Ultrastructural features of chondrogenesis in the human hand plate: a cytochemical and autoradiographic study

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SUMMARY

Ultrastructural features of chondrogenesis are correlated with the appearance of collagen and proteoglycan components of the cartilage matrix in the developing human hand (stages 16-18).

Prechondral mesenchymal cells comprising the digital plate (stage 16) exhibit euchromatic nuclei, sparse granular endoplasmic reticulum, few polysomes, broad cell to cell associations, little enhancement of electron density after postfixation with osmium tetroxide/potassium ferrocyanide and scanty incorporation of $^3$H-proline. Extracellular matrix is electron-lucent.

During stage 17, early chondroblasts acquire affinity for OsO$_4$/K$_4$Fe(CN)$_6$, develop filopodia, lose broad intercellular attachments, assemble cytoplasmic polysomes, incorporate $^3$H-proline and the extracellular matrix acquires electron-dense particles (10-20 nm in diameter).

By stage 18, chondrogenic cells of digital blastemata exhibit affinity for osmium-ferrocyanide, and particles (10-20 nm in diameter) and filaments (5 nm in diameter) are present in the extracellular matrix. Mature chondrocytes are present.

INTRODUCTION

Developing cartilage exhibits both extracellular and cellular phases. The main constituents of the extracellular matrix are (a) collagen and (b) acid mucopolysaccharides (Jackson, 1964), both secreted by developing cartilage cells (Revel & Hay, 1963). Electron microscopy reveals extracellular ground substance to contain electron-dense particles and filaments (Godman & Porter, 1960). Matukas, Panner & Orbison (1967) suggest that the particles consist of acid mucopolysaccharide and non-collagenous protein, whereas filaments represent procollagen. Ultrastructure of the developing cellular phase has been described in the mouse by Godman & Porter (1960), and in the chick by Goel (1970). Searls, Hilfer & Mirow (1972) reported the ultrastructure of developing chick limb cartilage during the early formation of precartilaginous blastemata. However, no similar study has explored early limb chondrogenesis in man.

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The upper limb of man first appears in embryos by stage 12, approximately 25–27 days after ovulation (O’Rahilly, Gardner & Gray, 1956). The lower extremity appears by stage 13 (27–29 days after ovulation). In this primitive period, limb-buds exhibit a thickened ectodermal epithelium which invests subjacent mesenchyme. Interaction between these cell layers, reviewed by Zwilling (1961), Milaire (1965), Rubin & Saunders (1972) and others, establishes the presumptive structure of the developing limb, including mesenchymal precursors of the cartilaginous skeleton (Zaaijer, 1958). O’Rahilly et al. (1956) report that the digital plate appears on the upper limb by stage 16, and precartilaginous condensations of the developing fingers are present by stage 17. Metachromatic matrix and cellular features of chondrification in the digits are visible by stage 18.

Since structural differentiation reflects changes occurring at the molecular level, it is important to record the onset and fate of subcellular events which accompany cytodifferentiation. If functional changes can be detected at the level of ultrastructure, then development of cell architecture might be correlated with those molecular events. This report combines techniques of electron microscope autoradiography and cytochemistry to investigate the sequence of events which reflect chondrogenesis in the human limb.

**MATERIALS AND METHODS**

Hand plates for this investigation were dissected from human embryos following therapeutic interruption of pregnancy. Developmental stage was determined by matching structures with corresponding horizons of Streeter (1948) and descriptions of O’Rahilly et al. (1956). Sequential specimens from stages 16–18 were studied (approximately the sixth through seventh post-ovulation weeks).

**Electron microscopy**

Specimens were fixed by immersion in 3% glutaraldehyde in 0.15 M cacodylate/HCl buffer (pH 7.3) at 4°C for 2–4 h. A postfixative was prepared by mixing equal parts of a 3% solution of potassium ferrocyanide (K₄Fe(CN)₆; w/v in 0.2 M cacodylate buffer) with aqueous 2% osmium tetroxide (Karnovsky, 1971). Tissues were rinsed in buffer and immersed in postfixative for 2 h at 4°C, rapidly dehydrated through an ethanol series to propylene oxide and embedded in Epon 812. Thin sections (mounted on uncoated grids) were stained for 30 min in either saturated aqueous or ethanolic uranyl acetate at 35°C, for 5 min in alkaline lead citrate at room temperature and examined in an Hitachi HU-11C electron microscope.

**Autoradiography**

Staged upper limbs were placed in Eagle’s Minimum Essential Medium supplemented with 10% tryptose phosphate broth (Difco), 10% fetal calf
serum (NABI), low concentrations of antibiotics (25 units/ml Nystatin, 50 μg/ml Neomycin Sulfate, U.S.P., and 10 μg/ml Aureomycin) and containing 25 μCi of L-proline-2,3-3H (New England Nuclear, Boston). Specimens (stages 16, 17 and 18) were incubated in a humidified incubator (37 °C) in an atmosphere of 7-5 % CO₂ for 1 h. One group of limbs (stages 16–17) were ‘pulsed’ in labeled medium for 15 min, ‘chased’ by incubation in medium containing unlabeled proline and specimens removed after 30 min, 1 h, 2 h and 4 h. All tissues were rinsed five to eight times with Hank’s balanced salt solution (warmed to 37 °C), fixed (as described in preceding paragraph) and prepared for electron microscope autoradiography after the method of Caro (1964).

RESULTS

Postfixation with osmium tetroxide containing K₄Fe(CN)₆ has a dramatic effect upon the contrast of chondrogenic cells in the developing human limb. Fig. 1 illustrates cells at the growing digital tip during stage 18 which exhibit three different electron densities: (1) a mesenchymal cell on the periphery of the digital blastemata, (2) an early chondroblast, and (3) a mature chondroblast surrounded by extracellular matrix. The latter cells exhibit greater electron density in nucleoplasm and cytoplasm than the former. Advantage has been taken of this phenomenon to discern the appearance of chondrogenic cells in predigital and digital areas of the developing hand plate.

Stage 16

Examination of the predigital plate reveals a uniform zone of closely associated mesenchymal cells (Figs. 2 and 3). These cells are rounded and extracellular spaces are minimized. Nuclei are predominantly euchromatic, spherical to ovoid in shape and exhibit small clusters of condensed chromatin. Nucleoli are particulate in appearance and usually located near the periphery of the nucleus. Cytoplasmic organization reveals mitochondria, short segments of granular endoplasmic reticulum (ER), free ribosomes, polysomes, and the flattened cisternae and vesicles characteristic of Golgi centers. Increased affinity for osmium/K₄Fe(CN)₆ has not been acquired at this stage. Existing microtubules (not illustrated) are associated with centrioles and are thought to be portions of mitotic spindles. Microfilaments (5–8 nm in diameter, arrows, Fig. 3) are present in random orientation throughout the cytoplasm.

Fig. 3 illustrates close cell association exhibited by mesenchyme in the predigital plate. Cell membranes are 20–25 nm apart and the intercellular space contains focal centers of electron-dense material. Specialized structures of contact (e.g. gap junctions, focal tight junctions, desmosomes) have not been observed. In addition, materials which could be interpreted as ‘matrix’ are not visible in extracellular spaces.
Stage 17

By stage 17, digital blastemata appear in the forelimb as five radiating zones of closely associated cells. Electron microscopy reveals that certain of the blastemal cells have acquired affinity for osmium/K₄Fe(CN)₆ and exhibit increased electron density (Figs. 4, 6 and 7). Furthermore, the unstained prechondroblast cells reveal subtle structural alterations from earlier stages. Nuclei remain euchromatic and ovoid, but perinuclear cytoplasm is notably diminished. Plasma membranes acquire a ruffled, irregular appearance (Fig. 4) and increased numbers of cytoplasmic polysomes are present (Fig. 5). Fig. 4 illustrates cells with broad cell to cell contacts at one surface whereas filopodia at free borders extend into increased amounts of extracellular space. Matrix materials appear as particles (10–20 nm in diameter) and a few fine fibrils (5.0–10.0 nm in diameter).

Fig. 6 demonstrates two cells with differing affinity for osmium/K₄Fe(CN)₆. The upper cell is thought to represent an earlier stage of cytodifferentiation than the lower (the more electron-dense cell). In the former cell, the nucleus exhibits characteristic features of mesenchyme. Mitochondria are spherical to elongated, glycogen is prevalent, cisternae of granular ER contain stainable products and cell borders are in focal association with filopodia of adjacent cells. In the latter cell, the nucleus has more condensed chromatin and an electron-dense nucleoplasm. The cytoplasm exhibits perinuclear Golgi complexes and higher magnification (Fig. 7) reveals that membranes of the Golgi cisternae appear more electron-dense than other vesicles within the Golgi center. Fig. 8 illustrates the presence of dense particulates (65 nm in diameter) which are prevalent in the cytoplasm of the differentiating cell population by stage 17.

**Figures 1–3**

Fig. 1 (stage 18). Cells at the growing tip of digital cartilage after immersion for 2 h in osmium tetroxide/K₄Fe(CN)₆ at 4 °C (see methods). Note electron density of cells which have acquired affinity for osmium/K₄Fe(CN)₆. Enhanced contrast is thought to reflect at least in part the presence of acid glycosaminoglycan synthesized by chondroblasts. Note cells showing three different electron densities: (1) mes, mesenchymal cell; (2) e.ch., early chondroblast; (3) m.ch., mature chondroblast surrounded by extracellular matrix (                     ) \( \times 12,500 \). Inset illustrates limb development at (A) stage 15; (B) stage 16; (C) stage 17; (D) stage 18. Upper limb, left; lower limb, right.

Fig. 2 (stage 16). Prechondral mesenchyme in developing digital plate (see inset). Cells have not developed an affinity for osmium/K₄Fe(CN)₆. Broad cell to cell contacts are present. Arrow designates a single cilium. \( m \), Mitochondrion; \( n \), nucleus; \( \mu \mu \), nucleolus. \( \times 5,500 \).

Fig. 3 (stage 16). Zone of broad intercellular abutment. Distance between cells is approximately 20 to 25 nm. Note electron-dense material between apposing cell membranes. Arrows denote microfilaments in a particulate cytoplasm. \( ger \), Granular endoplasmic reticulum; \( n \), nucleus. \( \times 50,000 \).
It is difficult to determine whether these represent rosettes of glycogen (Revel, Napolitano & Fawcett, 1960) or polysomes, their immediate product being heavily stained by OsO$_4$/K$_4$Fe(CN)$_6$.

Stage 18

A metachromatic matrix is visible with the light microscope in digital blastemata at this stage. Electron microscopy reveals that chondrogenic cells have acquired affinity for the osmium/K$_4$Fe(CN)$_6$ complex and exhibit features of chondroblasts (Fig. 9). Cells are generally separated from their neighbors although areas of close association remain. Nuclei are particulate and homogeneous in appearance and lipid droplets appear in the cytoplasm. The cell profile in Fig. 10 exhibits a prominent nucleolus containing electron-dense portions within a granular core. At higher magnification (Fig. 11), these structures assume an irregular, circular shape measuring approximately 330 nm in diameter. The particulate components of the nucleolus are about 20 nm in diameter and are thought to be ribonucleoprotein. The cytoplasm (Fig. 10) exhibits cisternae of granular ER which contain a fibrillar product. Glycogen is abundant.Microfilaments are present throughout the cytoplasm at this stage but microtubules are generally perinuclear in location (Fig. 11). Extracellular matrix (Fig. 12) consists of large particles (15–20 nm in diameter), smaller particles (approximately 10 nm in diameter) and a network of fine filaments (10–12 nm in diameter). Thought to consist of procollagen, the filamentous structures are frequently decorated with both large and small particles. Bundles of 10–15 nm filaments are occasionally seen in close association with surfaces of chondroblasts (Fig. 13) but similar clumps have not been observed in the developing cartilage matrix.

Figures 4–8

Fig. 4 (stage 17). Cells of developing digital blastemata (see inset). Prechondral cells with lesser electron density exhibit serrated borders (pm), euchromatic nuclei (n), and polyribosomes (circle). Cells with enhanced contrast are chondroblasts. Note the sparse particulate appearance of extracellular matrix (em). × 9000.

Fig. 5 (stage 17). Higher magnification of polyribosomes present in the cytoplasm of prechondral cells (circle, Fig. 4). × 75000.

Fig. 6 (stage 17). Two stages in the development of chondroblasts. Lower cell has greater affinity for OsO$_4$/K$_4$Fe(CN)$_6$ in both cytoplasm and nucleoplasm and may reflect an increased quantity of acid-glycosaminoglycan. Note prominent perinuclear Golgi center (gc) in lower cell. ger, Granular endoplasmic reticulum; m, mitochondrion; n, nucleus; nu, nucleolus. × 8500.

Fig. 7 (stage 17). Higher magnification of Golgi center illustrated in Fig. 6. Cisternae have stained with OsO$_4$/K$_4$Fe(CN)$_6$ (arrows). Compare contrast with membrane profiles of vesicles (v), mitochondria (m) and granular endoplasmic reticulum (ger). × 21 000.

Fig. 8 (stage 17). Electron-dense granules (arrows) 65nm in diameter present in cytoplasm of developing chondroblasts. × 45 000.
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Autoradiography

Mesenchymal cells in forelimbs (stage 16) incubated for 1 h in \(^{3}\)H-proline exhibit modest activity which is most prominent in nuclei (Fig. 14). Silver grains over polysome-rich cytoplasm, granular endoplasmic reticulum and Golgi centers are sparse. Furthermore, extracellular spaces lack the presence of labeled (or visible) material.

By stage 17, cells which have acquired affinity for osmium/K\(_{4}\)Fe(CN)\(_{6}\) also incorporate labeled proline during the 1 h of incubation (Fig. 15) and silver grains are visible over the perinuclear cytoplasm, periphery of the nucleus and intercellular zones.

Pulse-chase experiments with early chondroblasts in digital blastemata at stage 17 (cf. Fig. 4) demonstrate prevalence of label over perinuclear cytoplasm (Fig. 16) and Golgi centers (Fig. 17) after 30 min of culture. Two hours after removing blastemata from labeled medium, the majority of silver grains are extracellular (Fig. 18) overlying the particulate matrix (cf. Fig. 12).

Discussion

When an aqueous solution of osmium tetroxide interacts with the mild reducing agent, potassium ferrocyanide, presumably the osmium is partially reduced and forms a complex with the ferrocyanide (Karnovsky, 1971). Under different valence conditions, potassium ferricyanide \([K_{3}Fe(CN)e]\) becomes a strong oxidizing agent and has been used recently by de Bruijn (1973) to

Figures 9–13

Fig. 9 (stage 18). Chondroblasts of digital blastema (see inset) and associated extracellular matrix (em). All cells have acquired affinity for osmium/K\(_{4}\)Fe(CN)\(_{6}\) at this stage. Note bundles of filaments (arrows) in the developing matrix. \(l\), Lipid droplet; \(n\), nucleus. \(\times 5500\).

Fig. 10 (stage 18). Mature chondrocyte exhibiting density-enhanced nucleoplasm and cytoplasm, euchromatic nucleus \((n)\), nucleolus \((nu)\), cisternae of granular endoplasmic reticulum \((ger)\), glycogen \((g)\) and vacuole \((v)\) containing components with features of extracellular matrix \((em)\). \(\times 12500\).

Fig. 11 (stage 18). Circular, electron-dense structures in nucleolus of a mature chondrocyte. Particulate components of nucleolus are thought to be ribonucleoprotein. Note perinuclear microtubules \((mt)\) and microfilaments \((mf)\). \(\times 24500\).

Fig. 12 (stage 18). Components of extracellular matrix are (1) large particles \((p)\), 15–20 nm in diameter; (2) smaller particles \((sp)\), 10 nm in diameter; and (3) filaments \((f)\), approximately 10–12 nm in diameter associated with both large and small particles. Particles may be acid mucopolysaccharide whereas filaments are thought to consist of procollagen. \(\times 50000\).

Fig. 13 (stage 18). Bundle of 10–15 nm filaments (arrows) at periphery of chondroblast which may represent 'ecdysis' during fibrillogenesis. Alternate interpretations include degeneration of a cilium during cytodifferentiation of cartilage. \(\times 54500\).
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selectively stain for glycogen. Although the chemical nature and mechanism of staining of these iron-cyanide complexes remain unclear, the empirical use of \( \text{OsO}_4/K_4\text{Fe(CN)}_6 \) enhances the electron density of structures known to contain acid mucopolysaccharide (e.g. enteric glycocalyx and intercellular material; Kelley, 1973).

Chondrocytes are essentially differentiated forms of mesenchymal cells common to all connective tissues, but the principal distinguishing characteristic of cartilage is its ability to synthesize and secrete an extracellular matrix containing a high concentration of acid mucopolysaccharide (glycosaminoglycan) and collagen (Revel & Hay, 1963; Jackson, 1964). Shatton & Schubert (1954) and Partridge & Davis (1958) reported that the acid glycosaminoglycans chondroitin sulfate A and C comprise 15–20 % of the dry weight of hyaline cartilage. Furthermore, Meyer (1958) identified an additional mucopolysaccharide (termed keratosulfate) in human hyaline cartilage. Collagen is synthesized by a series of steps, one of which is the hydroxylation of prolyl and lysyl residues in the three polypeptide chains of the procollagen molecule (Grant & Prockop, 1972). The enzyme responsible for this step, prolyl hydroxylase, is localized in cisternae of granular endoplasmic reticulum (Olsen, Berg, Kishida & Prockop, 1973). Fernández-Madrid (1967) described the basic procollagen unit as a rod 280 nm in length and approximately 1.4 nm in diameter. Cartilage collagen is composed of three identical \( \alpha 1 \) type II chains (Miller & Matukas, 1969) and is the principal type synthesized by developing chondrocytes in the embryonic chick limb (Linsenmayer, Toole & Trelstad, 1973). Unfortunately, little is known of the structural association of collagen fibrils with the acid glycosaminoglycans of developing cartilage matrix, although it is clear that these two

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**Figures 14-18**

Fig. 14 (stage 16). Prechondral mesenchyme in digital plate after incubation for 1 h in L-proline-2,3-\(^3\)H. Incorporation is slight, localized in nuclei and may reflect non-collagenous protein synthesis. Not all cells of the digital plate are labeled. gc, Golgi center; m, mitochondrion; n, nucleus. × 12 500.

Fig. 15 (stage 17). Prechondral mesenchyme in digital blastema after incubation for 1 h in labeled proline. Increased numbers of silver grains are present over perinuclear cytoplasm, indicating initiation of collagen synthesis. Labeled cells have acquired affinity for osmium/K\(_4\)Fe(CN)\(_6\). × 12 500.

Fig. 16 (stage 17). Electron-dense digital chondroblast prepared for autoradiography 30 min after pulse-labeling with radio-isotope. Predominance of label is in perinuclear cytoplasm. × 11 500.

Fig. 17. Perinuclear Golgi complex and associated vesicles 30 min after pulse-labeling with \(^3\)H-proline. × 20 500.

Fig. 18 (stage 17). Hand plate prepared 2 h after pulse-label with L-proline-2,3-\(^3\)H. Silver grains are present over developing extracellular matrix. Note presence of electron-dense particles (arrows). × 11 500.
moieties are more than casually related in both embryonic (Kelley & Bluemink, 1974) and adult (Pease & Bouteille, 1971) matrices.

Since hydroxyproline occurs principally in collagen (Solomon, 1965), cells which incorporate $^3$H-proline may help to correlate synthesis and secretion of collagen precursors with developmental stage and ultrastructure. Fernández-Madrid (1967) reported synthesis of procollagen on polyribosomes of the granular ER and it is probable that the electron-dense material visible in cisternae of the ER in the present study contains procollagen. Although human limb mesenchyme in vitro secretes quantities of collagen (Kelley et al. 1973), prechondral cells during stage 16 (Fig. 14) incorporate little labeled precursor. This pattern changes by stage 17 (Fig. 15) and correlates with the appearance of increased numbers of membrane bound and free polyribosomes (Fig. 5). Hence initiation of synthesis of collagenous precursors may be dependent upon factors affecting polyribosome formation in limb mesenchyme.

The site of initial collagen formation during chondrogenesis is controversial. Godman & Porter (1960) suggest that intact filaments of procollagen are shed by ‘excortication’ into extracellular spaces and serve as foundations for attachment of other collagen molecules. Direct continuity of endoplasmic reticulum with the plasma membrane has been proposed (Ross & Benditt, 1965; Ross, 1968) and Goel (1970) presents one of his micrographs with this interpretation. One might expect to see ribosomes attached to the inner leaflet of the plasma membrane if the proposed mechanism was universal during export of collagen. On the other hand, autoradiographic studies (Revel & Hay, 1963) indicate that soluble precursors of collagen are concentrated in vesicles of the Golgi complex prior to release from chondroblasts. Vesicles fuse with the cell membrane and contents hence become extracellular. Observations reported in the present investigation (Figs. 16 and 17) agree with the results of Revel & Hay. During stage 17, when cells are observed to incorporate labeled proline, those tissues removed from medium 30 min after pulse-label exhibit silver grains over Golgi centers and associated vesicles. After 2 h, label is predominant over developing extracellular matrix. If $^3$H-proline is incorporated into procollagen at the level of the polyribosome, it is proposed that the granular ER-Golgi center-vesicle mechanism of merocrine secretion is used during release of collagenous protein from chondrogenic cells in the developing human limb.

Alterations of ultrastructure observed during chondrogenesis in man are in general agreement with the reports of Goel (1970) and Searls et al. (1972) in the chick embryo. Fine structure of predigital mesenchyme in embryonic human limbs (Kelley, 1970) is similar to that observed in the hand plate (stage 16) prior to condensation of digital blastemata. Granular endoplasmic reticulum is sparse and free ribosomes and mitochondria populate a particulate cytoplasm. The appearance of polysomes in cells of the digital blastemata during stage 17 may correlate with synthesis of procollagen (previously discussed) and the subsequent appearance of serrated cell borders which may reflect merocrine
secretion (Godman & Porter, 1960). Changes in intercellular association occur between stages 16 and 17 (Figs. 2, 4 and 6). The amount of extracellular space varies with concomitant decrease in broad cell to cell attachments (Fig. 4) and appearance of filopodia (Fig. 6).

By stage 18 at least three components are visible in the developing matrix (Fig. 12): (1) large particles, 15–20 nm in diameter; (2) smaller particles, approximately 10 nm in diameter; and (3) filaments, approximately 10–12 nm in diameter. Particles exhibit affinity for osmium/K$_4$Fe(CN)$_6$ and are thought to represent acid mucopolysaccharide (see Matukas et al. 1967) whereas filaments may be small aggregates of procollagen or procollagen-proteoglycan complexes. The particulate components of extracellular matrix become visible early in stage 17 when chondrogenic cells acquire an affinity for osmium/K$_4$Fe(CN)$_6$ (Fig. 4). Filamentous material is sparse at this stage, suggesting that mucopolysaccharide secretion may precede export of collagen during chondrogenesis. This hypothesis agrees with autoradiographic results presented in this study. Incorporation of labeled precursors of collagen is scanty in cells not stained by ferrocyanide-containing osmium (Fig. 15), whereas synthesis and secretion of labeled materials are localized only in cells which have developed affinity for the osmium/K$_4$Fe(CN)$_6$ complex. Furthermore, particles thought to represent mucopolysaccharide are present in the matrix upon the arrival of labeled procollagen (Fig. 17).

Interpretation of matrix components is complicated by the occasional appearance of bundles of fibrils (10–15 nm in diameter) adjacent to cell surfaces but within the extracellular space (Fig. 13). Similar micrographs have been presented by Godman & Porter (1960), Goel (1970) and others as examples of 'excortication' and 'ecdysis'. Since cilia are frequently seen in mesenchymal cells (arrow, Fig. 2) and chondrocytes (Scherft & Daems, 1967) and since disruption of the cell membrane (Fig. 13) is often associated with 'ecdysis', an alternative interpretation would be that such fibrils are components of a deteriorating cilium rather than evidence of a mechanism of fibrillogenesis. However, whether or not cilia deteriorate normally during chondrogenesis requires further study.

The present observations indicate that early structural differentiation of prechondral mesenchyme correlates closely in time with the appearance of protein-polysaccharides of the future matrix. Mesenchymal cells of the digital plate (stage 16) lack affinity for osmium/K$_4$Fe(CN)$_6$ but acquire the staining characteristics of chondroblasts early in stage 17 (during the cellular condensation which established the presence of digits).

It can be concluded that the fine structural and functional features of chondrogenesis begins early in stage 17 prior to the appearance of metachromatic matrix (stage 18). Furthermore, it is reasonable to suggest that the increased affinity of chondroblasts, chondrocytes and matrix components for osmium/K$_4$Fe(CN)$_6$ (Figs. 1, 4 and 9) may be associated with increased amounts of
protein-polysaccharide within and between those cells. If true, development of increased electron density in cells of the digital blastemata may reflect the appearance of molecules which accompany chondrogenesis.

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