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ORIGINAL ARTICLE

TEM and SEM observations on the extracellular matrix of the developing murine centra

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TEM and SEM application demonstrated that the shift from chondrification to ossification in the developing murine centra from day 15 to day 18 of gestational age is marked by typical structural variations of the extracellular matrix (ECM). During day 15 GA, typical matrix vesicles with crystalline contents appeared, as followed by single and fusing pleomorphic aggregates of a more regular crystalline structure. During days 17/18 GA, these structures disappeared, and the ECM now exhibited a network of collagen fibrils that had been less conspicuous before. During the time period studied, the ECM switched from a more acid (proteoglycans) to a rather neutral (glycoproteins) milieu.

key words: development centrum, extracellular matrix, mice, TEM, SEM, ontogenesis, vertebral column

INTRODUCTION

The vertebral column is the most conspicuous metameric system of the vertebrate body. The vertebrae emerge from the paraxial mesoderm that had been previously segmented into somites. Somatic cells surround the spinal cord and the chorda dorsalis (notochord) to finally differentiate into cartilage and bone, whereby the vertebrae and intervertebral discs arise from the most ventral part of the somite, the sclerotome. Moreover, morphological features distinguish different types of vertebrae (cervical, thoracic, lumbar, coccygeal) along the most anterior axis [16]. In this connection, it has to be emphasised that the knowledge about the normal development of the lumbar vertebral column, in particular, is still relatively small regarding mammals, and such important laboratory animals like mice. This is in contrast to other animals used in basic ontogenetical research, such as the chicken or the quail [6, 7, 14, 16], or even humans [3, 5, 23].

The present study was designed to give first information about some ultrastructural aspects of this problem in mice, taking into consideration that the shift from chondrification to ossification in the cellular population of the early vertebrae is one of the most important features in vertebral column development. In mice, this shift generally takes place between day 15 and day 18 of gestational age [21]. We have chosen the extracellular matrix as an example and characteristic of this phenomenon, because its typical and varying constituents — in our opinion — reflect unequivocally any change in the course of centra development.

MATERIAL AND METHODS

Mice embryos (Han: NMRI; SWISS) of 15, 16, 17, and 18 days of gestational age (GA) (vaginal plug at day 0) were used in this study. The material was not decalcified. For light microscopy and scanning electron microscopicy (SEM), total embryos were fixed in neutral buffered 5% formalin for one week, before they

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were processed with regard to specific purposes. For transmission electron microscopy (TEM), the lumbar vertebral column was dissected out under microscopical control from three embryos of each day of the gestational age mentioned beforehand. This tissue was then minced into 1 mm³ cubes and fixed for 8 hours in a mixture containing 2% formaldehyde, 2.5% glutaraldehyde, and 2 mg/ml $CaCl_2$, in 0.1 M sodium cacodylate — HCl buffer (pH 7.3) at 4°C. After rinsing in this buffer at 4°C, the tissue specimens were postfixed in 1% osmium tetroxide in aqua dest., or in osmium tetroxide-reduced potassium ferrocyanide [13] for 1.5 hours. After dehydration in graded ethanol (25–100%, 4°C), the specimens were embedded via propylen oxide in Epon 812 [15]. Ultrathin sections and semithin sections were cut with a diamond knife on an ultramicrotome (Reichert, Ultracut E). The semithin sections for the choice of relevant TEM areas were stained with 0.1% toluidine blue. The ultrathin sections were transferred to copper grids that had been coated with formvar, routinely stained with lead citrate and uranyl acetate, and viewed in an electron microscope (Zeiss 10 C/R) at 80 kV. For SEM preparation, the formalin fixed embryos were carefully cut in two at the lumbar re-

gion, the parts were then rinsed for several hours in 0.1 M PBS at 4°C, dehydrated in ethanol (25–100%, 4°C), and afterwards critical-point-dried through CO_2 in a drying apparatus (EMITECH K850). Finally, all specimens were sputtered with gold in a sputter coater (Edwards S 150 B), and viewed in a scanning electron microscope (LEO 435 VP) at 15 kV.

For control of the basic type of glycoconjugate milieu of the extracellular matrix during the development of the lumbar vertebral column, formalin-fixed embryos were embedded in the paraffin mixture Paraplast plus (Sherwood), with the help of an embedding system (Shandon, Hypercenter 2). 5 μm serial paraffin sections were then routinely processed to obtain a good alcianblue-PAS staining [17], using alcianblue 8GX (Sigma) and Schiff«s reagent (Merck). Photos were taken with a photomicroscope (Zeiss photomicroscope II).

RESULTS

The ventral centra could first be differentiated from the surrounding connective tissue about day 14 GA, because it was not until this time that the chondrification of the originally mesenchymal cells proceeded quite rapidly (Fig. 1). The centra consisted of hy-
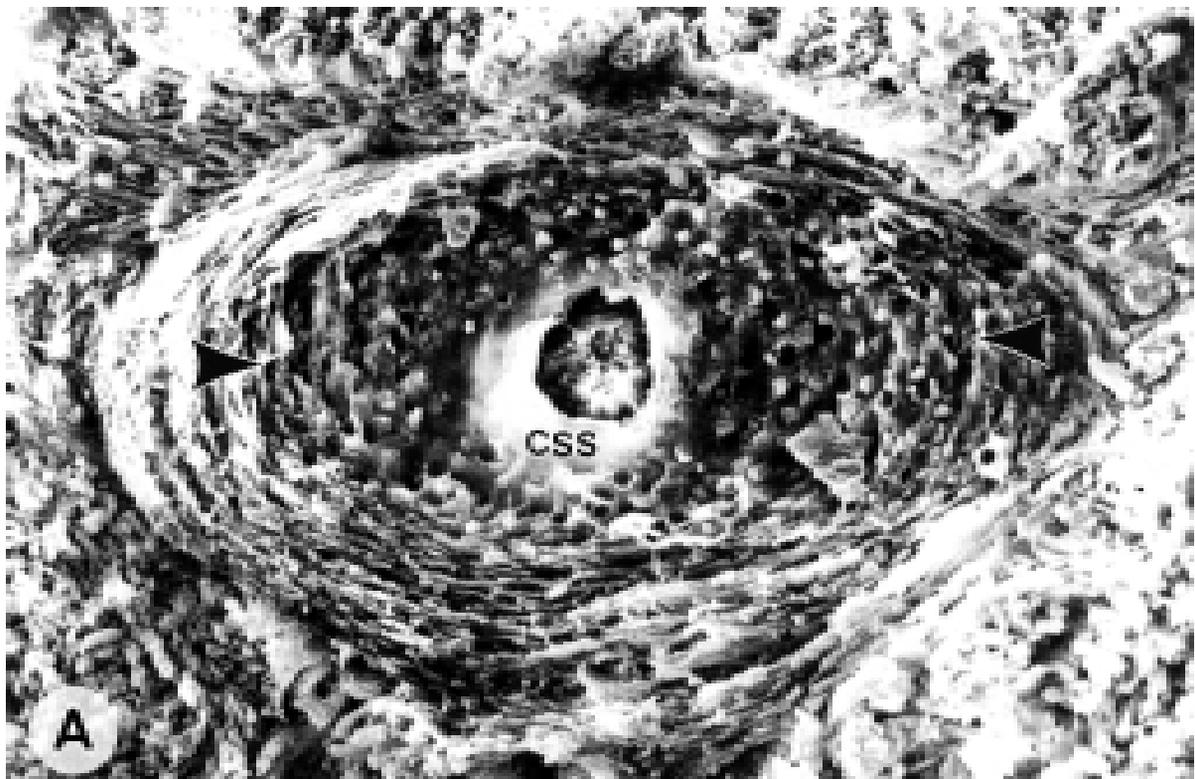
Figure 1. SEM; frontal section of the lumbar vertebral column; mouse day 17 GA; the two regions of the developing vertebrae studied are visible laterally to the chorda (notochord), and are marked by arrows; the chorda sheath system (CSS) is very distinct; × 2900.

aline cartilage and their surface exhibited a perichondrium, the numerous small and roundish chondrocytes, however, had not yet formed chondrons. The remnants of the chorda (notochord) within the developing centra were somewhat distended in the regions of the anlagen of the intervertebral discs. The extracellular matrix (ECM) between the chondrocytes showed an alcian blue-positive reaction, in contrast to the still alcian blue-negative connective tissue. PAS-positive staining was detectable in the chorda (notochord) cells, the chondrocytes, and the cells of the perichondrium. The chorda (notochord) cells were separated from the hyaline cartilage of the centra by a non-cellular alcian blue-positive sheath region (Fig. 2).

Beginning with days 14/15 GA, TEM revealed somewhat hypertrophic chondrocytes with vesicles adjacent to the plasma membrane, a few partly dilated cisterns of the rough endoplasmic reticulum, and several roundish mitochondria (Fig. 3A). The ECM between the large chondrocytes was mainly filled with numerous pleomorphic and membrane-bound vesicles (matrix vesicles). Some of these contained elongated crystalline and electron dense structures, others had amorphous electron dense material tightly adhering to both sides of the vesicle membrane (Fig. 4A). The vicinity of the matrix vesicles varied strongly in its structural composition, i.e. most of the vesicles were located near to thin collagen fibrils (Fig. 4B), and occasionally the vesicles were also found within a fine network of branching filaments. Such observations were first obvious close to the so-called chorda (notochord) sheath system within the developing vertebrae.
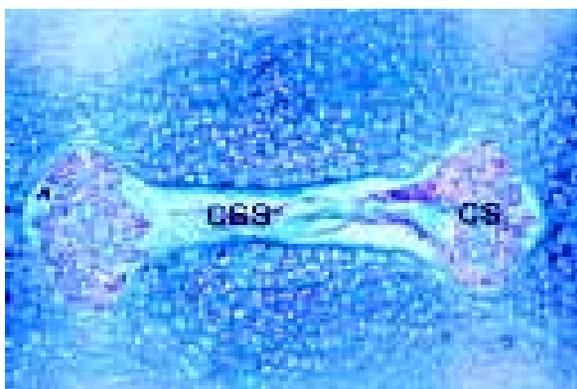
Figure 2. LM; longitudinal section of one developing vertebra in the lumbar vertebral column, showing also the rope-like chorda sheath system (CSS) deficient in cells, as well as two chorda segments (CS) rich in cells, that differentiate into intervertebral disks; mouse day 15 GA; paraffin section, alcianblue-PAS reaction; × 120.

During day 16 GA, pleomorphic aggregates could be detected in the ECM of the central cartilage parts, amongst hypertrophic chondrocytes (Fig. 5). These aggregates were visible as single systems, occasionally, however, they fused to form larger units. The crystalline structures described before, were now detected preferably at the periphery of the pleomorphic aggregates as being arranged radially (Fig. 5B). The aggregates, moreover, were associated frequently, but to a varying degree with matrix vesicles, the contents of which appeared predominantly homogeneous, and in some cases also crystalline. In the vicinity, as well as in the aggregates themselves, single thin collagen fibrils became obvious.

During day 17 GA, the most important changes took place within the centra, i.e. most of the chondrocytes had disappeared from the central parts, and only remnants of aggregates were found in the ECM. Now many osteoblasts could be discerned, together with some osteoclasts. The young and still roundish osteoblasts were lying in a fine and dense network of thin collagen fibrils (Fig. 6A, B).

During day 18 GA, TEM observations exhibited a strongly electron dense ECM material that trabecula-like interwove the newly constructed bony centres of the vertebral centra. At the surface of this bony structure, relatively thick collagen fibrils could be observed that were orientated into different directions and seemed to form bundle-like systems (Fig. 6C). Such typical and bone dependent structural development that had started about day 17 GA, was best documented by the SEM preparations made. Light microscopically, the collagen of the still tiny bone trabeculae reacted distinctly PAS-positive. The osteoblasts found were characterised by several cisterns of the rough endoplasmic reticulum in a parallel arrangement. Additionally, numerous polysomes, a distinct Golgi apparatus, single electron dense vesicles, and a cell nucleus with euchromatin were obvious in this cell type (Fig. 3B). The hypertrophic chondrocytes were restricted to the periphery of the ossified central parts and showed decreasing amounts of glycogen granules. Light microscopically this aspect was corroborated by a weak PAS staining intensity.

DISCUSSION

TEM and SEM applications have clearly demonstrated in the course of this study that the shift from chondrification to ossification in the developing murine centra from day 15 to day 18 GA is marked by typical structural variations of the extracellular
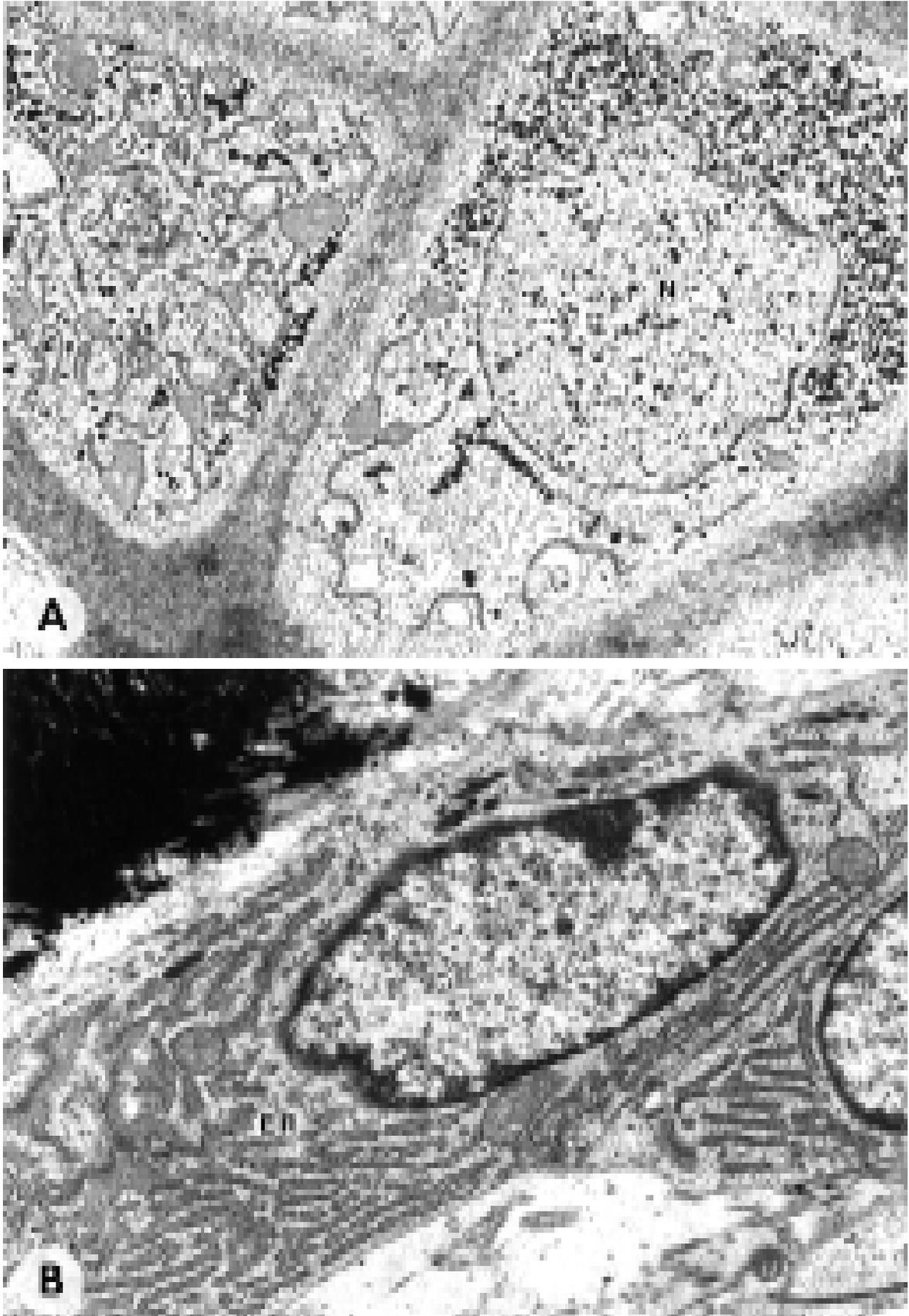
Figure 3. TEM; **A.** Hypertrophic chondrocytes with numerous glycogen granules (G) and a nucleus (N) with euchromatin; mouse day 16 GA; postfixation with reduced osmium tetroxide; × 6300; **B.** Oblong maturing osteoblast of the calcified peripheral border zone of the centra, with a distinct rough endoplasmic reticulum (ER); mouse day 18 GA; × 12400.
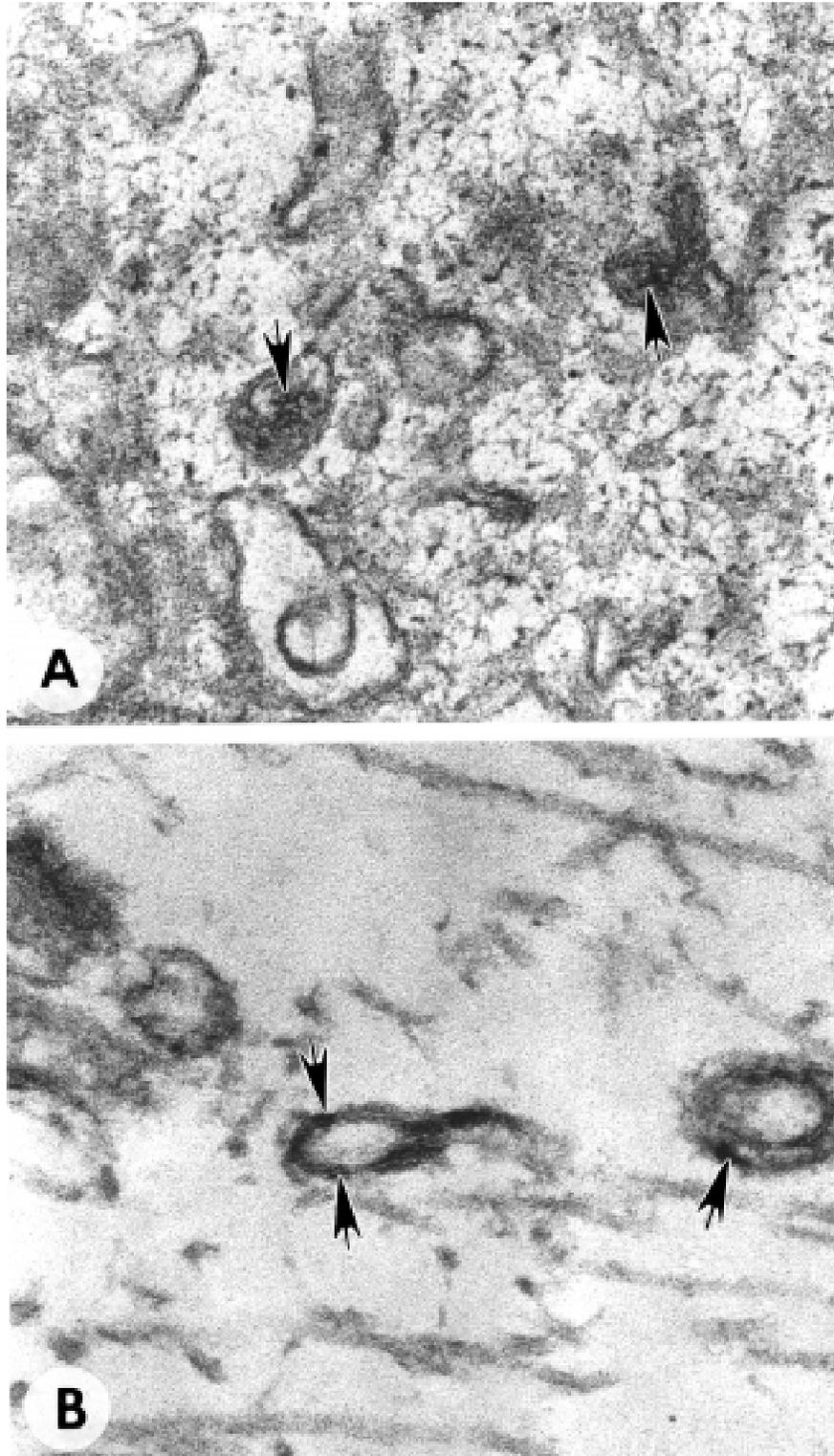
Figure 4. TEM; **A.** Matrix vesicles in a network of fine filamentous structures, intravesicular crystals are marked by arrows; mouse day 15 GA; × 126000; **B.** Matrix vesicles among thin collagen fibrils, amorphous electron dense accumulations adhering to the vesicle membrane are marked by arrows; mouse day 15 GA; × 160000.
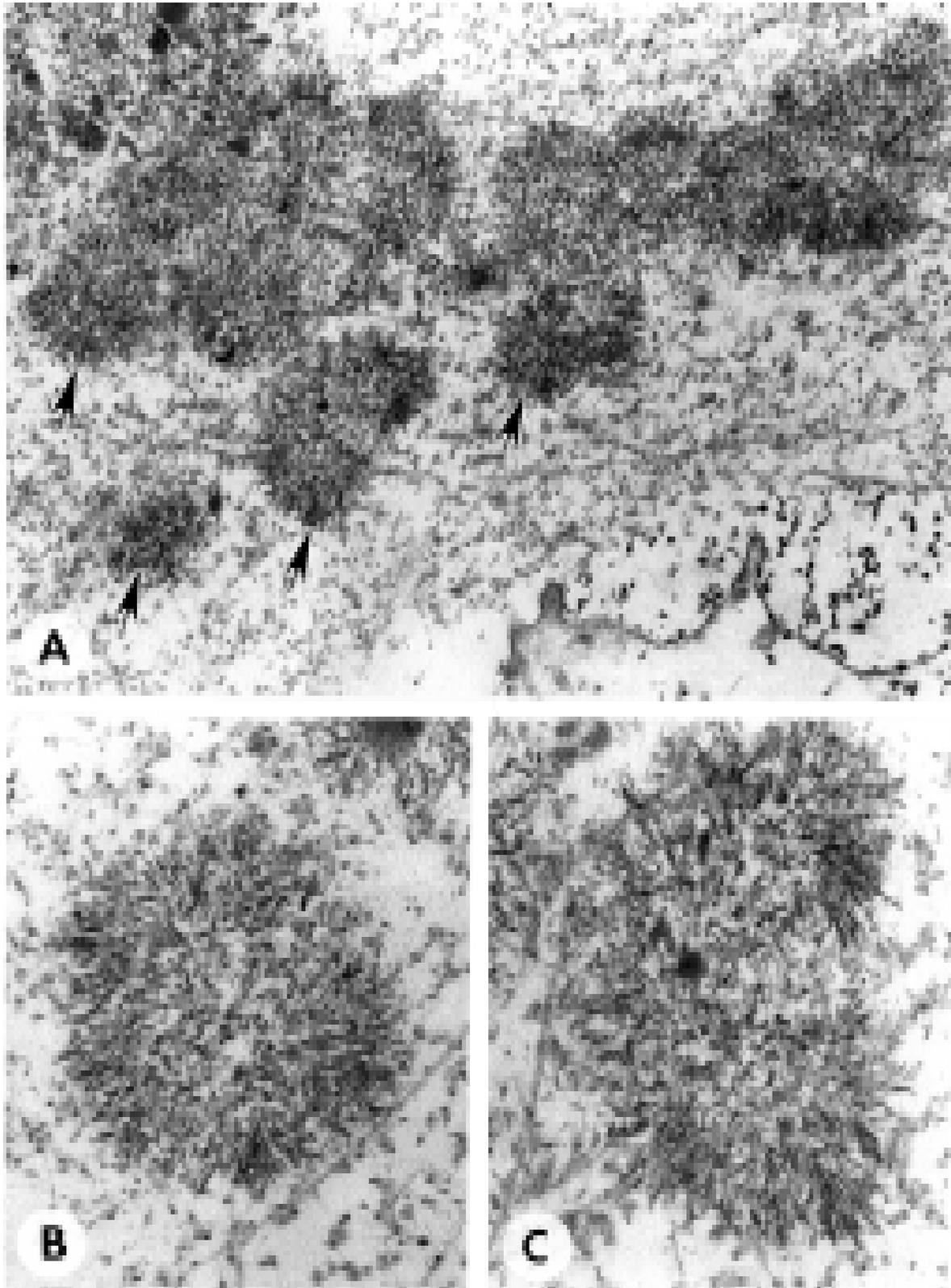
Figure 5. TEM; **A.** Grouping of pleomorphic aggregates (arrows) that contain predominantly crystalline structures; mouse day 16 GA; × 40000; **B** and **C.** Variations of aggregate morphology, aggregates with distinct peripheral accumulation of crystalline structures; mouse day 16 GA; B: × 100000, C: × 80000.
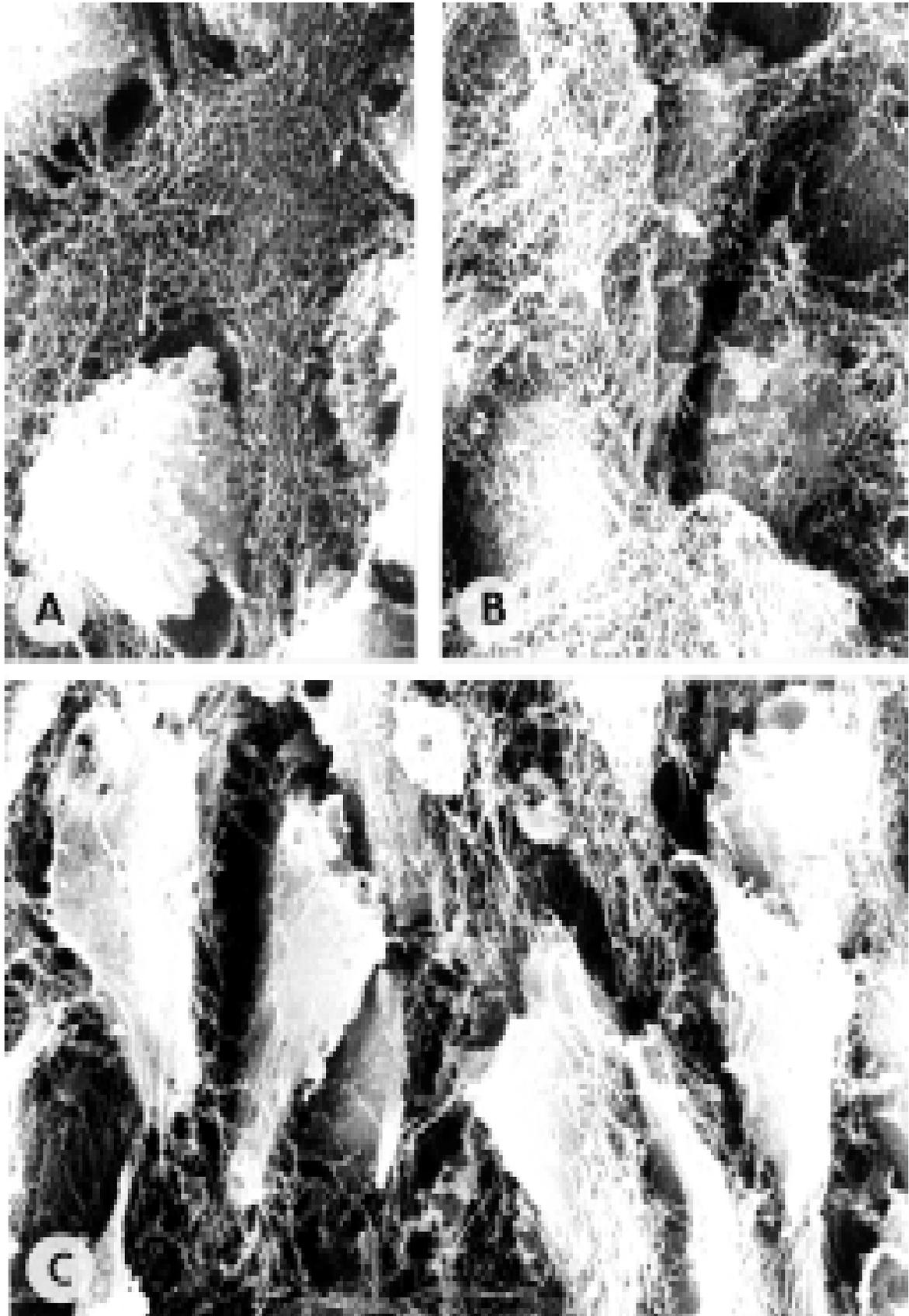
Figure 6. SEM; **A** and **B.** Distinct network of fine collagen fibrils surrounding young osteoblasts; mouse day 16/17 GA; A: × 5300, B: × 4600; **C.** Coarse network of thicker collagen fibrils surrounding maturing osteoblasts; mouse day 18 GA; × 4800.

matrix (ECM). This was firstly documented during day 15 GA by matrix vesicles with crystalline contents, as followed by single and fusing pleomorphic aggregates of a more regular crystalline structure. During days 17/18 GA, these structures more or less disappeared, and the ECM now exhibited increasing amounts of collagen fibrils that had been less conspicuous before. From the light microscopical/histochemical point of view, the ECM basically switched from a more acid (proteoglycans) to a rather neutral (glycoproteins) milieu.

With respect to all the components of the ECM involved, it seems quite reasonable that the crystalline and vesicle-bound electron dense structures seen first are hydroxyapatite, as also postulated for developing mineralisation in long bones [1, 2, 4, 10, 20]. The amorphous substances found adjacent to the membrane of the matrix vesicles have to be looked upon as accumulations of calcium and phosphate ions necessary for the deposition of hydroxyapatite crystals. This idea is supported by biochemical findings of Peress et al. [19] and Glaser and Conrad [9], emphasising the high affinity of membrane phospholipids for calcium ions. Together with other information available in this context [4, 11, 12, 22], it becomes quite evident that the matrix vesicles produced by the hypertrophic chondrocytes are initiating features of mineralisation. The subsequently arising crystalline aggregates in the ECM, thus, are the next normal step towards a regular mineralisation process, that afterwards proceeds along the collagen fibrils demonstrated in increasing amounts. The now also intensifying ossification is additionally characterised by a shift from collagen II and IX to collagen I in the ECM. Such an event could be markedly accelerated by the resorption of the first two collagen types by hypertrophic chondrocytes of the early centra, before or even when osteoblasts are active [18].

It is of general interest, moreover, that the first hydroxyapatite crystals are formed in direct contact with the chorda (notochord) sheath system, that is a relic of the original chorda. In this way, inhibiting properties of the (aging?) chorda (notochord), with regard to the differentiation of mesenchymal cells into chondrogenic tissue, are indirectly corroborated [8, 16].

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