Int. J. Exp. Path. (1997), **78,** 187–196

Intrinsic control of vascularization in developing cartilage rudiments

S.A. FENWICK, P.J. GREGG, S. KUMAR*, J. SMITH* AND P. ROONEY

*Department of Orthopaedic Surgery, University of Leicester, Glenfield Hospital NHS Trust, Leicester, *Department of Pathological Sciences, University of Manchester, Manchester, UK*

Received for publication 20 September 1996
Accepted for publication 12 March 1997

Summary. The vascularization of developing cartilage rudiments is temporally and spatially defined. By using an *in vivo* angiogenesis model, the chorioallantoic membrane (CAM) of the chick embryo and chick embryo cartilage rudiments, we conclude that the factors controlling the vascular invasion of cartilage rudiments are intrinsic. Intact rudiments, separate hypertrophic zones and separate rounded cell zones, when grafted onto the CAM, become vascularized in the same temporal and spatial manner as occurs *in ovo*. When grown as organ cultures prior to CAM grafting, rudiments still become vascularized in the same temporal and spatial manner. The integrity of the extracellular matrix and the presence of the periosteum are two physical factors regulating the control of vascularization. Removal of the periosteum from hypertrophic regions caused a cessation of the invasion. Insults to the matrix via brief enzymatic degradation of extracellular matrix components resulted in invasion and erosion of rounded cell zones at an earlier time than is ordinarily seen both *in ovo* and on the CAM.

Keywords: hypertrophic cartilage, vascularization, CAM graft, extracellular matrix, angiogenesis

Long bone development begins with a condensation of mesenchymal cells and their subsequent differentiation into chondrocytes to form a cartilaginous precursor for the future bone (Fell 1925; Price *et al.* 1994). The factors controlling this cellular condensation are unknown, but the process occurs in a pre-defined temporal and spatial manner (Hallmann *et al.* 1987). Cartilaginous long bone rudiments consist of hypertrophic, flattened and rounded chondrocytes. These are organized into five distinct zones, a central hypertrophic core, with a flattened then rounded cell zone at each end (Fell 1925). The rudiment is surrounded by a fibrous perichondrium.

Embryonic cartilage is similar to other cartilage types in terms of its composition. It consists of a meshwork of collagen types II, VI, IX, XI and XIV arranged as fibres which are embedded within a hydrated proteoglycan gel (Kuettner 1992). However, the hypertrophic cartilage zone of developing rudiments is unique in that it also contains type X collagen at certain stages of its development. The role of type X collagen is not fully understood, though it is believed to have a mechanical function (Aspden 1994; 1996) and to be involved in the processes of endochondral ossification, vascularization and calcification of cartilage (Schmid *et al.* 1990; Iyama *et al.* 1991, Rooney & Kumar 1993; Wallis 1993).

At a defined time during development, hypertrophic cartilage is invaded by vascular tissue and erosion begins (Roach & Shearer 1989; Price *et al.* 1994). In long bones a secondary centre of ossification appears within the epiphyses. As resorption proceeds, the epiphyseal cartilage

Correspondence: S.A. Fenwick, Department of Orthopaedic Surgery, University of Leicester, Glenfield Hospital NHS Trust, Groby Road, Leicester, LE3 9QP, UK.

is gradually replaced by bone until only a thin layer remains at the articular surface (Price *et al*. 1994). Although the general process of endochondral ossification is similar in birds and mammals, avian long bone development differs from that in mammals in that a larger proportion of the cartilage is removed prior to extracellular matrix calcification and the resorbed tissue is mainly replaced with a larger bone marrow cavity. There is therefore a lack of true endochondral ossification in avian bone development, except at the extremities of the diaphysis (Fell 1925).

Due to the ease of obtaining specimens and the accuracy to which development can be staged, many studies have been performed on the developing chick embryo. Hypertrophy of the central zone chondrocytes begins, in the presumptive femur, at Hamburger and Hamilton stage 30 (6 days) (Hamburger & Hamilton 1951; Rooney 1984; Rooney *et al*. 1984; Pechak *et al*. 1986). Between stages 32 and 34 (7–8 days) there is a progression of hypertrophy of the chondrocytes, as determined by an increase in cell size, loss of cell density and increased vacuolation (Rooney *et al*. 1984; Pechak *et al*. 1986). In the leg rudiments at stage 35 (9 days) vascular elements and associated cells invade the hypertrophic core at a central point (Fell 1925; Rooney & Kumar 1993). By stage 36 (10 days) invasion is such that the central section of the hypertrophic cartilage has been eroded and the marrow cavity is forming (Pechak *et al*. 1986). This process occurs at a slightly later time in the wing bone rudiments.

Approximately five days after the initiation of diaphyseal vascularization, medullary spaces begin to form in the epiphyseal cartilage containing rounded cells. Although diaphyseal vascularization is continuing, the invading epiphyseal vessels are quite separate (Fell 1925). Eventually, expansion of both ossification centres leaves a narrow belt of cartilage, the epiphyseal growth plate (Fell 1925).

Vascular invasion always begins within the central hypertrophic cell region. The control of this process is not well understood. The aims of this study were to determine if only hypertrophic cells are eroded, if all hypertrophic cells can stimulate or allow vascularization and whether the process is temporally controlled. To do this, we have used the well-established model of the chick chorioallantoic membrane (CAM) to provide an *in vivo* model of vascularization.

The results presented here demonstrate that control of vascular invasion in cartilage rudiments in the chick embryo is both temporally and spatially controlled within hypertrophic cartilage. This remains in force, even when the cartilage is removed from *in ovo* and placed into both an *in vivo* or *in vitro* culture system.

Materials and methods

Eggs

Fertilized White Leghorn chicken eggs were obtained from a registered breeder. All eggs were wiped with 70% methanol on arrival and incubated in a humidified, tilting egg incubator at 37°C. At 3 days of incubation, the eggs were windowed and the embryos staged according to Hamburger & Hamilton (1951). The eggs were returned to the incubator until the desired stages of development were reached.

Harvesting of tissue

When the desired stage was reached, embryos were removed from eggs and placed into sterile petri dishes in unsupplemented α-MEM medium (Gibco Life Technologies, Paisley, U.K.). Cartilage long bone rudiments were removed from both wings and legs and cleared of all connective tissue. Intact long bone rudiments and individual hypertrophic and rounded cell zones, excised using a scalpel, were utilized in CAM grafts onto 10 day host embryos.

Grafting of intact rudiments

Intact stage 32, 33, 34, 35 and stage 36 rudiments (approximately 7, 7½, 8, 9 and 10 days respectively) were grafted immediately onto the CAM of 10 day hosts. The eggs were sealed and returned to the incubator for a further 3 days when the CAM surrounding the grafted tissue was excised, fixed in 10% buffered formalin and processed for wax histology. Three rudiments of each stage were added to each CAM and a minimum of five eggs for each stage of rudiment were used, i.e. at least 15 rudiments of each stage were grafted.

In a separate series of experiments, intact stage 32 and stage 36 rudiments were grown in organ culture in BGJb Fitton-Jackson modified medium, supplemented with 10% foetal bovine serum, L-glutamine (1.4 mM), Penicillin (50 IU/ml) and Streptomycin (50 μg/ml) (all Gibco Life Technologies, Paisley, UK), for 3 days prior to CAM grafting for a further 3 days. At the end of the graft period, tissues were fixed and processed as above.

Grafting of hypertrophic zones

Hypertrophic cell zones were isolated from rudiments at each stage between stages 32 and 36 and grafted onto the CAM of 10 day host embryos. Three zones were grafted onto each CAM and a minimum of 8 eggs were

Table 1. Table to show the time period of grafting of hypertrophic zones

Age of hypertrophic zone *in situ*	Time on CAM (days)
Stage 32 (7 days)	1–4
Stage 34 (8 days)	1–3
Stage 35 (9 days)	1–2
Stage 36 (10 days)	1

used for each starting stage of the rudiment i.e. at least 24 hypertrophic zones of each stage were grafted. Samples of grafted hypertrophic zones were fixed at half daily intervals. The maximum time of grafting was such that for each starting stage, the total time, i.e. time of development *in ovo* plus time on the CAM equaled 11 days (Table 1). Some individual hypertrophic zones were isolated, fixed and processed directly for histology to ensure the efficiency of isolation of hypertrophic cartilage.

In one series of experiments, a detailed study of vascularization was performed by removing and processing samples of stage 32 hypertrophic zones at 4 hours, 1 day, 1½ days, 2 days, 2½ days, 3 days and then at 4 hourly intervals until 5 days on the CAM.

In an attempt to determine the role played by the periosteum in vascularization, periostea were removed from stage 34 (approximately 8 day) and stage 36 (approximately 10 day) hypertrophic zones prior to grafting for up to 4 days. Removal of the periostea was achieved by carefully cutting along its length with either a scalpel or by tearing the periosteum with a pair of sharp forceps and gently teasing the cartilage out of the opened zone.

To investigate the effect of enzymatic degradation on vascularization, hypertrophic zones from stage 36 embryos, both with and without their periosteum, were pre-treated with either collagenase (Type 1A, 0.2% w/v, Sigma), or hyaluronidase (Type 1-S, 0.05% w/v, Sigma) or a combination of both in unsupplemented α-MEM medium for 20 minutes and then grafted onto the CAM for 2–3 days.

Grafting of rounded zones

Rounded zones were isolated from stage 36 rudiments and grafted onto the CAM of 10 day hosts. As above, three grafts were made per CAM and samples were fixed daily at intervals for up to 5 days.

In one series of experiments, rounded cell zones were pre-treated with collagenase and/or hyaluronidase as described above.

Histological and histochemical examination

Sections were cut at 5–7 μm and general histological staining was performed using haemotoxylin & eosin (H&E). Various histochemical stains were also employed, safranin-O for proteoglycans (0.5% in 1 M acetate buffer, pH 4.6 for 10 min), toluidine blue (1% aqueous solution for 1 min) and alcian blue at critical electrolyte concentration of 0.06 M $MgCl_2.6H_2O$ for non-specific staining of sulphated glycosaminoglycans (GAG) and 0.7 M or 0.9 M $MgCl_2.6H_2O$ for specific staining of cartilage GAG (0.05% alcian blue + $MgCl_2.6H_2O$ in 0.2 M acetate buffer, pH 5.8, overnight) (Scott & Dorling 1965).

Results

Macroscopic observations

Despite being placed gently on the CAM, approximately 35% of the intact rudiments encouraged a slight leakage of blood around the graft site. Only 60% of the rudiments attached to the CAM and as observed by Thorogood (1983) the rudiments, instead of growing longitudinally, formed hoops and arches. A similar transition is seen when rudiments are maintained in organ culture.

The majority (approx. 70%) of the hypertrophic zones grafted encouraged a relatively large leakage of blood around the graft site after the first 24 hours irrespective of the *in situ* age of the zone (Figure 1). Zones removed at 4 hours did not adhere to the CAM, however, those removed after 24 hours were attached and were

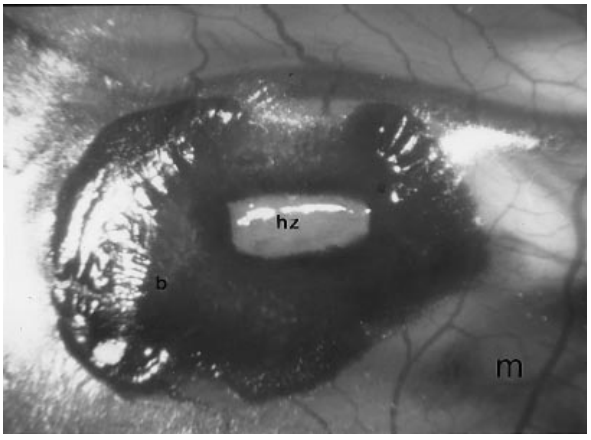


Figure 1. A hypertrophic zone (hz) grafted onto the CAM (m) for 36 hours, showing a large quantity of blood (b) having leaked out around the zone. This is never seen within the first 24 hours. (×30).

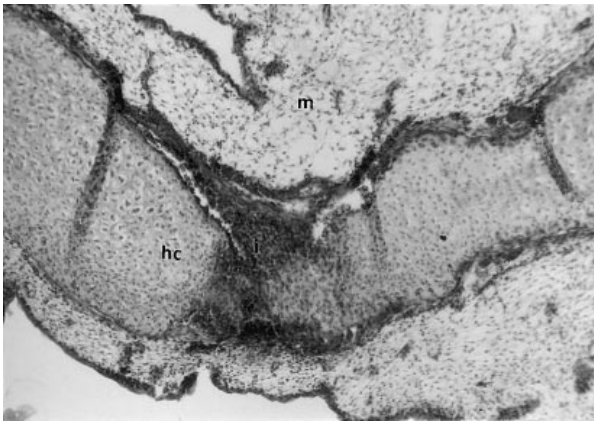


Figure 2. Invasion (i) of the hypertrophic cartilage (hc) of an intact stage 34 rudiment after being grafted on the CAM for 2 days. The amount of erosion seen is similar to that seen in a normal stage 36 tibia. (H&E, ×100).

enveloped by the CAM. Blood vessels could often be seen growing over the cartilage.

Periosteum-free hypertrophic zones did not cause blood leakage, but still became enveloped in the CAM. Blood vessels were observed growing over and around the cartilage. Those treated with collagenase were no longer visible after 3 days on the CAM, and as such, no histological examination could be performed.

Rounded zone cartilage generally encouraged a small leakage of blood and were enveloped by the CAM with blood vessels on and around the cartilage. This was also seen with enzymatically treated rounded cell zones.

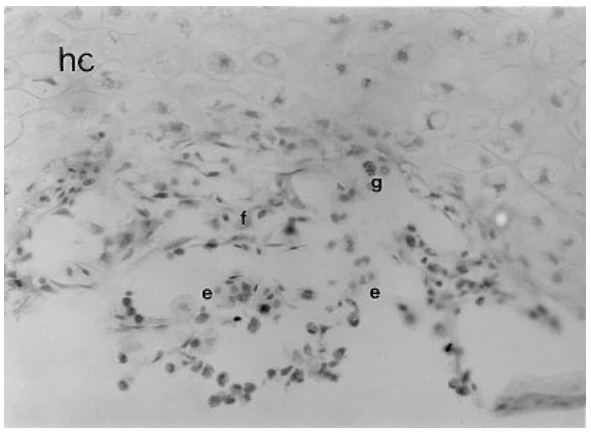


Figure 3. Invasion into the hypertrophic cartilage of a CAM grafted intact rudiment (hc) showing the different cell types, fibroblasts (f), erythrocytes (e), and granular or possibly multinucleated cells (g). (H&E, ×400).

Histological observations

Intact rudiment grafts. Stage 32 rudiments grafted for 1–2½ days (8–9½ days total age) showed no indication of becoming vascularized, either within the hypertrophic cell zone or the rounded cell zone. The cartilage appeared healthy and remained intact. Those grafted for 3 days (10 days total age) showed the first stages of vascularization within the hypertrophic zone (Data not shown). Stage 33 rudiments grafted for 2½ days (10 days total age) and stage 34 rudiments grafted for 2 days (10 days total age) showed the primary stages of invasion (Figure 2).

Stage 35 and 36 rudiments grafted for 3 days (12 and 13 days total age respectively) showed a pattern of vascularization similar to that observed in stage 38 and 39 rudiments (12 and 13 days old respectively) *in situ* i.e. most of the central portion of the hypertrophic zone was extensively eroded. The extent of invasion was greater than that seen in a stage 36 rudiment fixed immediately upon removal, indicating that invasion continued when the rudiment was grafted. The invasion site contained a mixture of cell types, including erythrocytes, fibroblastic cells and a number of cells containing darkly staining granules, or which were possibly multinucleated (Figure 3). In contrast rounded cell zones showed no signs of vascular invasion during the 3 day culture period (Data not shown).

Rudiments grown in organ culture prior to CAM grafting also showed cellular invasion when placed on the CAM. Stage 32 rudiments, organ cultured for 3 days followed by a further 3 days on the CAM (total age therefore 13 days) showed a degree of vascular invasion virtually identical to that seen in a stage 35–36 rudiment grafted immediately for 3 days (12–13 days total age) (Figure 4). In contrast, stage 36 rudiments organ cultured for 3 days then CAM grafted for a further 3 days (16 days total age) were only vascularized to a similar degree as a stage 36 rudiment grafted directly for 3 days (13 days total age) (Figure 4).

Hypertrophic zone grafts. Histological staining using H&E of stage 32 hypertrophic zones grafted for between 0 and 2½ days showed no sign of cartilage vascularization. However, approximately 80% of stage 32 grafts removed after 3–5 days of incubation (total age of 10–12 days) exhibited vascular invasion (see Table 2). All stage 34 zones grafted for 3 days and stage 35 zones grafted for 2 days showed varying degrees of vascularization indicating that vascularization began while the cartilage was on the CAM. Stage 36 zones grafted for 1 day also showed extensive vascularization but a stage 36 zone will be partially vascularized prior to grafting due

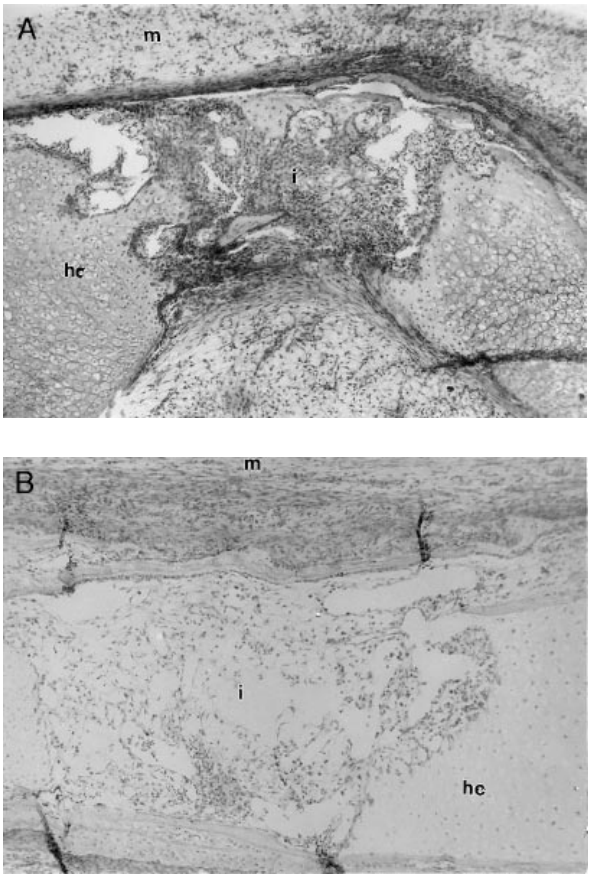


Figure 4 a. A stage 32 rudiment, having been organ cultured for 3 days then CAM grafted for a further 3 days. Most of the central portion of the hypertrophic zone (hc) has been eroded as is normally seen in a stage 35/36 rudiment grafted for 3 days. CAM tissue (m), invasion site (i). (H&E, ×100). b. The central portion of a stage 36 rudiment having been organ cultured for 3 days then grafted for a further 3 days. Again, the extent of erosion is similar to that seen in a stage 35/36 rudiment grafted for 3 days. CAM tissue (m), hypertrophic cartilage (hc), invasion site (i). (H&E, ×100).

to the process beginning during stage 35 (Pechak *et al.* 1986). Our own observations of stage 36 rudiments agree with these findings.

The onset of hypertrophic cartilage erosion always occurred at a central point along the long axis of the zone (Figure 5). The site of invasion contained the same mixture of morphologically distinct cells as described earlier (Figure 3). A number of lacunae were often seen along the edge of the invasion site that had been breached by the process of invasion and appeared to be empty. However, several intact lacunae contained vacuolated chondrocytes indicating possible cellular degeneration or death (see Figure 7).

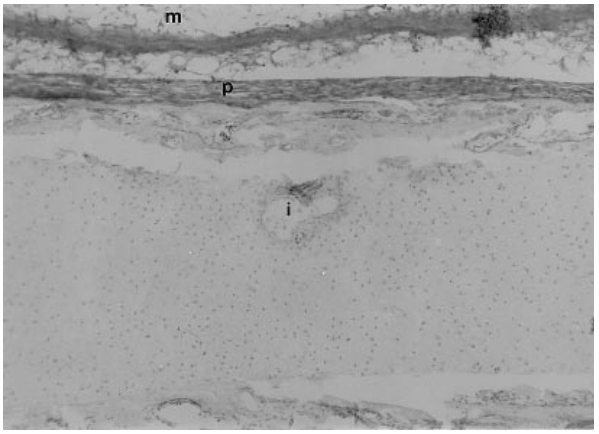


Figure 5. The early stages of hypertrophic zone invasion (i). This is always seen to occur at a central point along the long axis of the zone. CAM tissue (m), periosteum (p). (H&E, ×100).

The site of invasion was always restricted to the central portion of the long axis. The exposed cut ends did not exhibit vascularization, despite the presence of blood vessels 1–2 cell widths from the cartilage (Figure 6).

Metachromatic staining with safranin-O (Figure 7) and toluidine blue (Data not shown) showed a loss of staining intensity in the cartilage along the leading edge of the invasion site indicating a loss of proteoglycans within the matrix. This loss of staining only penetrated 1–2 lacunae widths into the cartilage. A similar loss of staining was seen with alcian blue CEC at 0.9 M (Data not shown), 0.7 M and 0.06 M $MgCl_2$ (Figure 7), indicating a specific loss of keratan sulphate, heparan sulphate and general loss of all cartilage GAG respectively. The depth of

Table 2. Table to show the times at which isolated hypertrophic zones become vascularized on the CAM

Age of zone	Number of zones grafted	Time on CAM (days)	Total age of zone (days)	% of zones vascularized
Stage 32 (7 days)	30	0–2½	7–9½	0
Stage 32 (7 days)	30	3–5	10–12	80
Stage 34 (8 days)	24	3	11	100
Stage 35 (9 days)	24	2	11	100
Stage 36 (10 days)	24	1	11	100

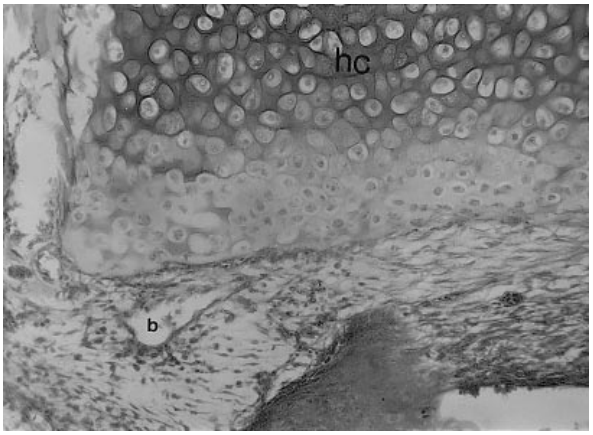


Figure 6. Cut end of a CAM grafted hypertrophic zone (hc). Despite the presence of a blood vessel (b) within 2–3 cell widths of the cartilage, there is no evidence of any vascular invasion in this part of the cartilage, or in any other part except for the central point. Note also the loss of metachromatic staining along the edge of the end of the zone. (Safranin-O, ×400).

staining loss did not occur to as great an extent as seen with safranin-O and toluidine blue. A loss of staining with safranin-O was also seen at the ends of the hypertrophic zones, although no invasion occurred (Figure 6). Enzymatic treatment with either hyaluronidase, collagenase or both had no effect on the pattern of vascular invasion of hypertrophic zones (Data not shown).

Periosteum-free zones. Stage 34 periosteum-free zones, grafted for 4 days (total age of 12 days) showed no sign of vascular invasion. The chondrocytes were morphologically normal with only a few vacuoles (Data not shown). Sections of stage 36 periosteum-free hypertrophic zones, fixed directly after dissection, showed the early stages of invasion at the central point. The chondrocytes contained many vacuoles and often appeared crescent shaped, lying compressed against their lacunae walls (Figure 8).

When grafted, stage 36 periosteum-free zones showed no evidence of active vascularization on the CAM. At the end of the 3 day culture period, chondrocytes throughout the entire zone appeared more healthy than chondrocytes from non-grafted stage 36 hypertrophic regions, in that they showed a normal rounded chondrocytic phenotype and contained few vacuoles. Some of the chondrocytes were undergoing mitosis. A small proportion of the cells, which increased in concentration towards the invasion site, contained darkly staining granules (Figure 8).

Hyaluronidase treatment of stage 34 and 36 periosteum-free zones had little effect on vascularization.

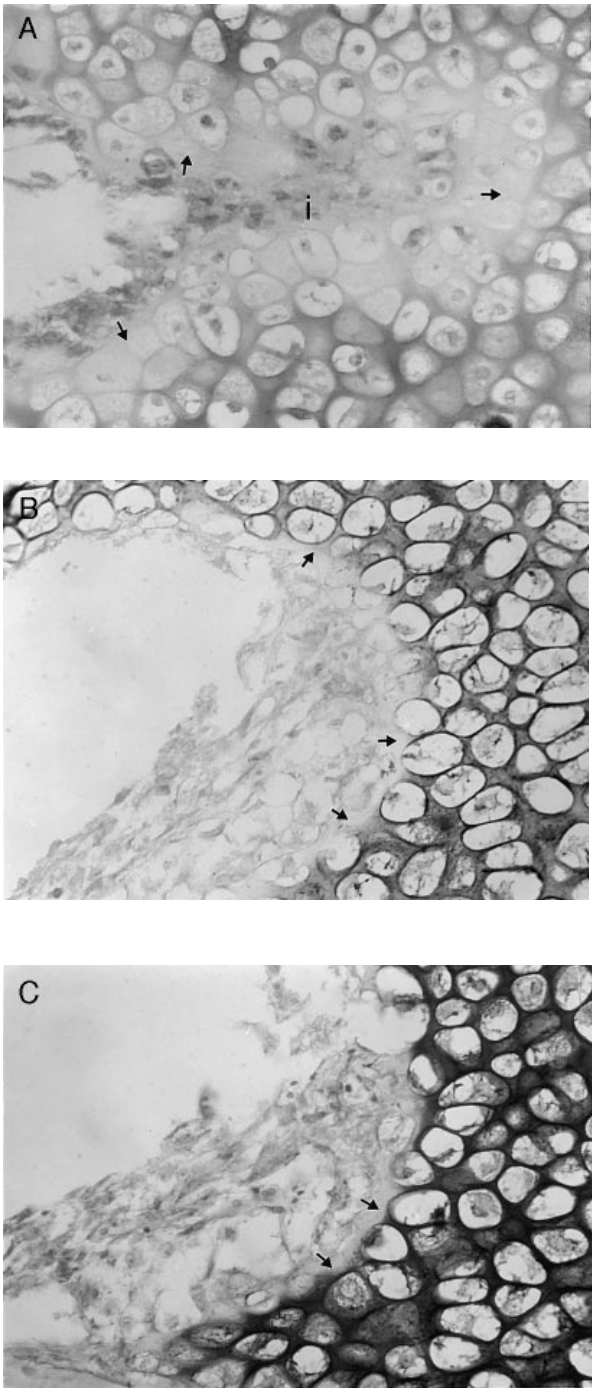
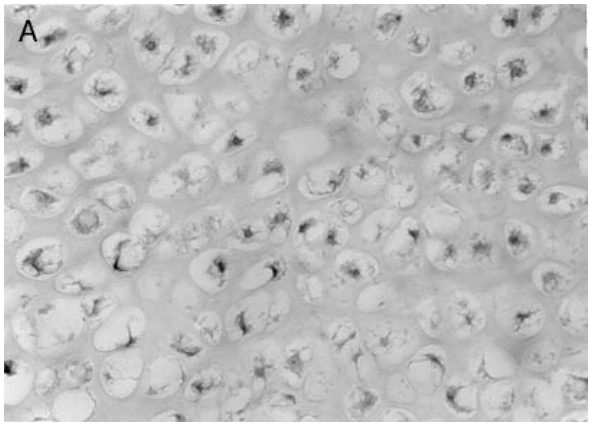


Figure 7. Loss of metachromatic staining along the rim of invasion (arrows). Invasion front (i). a. Safranin-O – staining loss occurs up to 2 lacunae widths in depth. (×400) b. Alcian Blue CEC 0.06 M $MgCl_2$ – staining loss only occurs up to approximately 1 lacunae width in depth. (×400) c. Alcian Blue CEC 0.7 M $MgCl_2$ – stains specifically for heparan and keratan sulphate which are found in high amounts in cartilage. (×400) Many of the chondrocytes around the invasion site are very degenerate.



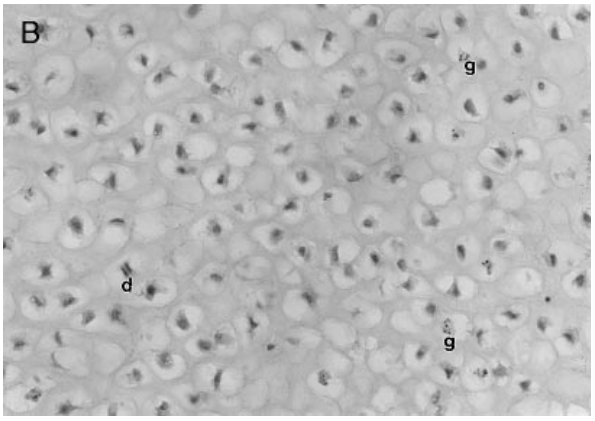
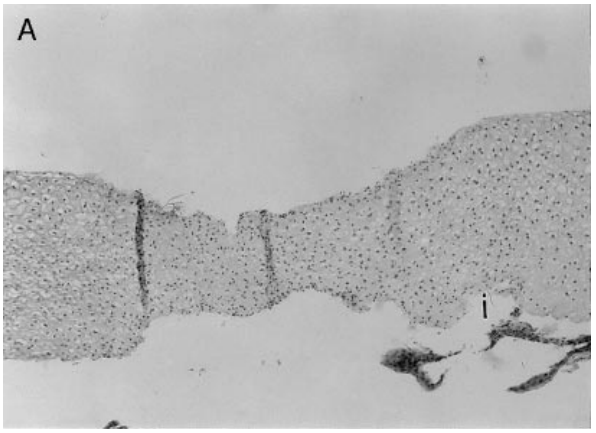


Figure 8 a. Hypertrophic chondrocytes from a non-CAM grafted stage 36 hypertrophic zone. b. Hypertrophic chondrocytes from a CAM grafted, periosteum-free hypertrophic zone. Cells from non-CAM hypertrophic zones appear very vacuolar and degenerate. CAM grafted cells are more chondrocytic, with occasional cells dividing (d). A number of cells contain darkly staining granules (g). (H&E, ×400).



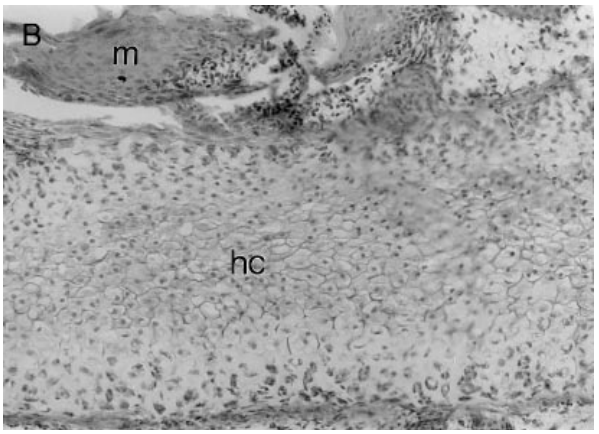


Figure 9 a. Hyaluronidase treated, periosteum-free stage 36 hypertrophic zone CAM grafted for 3 days. The likely primary invasion site can be seen (i), but no further vascularization has occurred. (H&E, ×100) b. Hyaluronidase treated, periosteum-free stage 34 hypertrophic zone (hc) CAM grafted for 3 days, shows no sign of vascular invasion. CAM (m). (H&E, ×200).

There was no indication of vascularization at stage 34 or any continued vascularization in the stage 36 zones (Figure 9). The chondrocytes appeared intact with few vacuoles. Periosteum-free zones treated with collagenase or both collagenase and hyaluronidase disappeared while on the CAM during the 3 day culture period and consequently no histological examination could be performed.

Rounded cell zones. Stage 36 zones grafted for 3 days showed no sign of vascular invasion. The cells were healthy and the cartilage was intact (Figure 10). Zones grafted for 5 days showed the first stages of vascular invasion, similar to that seen in cartilage canal formation *in situ* (Figure 11). One or two small vessels appeared

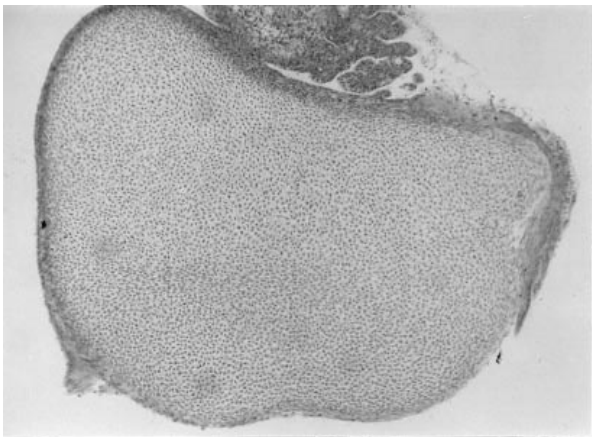


Figure 10. A stage 36 rounded cell zone, CAM grafted for 3 days, showing no vascular invasion. (H&E, ×100).

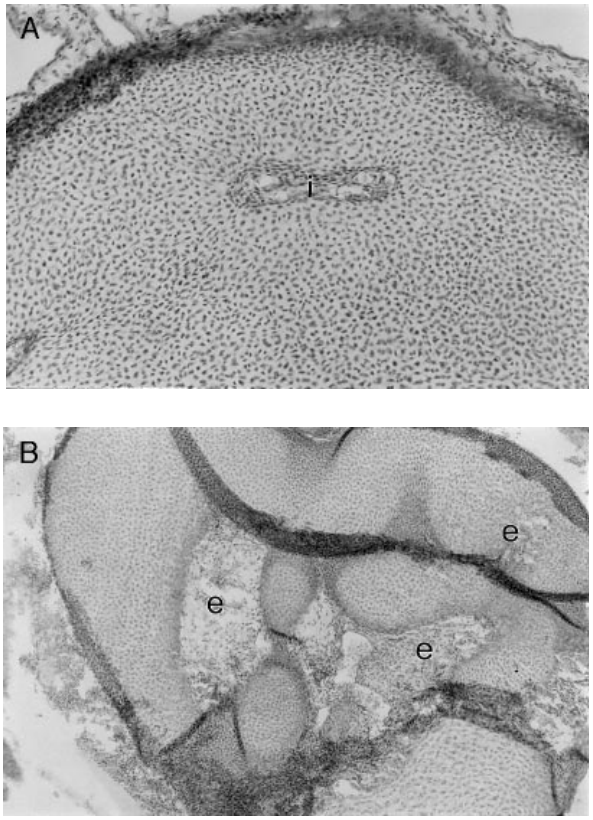


Figure 11 a. The initiation of invasion (i) of a stage 36 rounded cell zone after being CAM grafted for 5 days. A similar pattern is seen in rounded cell zones after hyaluronidase treatment, though at an earlier time. (H&E, ×200) b. Invasion of a single stage 36 rounded cell zone after being treated with collagenase and grafted for 3 days. Much of the zone has been invaded and eroded (e). (H&E, ×100).

within the rounded cell cartilage containing a similar mix of cells seen during hypertrophic zone invasion. Also, a noticeable rim of cells, thought to be endothelial cells, was seen lining the edge of the hole.

Enzymatic treatment of stage 36 rounded zones caused a change in the pattern of vascularization. Hyaluronidase treated zones became invaded in an identical way to untreated zones grafted for 5 days as seen in Figure 11, except that the invasion occurs within 2–3 days of grafting. Treatment with collagenase caused extensive tissue disruption and random general invasion and erosion of the rounded cell zone within 2–3 days of grafting (Figure 11). Zones exposed to both hyaluronidase and collagenase behaved in a similar way to those treated with collagenase alone, however, occasionally, a site of primary invasion was seen similar to that in hyaluronidase treated zones.

Discussion

Cartilage vascularization is an important step in the formation of bone. In this present study, we have examined temporal and spatial aspects of the vascular invasion of chick embryo long bone rudiments using an *in vivo* model of vascularization. We have shown that the temporal and spatial pattern of the invasion of hypertrophic and rounded cell cartilage is maintained even when the rudiments are removed from the embryo and grafted onto the CAM. Irrespective of the age at which the rudiments were taken, hypertrophic cartilage vascularization always occurred at a total age of approximately 10 days and always began at a central point, as is seen *in ovo* (Pechak *et al.* 1986). This has been reported to be due to a decrease in the production of anti-angiogenic factors by the embryonic cartilage (Pechak *et al.* 1986) which have been shown to be produced by the hypertrophic core (Sorgente *et al.* 1975).

This result is similar to those obtained by Gibson *et al.* (1995) who found that sterna from 20 day chick embryos, when cultured on the CAM, were resorbed specifically at the primary ossification centre, suggesting that only a specific region of cartilage, at a specific time is able to become vascularized.

This is most clearly seen with isolated hypertrophic cartilage. Interestingly the ends of these zones expose large areas of hypertrophic cartilage, yet invasion still only occurs from a central point, never from the exposed ends. *In ovo*, however, this cartilage is resorbed, once the invasion has worked its way from the centre to the extremities of the cartilage (Rooney & Kumar 1993). We suggest then, that all hypertrophic cartilage has the capacity to become resorbed, but not all hypertrophic cartilage, at a given time, has the capability to trigger the resorption process.

Gibson *et al.* (1995) also showed that sterna from 18 day embryos did not become vascularized when grafted onto the CAM. It was suggested that only cartilage that has started to become resorbed will continue the process on the CAM. This suggestion disagrees with our own observations. We have shown here that hypertrophic cartilage, still 2–3 days away from the initiation of resorption, still becomes resorbed in an identical spatio-temporal manner when grafted.

It appears therefore that a small subset of hypertrophic chondrocytes may have an intrinsic mechanism that can trigger vascular invasion and subsequent resorption. This is most clearly demonstrated from rudiments which are the first organ cultured and then CAM grafted. Organ culturing the rudiments allows normal development of the pre-vascularization processes. However

once at a certain stage, the process cannot continue any further without the external influences of invasive tissue e.g. the CAM. It is also apparent that the 'trigger' for invasion does not disappear when rudiments are organ cultured, but remains within the cartilage until the rudiment is placed in an environment conducive to vascular invasion.

Endothelial cells are thought to have a central role in the process of vascularization (Roach & Shearer 1989; Gibson *et al*. 1995). The first stage in vascular invasion occurs outside of the cartilage and within the periosteal bone (Silvestrini *et al*. 1979; Pechak *et al*. 1986). The vascular cells move through a hole in the inner periosteal layer, which is thought to be formed by phagocytic cells (possibly osteoclasts) which resorb a restricted region of this inner periosteal layer (Caplan & Pechak 1987). The cells associated with the vasculature subsequently dissociate the underlying cartilage (Pechak *et al*. 1986). This implies that vasculature-associated cells are important in triggering the invasion and resorption process. The majority of the cells in the invasion cluster are not present at the earlier stages, and are presumably recruited from extra-skeletal haemopoietic sites (such as the spleen or the liver) along with cells containing a granular cytoplasm.

This mixture of cells, or possibly factors produced by cells within the periosteum, appear vital for the initiation and continuation of the vascularization and resorption process. Removal of the periosteum prior to, or immediately after, the primary invasion prevents the resorption process occurring or continuing respectively. This provides further evidence that only a specific portion of hypertrophic cartilage can trigger invasion or can be triggered into becoming invaded. It also indicates that a periosteal specific mixture of cells is required in the initiation and early stages of invasion.

An important observation is that periosteum-free cartilage cells resist the degeneration processes induced by vascularization when grafted onto the CAM. We postulate that removal of the periosteum removes part or all of the vascularization 'signal' and in turn, chondrocytes which have begun to degenerate in response to, and in readiness for cartilage resorption, are able to recover and can continue to grow and divide.

The role of the cartilage matrix was investigated by using rounded cell zones from Hamburger & Hamilton (1951) stage 36 embryos. In these zones vascularization begins approximately five days after the hypertrophic zones, to form the secondary centre of ossification at approximately 15 days of age (Fell 1925). When grafted onto the CAM, stage 36 rounded cell zones did not become vascularized until the fifth day of culture. Treatment with hyaluronidase allowed apparently normal vascular invasion to begin on the CAM after only 2 days. Treatment with collagenase, or both collagenase and hyaluronidase allowed much cellular invasion and resorption of the entire zone, again after only 2 days on the CAM. Insults to the integrity of the matrix therefore allowed more rapid invasion of the vasculature. In particular, hyaluronidase treatment allowed the normal invasion process to occur, but at an earlier time than would normally occur *in ovo*, or if grafted without enzymatic treatment. It has been shown that breakdown products of hyaluronan are angiogenic (Rooney & Kumar 1993). This suggests that damage to the matrix components is a primary step in cartilage invasion, and it is known that chondrocytes do have the ability to degrade the proteoglycan component of the matrix (Gibson *et al*. 1995). It is also believed that endothelial cells can initiate cartilage resorption via the release of proteases (Roach & Shearer 1989).

In conclusion, hypertrophic cartilage zones from developing long bone cartilage rudiments in the chick embryo become vascularized in a pre-defined temporal and spatial manner, even when removed from *in ovo* and placed onto the CAM. We postulate an intrinsic mechanism within the hypertrophic cartilage as to when and where it becomes vascularized, which at least partly relies on cells present within the periosteum and on the integrity of the matrix.

Acknowledgements

We would like to acknowledge the University of Leicester for funding this work.

References

Aspden R.M. (1994) Fibre reinforcing by collagen in cartilage and soft connective tissues. *Proc. Royal Soc. Lond.* B **258** (1352), 195–200.

Aspden R.M. (1996) Could type X collagen have a mechanical function? *Int. J. Exp. Pathol.* **77,** A3–A4.

Caplan A.I. & Pechak D.G. (1987) The cellular and molecular embryology of bone formation. In *Bone and Mineral Research*, 5th edition. Ed. W.A. Peck. Elsevier Science Publishers, pp. 117–181.

Fell H.B. (1925) The histogenesis of cartilage and bone in the long bone rudiments of the embryonic fowl. *J. Morph. Phys.* **40,** 417–459.

Gibson G.J., Lin D.L., Schaffler M.B. & Kimura J.H. (1995) Endochondral resorption of chick sterna in culture. *J. Orth. Res.* **13,** 542–552.

Hallmann R., Feinberg R.N., Latker C.H., Sasse J. & Rissau W. (1987) Regression of blood vessels precedes cartilage differentiation during chick limb bud development. *Differentiation* **34,** 98–105.

HAMBURGER V. & HAMILTON H.L. (1951) A series of normal stages in the development of the chick embryo. *J. Morph.* **88,** 49–92.

IYAMA K., NINOMIYA Y., OLSEN B.R., LINSENMAYER T.F., TRELSTAD R.L. & HAYASHI M. (1991) Spatiotemporal pattern of type X collagen gene expression and collagen deposition in embryonic chick vertebrae undergoing endochondral ossification. *The Anatomical Record* **229,** 462–472.

KUETTNER K.E. (1992) Biochemistry of Articular Cartilage in Health and Disease. *Clin. Biochem.* **25,** 155–163.

PECHAK D.G., KUJAWA M.J. & CAPLAN A.I. (1986) Morphology of bone development and bone remodelling in embryonic chick limbs. *Bone* **7,** 459–472.

PRICE J.S., OYAJOBI B.O. & RUSSELL R.G.G. (1994) The cell biology of bone growth. *Eur. J. Clin. Nutrition* **48**, (Suppl. 1), S131–S149.

ROACH H.I. & SHEARER J.R. (1989) Cartilage resorption and endochondral bone formation during the development of long bones in chick embryos. *Bone and Mineral* **6,** 289–309.

ROONEY P. (1984) The cellular basis of cartilage morphogenesis. PhD Thesis, University of London.

ROONEY P., ARCHER C.W. & WOLPERT L. (1984) Morphogenesis of cartilaginous long bone rudiments. In *The Role of Extracellular Matrix in Development.* Ed. Trelstad R.L. New York Academic: Academic Press. pp. 305–322.

ROONEY P. & KUMAR S. (1993) Inverse relationship between hyaluronan and collagens in development. *Differentiation* **54,** 1–9.

SCHMID T.M., POPP R.G. & LINSENMAYER T.F. (1990) Hypertrophic cartilage matrix. Type X collagen, supramolecular assembly and calcification. *Annals New York Academy of Sciences* **580,** 64–73.

SCOTT J.E. & DORLING J. (1965) Differential staining of acid glycosaminoglycans (mucopolysaccharides) by alcian blue in salt solutions. *Histochemie* **5,** 221–233.

SILVESTRINI G., RICORDI M.E. & BONUCCI E. (1979) Resorption of uncalcified cartilage in the diaphysis of the chick embryo tibia. *Cell Tissue Res.* **196,** 221–235.

SORGENTE N., KUETTNER K.E., SOBLE L.W. & EINSTEIN R. (1975) The resistance of certain tissues to invasion. II. Evidence for extractable factors in cartilage which inhibit invasion by vascularised mesenchyme. *Laboratory Investigation* **32,** 317–322.

THOROGOOD P. (1983) The morphogenesis of cartilage. In *Cartilage* vol. 2. Ed Hall B.K. New York: Academic Press. pp. 223–254.

WALLIS G.A. (1993) Here today, bone tomorrow. *Current Biology* **3,** 687–689.