; but a wide fracture gap posteriorly where collagen fibres were sparse and osteogenesis was restricted to the fractured bony ends.

Osteogenesis was enhanced if vital bony spicules were present within the fracture callus (Figs. 6, 16), and conversely was retarded by large bony sequestra. Occasionally, bony spicules had been pushed into the underlying brain where they were walled off by fibrous tissue. There were no differences in the sequences of fracture repair between the different species of lizard examined in this study. If similar fracture types (i.e. narrow or wide) were compared then there were also few differences in the chronology of fracture repair between the different lizard species. For example, the nature of fracture repair at day 12 was almost identical in both the *Scincidae scincomorphus* and the *Lacerta muralis* specimens.

If anything, the *Lacerta muralis* callus tended to be better vascularised than the *Scincidae scincomorphus* at all stages and so healing was marginally more rapid. The *Agamidae* butterfly lizard normally has very vascular diploe (Fig. 15). These resulted in a rapidly developing, highly vascularised callus (Fig. 16) so that fracture repair occurred more quickly in this species than in either *Lacerta* or *Scincidae*. The unoperated control animals exhibited no changes in skull histology during the experimental period (Figs. 1, 2). The effect of age on fracture repair could not be ascertained as the lizards, although of similar lengths (Table 1), were of unknown age.

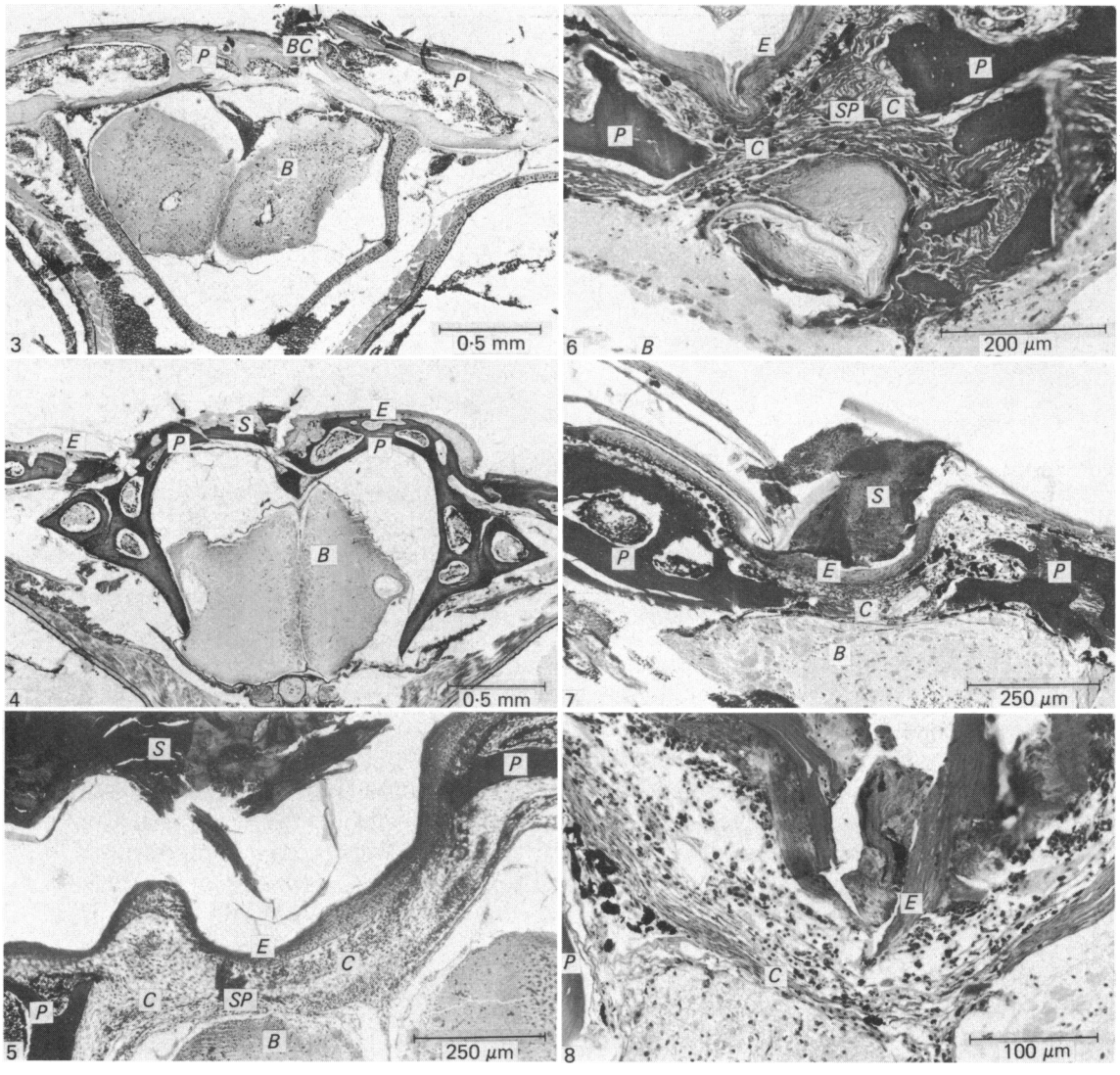

Fig. 3. Fracture site at Day 0 in *Scincidae*. Note the haemorrhage and the brain case.

Fig. 4. Fracture site at Day 2 in *Scincidae*. An incision has been made parallel to the fracture site (arrows). Note the downgrowth of the epithelium (*E*) at the fracture edge beneath the sequestrating bone (*S*).

Fig. 5. Fracture site at Day 6 in *Scincidae*. Note the sequestrating bone (*S*), re-epithelialisation (*E*), bony spicule (*SP*) and collagen fibres (*C*) particularly on the dorsal aspect of the callus.

Fig. 6. Fracture site at Day 10 in *Lacerta*. The fracture gap is small and filled with dense collagen fibres (*C*).

Fig. 7. Fracture site at Day 11 in *Lacerta*. The fracture gap is wide, there is a large bony sequestrum and osteoblasts are present adjacent to the bony ends.

Fig. 8. Higher magnification of an even wider fracture site at Day 11 in *Lacerta*. The callus consists of loose connective tissue with collagen fibres concentrated on the dural aspect. Compare with Figure 7. *B*, brain; *BC*, blood clot; *C*, collagen fibres; *E*, epithelium; *P*, parietal bone; *S*, sequestrating bone; *SP*, bony spicule; arrows indicate areas of fracture incisions. All specimens sectioned in the coronal plane.

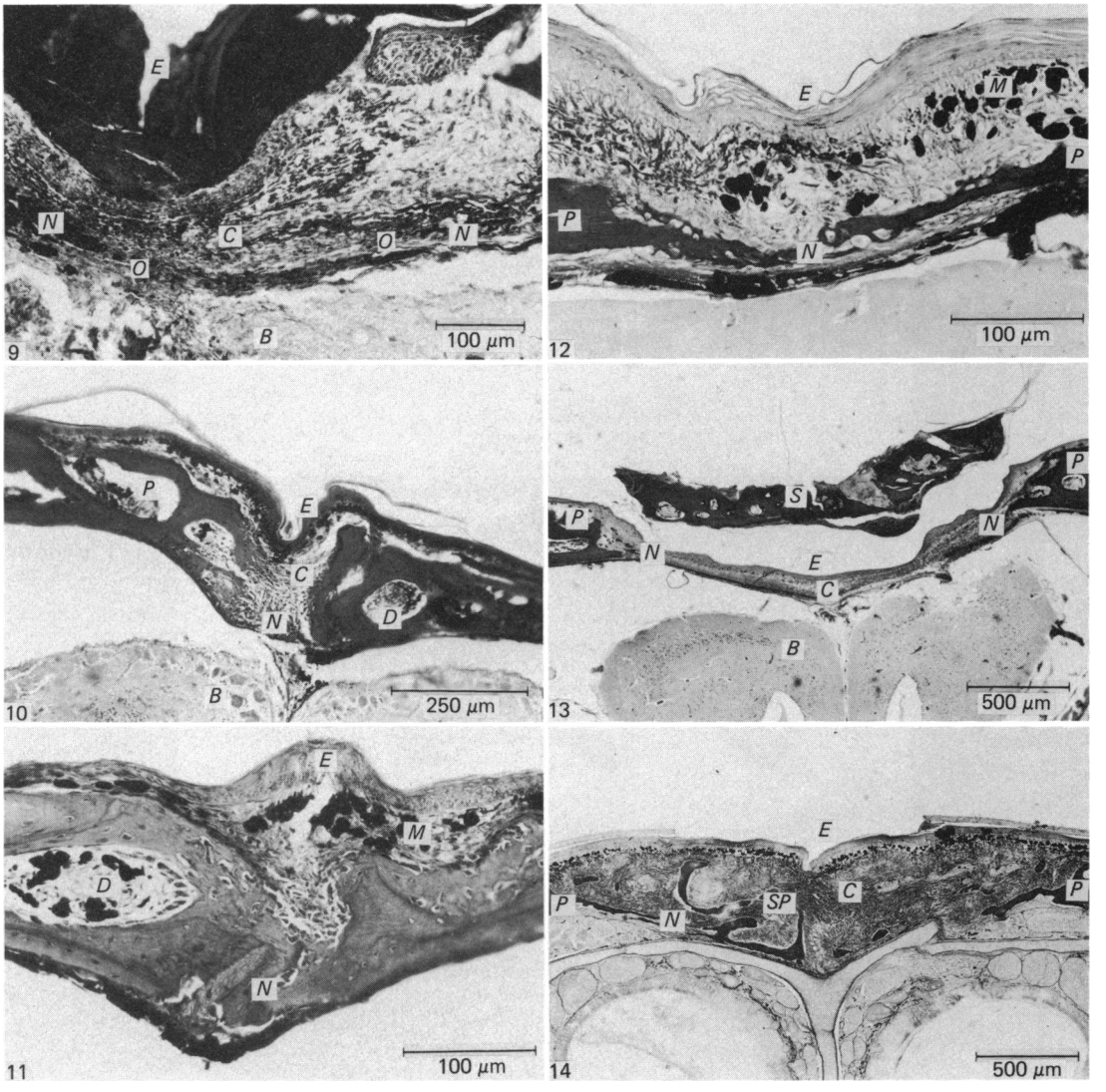

Fig. 9. Fracture at Day 12 in *Scincidae*. Note the collagen fibres (*C*) particularly on the dural aspect of the callus, and the osteoblasts (*O*).

Fig. 10. Fracture site at Day 14 in *Lacerta*. The fracture gap is small and almost bridged by new bone (*N*). The callus is fibrous and vascular.

Fig. 11. Fracture at Day 16 in *Lacerta*. Note that the small fracture gap has been bridged by new bone on the dural aspect.

Fig. 12. Fracture site at Day 18 in *Lacerta*. Note the bony union by a single bony lamella.

Fig. 13. Fracture site at Day 18 in *Scincidae*. Parallel incisions were made on either side of the fracture, and a large bony sequestrum resulted in a wide fracture gap. The callus is fibrous and has new bone at its ends.

Fig. 14. Fracture site at Day 26 in *Scincidae*. The fracture site was wide due to parallel incisions and bony sequestration. The fracture site is very fibrous with osteogenesis occurring around the ends of the bones and bone spicules. *B*, brain; *C*, collagen fibres; *D*, diploe in skull; *E*, epithelium; *M*, melanin pigment; *N*, new bone formation; *O*, osteoblast; *P*, parietal bone; *S*, sequestrating bone; *SP*, bony spicule. All specimens sectioned in the coronal plane.

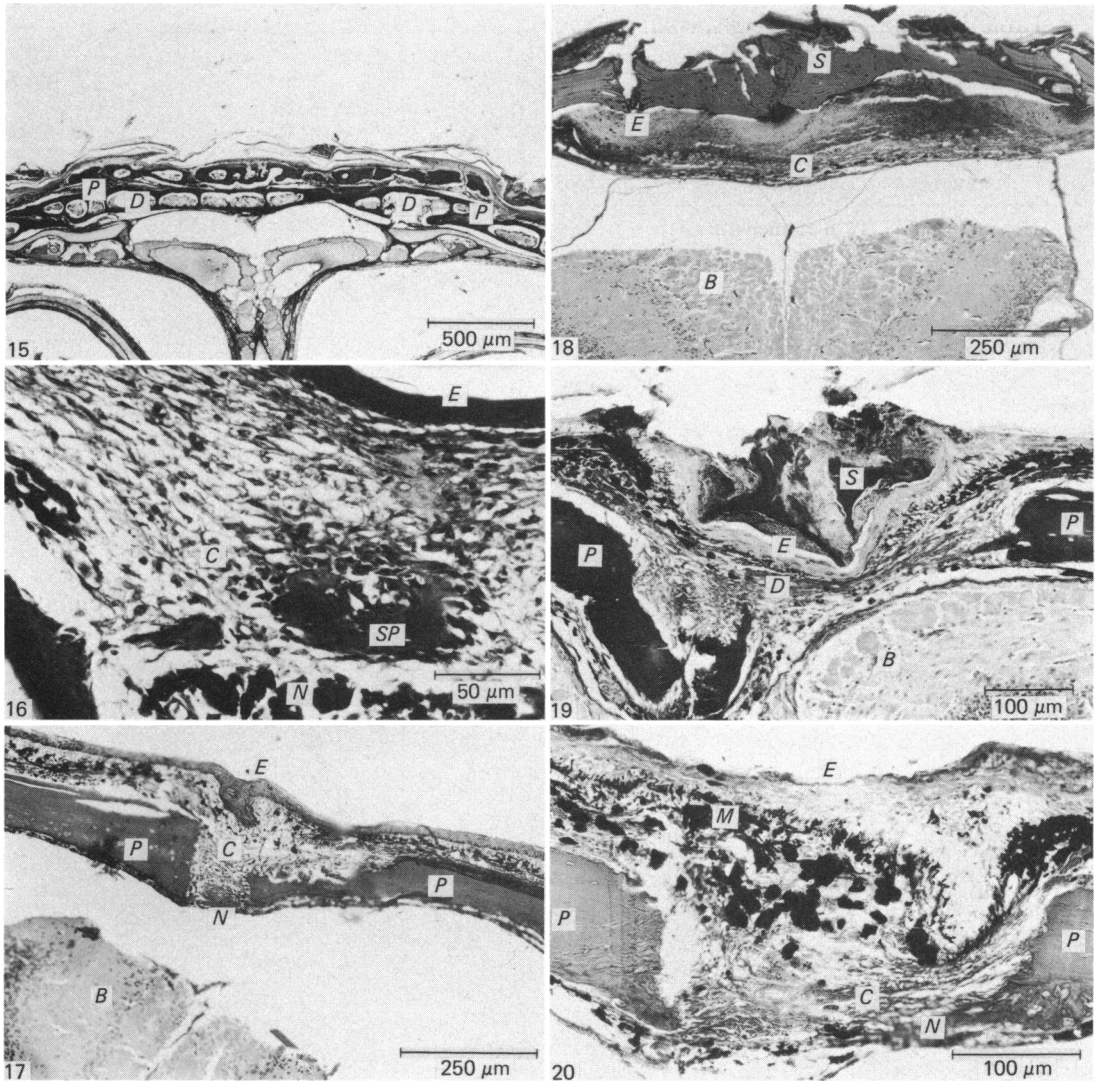

Fig. 15. Coronal section through the parietal bones of the *Agamidae* lizard illustrating the vascular diploe.

Fig. 16. Fracture site at Day 24 in *Agamidae*. The callus is collagenous with new bone on the dural aspect.

Fig. 17. Fracture site at Day 15 in the snake *Thamnophis radix*. Note the small fracture gap, the fibrous callus with some new bone on the dural aspect.

Fig. 18. Posterior fracture site at Day 15 in *Thamnophis*. Note the large bony sequestra (due to incisions parallel to the fracture), incomplete re-epithelialisation and fibrous callus. Compare with Figure 17.

Fig. 19. Posterior fracture site at Day 20 in *Thamnophis*. Note the bony sequestra, re-epithelialisation and collagen fibres in the callus.

Fig. 20. Anterior narrow fracture site at Day 20 in *Thamnophis*. Note the coarse collagen fibres and new bone formation. *B*, brain; *C*, collagen fibres; *D*, diploe in skull; *E*, epithelium; *M*, melanin pigment; *N*, new bone formation; *P*, parietal bone; *S*, sequestrating bone; *SP*, bony spicule. All specimens sectioned in the coronal plane.

Table 2. *Summary of the principal features of fracture repair in lizard parietal bones, where the width of the fracture gap was small* (0·5–0·75 *mm*)

Days after fracture	Principal histological features of the fracture site	Presence (+) or absence (−) of secondary cartilage	Figure
0	Torn epithelium Blood clot in fracture gap Red blood cells in diploe Bony spicules in fracture gap Torn, bleeding dura mater	—	3
2	Proliferation of epithelium at the edges of the fracture Proliferation and thickening of periosteum on the dural surface of the fractured bony ends Blood clot, macrophages and loose connective tissue in the fracture gap Poor vascularisation of the fracture site	—	4
6	Re-epithelialisation complete Epithelium which has grown beneath bony sequestra is poorly differentiated and not keratinised Melanin pigment present in basal epithelial layers and connective tissue Callus compact, poorly vascularised, no blood clot remaining, a little collagen present	—	5
9–42	Epithelia beneath bony sequestra keratinised Dense collagenous connective tissue abundant in callus Coarse collagen fibres more numerous on the dural aspect of the callus Osteoclasts prominent at the ends of the fractured bones Osteoblasts discernible particularly in callus adjacent to the fractured bony ends	—	6–9
14–15	New bone formation particularly at the ends of the fractured bones Osteoblasts lining up across the fracture gap Callus more vascular	—	10 17 18
16	Single lamella of new bone across the fracture gap, usually on the dural aspect Thin collagen fibres surround the bony lamella	—	11
18–26	Bony union	—	12–14 16 19, 20

Fracture repair in the two snakes (*Thamnophis radix*) studied followed the same sequence as in Table 2 but took approximately 5 days longer at each stage (Figs. 17–20).

Full descriptions of the appearance of the fracture callus in each individual animal can be found in Irwin (1984).

REVIEW AND DISCUSSION

Secondary cartilage

Bones have long been classified as being either endochondral, formed by replacing a cartilage model, or intramembranous, their origin being in a mesenchymal condensation without any preceding cartilage model. Nevertheless, certain bones which

are membranous in the phyletic sense develop by the replacement of preformed cartilage, as do endochondral bones. This cartilage is termed secondary (or adventitious) as it appears after, and has no obvious connection with, the primary cartilaginous skeleton (Schaffer, 1930). Beresford (1981) defined secondary cartilages as those "formed by the periosteum of an existing bone with the implication, or explicit stipulation that the bone is of dermal origin". Histologically, secondary cartilage differs from primary cartilage in having very little extracellular matrix and a pericellular pattern of mineralisation, whilst DNA synthesis and cell division continue after the secondary chondrocytes are embedded in the extracellular matrix, so that growth is both appositional and interstitial (Hall, 1968, 1984; Durkin, 1972). Moreover, secondary cartilage differentiates only in response to mechanical stimuli: chorio-allantoic membrane transplants (Murray & Smiles, 1965), organ culture (Hall, 1967, 1968), detachment of local muscles (Hall & Jacobsen, 1975), or neuromuscular blocking agents (Murray & Smiles, 1965; Hall, 1979) all cause paralysis of developing bony sites and all totally inhibit secondary chondrogenesis. Apart from mechanical stimuli, other factors which enhance secondary chondrogenesis, especially during fracture repair, include a poor blood supply (Ham, 1930; Girgis & Pritchard, 1958; Richman & Laskin, 1964; Hall & Jacobsen, 1975), a slow healing rate, the presence of foreign bodies and wound infection (Richmond & Laskin, 1964). These and the mechanical factor itself may act through a common, as yet unknown, agency which is the direct evocator of chondrogenesis.

Secondary cartilage in birds and mammals forms at the sutures and articulations of many dermal bones, particularly during their development, and in the fracture repair of such bones (Murray, 1963; Hall, 1970, 1984). It does not form in any of these locations in fish (Moss, 1961, 1962; Goss, 1969; Murray, 1963; Beresford, 1981; Huysseune, Ismail & Verraes, 1981; Ismail, Verraes & Huysseune, 1982) or amphibians (Goss & Stagg, 1958; Goss, 1983; Hall & Hanken, 1985). No unequivocal evidence has been presented for the existence of secondary cartilage in adult or embryonic reptilian skulls (Hall, 1984), but data are sparse. Likewise, Nissenbaum's (1971) abstract on repair of fractured reptilian dermal bones makes no mention of secondary cartilage formation. Patterson (1977) contended that secondary cartilage was confined to endothermic tetrapods (birds and mammals) and called for studies of fracture repair in amphibian and reptilian dermal bones to test his contention. Advantages said to accrue from the ability to form secondary cartilage include: formation of shock absorbing articulations, reduction of damage to periostea at points of attachment of muscles and ligaments, quick immobilisation of fractures, and developmental plasticity (Hall & Hanken, 1985); why these features should not be equally advantageous in amphibians and reptiles is unclear!

Another important reason for looking for secondary cartilage in reptiles relates to the evolution of periosteal cells, an important event underlying tissue and morphological change during development and evolution (Hall, 1984). The periosteal cells which form secondary cartilage on avian and mammalian dermal bones can also differentiate into osteoblasts and deposit bone should the stimulus required for secondary chondrogenesis be removed (Hall, 1967, 1968, 1979; Thorogood, 1979). If reptiles lack secondary cartilage either (i) cells capable of chondrogenesis are absent in the periostea of dermal bones or (ii) such cells are present but the environment is inappropriate for the expression of secondary chondrogenesis. These alternatives can be distinguished by experiments which create an environment conducive to secondary chondrogenesis, for example, a fracture site with a poor blood supply but

experiencing mechanical movement as in the present study. The results are important as they should indicate whether the evolution of secondary cartilage in birds and mammals has involved genetic changes in the periosteal cell population *per se* (i.e. the expression of cartilage-specific molecules, for example, Type II collagen, etc.) or in the environment of the cells. Moreover, the presence or absence of secondary chondrogenesis in modern reptiles should indicate whether this process arose in the common reptilian ancestors of birds and mammals or later, independently, in the two groups.

Dermal fracture repair

Pritchard (1946) experimentally fractured the parietal bones of rats and documented a fibrous union in nearly all cases, but some new bone formation occurred especially if the fracture gap was small or if bony fragments, functioning as grafts, remained at the fracture sites. The incidence of secondary cartilage formation was increased (maximal 7–12 days after fracture) if the blood supply to the fracture site was decreased by incisions made either parallel or at right angles to the latter (Girgis & Pritchard, 1958). Similar results were obtained in fractures of the dog zygomaticomaxillary complex (Richman & Laskin, 1964). In chickens, fracture repair of dermal bones involved fibrous union, osteogenesis and secondary chondrogenesis particularly when the fracture gap was small (Hall & Jacobsen, 1975). Similar events occur in amphibians but secondary cartilage does not form in the fracture callus (Hall & Hankin, 1985).

Present study

The results obtained in this study show many similarities to those obtained by Pritchard (1946), who examined the repair of a single, experimentally produced, fracture in the dermal bones of the rat's skull. Surprisingly, the chronology of dermal fracture repair in reptiles is also similar to that previously reported for rats (Pritchard, 1946) and chickens (Hall & Jacobsen, 1975), providing similar sized fracture gaps are compared. A slower rate of repair might have been expected in view of the ectothermic constitution of reptiles. Fracture repair in the lizard and snake dermal bones occurred much faster than in the fractured lower jaws of amphibians (*Ambystoma maculatum*) where the fracture sites were wider and more mobile (Hall & Hanken, 1985).

Summarised, the major events in reptilian dermal fracture repair are rapid re-epithelialisation, sequestration of dead bony fragments, proliferation of the dural periosteum, organisation and fibrosis of the callus, osteoblast differentiation, osteogenesis and bony union (Table 2). The one striking feature of this study was that regardless of the width of the fracture gap, the degree of vascularity, the mobility of the fracture site, the stage of repair or the species of reptile, no specimen showed any sign of secondary cartilage in the fracture callus.

Thus, even when a permissive environment of avascularity and increased potential for movement is experimentally created, the progenitor cells of reptilian dermal periostea fail to initiate secondary chondrogenesis. Two possible reasons are advanced to explain why amphibia, and now also reptilia, cannot form secondary cartilage whereas birds and mammals can (Hall, 1984; Hall & Hanken, 1985). By analogy with embryonic induction, the choice is between evolution of the inducer (the environment) and evolution of the responding tissue (the progenitor cells). The present experiment set out to create an environment known to induce secondary

chondrogenesis in aves and mammalia, so that the resulting lack of secondary cartilage in reptilia would most likely derive from differences in the progenitor cells rather than from differences in the environment of such cells. This contention is reinforced by preliminary data (M. W. J. Ferguson, unpublished observation) on the ontogeny of the skull of *Alligator mississippiensis* where secondary cartilage was not observed on developing dermal bones. The periosteal cells of dermal bones in birds and mammals can form both secondary cartilage and bone. Either progenitor cells are bipotential, their differentiative fate depending on the environment (Murray, 1963; Hall, 1967, 1968, 1978, 1979; Meikle, 1973; Thorogood, 1979) or there are at least two stem cell populations: one osteogenic and one chondrogenic (Stutzmann & Petrovic, 1975), Clonal cell culture is required to unravel this problem. Reptiles, therefore, could lack secondary cartilage for several reasons: (i) periosteal cells on dermal bones are unipotential, and lack the genetic information to express cartilage-specific molecules (Type II collagen, cartilage type proteoglycans and glycosaminoglycans), (ii) only one stem cell population (for osteogenesis) is present, (iii) cells with the genetic information to produce cartilage are present, but are unable to respond to the appropriate environmental signals, for example, due to lack of appropriate receptor mechanisms, (iv) cells capable of making cartilage are present but the environmental stimuli to evoke chondrogenesis are absent and differ from those in birds and mammals. These alternatives may be distinguished by use of appropriate molecular probes and clonal cell culture. Such studies are important for an understanding of the evolution of the skeletal system, itself the basis of much phylogenetic morphological change.

The absence of secondary cartilage in reptiles, amphibians (Hall & Hanken, 1985) and fish (Beresford, 1981; Huysseune *et al.* 1981) lends support to Patterson's (1977) suggestion that this tissue arose late in vertebrate evolution and is currently limited to endotherms. However, Gardiner's (1982) radical proposal that in their phylogeny birds and mammals are closer to each other then either is to reptiles, based on the distribution of secondary cartilage within these groups, is premature. First, lamentably few species have been studied and second, secondary chondrogenesis in birds and mammals may have arisen independently. Of great interest would be a search for secondary cartilage amongst the mammal-like reptiles and dinosaurs, particularly now that fossilised embryonic, juvenile and fractured material has been discovered. Revealing histological details of bone and cartilage organisation can be obtained from such fossilised specimens (Reid, 1984).

SUMMARY

The fracture repair of reptilian dermal bones has not previously been reported. Moreover, repair of fractured dermal bones in birds and mammals involves secondary chondrogenesis whereas that of amphibians does not. Therefore an investigation into the repair of fractured reptilian dermal bones could reveal the stage during vertebrate evolution at which the process of secondary chondrogenesis appeared. Experimental incisions were made in the parietal bones of seventeen lizards (3 species) and 2 snakes (1 species). These resulted in a fracture environment of limited vascularity and increased movement – two known stimuli of secondary chondrogenesis in birds and mammals. Re-epithelialisation was rapid and dead bony fragments quickly sequestered. The blood blot was quickly organised into connective tissue, the dural periostea proliferated, osteoblasts differentiated and bony union was

effected after 18 days. The width of the fracture gap was the principal variable affecting the chronology of fracture repair. Secondary cartilage was not detected in any specimen, of any species, at any stage of the fracture repair. It therefore appears that the progenitor cells on reptilian dermal bones are not capable of forming secondary cartilage and that this tissue arose comparatively late in vertebrate evolution.

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