Regulated changes in chondroitin sulfation during embryogenesis: an immunohistochemical approach

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ABSTRACT Chondroitin sulfate proteoglycans, which represent the main class of non-fibrous macromolecules found in the extracellular matrix of connective tissues, have been implicated in the control of a variety of cell activities during ontogenesis. The respective contributions of the chondroitin sulfate chains and of the protein moiety of the proteoglycan in morphogenesis and cytodifferentiation are not known. In this context, monoclonal antibodies identifying specific chondroitin sulfate chains are interesting new tools. A panel of well characterized monoclonal antibodies recognizing distinct epitopes present only in chondroitin sulfate chains was used in conjunction with immunohistochemical techniques for the purpose of identifying and mapping chondroitin sulfate isoforms during development in the mouse and rat fetus. Expression of chondroitin sulfate isoforms occurred in the tissues according to specific spatio-temporal patterns, suggesting that chondroitin sulfates differing in sulfation position and degree perform distinct functions in development.

KEY WORDS: *chondroitin sulfates, proteoglycans, odontogenesis, monoclonal antibodies, immunohistochemistry*

Introduction

Immunochemical studies with monoclonal antibodies (Mabs) have shown that a number of epitopes exhibiting stage-specific patterns of expression during embryogenesis are carbohydrate structures occurring in glycoproteins and glycolipids (Childs *et al.*, 1983; Feizi and Childs, 1987; Muramatsu, 1988; Thorpe *et al.*, 1988). Chondroitin sulfates (CS) are polysaccharides which characterize a family of proteoglycans designated as chondroitin sulfate proteoglycans (CSPG). Recently, several monoclonal antibodies recognizing distinct epitopes on CS chains have been developed. Employing some of these probes in conjunction with indirect immunofluorescence or immunoperoxidase labeling, it was possible to visualize developmental changes in chondroitin sulfation in rat and mouse fetuses (Mark *et al.*, 1989, 1990). Most of the data presented in this review will concern the tooth germ which is used as a model system in our laboratory.

Structure of chondroitin sulfates

Glycosaminoglycans are polysaccharides of variable length composed of repeating disaccharide units one residue of which is always hexosamine (Sharon, 1986). Four major types of glycosaminoglycans occur in mammalian tissues: hyaluronic acid, keratan sulfate, the heparan sulfate/heparin type and the chondroitin sulfate/dermatan sulfate (CS/DS) type (reviews: Lindahl and Höök, 1978; Scott, 1988; Conrad, 1989).

Glycosaminoglycans of the CS/DS type are constructed from uronic acid-N-acetyl-D-galactosamine disaccharide units. Generally 80 - 90% of the disaccharide units are sulfated either at C-4 or at C-6 of the N-acetyl-D-galactosamine (GalNAc) residues. The uronic acid can either be D-glucuronic acid or L-iduronic acid. CS, by definition, contains no L-iduronic acid. In earlier studies, two isomers of CS were distinguished: chondroitin-4-sulfate (C4-S) and chondroitin 6-sulfate (C6-S), containing sulfate ester groups on the fourth and on the sixth carbon of the GalNAc residues respectively. However, it is now apparent that most CS chains are copolymers in which the sulfate group occupies one or the other isomeric position (Kimata *et al.*, 1974). DS is an isomer of CS in which a variable portion of the D-glucuronic acid residues have been epimerized into L-iduronic acid residues. Glycosaminoglycans of the CS/DS type may also contain a small percentage of 4,6 disulfated GalNAc residues. In addition, D-glucuronic acid residues or L-iduronic acid residues can be sulfated at either position 2 or position 3 (see Suzuki *et al.*, 1968). CS and DS chains are linked to proteins by means of a glucuronosyl-galactosyl-galactosyl-xylosyl-O-serine sequence (Roden and Smith, 1966).

Abbreviations used in this paper: CS, chondroitin sulfate; C4-S, chondroitin 4-sulfate; C6-S, shondroitin 6-sulfate; CSPG, chondroitin sulfate proteoglycan; DS, dermatan sulfate; ECM, extracellular matrix; GalNAc, N-acetyl-D-galactosamine; Mab, monoclonal antibody.

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In the tissues CS and DS occur as CSPG which are composed of one or more glycosaminoglycan chains covalently attached to a core protein.

Functional aspects of chondroitin sulfates: the proteoglycans

CSPG are exceptionally diverse: this is due both to the number of different core proteins and to the considerable variety of post-translational modifications required to construct the macromolecule (Hassell *et al.*, 1986). CSPG are ubiquitous components of the extracellular matrix (ECM) of connective tissues and are also found at the surface of many cell types and in intracellular secretory granules (Reviews: Hassell *et al.*, 1986; Poole, 1986; Fransson, 1987; Ruoslahti, 1988).

This diversity of structure and distribution suggests a diversity of functions. For example, CSPG of cartilage have large hydrodynamic sizes and are reversibly compressible when subjected to a compressive load. These physico-chemical properties of CSPG provide hyaline cartilage with its essential properties of resiliency and stiffness (review: Hascall and Hascall, 1981). Moreover, CSPG interact specifically with collagen fibers *in situ* and modulate the rate of collagen fibril formation *in vitro* (Ruoslahti, 1988; Scott, 1988). For example, CSPG-collagen interactions might be critical for the dilatation of the uterine cervix (Kokenyesi and Woessner, 1989), for the visco-elastic properties of the aorta wall and for the transparency of the corneal stroma (review: Hascall and Hascall, 1981).

Besides this role in filling or organizing the ECM, certain CSPG might function as modulators of cell adhesion, cell migration, cell proliferation and cell differentiation during ontogenesis. Syndecan, an integral membrane proteoglycan containing CS and heparan sulfate chains, can promote the attachment of mouse mammary epithelial cells to collagen types I, III and V (Koda *et al.*, 1985) and to fibronectin (Saunders and Bernfield, 1988). On the other hand, those CSPG which do not occur as constituents of the cell membrane appear to inhibit cell adhesion (review: Ruoslahti 1989; Yamagata *et al.*, 1989). Interstitial CSPG are potent inhibitors of embryonic cell migration on fibronectin substrates (Perris and Johansson, 1987) and in three-dimensional collagen gel lattices (Funderburg and Markwald, 1986). Expression of recombinant CSPG "decorin" in Chinese hamster ovary cells inhibits cell proliferation (Yamaguchi and Ruoslahti, 1988).

Distinct molecular species of CSPG appear sequentially during chondrogenesis (Shinomura *et al.*, 1984; Kimata *et al.*, 1986) and myogenesis (Carrino *et al.*, 1988).

A large muscle-specific CSPG which is synthesized during early myogenesis in the chick is not synthesized by adult skeletal muscle *in vivo* nor by myotubes in advanced cultures (Carrino *et al.*, 1988). Similarly, prechondrogenic mesenchymal cells of embryonic chick limb buds produce a large CSPG, termed PGM, which is no longer expressed by mature chondrocytes (Kimata *et al.*, 1986). Syndecan, is expressed by embryonic mesenchymal cells only during definite periods of tooth or kidney morphogenesis (Thesleff *et al.*, 1988, 1989; Vainio *et al.*, 1989 a, b) and is absent from adult mesenchyme (Hayashi *et al.*, 1987).

In vitro experiments using ß-D-xyloside have provided further evidence for a role of CSPG in morphogenesis. ß-D-xyloside is a molecule which competes with the xylosilated protein core for CS/DS or heparan sulfate chain elongation, thereby uncoupling the proteoglycan core protein from glycosaminoglycan synthesis. ß-D-xyloside, when added to the culture medium of embryonic organs, inhibits branching morphogenesis in kidney and salivary glands from fetal mouse (Thompson and Spooner, 1982; Platt *et al.*, 1987) and disrupts feather pattern formation in chick embryo skin (Goetinck and Carlone, 1988). It has been shown, in the kidney model, that dose-dependent inhibition of morphogenesis by ß-D-xyloside did not involve heparan sulfate proteoglycans. (Platt *et al.*, 1987).

It is also of interest to note that transforming growth factor-ß (TGF-ß) is a selective inducer of CSPG synthesis in many cell types (Bassols and Massague, 1988) and can increase the ratio between CS and heparan sulfate chains on the core protein of syndecan (Rasmussen and Rapraeger, 1988). The high molecular weight receptor to TGF-ß is a proteoglycan which contains CS and heparan sulfate chains (Segarini and Seyedin, 1988). These observations suggest that some of the effects of TGF-ß on cell growth and cell differentiation are mediated by CSPG.

The respective contributions of glycosaminoglycan chains and core protein to the function of CSPG in ontogenesis are not known. The properties of CSPG such as binding large amounts of water and interacting with collagens and fibronectin depend primarily on the sulfate density of its glycosaminoglycan constituents (Ruoslahti, 1989). On the other hand, it is often accepted that the specific characteristics of a CSPG are primarily determined by the sequence of its core protein (see example: Krusius *et al.*, 1987; Zimmermann and Ruoslahti, 1989). However, it cannot be excluded that some interactions of CSPG with other molecules present in their environment might require precise monosaccharide sequences and sulfate substitution patterns of the CS chains (Casu *et al.*, 1988). It might therefore be assumed that variations in the structure of the glycosaminoglycan chain are of physiological significance. Since histochemical methods employing cationic dyes to visualize glycosaminoglycans on tissue sections (Scott, 1985) do not permit identification of CS of differing sulfation, Mabs recognizing specific determinants in CS chains are of great interest.

Monoclonal antibodies to chondroitin sulfates

Several Mabs recognizing determinants present in CS have been recently prepared (Jenkins *et al.*, 1981; Avnur and Geiger, 1984; Couchman *et al.*, 1984; Caterson *et al.*, 1985; Yamagata *et al.*, 1987; Sorrel *et al.*, 1988; Mark *et al.*, 1989) which provide new specific tools for identification and mapping of CS in tissues, when employed in association with immunohistochemical methods.

This possibility is particularly interesting in embryonic tissues where the amount of available materials is often too low for biochemical analysis. The characteristics of the anti-CS Mab employed in our laboratory for immunohistochemical investigations are summarized in Table 1.

This approach of localizing CS of differing sulfate substitution pattern in situ during ontogenesis is best exemplified with MC21C. MC21C is a Mab raised in our laboratory which specifically recognizes 6-sulfated segments in intact CS chains.

The MC21C-epitope is not detected in adult tissues but exhibits a site-restricted expression during development in rat and mouse fetuses.

MC21C identifies an embryonic isoform of C6-S

Immunoperoxidase staining of mouse fetuses at different ages

TABLE 1

MONOCLONAL ANTIBODIES (Mabs) TO CS

Mab designation	2B6	1B5	3B3	MC21C	MO-225
Immunogen	Chase-treated bovine articular cartilage CSPG	Chase-treated rat chondrosarcoma CSPG	As for 1B5	Rat bone proteins	PG-M of chick origin
Immunoglobulin subclass	IgG1	IgG1	IgM	IgM	IgM
Mab specificity	Unsaturated uronic acid-GalNAc 4-sulfate disaccharide unit	Unsaturated uronic acid-GalNAc disaccharide unit	Unsaturated uronic acid-GalNAc (6-sulfate) disaccharide unit	6-sulfated segments in intact CS chains	GlcA2-sulfate - GalNAc6-sulfate - containing determinant in intact CS chains
Classification for immunohistochemistry	anti-C4-S/anti DS	anti-chondroitin	anti-C6-S/anti-chondroitin	anti-C6-S	anti-C6-S
References	Couchman *et al.*, 1984; Caterson *et al.*, 1985			Mark *et al.*, 1989, 1990	Yamagata *et al.*, 1987

GlcA2-sulfate, D-glucuronic acid 2-sulfate; Chase, chondroitinase ABC; other abbreviations as in the text. Immunohistochemical staining with 1B5, 2B6 and 3B3 requires digestion of the histological sections with glycosaminoglycan-lytic enzymes, such as chondroitinase ABC in order to create an epitope which does not exist *in vivo*.

of gestation demonstrates that the MC21C-epitope is strongly expressed in embryonic skeletal tissues (bone, muscle, cartilage) and in organs undergoing epithelial-mesenchymal interactions (such as lung and intestines) but is progressively lost during maturation of these tissues and organs.

Expression of the MC21C-epitope correlates with morphogenesis

Specific examples concern bone, cartilage, lung and gut.

Bone

Strong staining with MC21C is observed at the peripheral, growing edge of the mandibular bone anlage in young mouse fetuses (days 14-16), when this tissue is rapidly expanding. In contrast, this staining is always weak or absent in the central region of the mandibular bone anlage (Fig. 1 a-e). The MC21C-epitope progressively disappears from all bone forming cells as morphogenesis proceeds (Fig. 1e). Preosteoblasts in the periosteum of long bones and vertebrae do not react with MC21C (Fig. 1f, g). This finding was not expected since bone forming cells in endochondral and membrane bones behave identically with respect to the timing of expression of ECM molecules, such as osteopontin, implicated in early bone formation (Mark *et al.*, 1988). However, there is an obvious difference in the development of these two types of bones. In membrane bones, morphogenesis starts concomitantly with the differentiation of the first bone forming cells. In endochondral bones, appearance of the first preosteoblasts occurs on a preexisting cartilage model which dictates the shape of the future bone (Fig. 1f, g).

Thus, bone morphogenesis and bone cell differentiation are essentially interdependant in membrane bone and uncoupled in endochondral bone. The differences in expression of the MC21C-epitope observed between membranous and endochondral ossification are consistent with a role of C6-S in morphogenesis.

Cartilage

The distribution pattern of the MC21C-epitope correlates with morphogenesis of the vertebral column in the rat fetus (Mark *et al.*, 1989). The sequence of immunostaining which is observed during sclerotome evolution (Fig. 2) can be summarized as follows: the sclerotome of late somites is uniformly immunostained with MC21C. During maturation of the sclerotomal mesenchyme, the reactivity towards MC21C disappears rapidly from the central part of these structures while remaining associated with the cephalic and caudal parts of the sclerotomes. Upon fusion of the caudal region of one sclerotome with the cephalic region of the sclerotome immediately behind, the immunostaining in the precartilaginous vertebrae becomes uniform (Figs. 2, 3a). During somite to vertebra transformation similar changes occur in the distribution patterns of the MC21C-epitope (Mark *et al.*, 1989) and of TGF-ß (Heine *et al.*, 1987). In all "bone forming" cartilages immunostaining with MC21C decreases as morphogenesis proceeds and disappears definitively once they have reached their final shape (Fig. 3a-c).

Lungs

During lung development, bronchial ramifications are formed as a result of sequential branching of the endoderm: primary (stem) bronchi are developing secondary (lobar) bronchi which will later give rise to tertiary (segmental) bronchi. Immunostaining by MC21C of the lung mesenchyme is strong and uniform at early stages of development (mouse, day 12; Fig. 4a) but becomes rapidly heterogeneous as branching morphogenesis progresses, disappearing selectively around the bronchi which were formed first (Fig. 4b, c). The lung parenchyma of the newborn mouse is completely devoid of immunostaining by MC21C (Fig. 4d).

Small intestine

Changes in the distribution of the MC21C-epitope during morphogenesis of the mouse small intestine occur in a proximal-distal sequence. At day 10, the mesenchyme of the primitive intestine is uniformly immunostained by MC21C (Fig. 5a). The duodenum, at day 12, demonstrates an accumulation of the staining in the inner mesenchymal layers (Fig. 5c). In the mesenchyme of the more distal

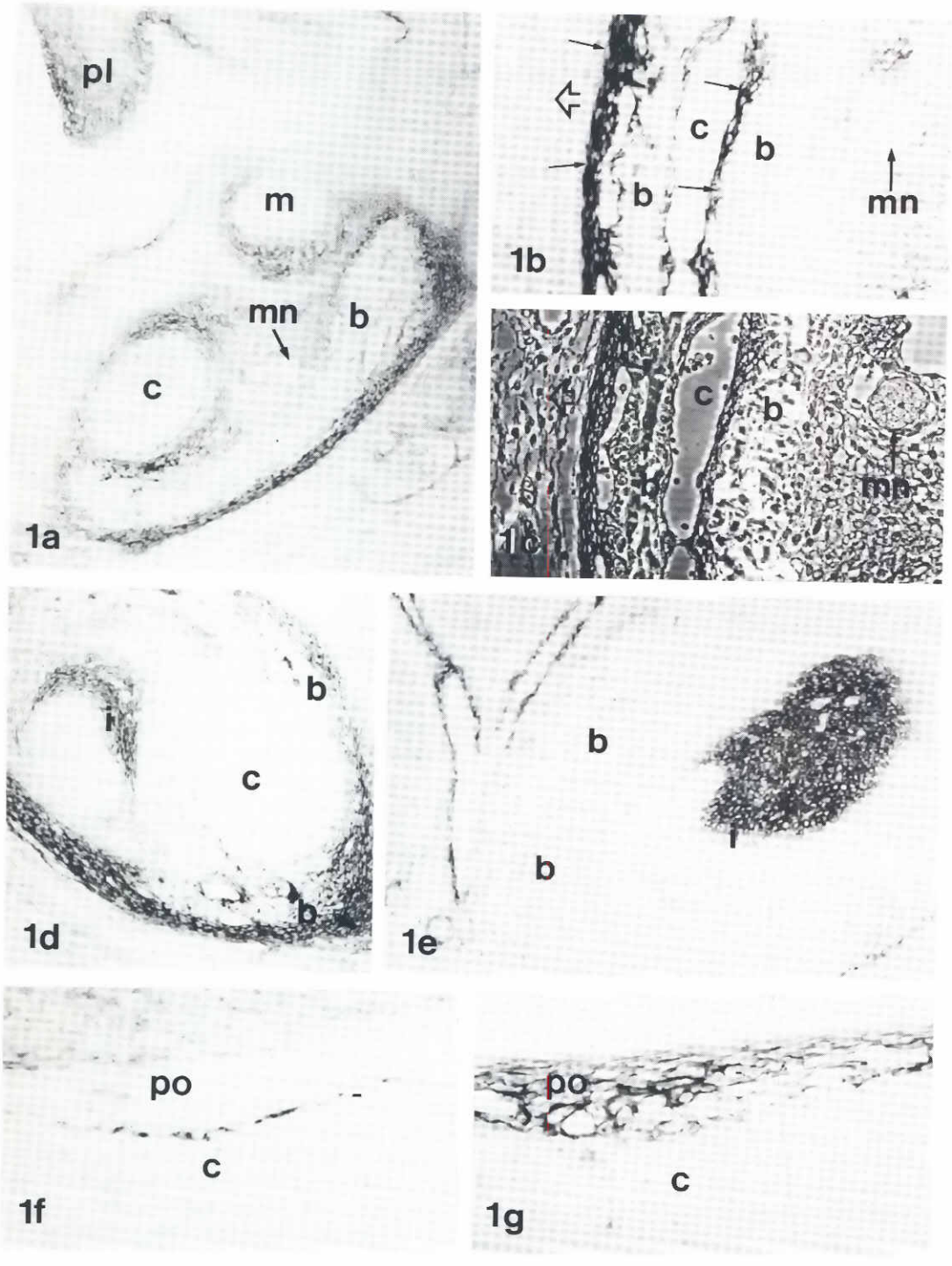


Fig. 1. Expression of the MC21C-epitope during osteogenesis in the mouse. (a) *Frontal section through the lower jaw at day 14: strong immunoperoxidase reaction is observed at the growing edge of the bone anlage b. m, first lower molar; c, Meckel's cartilage; mn, mandibular nerve; pl, palatal process. x90.* **(b, c)** *High magnification (x200) and phase contrast of a frontal section of the mandibular bone, in the molar region, at day 15, demonstrating the preponderance of the immunoperoxidase staining around the preosteoblasts (arrows) localized at the outer surface of the bone trabeculae b. c, capillary; mn, mandibular nerve. The open arrow points to the direction in which the bone expands.* **(d, e)** *Frontal sections through the alveolar bone at the posterior region of the incisor (i) at days 15 and 19 respectively: immunoperoxidase staining by MC21C disappears from the bone (b) during that period. c, Meckel's cartilage. x100.* **(f, g)** *show respectively immunoperoxidase staining by MC21C and histochemical staining for alkaline phosphatase on consecutive longitudinal sections through the diaphysis of the tibia at day 15: the MC21C-epitope is detected only in the peripheral layer of chondrocytes which limits the cartilage model (c); preosteoblasts (po) which are identified by a positive reaction for alkaline phosphatase are not immunostained. x200.*

regions of the gut, the MC21C-epitope is still evenly distributed (Fig. 5d). At day 15, villi have developed only in the duodenal region: the presumptive lamina propria, which includes the core of the villi, demonstrates expression of the MC21C-epitope; this is in contrast with the presumptive muscular layers which are MC21C-negative (Fig. 5e). At birth (day 19), the MC21C-epitope is absent from the duodenum (Fig. 5f). In the ileum, where histomorphogenesis is delayed as compared to the duodenum, the lamina propria still reacts weakly with MC21C (Fig. 5g). The small intestine of the adult mouse is devoid of immunolabeling by MC21C (not shown). On the whole, this sequence demonstrates the segregation of the MC21C-epitope in the presumptive lamina propria (Fig. 5a-c) and its disappearance after the achievement of villus formation (Fig. 5 d-f). Changes in the distribution pattern of the MC21C-epitope during

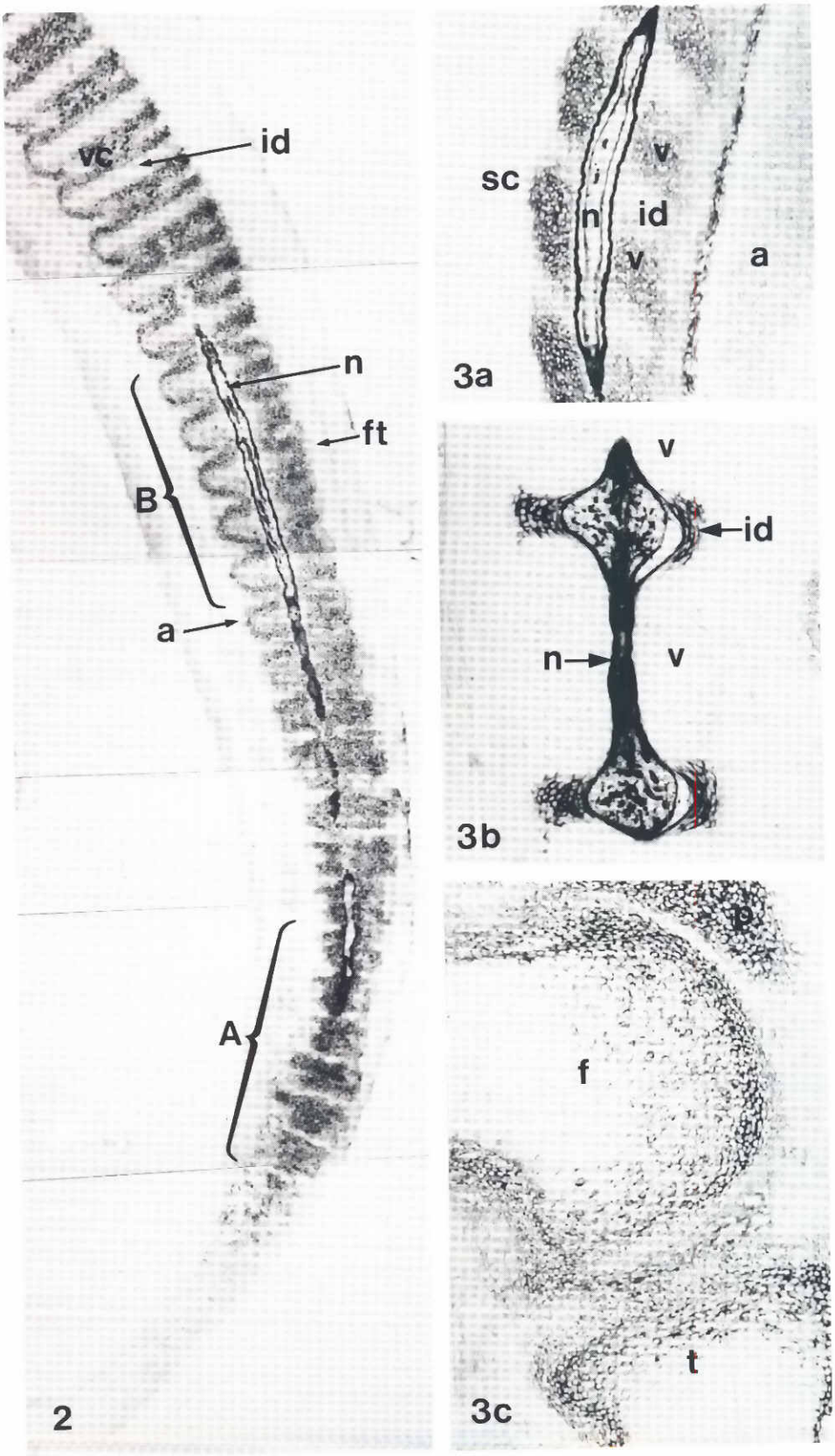


Fig. 2. Median sagittal section through the tail region of a 16-day old rat fetus immunostained by MC21C. *Peroxidase substrate deposits are evenly distributed in the mesenchyme of the most caudal sclerotomes (A) but then accumulate in the cephalic and caudal regions of the sclerotomes (B). After fusion of the sclerotomes (upper part of the figure), the anlage of the vertebral column demonstrates an alternance of positive (dark) bands corresponding to the vertebral centra (vc) and of negative (clear) bands corresponding to the primitive intervertebral discs (id). a, aorta; n, notochord, ft, filum terminale. x45.*

Fig. 3. Expression of the MC21C-epitope during chondrogenesis in the mouse. (a, b) *are sagittal median sections at comparable levels of the vertebral column of a mouse fetus at day 12* **(a)** *and 15* **(b):** *immunoperoxidase staining by MC21C is strong in the prevertebrae but disappears as chondrification proceeds. v. vertebra; id, intervertebral disc; n, notochord; a, aorta; sc, spinal column. x100.* **(c)** *Longitudinal section through the knee joint of a day 15 mouse fetus: immunoperoxidase staining by MC21C is restricted to the epiphysis of the tibia (t) and femora (f) where morphogenesis is retarded as compared to the diaphyseal region. The diaphysis of the same tibia anlage is shown in Fig. 1f. p, patella. x100.*

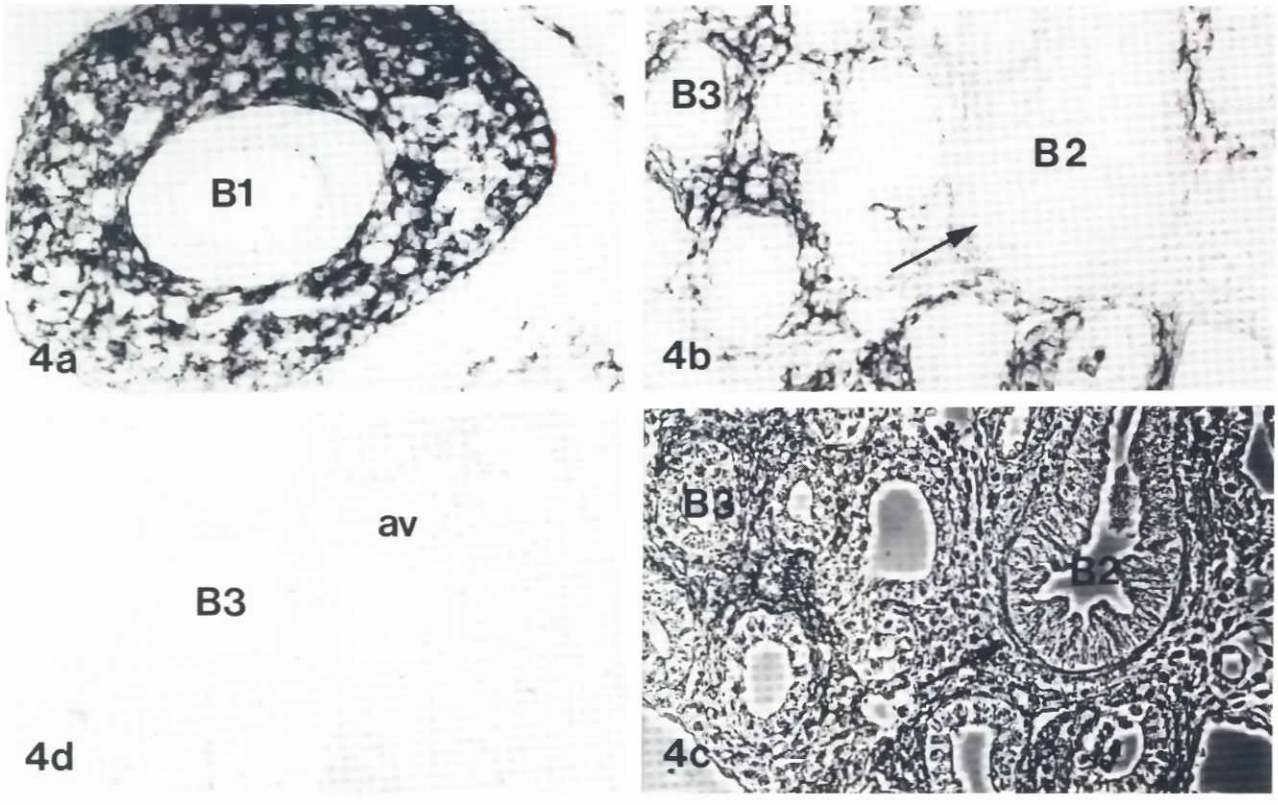


Fig. 4. Sections through the lung parenchyma of the mouse *at day 12* **(a)**, *day 15* **(b, c)** *and day 19* **(d)**. *Immunoperoxidase staining with MC21C is uniformly distributed in the lung parenchyma at day 12 but has disappeared from the mesenchyme of the secondary bronchi at day 15 (arrow). At birth (day 19), the lung parenchyma is devoid of immunostaining. B1, B2, B3 primary, secondary and tertiary bronchi respectively; av, alveoli. x260.*

morphogenesis of the small intestine are not mimicked by any other known macromolecule of the ECM (Simon-Assman *et al.*, 1986, 1989; Aufderheide and Ekblom, 1988).

MC21C does not detect all the C6-S present in the organism

3B3 is another Mab which can be employed to identify C6-S in tissues. The epitopes recognized by MC21C and 3B3 are different: the former is localized near the non-reducing terminus of the CS chains while the latter binds to the "stub" of the CS chain produced by chondroitinase digestion (i.e. close to the proteoglycan protein core). The MC21C and 3B3 epitopes are coexpressed in many tissues during development in the mouse (Fig. 6 a-e). However immunostaining with 3B3, but not with MC21C, identifies C6-S in a variety of basement membranes in the newborn mouse (Fig. 7 a-d). One possible explanation is that during synthesis of CSPG, sulfation near the reducing terminus of the CS chains might be controlled independently from sulfation at the non-reducing terminus. Alternatively the possibility that MC21C has a preferential reaction with certain structural variants of 6-sulfated segments in CS chains cannot be ruled out.

These observations emphasize the usefulness of Mabs to identify CS in immunolocalization studies. They also outline the need for further investigating the structural basis of antigenic differences in CS chains.

In order to gain more information on chondroitin sulfation during development, we have established the patterns of distribution of five different epitopes present in CS chains in the developing tooth germ, a model system for the study of epithelial-mesenchymal interactions mediated by the ECM (reviews: Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1983).

The tooth germ: a model system for the study of regulated changes of CS sulfation

The development of the tooth starts with a thickening of the oral epithelium called the dental lamina. This is followed by the ingrowth of the dental lamina into the jaw mesenchyme forming the dental bud. During this period the neural crest-derived mesenchymal cells underlying the epithelium condense and this condensation identifies the dental mesenchyme. The dental lamina is the presumptive anlage of the enamel organ which gives rise to the ameloblasts, secreting enamel; the dental mesenchyme contains the presumptive anlage of the dental papilla which will ultimately give rise to the odontoblasts, secreting predentin and dentin. The terminal differentiation of odontoblasts and ameloblasts occurs in each tooth according to a specific temporo-spatial pattern (review Ruch *et al.*, 1983). Odontoblast terminal differentiation begins at the tip of the cusps in the molars and at the anterior end of the incisor and progresses towards the cervical region of the teeth. Ameloblast terminal differentiation begins 24 to 36hr later and follows the

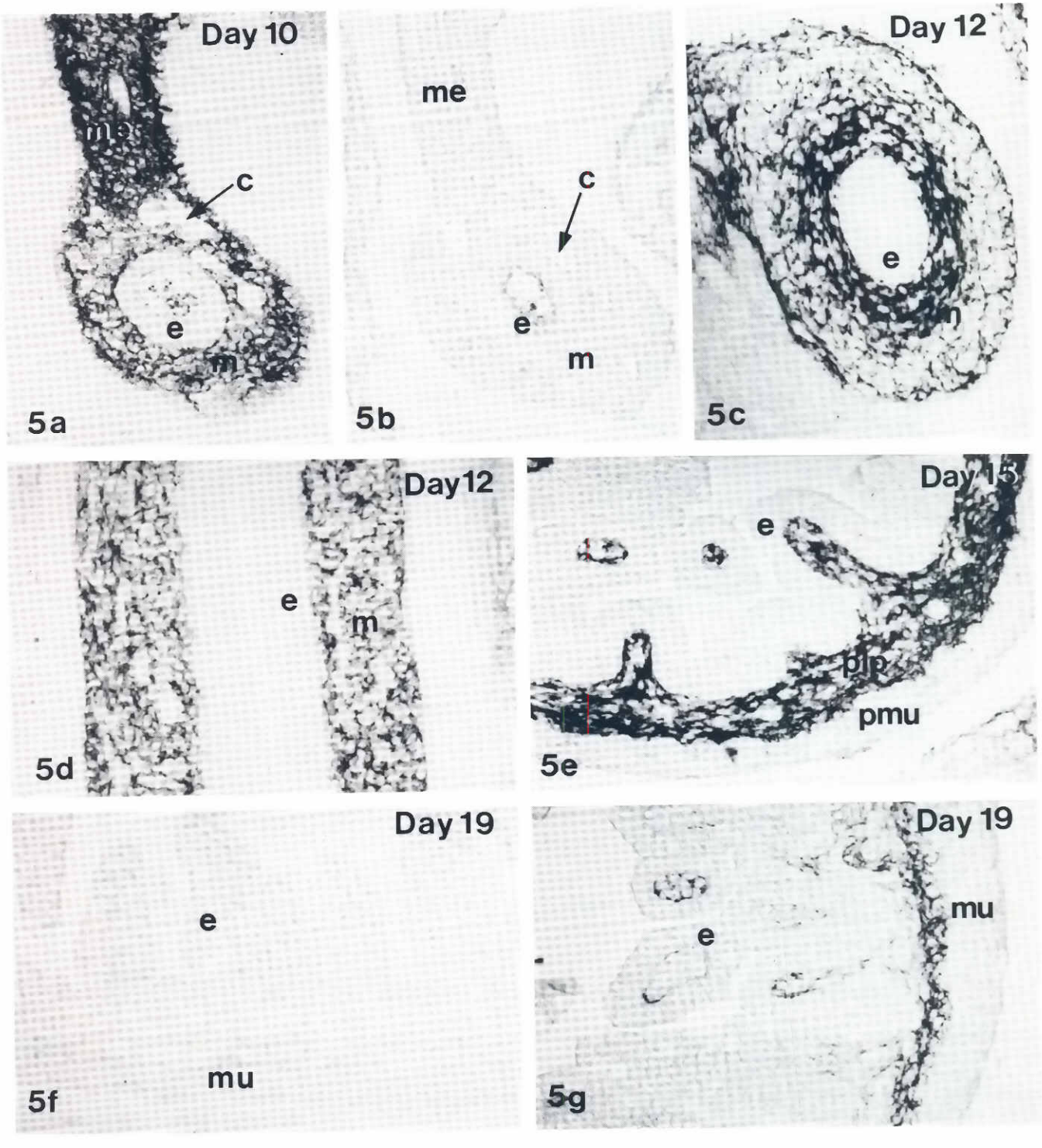


Fig. 5. Localization of the MC21-C epitope during morphogenesis of the small intestine by immunoperoxidase staining *at days 10* **(a)**, *12* **(c, d)**, *15* **(e)** *and 19* **(f, g)**. **(b)** *is a section adjacent to* **(a)** *which has been immunostained with a monoclonal IgM not-recognizing CS.* **(c, e, f)** *are sections of the duodenum.* **(d, g)** *were obtained from the distal segment of the small intestine. e, epithelium (endoderm); m, mesenchyme; me, mesentery; c, capillary; plp, presumptive lamina propria; pmu and mu, presumptive muscle layers and muscle layers, respectively. x260.*

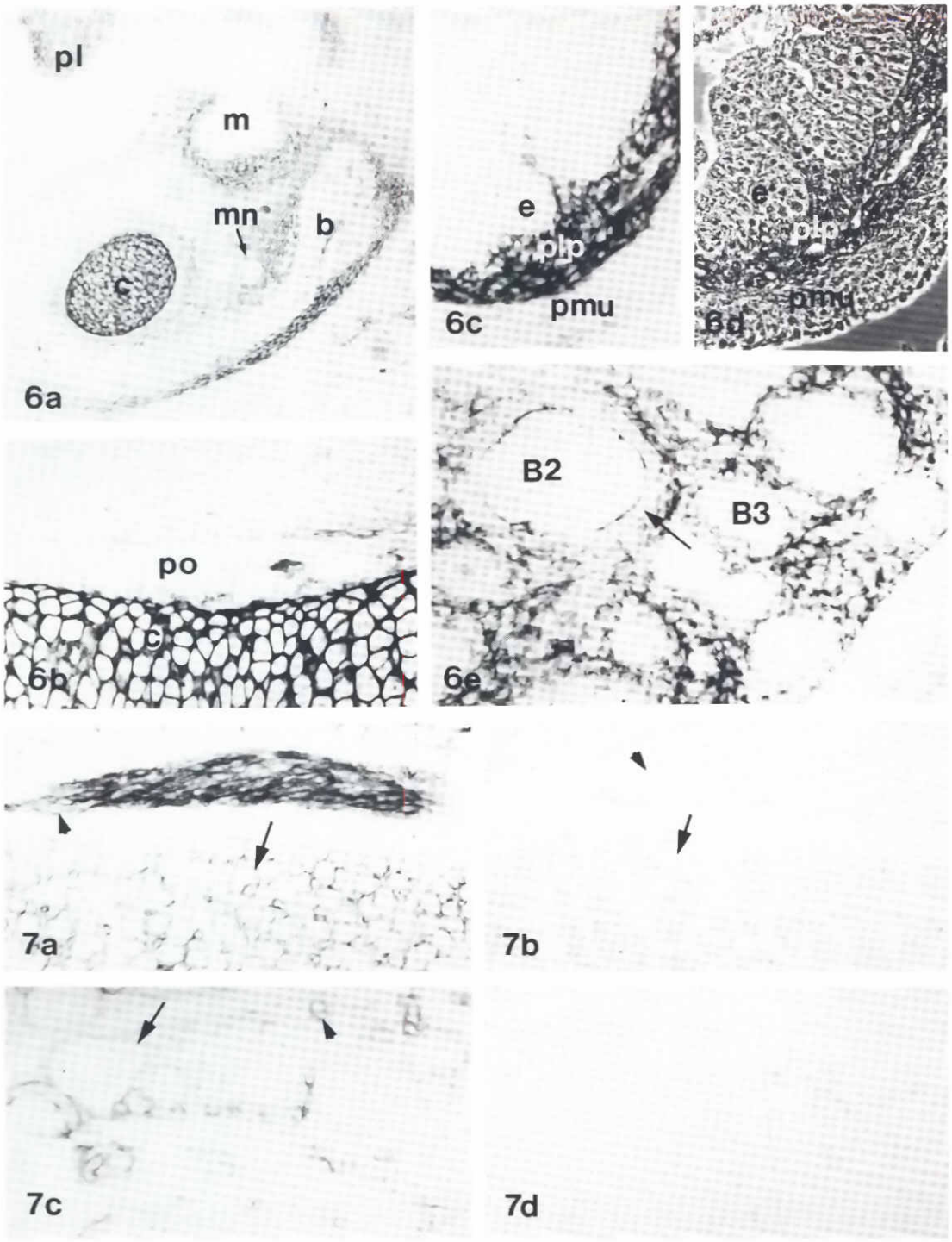


Fig. 6. The patterns of expression of MC21C and 3B3 in the mouse fetus are similar in many developing systems. (a) *Frontal section of the mandibular bone at day 14; immunoperoxidase staining by 3B3: compare with Fig. 1a, immunostaining by MC21C.* **(b)** *Longitudinal section through the diaphysis of the tibia, immunostaining by 3B3: compare with Fig. 1f, immunostaining by MC21C.* **(c, d)** *Duodenum at day 15, immunostaining by 3B3 and phase contrast respectively: compare with Fig. 5e, immunostaining by MC21C.* **(e)** *Lung, day 15 immunostaining by 3B3: compare with Fig. 4b immunostaining by MC21C. Abbreviations and magnifications as in the corresponding figures immunostained by MC21C. Comparison of the immunostaining patterns produced by these two antibodies in cartilage was voluntarily omitted from this compilation since this tissue contains unsulfated chondroitin which cannot be distinguished from C6-S by immunostaining with 3B3.*

Fig. 7. Immunoperoxidase staining with 3B3 (a, c) or MC21C (b, d): 3B3, but not MC21C, identifies C6-S in basement membranes of the newborn mouse. (a, b) *Tongue: basement membranes of muscle fibers (arrows) and nerve fibers (arrowheads) are stained by 3B3 but not by MC21C.* **(c, d)** *Salivary glands: the basement membranes surrounding the glandular epithelium (arrows) and the capillaries (arrowheads) react with 3B3 but not with MC21C. x260.*

same pattern.

It is well established that, from the initiation of tooth development to the completion of crown and root formation, tooth morphogenesis and cytodifferentiation are controlled by reciprocal epithelial-mesenchymal interactions in which the ECM appears to play an important role.

With the aims of characterizing the sulfation patterns of the CSPG present in the ECM of embryonic teeth we have incubated histological sections from lower molars and lower incisors at different stages of development with five distinct Mabs: 1B5, 2B6, 3B3, MC21C and MO-225 (Table 1). Immunostaining patterns of 1B5, 2B6, 3B3 and MC21C have been reported elsewere (Mark *et al.*, 1990).

CSPG of differing sulfation exhibit distinct patterns of distribution during odontogenesis (Table 2)

The 2B6-epitope (generated from C4-S) was associated with the basement membrane separating the epithelium and mesenchyme of the tooth from the initiation of tooth morphogenesis to the completion of odontoblasts terminal differentiation (Table 2 and Fig. 8).

During the bell stages of morphogenesis, C4-S was also detected in the dental papilla of the occlusal (upper) region of the molars and of the anterior region of the incisors. The predentin, produced by the odontoblasts, was rich in C4-S (Figs. 8, 9a).

MC21C, 3B3 and MO-225 recognize segments on the CS chain which contain N-acetyl-D-galactosamine 6-sulfate (Table 1). Their epitopes were first detected in the condensing dental mesenchyme at the bud stage. The presence of C6-S-related epitopes in the dental basement membrane of the forming cusps was observed during a brief period. Shortly after this, C6-S disappeared from the most developed region of the dental papilla: cuspal mesenchyme in the molars (Table 2) and anterior region of the dental papilla in the incisor (see example Fig. 11 a-c).

Futhermore, the immunostaining pattern produced by each of the three Mabs (MC21C, 3B3 and MO-225) were not identical (Table 2, Figs. 9 c, d and 10a-c).

Specific examples are: 1) The absence of the MC21C and MO-225 epitopes in the dental papilla of the molar at day 15 contrasting with the strong staining of this structure after incubation with 3B3 2) The antagonistic gradients of immunostaining by MC21C and 3B3 observed in the dental papilla during cusp formation (Fig. 10 b, c). 3) The presence of the MC21C and MO-225-epitopes in the basement membrane of the teeth at a specific stage (molar: days 16-17), whereas the 3B3-epitope is absent from this structure at all stages studied (Fig. 10 a-c). 4) The presence of the sole MO-225 epitope in the predentin secreted by young odontoblasts (Figs 9c, 11 a,b).

The 1B5-epitope (chondroitin) was never detected during odontogenesis (see example Fig. 9b). This suggests that, unlike the CSPG from precartilage (Mark, unpublished data) and cartilage (Couchman *et al.*, 1984; Caterson *et al.*, 1985), the CSPG expressed by the dental mesenchyme do not contain unsulfated uronic acid-Gal NAc disaccharide units.

Changes occurring in chondroitin sulfation during odontogenesis are coordinated

Comparison of the immunostaining patterns produced by 2B6 and 3B3 on consecutive sections demonstrate that each Mab reacts with a distinct region of the dental papilla which strictly complements the region immunostained by the other Mab. Therefore, the expression patterns, during odontogenesis, of proteoglycans carrying 6-and 4-sulfated hexosamine residues near the reducing end of their CS chains appear mutually exclusive (Mark *et al.*, 1990).

Changes in CSPG sulfation correlate with the distribution of the mitotic indices in the lower incisor

The incisors of rodents, contrary to the molars, are continuously growing. The continiously growing lower incisor of the mouse is subdivided into three distinct zones with respect to the distribution pattern of mitosis along its antero-posterior axis (Osman and Ruch, 1976; Ruch, 1984). This subdivision is established at the early bell stage (day 15) and persists in the adult. In the anterior region, the mitotic activity of mesenchymal cells and epithelial cells is low; progressively post-mitotic odontoblasts and ameloblasts appear in this region. In the middle, or transition zone the mitotic indices increase in the posterior direction. In the posterior zone, the mitotic activities of the dental papilla and of the inner dental epithelium are high. This posterior zone, of probably constant dimension, corresponds to the compartment of proliferation.

In the mouse lower incisors at the bell stage, a striking positive correlation was found between immunostaining patterns with antibodies that recognize C6-S-related epitopes (3B3, MC21C, MO-225) and the distribution of the mitotic indices along the anterior-posterior axis of the tooth: C6-S was abundant in the posterior, growing region of the dental papilla and absent in the anterior region which demonstrates low rate of mitotic activity. On the other hand, immunostaining for C4-S was high in the compartment of maturation and decreased toward the compartment of proliferation (Mark *et al.*, 1990).

Changes in CSPG sulfation correlate with odontoblast terminal differentiation

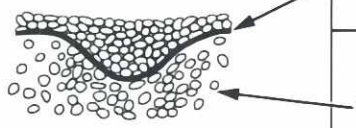
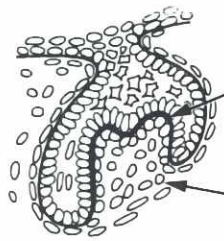
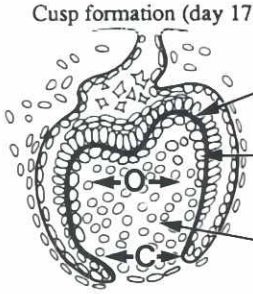
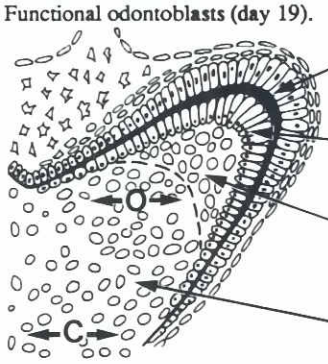
The epithelial mesenchymal interactions involved in differentiation of the odontoblastic cell lineage are mediated by the basement membrane interposed between the inner dental epithelium and the dental papilla (Thesleff and Hurmerinta, 1981; Ruch *et al.*, 1983). During odontogenesis in the first lower molar, C4-S which was uniformly distributed in the basement membrane during the dental lamina and dental bud stages accumulated in the occlusal part of this structure at the early bell stage. Twenty four hours prior to the onset of odontoblast terminal differentiation, the MC21C and MO-225 epitopes were found in the basement membrane for a brief period after which C6-S disappeared definitively from the preodontoblasts (Table 2). During terminal differentiation, C4-S which completely surrounded the preodontoblasts was confined to the epithelial mesenchymal junction when odontoblasts polarized (Fig.8). These rapid changes in the distribution of specific CS-epitopes during differentiation of the odontoblastic cell lineage suggest that proteoglycans bearing CS chains of differing sulfation position play an important role in this process.

Conclusion

Chondroitin isoforms present in developing mouse tooth germs are sulfated at either position 6 or 4 of the hexosamine residue. CSPG of embryonic mouse teeth also possess D-glucuronic acid 2-

TABLE 2

DISTRIBUTION OF CS-RELATED EPITOPES IN THE DENTAL MESENCHYME AT DIFFERENT STAGES OF MORPHOGENESIS IN THE FIRST LOWER MOUSE MOLAR

		1B5	2B6	3B3	MC21C	MO-225
Dental lamina stage (day 12). 	bm	–	++	–	–	–
	dm	–	–	–	–	–
Dental bud stage (day 14). 	bm	–	++	–	–	–
	dm	–	–	++	++	–
Early bell stage (day 15). 	bm	–	O:++ / C:–	–	–	–
	p	–	–	++	–	–
Cusp formation (day 17). 	bm	–	O:++ / C:+	–	++	++
	pod	–	O:++ / C:+	–	++	++
	p	–	O:++ / C:–	O:– / C:++	O:++ / C:+	O:++ / C:+
Functional odontoblasts (day 19). 	pd	–	++	–	–	+
	od	–	–	–	–	–
	p(t)	–	++	–	–	–
	p	–	O:+ / C:–	O:+ / C:++	O:++ / C:+	O:++ / C:+

bm, basement membrane; dm, dental mesenchyme; p, dental papilla; p(t) designates the dental papilla at the tip of the cusps at day 19: this region behaves differently with respect to immunostaining with anti-CS antibodies as compared to the rest of the papilla. O, occlusal region of the dental papilla; C, cervical region of the dental papilla; pod, preodontoblastic layer; od, odontoblastic layer.

-: no staining
+ and ++: weak and strong staining, respectively.
o/c: indicate an occlusal-cervical gradient of immunostaining.

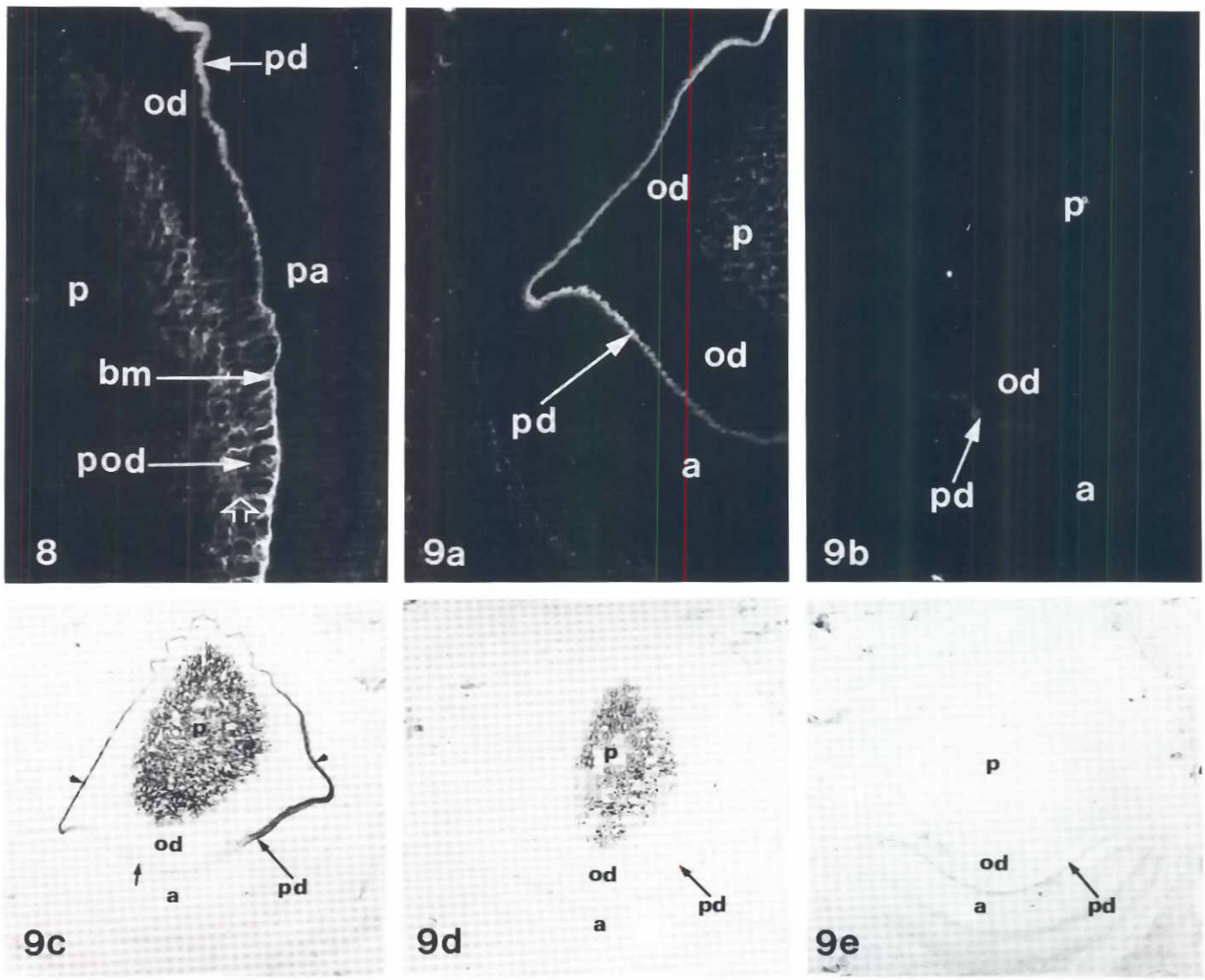
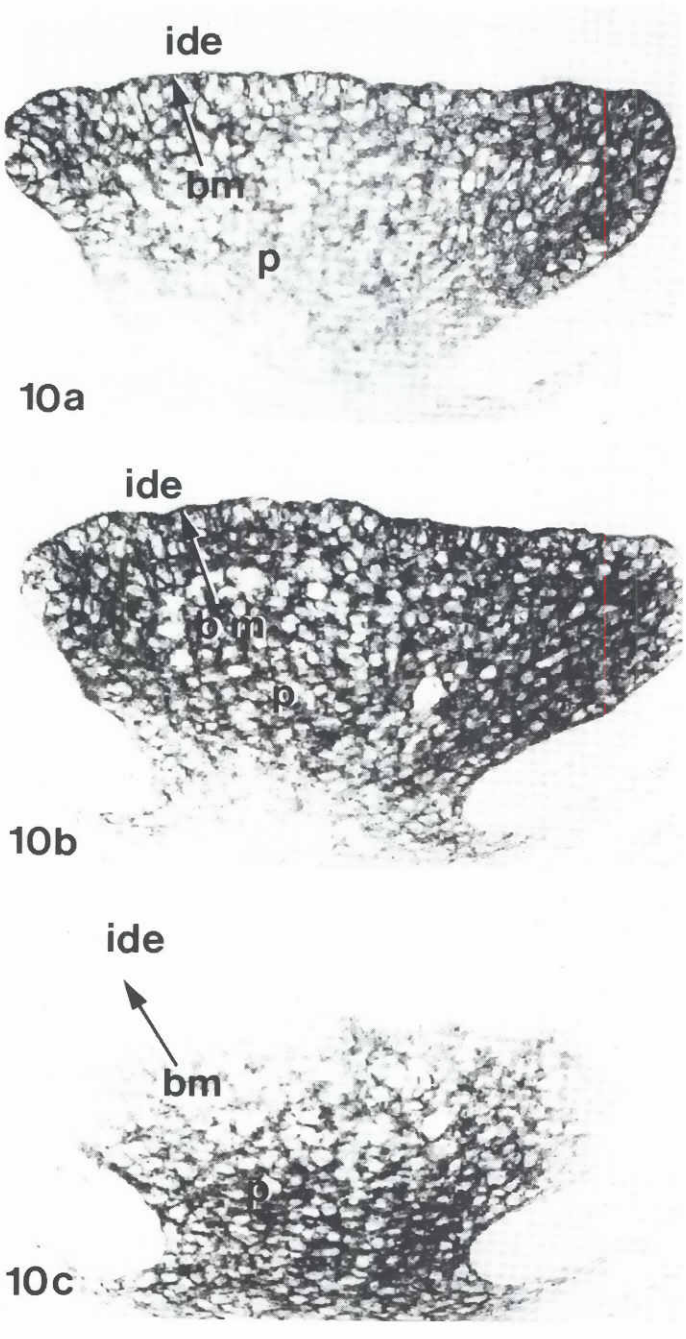


Fig. 8. Frontal section of the first lower molar at day 19. *Immunofluorescence staining with 2B6, showing the redistribution of C4-S in the odontoblastic layer. The open arrow points towards the tip of the cusp, indicating the progression of cytodifferentiation. pod, preodontoblasts; od, odontoblasts, bm, basement membrane; p, papilla; pd, predentin; pa, preameloblasts. x260.*

Fig. 9. Detection of glycosaminoglycans in early predentin on adjacent frontal sections through the median region of a lower incisor at day 19. **(a)** *Immunofluorescence with 2B6 stains the predentin layer uniformly.* **(b)** *Immunofluorescence staining with 1B5 (negative control).* **(c)** *Immunoperoxidase staining with MO-225: at this developmental stage, the MO-225 epitope has disappeared from the predentin at the labial side of the tooth (arrow) but remains associated with the more recently deposited predentin of the lateral sides (arrowheads).* **(d)** *Immunoperoxidase staining with MC21C: this Mab never stains predentin.* **(e)** *Immunoperoxidase staining with a monoclonal IgM not recognizing CS (negative control). od, odontoblasts; a, ameloblasts; p, dental papilla; pd, predentin. x260 (a, b) and x100 (c, d, e).*

sulfate containing disaccharide units. Different epitopes present in CS display stage-specific patterns of distribution during odontogenesis. C6-S is the only isoform found in the dental mesenchyme at early stages of morphogenesis. C4-S expression by the cells of the dental papilla is associated with a decrease in mitotic activity in the incisors and with the achievement of morphogenesis in the molars. On the other hand C4-S is the main isoform in the tooth basement membrane where it can be detected as soon as the dental lamina stage. Loss of C6-S expression and redistribution of C4-S antigenicity are associated with odontoblast terminal differentiation. Changes occurring in the distribution pattern of two C6-S related epitopes (MC21C and 3B3 epitopes) during odontogenesis parallel the changes occurring in the distribution pattern of syndecan, which is the only CSPG identified so far in the embryonic tooth (Thesleff *et al.*, 1988). This suggests that syndecan is one of the CSPG recognized by these antibodies. Changes in the distribution of C4-S and fibronectin during terminal differentiation of odontoblasts are identical (Thesleff *et al.*, 1979; Lesot *et al.*, 1981; Mark *et al.*,



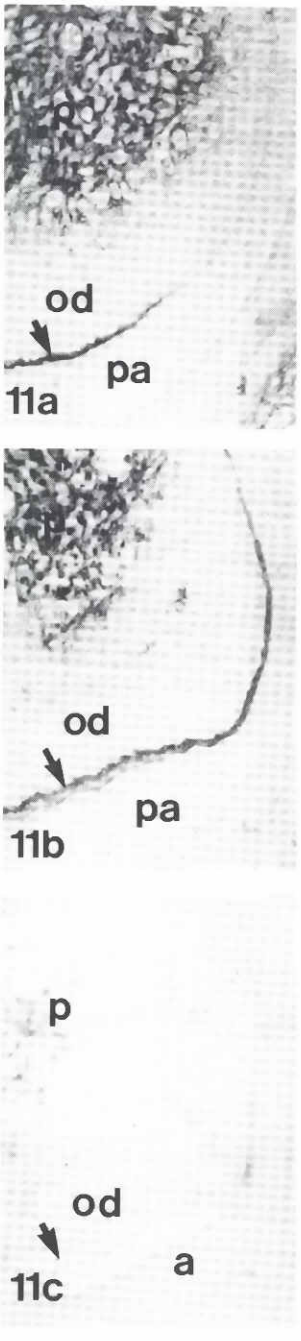


Fig. 10. Three consecutive frontal sections through a lower molar, 24h prior to the onset of odontoblast terminal differentiation. (a) *Immunostained with MO-225: staining is maximum in the occlusal (upper) region of the dental papilla (p) and is also found in the basement membrane (bm) at this stage.* **(b)** *Immunostained with MC21C.* **(c)** *Immunostained with 3B3: staining is restricted to the cervical (lower) region of the dental papilla and is absent from the basement membrane. The dental papilla of the molar appears highly heterogeneous with respect to the distribution patterns of C6-S related epitopes. ide, inner dental epithelium. Immunoperoxidase. x260.*

Fig. 11. Frontal sections at three different levels of the lower incisor at day 17: *posterior* **(a)**, *median* **(b)**, *anterior* **(c)**. *Immunoperoxidase staining with MO-225. The continuously growing incisor of the mouse has the advantage over the molar of offering a continuous gradient of cytodifferentiation progressing from its posterior end to its anterior end. This figure illustrates the transient presence of the MO-225-epitope in the predentin (arrow) facing the newly polarized odontoblasts (od) and its disappearance from this ECM during subsequent maturation. The absence of dentin (which might have interfered with the immunostaining) was ascertained by Von Kossa staining (not shown). Also note that the dental papilla (p) in the most anterior section is almost devoid of labeling by MO-225; pa, preameloblasts; a, ameloblasts. x280.*

1989a): fibronectin and C4S surround preodontoblasts and become restricted to the apical pole of polarizing odontoblasts. The redistribution of fibronectin, interacting with odontoblasts, has been suggested to be causally related to odontoblast terminal differentiation (Lesot *et al.*, 1988) and specific CSPG might be involved in fibronectin redistribution.

Our immunohistochemical data suggest: 1) that CSPG play important roles during ontogenesis on the whole and particularly during odontogenesis and 2) that the position of the sulfate ester groups and their distribution along the CS chain has a physiological significance.

These immunohistochemical data confirm the view that, *in vivo*, cells might produce CSPG carrying predominantly one type of CS chain with respect to sulfation in C-4 or C-6 of the Gal NAc residues (Couchman *et al.*, 1984). They clearly demonstrate that the position of the sulfate ester groups along the CS chain is developmentally regulated. They also support the existence of specific CS sequences found predominantly or exclusively in embryonic tissues and provide the basis for designing new experiments aimed at clarifying the biological significance of CSPG sulfation during ontogenesis and disease processes.

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