An experimental investigation into the early development of the chick elbow joint

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SUMMARY

The theory that differential growth of opposed chondrogenic centres is important in early joint formation has been tested experimentally by removing structures in relation to the chick elbow joint. The humerus and its cap of differentiating joint cells were found to develop independently of structures distal to them. Removal of the presumptive joint region at early stages resulted in fusion of the humerus with the radius and ulna. Results are discussed in terms of concepts concerning pattern formation of cell types in the early wing-bud.

INTRODUCTION

Other than the organ culture experiments of Fell & Canti (1934) most of the investigations into the early development of embryonic joints have been descriptive, using the light and electron microscopes. Excellent light microscope studies of human limb joint development (Gray & Gardner, 1950, 1951; Gardner & Gray, 1953; Andersen, 1961, 1962a, 1962b, 1963; Andersen & BroRasmussen, 1961; Gardner & O’Rahilly, 1968) have given a good understanding of their morphology but not the cellular processes involved in creating these structures.

It is convenient to split up the course of the development of the elbow joint into two phases. Firstly, the differentiation of the cell types begins in the wing-bud and the cartilage condensations appear (stage 25, Hamburger & Hamilton 1951). The presumptive joint becomes increasingly more localized until the shapes of the opposing cartilage elements are clear (stage 36). Secondly, the joint cleft begins to form and the associated synovial structures differentiate. The cellular origins and mechanisms concerned with the second phase have been discussed elsewhere; (Haines, 1947; Lelkes, 1958; Hendrikson & Cohen, 1965; Drachman & Sokoloff, 1966). This study is concerned with the first phase which can be experimentally investigated more readily.

At stage 36 the cartilages of the humerus, radius and ulna are separated by a region of flattened densely staining cells in which the synovial space will develop. An interesting question is whether these cells are differentiating according to instructions given to them when the specification of the pattern of cell types

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occurred in the early limb-bud (Wolpert, Lewis & Summerbell, 1975), or whether they are physically modelled and their differentiation governed by mechanical forces resulting from the growth properties of the cartilage elements around them.

Several authors have stressed that a growth differential occurring between the expanding chondrogenic centres and the presumptive joint region is important in early joint development (Carey, 1922; Whillis, 1940; Andersen, 1961, 1962a, 1962b, 1963; and more recently Hendrikson & Cohen, 1965). It is important to understand exactly the nature of this differential growth and just how this mechanical process is involved in early joint morphogenesis. Fell & Canti (1934) have shown that the form of the developing chick knee joint is determined by stage 25, and they also suggest that a small piece of shaft tissue is necessary to obtain separation of the articular surfaces in culture. Their interpretation of these experiments is that although the joint cells may be behaving according to instructions given to them at early stages, the mechanical stresses brought about by matrix secreted by the cartilage cells within each developing cartilage element play a part in the flattening process of the joint cells at the distal surface of that element. A similar mechanical mechanism has been suggested for the formation of the perichondrium (Gould, Selwood, Day & Wolpert, 1974) which forms as an inevitable result of the secretion of matrix by the developing cartilage cells. This kind of process differs from the idea that differential growth of the opposed cartilage elements causes the flattening of the presumptive joint cells.

The experiments described in this paper are designed to shed light on two main points. If the structures distal to the elbow joint are surgically removed at stage 24 and the humerus and its joint cap develop normally, opposed growth can be excluded as a causal event in joint development. In addition, if the presumptive elbow joint region is surgically removed at stage 24 and fusion of the humerus, radius and ulna occurs it will be clear that a joint will only result if cells programmed to form a joint are present.

METHODS

General: fertilized White Leghorn embryos were incubated at 38 °C and windowed on the third and fourth days of incubation. Stage 24–26 embryos were selected and the elbow joint of the right wing-bud marked, by making a very small cut, using the technique described below.

Experiment 1: the limb was cut squarely across the proximo-distal axis at varying positions relative to the presumptive joint, and the distal piece discarded (Fig. 1A).

Experiment 2: slices, varying in width between 100 μm and 150 μm containing the presumptive elbow joint region were cut out and discarded. The distal piece was transfixed with two platinum pins and pinned back onto the stump (Fig. 1B).
Fig. 1. Diagrammatic representation of the operations performed. (A) Experiment 1. A cut was made across the elbow at stage 25 and the distal piece discarded. (B) A slice about 150 \( \mu \)m wide containing the presumptive joint was removed and discarded at stage 24 and the distal piece pinned back onto the stump. H, developing humerus; R, developing radius; U, developing ulna.

The eggs were then re-sealed and returned to the incubator. Embryos were sacrificed at 10 days (stage 36) because by then the elbow has reached the end of its first phase of development, and the humerus is a miniature version of the adult.

Operated and control (left) wing-buds were excised from the embryos. Those for whole mounts were fixed in 5% trichloracetic acid, stained in 0.1% Alcian green 8GX in 70% acid alcohol, differentiated in acid alcohol, dehydrated and cleared in methyl salicylate. Limbs for sectioning were fixed in half-strength Karnovsky fixative, dehydrated and embedded in Araldite. 2 \( \mu \)m sections were cut on a Huxley Cambridge microtome and stained with toluidine blue.

*Elbow joint marker*

To pinpoint the elbow joint so that accurate excisions could be made, a reproducible marker had to be obtained. Although the elbow joint region is not histologically distinguishable at stage 24, its position can be clearly seen in whole mounts.

Wings of chick embryos between stages 24 and 28 were measured in length using an eye piece graticule, before being excised and whole mounted. The length of the whole limb, and the distance of the elbow joint to distal wing tip
were measured (Fig. 2), and assuming that shrinkage was constant over the whole limb, the distance of the joint to the tip in the unfixed wing-bud can be found by the following simple formula:

\[
\frac{\text{Elbow/tip (fixed)}}{\text{Wing length (fixed)}} = \frac{\text{Elbow/tip (unfixed)}}{\text{Wing length (unfixed)}}
\]

therefore, \( \text{Elbow/tip (unfixed)} = \frac{\text{Wing length (fixed)}}{\text{Wing length (unfixed)}} \cdot \text{Elbow/tip (fixed)} \).

By plotting unfixed elbow/tip length against unfixed limb length the elbow position can be accurately determined if the wing length is known (Fig. 3).

To test the accuracy of the marker, 150 \( \mu \text{m} \) squares were excised at the points given as the elbow by Fig. 2 from stage 25- and stage 26- embryos and these were immediately cut off and whole mounted. In nine out of ten cases the whole joint had been accurately removed.

RESULTS

Table 1 summarizes the experiments that were completed.

Normal development of the elbow at stage 36

At stage 36 the shape of the elements comprising the elbow joint are essentially replicas of the adult. The humerus has two large condyles which lie in a dorso-ventral line; the lateral epicondyle being dorsal to the ulna articulation. The radius and ulna curve away distally to lie in the antero-posterior plane, as seen in Fig. 4. Essentially the epiphysis has a flattened appearance with the lateral epicondyle being the more pronounced and curved of the two extensions.
Fig. 3. Graph showing the relation between the measured length of the unfixed wing-bud and the predicted position of the elbow, as calculated from equation (1).

Fig. 4. Whole mount of a stage-36 wing showing the normal appearance of the elbow joint. Mag ×10.

In section the radius and ulna are separated by a region of closely packed rounded cells that are continuous with and resemble the cells separating these elements and the humerus. The perichondria of the individual elements are also continuous with this layer but differ in appearance: the two merge on each element on the proximal part of the epiphysis. The perichondrium lies at right angles to the flattened cartilage cells of the diaphyses. Figure 5 shows a high power view of the perichondrium of the radius merging with the cells which cap the epiphysis. These cells are not so flattened and are darker staining. Unlike the
Fig. 5. High power view of the radius of a control limb, showing the perichondrium merging with the joint cap cells. C, cartilage; M, mesenchyme; P, perichondrium; J. joint cap. Mag × 72.

Fig. 6. Diagrammatic representation of the cellular regions of the humerus at stage 36.

perichondrial cells they merge with the new rounded cells of the epiphysis and are continuous with the corresponding layers of the other elements where they come into contact. It is these regions that will later form the articulations, and it is these cells, by nature of their position and special character that we have designated as joint cells.
The cartilage cells of the humerus at stage 36 can consistently be split into regions according to their size and shape (Fell, 1925), as shown in Fig. 6, which acts as a useful marker for the normal appearance of this element. The development of the joint cells from their first appearance, at stage 28, up to stage 36 is being studied in the light and electron microscopes.

**The operated limbs**

**Whole mounts**

*Experiment 1.* In 12 out of 12 cases when the wing was cut across the elbow joint at stage 25, the humerus appeared to be of normal shape at stage 36. The operated limbs were on average 5%, but in no case more than 10%, shorter than the contralateral controls but because the form in whole mount and in section was normal this was assumed to be due to trauma. Figure 7A shows an operated limb where the lateral and medial epicondyles are normal and the epiphysis is flattened in the dorso-ventral plane. Figure 7B contrasts this with a normal limb of the same stage with the radius and ulna dissected away. The kink in the diaphysis of the humerus is also normal (see Fig. 4). Figures 7C and D show operated limbs that have been cut at successively more proximal positions relative to the elbow joint. The least proximally cut limb shows a portion of the epiphysis with the medial and lateral supracondylar ridges but the condyles themselves are missing.

*Experiment 2.* Fusion of the radius, ulna and humerus occurred when slices were removed from stage 24–26 embryos (Fig. 8). Success was greater at stage 24 (Table 1) because the operation is physically easier at this stage than stage 26 when the wing-bud is not only larger but the embryo lies at an awkward angle for operating. Failure of the operations at stage 26 was due to the distal piece not healing to the stump properly and a large blood clot developing, which resulted in abnormal development of the distal parts. However, in most cases fusion of the elbow could be seen, and in no case did a recognizable joint develop. The control experiment, where the slice was cut out but repinned with the distal piece on to the stump, resulted in normal limbs that were slightly smaller than their contralaterals; this was presumably due to trauma.

**Sections**

The cellular orientation of the humerus diaphysis and epiphysis was normal (Fig. 9A). When the cut was made proximal to the joint the humerus behaved as a mosaic and the truncation showed the predictable cellular arrangement at the distal end.

The perichondrium of the humerus was of normal thickness and at right angles to the flattened cartilage of the distal diaphysis. It merged with the cap of joint cells in the correct position. These cells were continuous with the rounded cartilage cells of the epiphysis and had the flattened darker staining properties of the joint cells (Fig. 9B). In the normal joint when these cells do not come into
Fig. 7. Whole mounts of humeri at stage 36. (A) A wing that was cut across the elbow at stage 25. Mag × 30. (B) A normal humerus from a left wing control with the distal structures dissected away. Mag. × 20. (C) A wing cut just proximal to the elbow with the condyles missing. Mag × 15. (D). A wing cut 100 μm proximal to the elbow showing truncation at the distal diaphysis. Mag × 15. S, supracondylar ridge; L, lateral epicondyle; M, medial epicondyle; U, proximal ulna.

Fig. 8. Stage-36 wing that had a 150 μm slice containing the presumptive elbow removed at stage 24. The elements of the elbow have fused. H, humerus; R, radius; U, ulna. Mag. × 10.
Fig. 9. (A). Low power longitudinal section of a wing truncated at the elbow. Note the normal cellular arrangement of the humerus. The numbers correspond to those in Fig. 6. Mag × 23. (B). High power view of the joint cap region of a similar specimen. Mag × 100. M, mesenchyme; J, joint cap region; C, epiphyseal cartilage of the humerus.

Table 1

<table>
<thead>
<tr>
<th>Operation performed</th>
<th>Stage</th>
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<th>Radius and ulna present</th>
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<td>24</td>
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<td>3</td>
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<td>12</td>
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<td></td>
<td>26</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Cut distal to the elbow</td>
<td>25</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>100 μm proximal to the elbow</td>
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<td>5</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>150 μm proximal to the elbow</td>
<td>25</td>
<td>3</td>
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<tr>
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<tr>
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contact with the corresponding layers of the other elements they contact diffuse mesenchyme, except between the radius and ulna, as mentioned previously. The cap forms a distinct compact layer which is seen on the operated limbs where these cells are continuous with mesenchyme all the way across the epiphysis. This layer of mesenchyme in some cases was very thin with the outer barrier being not the ectoderm, which has failed to grow across; but a single layer of flattened mesenchyme cells. Other sections show a thicker layer of
mesenchyme which has an ectoderm and feather germs over the distal surface. These have been pulled round with the ectoderm which has grown round the cut surface.

In two of the operated limbs that were sectioned, splits occurred in the mesenchyme surrounding the epiphysis of the humerus. These were small and thin and occurred along the anterior and posterior edges of the cartilage as well as over the articular surfaces. They may be caused by the growth of the humerus tearing the mesenchyme, especially where no ectoderm was present. They do not resemble, in their cellular nature or time of appearance, the joint cleft.

DISCUSSION

The striking result of these experiments is that the morphology and histology of the humerus, including its cap of differentiating joint cells, is normal at 10 days of incubation when all the structures distal to it have been removed at an early stage in its development. This is persuasive evidence that the elbow joint is not localized or influenced by the structures distal to it up to stage 36.

This result distinguishes between the two processes of differential growth that have been suggested as being important in the first phase of joint development. It is clear that opposed growth of adjacent cartilage elements is not necessary for normal joint morphogenesis. The flattening of the cells of the joint cap may result from localized mechanical pressures exerted by the matrix secreting cartilage cells in the near vicinity of the joint cap. This interpretation agrees with that of Fell & Canti (1934). However, these authors showed that if a thin slice including the presumptive knee joint region was removed and the femur placed adjacent to the tibia and fibula the normal condyle shapes were lost but an articulation still formed. If they removed a larger slice, fusion did occur. This is the result reported in this paper where slices of 100–150 μm were removed at stage 24. The question is therefore how exact is the specification of the elbow joint? These experiments suggest that only the cells in the distal epiphyseal regions of the developing cartilage elements have the capabilities of forming an articulation. It is possible that only the cells in a very restricted zone of this region are capable of forming the synovial cavity during the second phase of joint development.

It can be said therefore that the cells of the joint follow a specific pathway of differentiation that is governed by the instructions given to them during the early stages of limb development (Wolpert et al. 1975). The early determination of the joint has also been reported by Wolff (1958) and Hampe (1956), who explanted slices of leg-buds from stage-20 to stage-22 chick embryos which contain the presumptive knee joint region onto the chorioallantoic membrane, where a normal joint developed. It is clear from this experiment that the cells contained in the transplanted slice of leg-bud contain the information necessary to develop into a normal knee joint region by stage 20.
Development of the chick elbow joint

It is interesting that the leg-bud, after removal of this slice, is capable of regulation along the proximo-distal axis and the missing knee joint is replaced by a new knee joint which is re-specified in some way from the cells in the now reduced leg-bud. Regulation of the elbow joint regions occurs in the wing-bud at stage 23 when it is rotated through 180° (Stark & Searls, 1974), although by stage 24 (the earliest stage of operating reported here) the elbow behaves in a mosaic fashion. There are some apparently conflicting results concerning the regulative ability of the chick wing-bud. Summerbell (reported in Wolpert et al. 1975) has removed slices from stage-21 wing-buds and obtained predictable deletions of the skeletal elements after 10 days of development. In contrast to these non-regulatory results, several authors have shown clear regulation after deletion or addition of presumptive tissues in the chick limb-bud (Kiény, 1964a, b; Sengel, 1975). What is absolutely clear however is that the chick elbow joint is determined by stage 24. Specification may have occurred by stage 19 for the elbow joint in the wing-bud (Summerbell, 1974) and by stage 20 for the knee joint in the leg-bud (Wolff, 1958). The major part of this paper is concerned not with the mechanism of initial cell specification in the wing-bud but with growth processes that are occurring between the fourth and tenth days of incubation.

Fell & Canti concluded that the presumptive joint region was merely a region of cartilage cells continuous with the faster growing chondrogenic centres either side. The descriptions of this paper and others (see Introduction) have shown this not to be the case; in fact the joint cells have cellular characteristics quite distinct by stage 36. Further, Hendrikson & Cohen (1965) have shown that the matrix found in the joint region, which was taken by Fell & Canti to be indicative of cartilage, is distinct from that of cartilage.

It is clear that joint cells can be thought of as a distinct cell type differentiating according to information acquired at early stages. One could then be led to assume that the mechanism specifying the cellular pattern in the early limb-bud (Wolpert, 1969) would need to be exceptionally accurate if a structure as relatively small as the stage-36 elbow joint region was laid out at stage 19 (Summerbell, 1974; Summerbell & Lewis, 1975). However, Lewis (1976) has shown this not to be the case; by using tritiated thymidine labelling at these early stages and looking at the distribution of label at later stages he has shown that the forearm region grows considerably more than the elbow region; therefore, at the early stages they were of comparable size. The mechanisms necessary to determine the pattern need not therefore be on such a minute scale as the eventual structure would suggest.

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