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Role of the notochord in the development of cephalic structures in normal and anencephalic human fetuses

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Summary. Normal and anencephalic human conceptuses were analysed histologically to investigate the role of differentiation of the intracranial notochord and its relation to the formation of the basichondrocranium. We have examined 16 normal embryos and fetuses and 4 anencephalic fetuses. Each developmental stage of formation of the normal basichondrocranium presented specific morphological changes during the course of notochord depletion. In contrast with normal specimens, anencephalic fetuses presented malformations of the basichondrocranium which were always related to an abnormal position of the notochord. Macroscopical differences between craniorachischisis and cranioschisis in fetuses with anencephaly correlated with the existence of two histologically different degrees of malformation. In fetuses with craniorachischisis we found severe disturbances in the shape, position and ossification of the basichondrocranium and in the course of the intracranial notochord. In fetuses with cranioschisis the described disturbances of the basichondrocranium and intracranial notochord were mild. In addition, marked differences in affection of the central nervous system and the hypophysis were observed. These findings suggest different periods of dysmorphogenesis. Our results underline the importance of the chordal mesoderm in the differentiation for the formation of cephalic structures in Man.

Key words: Human notochord – Basichondrocranium – Anencephaly

Introduction

Dysraphia is a faulty closure of neural and skeletal structures that may involve the spinal or the cerebral region, or both (Muller and O'Rahilly 1991). In an extensive study of human anencephaly published by Marin-Padilla (1965, 1966b) macroscopic examination of the skull bones and serial histological sections of the vertebral segments in human craniorachischisis revealed a morphological relationship between malformations of the bones of the skull and vertebral bodies and underlying notochordal alterations. The chordal mesoderm disturbances were considered to be essential for these malformations.

In contrast to these findings, a histological analysis of spinal malformations provided by Goto and Uhthoff (1985, 1986) suggested that the developing human notochord has no influence on either the normal differentiation of the spine or its malformations. Congenital vertebral malformations were believed to occur during the stage of resegmentation and were thought to be related to the abnormalities of intersegmental arteries rather than to those of the notochord (Tanaka and Uhthoff 1981a, b).

In animal experiments it was recently shown that the notochord induces formation of the floor plate of the neural tube (van Straaten and Drukker 1987; Smith and Schoenwolf 1989; Placzek et al. 1991; Yamada et al. 1991) and regulates the pattern of local neurogenesis (Lumsden 1991). Mutational analysis of animal embryos indicated that homeobox-containing genes play a key role in determining the anteroposterior axis of the body. Progressive subdivision of the body into segments is controlled by several classes of gene, including pair-rule genes (Alberts et al. 1989). The products of genes were spatially and temporally expressed in specific areas along the axis. XIHbox1 protein was found in overlapping areas of both the neural tissue of the hindbrain and the spinal cord and within the mesodermal tissue, including notochord (De Robertis et al. 1989). Recent investigations associate the differentiating notochord with anteroposterior regionalization of the nervous system rather than with neural induction (Storey et al. 1992).

Until now, the reports on malformed human conceptuses have lacked a histological reconstruction of the complete skull base during the fetal period. Therefore we undertook this investigation in order to analyse histo-

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logically the relationship between the notochord and the developing bones of the whole skull base in normal and anencephalic human fetuses. Our data suggest the importance of the notochord in normal and abnormal development of human cephalic structures.

Materials and methods

Human embryonic and fetal tissues were obtained after legal abortions or at autopsies. The post-ovulatory age was estimated from menstrual data and correlated with the crown-rump length (CRL) and Carnegie stages (O'Rahilly and Gardner 1971). Besides eight normal embryos of post-ovulatory age between 5 and 8 weeks (CRL 8–30 mm, Carnegie stage 15–23) and eight fetuses of post-ovulatory age between 9 and 22 weeks [crown-heel length (CHL) 3–28 cm], four malformed anencephalic fetuses of post-ovulatory age between 16 and 22 weeks (CHL 13–26 cm) were collected (Table 1). Malformations in anencephalic fetuses were revealed using ultrasound examination during the routine control of pregnant women. The ultrasound measurements were also used to determine the age of these fetuses.

Conceptuses from 5 to 8 weeks of development were fixed in 4% formaldehyde in phosphate buffer and sectioned in toto. Central parts of the skull bases from older fetuses were dissected together with the first two cervical vertebrae, parts of adjacent central nervous system and the supporting soft tissues. Samples were fixed in 10% formaldehyde in phosphate buffer, decalcified with 36% hydrochloric acid in formic acid and embedded in paraffin. Tissue blocks were serially, sagitally sectioned and stained with haematoxylin and eosin, alcian blue and kernechtrot. Three-dimensional reconstructions were performed by use of these serial sections.

Table 1. Age and number of normal and anencephalic conceptuses used

Normal embryos (*n*)	CRL (mm)	Carnegie stages	Post-ovulatory age	
			Days	Weeks
1	8	15	33	5
2	9–11	16	37	5–6
1	13	17	41	6
1	16	18	44	6–7
2	22–25	21–22	52–54	7–8
1	30	23	56,5	8

Normal fetuses (*n*)	CHL (cm)	Carnegie stages	Post-ovulatory age	
			Days	Weeks
1	3,2	/	/	8–9
2	4–5	/	/	9
2	8–9	/	/	12
1	12	/	/	14
1	16	/	/	16
1	28	/	/	22

Anencephalic fetuses (*n*)	CHL (cm)	Carnegie stages	Post-ovulatory days	
			Days	Weeks
With chraniorachischisis				
1	13	/	/	16
1	14	/	/	16
With cranioschisis				
1	16	/	/	16
1	26	/	/	22

CRL, Crown-rump length; CHL, crown-heel length

Results

Normal embryos and fetuses

The mesenchymal period of the developing skull base is observed during the 5th week of development: the notochord occurs as a continuous narrow cylinder in the condensed mesenchyme of the future skull base primordium (basichondrocranium). It is situated between the neuroectoderm of the central nervous system and the endodermal pharyngeal epithelium. The notochord parallels the pharyngeal epithelium in a slightly wavy course and comes into contact with it at different places, most cranially at the point of Rathke's pouch invagination (Fig. 1a, b). At more advanced embryonic stages the notochord grows cranially and ends near the top of Rathke's pouch. At the end of the 5th developmental week first signs of chondrification appear in the mesenchymal bone primordia. The outlines of the future skull bones and the notochord itself become readily identified in the surrounding mesenchyme (Table 2).

At the beginning of the 6th week of development the bone primordia are chondrifying and the notochord starts changing its course. One part becomes incorporated in the bone primordia, while the other is found in the mesenchyme underlying the skull base. Several side branches emerge from the main stream of the notochord and establish intimate contacts with the pharyngeal epithelium. During the 6th and 7th weeks of development the notochord enlarges within the loose mesenchyme between the first cervical vertebra and occipital part of the skull base. At areas of contact with the pharyngeal epithelium several invaginations can be seen (Table 2).

The first signs of ossification are visible during the 9th and 10th weeks of development. The ossification centre appears in the basilar part of the occipital bone. At later stages, its progressive development is followed

Table 2. Developmental characteristics of the cephalic structures in normal human embryos and fetuses 5–15 weeks old

SB	Embryos (weeks)		Fetuses (weeks)	
	5	6–8	9–12	12–15
Developmental stage	Mesenchymal	Chondrogenesis	Osteogenesis	Osteogenesis
Nothocord inside SB	+	+	±	−
Nothocord around SB	+	+	+	±
Ossification centres	−	−	±	+

SB, Skull base; +, present/developed; ±, partially present/partially developed; −, absent/undeveloped

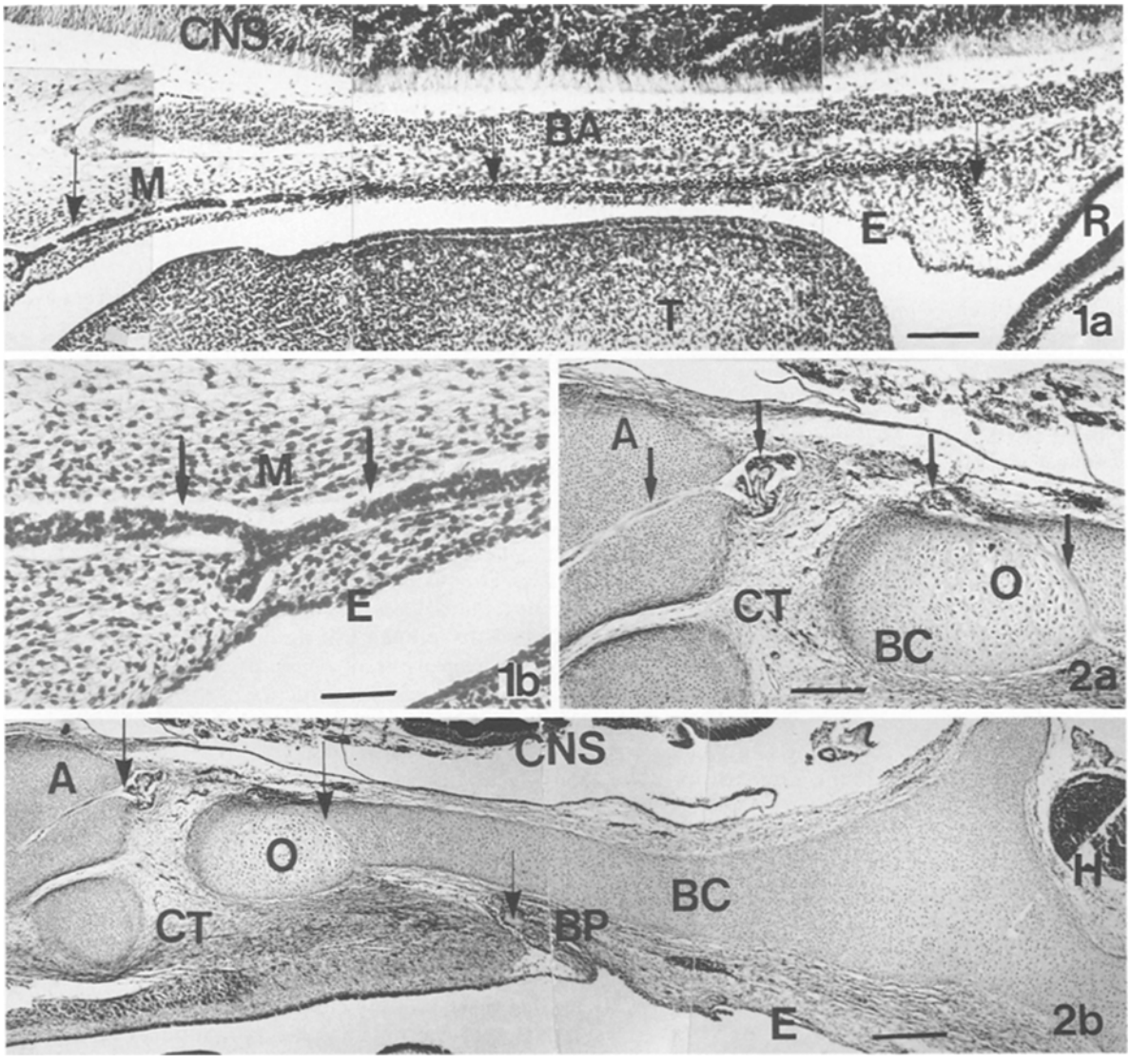


Fig. 1a. a Reconstruction of the mid-sagittal sections through the basichondrocranium of a 5th week human embryo. The notochord (*arrows*) is visible within the condensed mesenchyme (*M*) of the future basichondocranium. It parallels the pharnygeal epithelium (*E*) in a wavy course and its cranial end comes close to the Rathke's pouch invaginations (*R*). Other cephalic structures: basilar artery (*BA*), part of the central nervous system (*CNS*), tongue (*T*). The contrast to the cranial part of the notochord is highlighted by using black ink. H&E, ×100; *bar*=100 μm. **b** Higher magnification showing relationship of the notochord (*arrow*) to the pharyngeal epithelium (*E*) and the mesenchyme (*M*) of the future basichondrocranium. H&E, ×220; *bar*=450 μm

Fig. 2. a Head area of a 12th week fetus. Notochord is visible (*arrows*) inside the odontoid process of the axis (*A*), in the surrounding connective tissue (*CT*) and within the basichondrocranium (*BC*), where it is reduced to a thin thread of extracellular matrix (*arrow*). Developing ossification centre (*O*). H&E, ×60; *bar*=166 μm. **b** Reconstruction of the mid-sagittal sections through the basichondrocranium of a 12th week human fetus. It illustrates the intracranial course of the notochord (*arrows*) and its relation to the odontoid process of the axis (*A*), developing basichondrocranium (*BC*), surrounding connective tissue (*CT*), epithelium of the pharynx (*E*) and bursa pharyngea (*BP*). Developing ossification centre (*O*), central nervous system (*CNS*), hypophysis (*H*). H&E, ×30; *bar*= 330 μm

by degeneration of the notochordal cells within the skull base, while the notochordal enlargements in the connective tissue between the odontoid process and the occipital part of the skull base still persist (Fig. 2a). By 12–16 weeks of development only a thin thread of extracellular matrix represents remnants of the notochord inside the skull base, while the notochordal tissue lining the skull base remains in contact with a deep invagination of the pharyngeal epithelium (bursa pharyngea). During the degeneration process, the notochordal cells rearrange and form discontinuous cell groups separated by increasing amounts of notochordal extracellular matrix. Outlines of disrupted notochordal tissue become hardly detectable from the surrounding tissue (Fig. 2b). By the 16th developmental week no notochordal tissue is detectable inside or around the skull base (Tables 2, 3).

Table 3. Characteristics of cephalic structures in normal and anencephalic human fetuses during the 16th developmental week

SB	Normal fetus	Anencephaly + chranioschisis	Anencephaly + chranio-rachischisis
Notochord inside SB	−	±	+
Notochord around SB	−	−	+
Ossification centres	+	+	±
Shape changes	−	±	+
Positional changes	−	±	+
Sella turcica	+	+	±
Adenohypophysis	+	+	+
Neurohypophysis	+	+	−
CNS	+	±	−

SB, Skull base; +, present/developed; ± partially present/partially developed; −, absent/undeveloped; CNS, central nervous system

Fetuses with anencephaly and craniorachischisis

The bones of the skull vault were present as small fragments and the foramen magnum was lacking. Vertebral arches were incompletely developed down to the level of the lumbar region (Fig. 3).

In comparison with normal fetuses of the same age the base of the skull (basichondrocranium) appears abnormal: it is curved and positioned vertically towards the vertebral column axis. The ossification centres appear under-developed. The notochord is still present inside and around the skull base (Fig. 4). The basichondrocranium is mostly cartilaginous and contains well-developed cords of notochordal tissue situated inside the wide perichordal space, extending dorsally almost up to the hypophysis. The sella turcica is only partially developed as is the abnormally positioned hypophysis (Fig. 5). While notochordal tissue can be easily recognized inside the basichondrocranium, it is much harder to detect the disintegrating notochord in connective tissue. Nevertheless, groups of degenerating notochordal cells and extracellular matrix can be found dorsally (Fig. 6) and caudally (Fig. 7) to the curved basichondrocranium. Similar structures cannot be identified within the connective tissue of normal fetuses at the same stage. The cerebellum and cerebrum are not detectable and are replaced by blood vessels, glial-vascular and connective tissue. In the spinal cord, irregular strands of nervous tissue, blood vessels and connective tissue are found (Table 3).

Fetuses with anencephaly and cranioschisis

The bones of the skull vault were present as small fragments and the foramen magnum was developed. Vertebral arches appeared normal (Fig. 8).

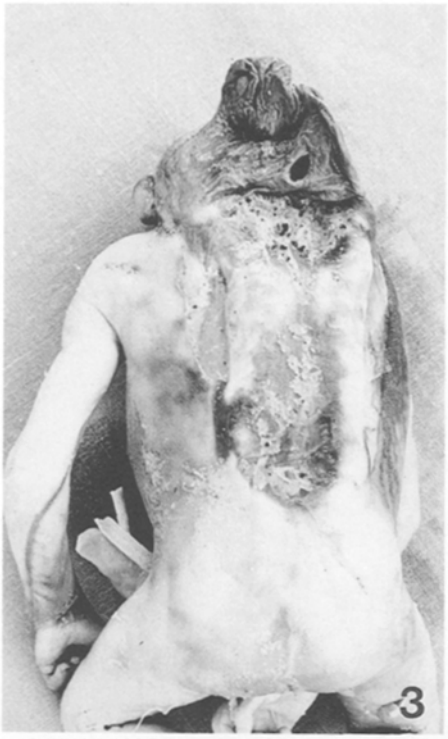
During the 16th developmental week remnants of the notochord can be detected only in the sphenoidal part of the basichondrocranium, ending close to the normally situated hypophysis (Fig. 9). Typical notochordal tissue lies inside a wide perichordal space (Fig. 10). At later stages, further development of ossification centres is accompanied by complete disappearance of notochordal remnants inside the basichondrocranium. When compared with normal samples, slight changes in the shape and position of the skull base towards the vertebral column are found. There are no notochordal remnants around the skull base (Fig. 11). Within the central nervous system, the cerebrum is reduced to amorphous tissue containing blood vessels and glial-connective tissue. The cerebellum and the spinal cord are only partly developed (Table 3).

Discussion

Our histological analysis of all developmental stages (mesenchymal, chondrogenesis, osteogenesis) of skull base formation has shown that each stage presented specific morphological changes in the course of notochord depletion. The peculiar characteristic of the cranial end of the notochord was its branching, which reached a maximum between the 6th and 12th week of development. The progressive disappearance and degeneration of the notochord and its branches in the head area were partly due to the development of the ossification centres in the basichondrocranium.

Earlier studies on the developmental events in the human head area described only bones from the skull base (Patten 1948) or were done with very small numbers of human embryos and fetuses (Marin-Padilla 1979). Except for the work of Huber (1912) the relationship of the notochord to the basichondrocranium during its mesenchymal stage has not previously been described in detail. Williams (1908) claimed the complete notochord lay inside the developing basichondrocranium. This contrasts with our findings showing a great part of the notochordal tissue lining primordial basichondrocranium during the early chondrification stage. Interactions between this part of the notochord and the pharyngeal epithelium lead to the formation of the bursa pharyngea (Saraga-Babić 1990). Similar descriptions of the intracranial notochord were reported by Marin-Padilla (1979).

Our investigation also revealed persistence of notochordal tissue in the head when compared with other levels of the body axis and parallel delayed appearance of ossification centres in the skull primordia. This may explain the fact that even in adults, remnants of the notochordal tissue can be found in the area of the nasopharynx, clivus and cervical vertebra (Perzin and Puspharaj 1986). In contrast with our findings Goto and Uhthoff (1986) reported that the development of the tissue surrounding the notochord was identical over the entire extent of the spine, denying the correlation between the development of the notochord and the surrounding skeletal tissue. In our specimens regional differences in the differentiation and disappearance of the notochord within the basichondrocranium and cervical spine were always accompanied by parallel changes in the surrounding skeletal tissue. Such findings implicate the impor-



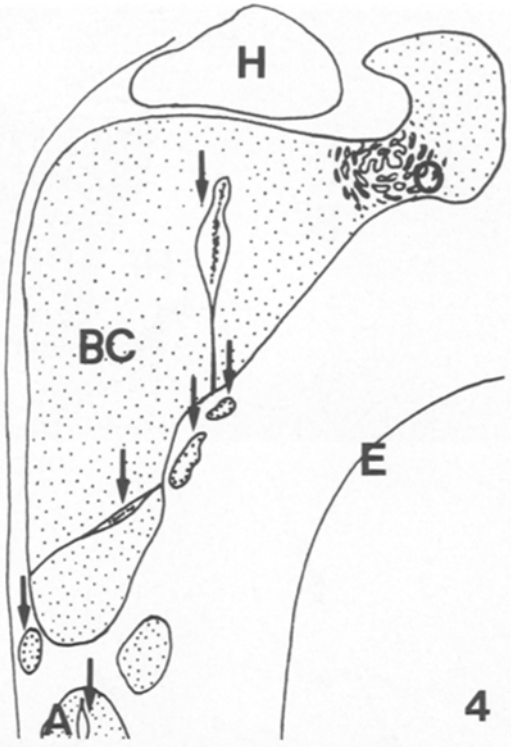


Fig. 3. Dorsal view of an anencephalic fetus with craniorachischisis in the 16th week of development. Vertebral arches are affected down to the level of lumbal vertebrae

Fig. 4. Three-dimensional reconstruction of the malformed skull base in human anencephalic fetus with craniorachischisis in the 16th week of development. The relation of the notochord (*arrows*) to the odontoid process of the axis (*A*), basichondrocranium (*BC*), pharyngeal epithelium (*E*) and hypophysis (*H*). Only one ossification centre (*O*) is developing in the ventral part of the basichondrocranium

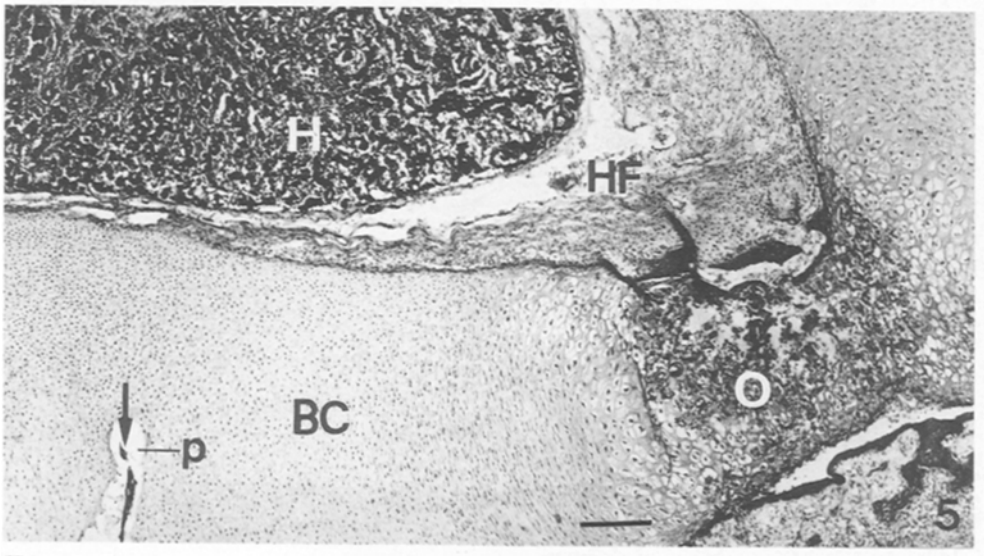
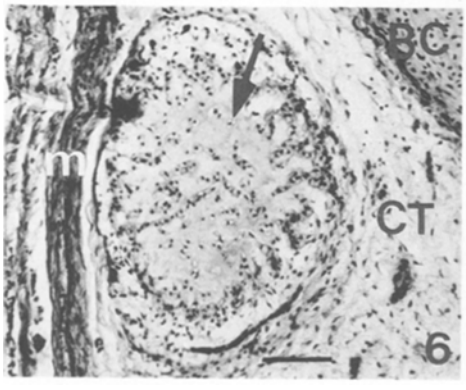


Fig. 5. Part of the skull base in human anencephalic fetus with craniorachischisis in the 16th week of development. Mostly cartilaginous basichondrocranium (*BC*) contains remnants of the notochordal tissue (*arrow*) surrounded by a wide perichordal space (*p*), which end near the hypophysis (*H*). One ossification centre (*O*) is seen around the partly developed hypophyseal fossa (*HF*). H&E, ×30; *bar* = 330 μm



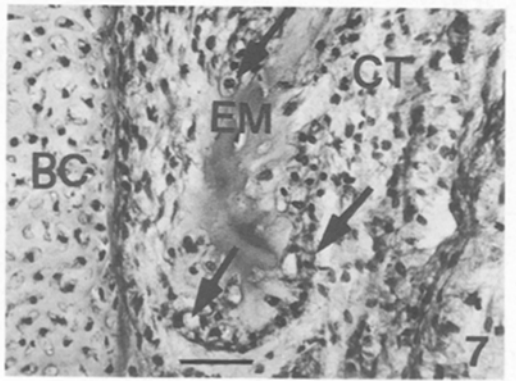
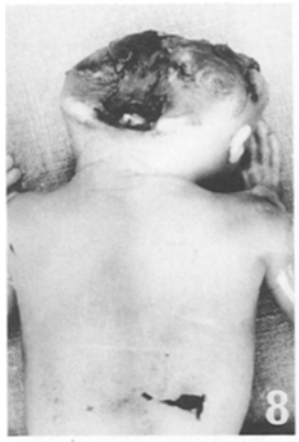
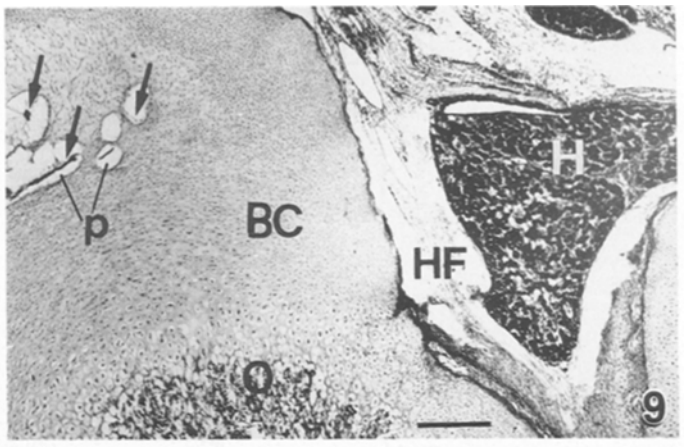
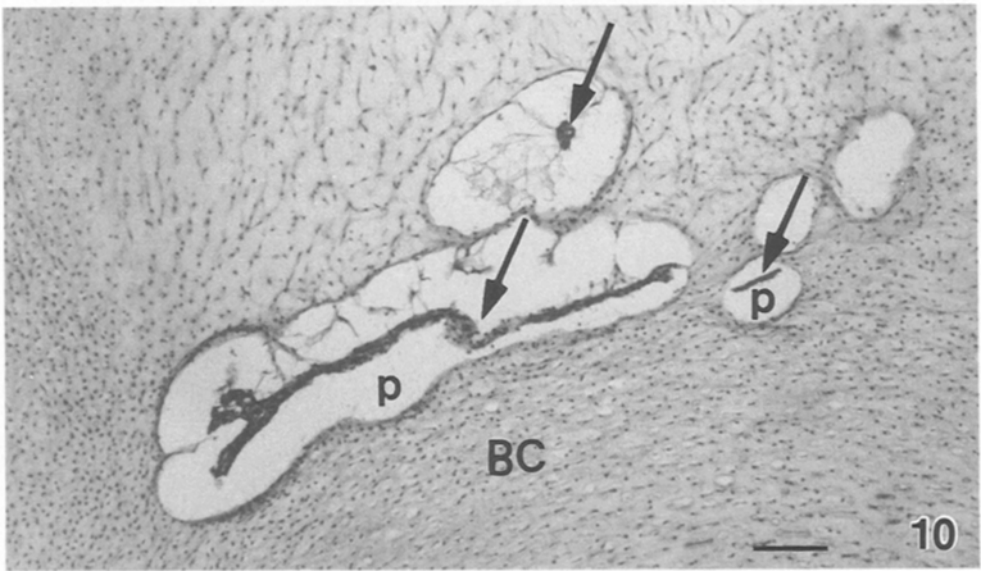


Fig. 6. Notochordal tissue (*arrow*) is situated in the connective tissue (*CT*) between the cartilaginous basichondrocranium (*BC*) and the meninges (*m*). Same embryo as shown in **Fig. 3**. H&E, ×60; *bar* = 166 μm

Fig. 7. Cords of the degenerating notochordal cells (*arrows*) which surround the extracellular matrix (*EM*) are visible within the connective tissue (*CT*) ventrocaudally to the basichondrocranium (*BC*). Same embryo as shown in **Fig. 3**. H&E, ×100; *bar* = 100 μm







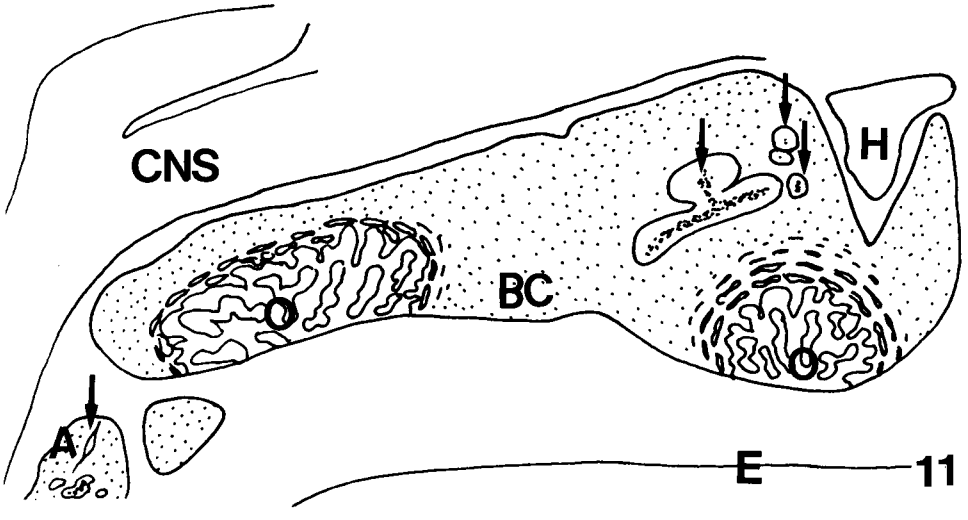


Fig. 8. Dorsal view of a human anencephalic fetus with cranioschisis in the 16th week of development. Vertebral arches are not affected

Fig. 9. Part of the basichondrocranium of the human anencephalic fetus with craniochisis in the 16th week of development. Remnants of the notochordal tissue (*arrows*) surrounded by the perichordal space (*p*) end near the hypophysis (*H*), which is normally positioned within the well-developed hypophyseal fossa (*HF*). Ossification centre (*O*). H&E, ×20; *bar* = 500 µm

Fig. 10. Notochordal remnants (*arrows*) surrounded by a wide perichordal space (*p*) lay inside the ossifying basichondrocranium (*BC*) of the 16th week anencephalic fetus with cranioschisis. Same embryo as in Fig. 8. H&E, ×60; *bar* = 166 µm

Fig. 11. Three-dimensional reconstruction of the skull base in human anencephalic fetus with cranioschisis in the 16th week of development. The relationship of the notochord (*arrows*) to the odontoid process of the axis (*A*), basichondrocranium (*BC*), pharyngeal epithelium (*E*) and hypophysis (*H*). Partially developed central nervous system (*CNS*)

tance of a notochord-mesenchyme interaction during the normal development of the skull base that additionally might be of essential importance for the normal formation of the overlying central nervous system.

The data described were used for comparison with features occurring in anencephalic fetuses. Previous studies of the abnormal formation of the skull base were carried out on a small number of human embryos (Muller and O'Rahilly 1991) or on human fetuses in the second half of pregnancy and newborns. Investigations by Marin-Padilla (1965) only described macroscopic aspects. Malformed fetuses reported in the present

investigation were younger (16th–22nd week of development) and smaller, with less developed ossification centres which made it possible to investigate the whole basichondrocranium and central nervous system of the anencephalic fetuses histologically. This revealed the existence of two different degrees of the same malformation:

1. Anencephaly with craniorachischisis – this variation showed severe disturbances in the development and formation of the basichondrocranium and in the course of the notochord, as well as in the development of the central nervous system (Table 3).

2. Anencephaly with cranioschisis – this variation showed mild disturbances in the formation of the basichondrocranium and in the course on the notochord. The central nervous system and the hypophysis were also less affected than in the case of craniorachischisis.

Our findings on human samples confirmed previously established experimental data provided on animals by Marin-Padilla (1966a, 1979) and Marin-Padilla and Ferm (1965) who considered cranioschisis occulta with encephalocoele and cranioschisis aperta with exencephaly as two different degrees of the same anomaly in golden hamsters. Investigations on human axial disorders and chondrodystrophies indicated the importance of primary paraxial insufficiency in their genesis (Marin-Padilla and Marin-Padilla 1977; Marin-Padilla 1991). The extent of axial defect was found to be dependant on the severity of mesodermal affection. Obviously, the appearance of different degrees of the same malformation in anencephaly does not seem to be species-specific, but common for different mammals.

Like our morphological findings, data from numerous experimental models have proven that disturbances in the course and position of the notochord during development may influence the formation of neighbouring skeletal structures (Zilliken 1967; Chenney and Lash 1981) and adjacent neural tissue (van Straaten and Drukker 1987; Center et al. 1988; Lumsden 1991; Yamada et al. 1991). It was recently found that local signals from the notochord induce functional properties of the floor plate, which is a source of chemoattractant for commissural axons of the spinal cord (Tessier-Lavigne et al. 1988; Placzek et al. 1991; Yamada et al. 1991). In mouse mutants, the appearance of accessory or extraneural tubes was combined with supernumerary notochordal masses (Center et al. 1988). Embryos with homozygous *Brachyury* gene mutation showed gross disturbances of the primitive streak, while in heterozygotes only sacral vertebrae were affected (Herrmann 1991).

Development of human neurospinal dysraphism results from interactions between the genetic background of an embryo and the environmental factors. The gross abnormalities of the central nervous system are determined very early during development and the failure of mesodermal induction seems to be of major significance in their genesis (Berry 1992a, b). Experiments with animal mutants disclosed that homeobox genes are expressed in both neuroectoderm of the neural tube and the underlying mesoderm. Growth factors seem to be necessary for initial specification of mesoderm with subsequent homeobox activity (Berry 1992a, b). Among different teratogens, vitamin A and retinoic acid produced combined skeletal and neural defects (Marin-Padilla and Ferm 1965; Morriss 1972; Alles and Sulik 1990). Spatial and temporal expression pattern of *Evx*1, *Hox* and *Pax* genes was compatible with a role specifying certain steps during the formation of the axial skeleton (Kessel and Gruss 1990). The combination of *Hox* gene products was found to specify individual body segments (Bastian and Gruss 1990), while *Pax*1 was involved in correct differentiation from sclerotome to vertebrae and intervertebral discs (Balling et al. 1988; Deutch et al. 1988). The described experimental data would suggest that failure of appropriate homeobox activity could result in neurospinal dysraphism in Man. Growth factors which provide positional information for graded activation of these genes seem to have an important role during mesodermal induction. Via homeogenetic induction across germ layers, mesoderm could act on the neural plate. If the induction process fails, dysmorphogenesis of the neural tube may appear.

In vertebrate embryos, a process of segmentation occurs in the somites and in the hindbrain, where transient repeated structures called rhombomeres develop. The rapid growth and segmentation of the human hindbrain appears during the 3rd developmental week, which is also the period of the most intensive development of the notochord and the paraxial mesoderm (O'Rahilly et al. 1984). The timing of mesenchymal defects is probably as early as stages 8 and 9 (18–20 days) (Muller and O'Rahilly 1991). We could speculate that the teratogenic action during the 3rd developmental week, which is the most vulnerable period of axial formation, could cause a more severe form of malformation. Teratogenic action during the 4th developmental week could cause a mild form of the same malformation, as the notochord is already formed in the future head area and the paraxial mesoderm shows segmentation into somites. Marin-Padilla (1979, 1991) also associated the timing and duration of the mesodermal insufficiency with the final type of axial malformation. His conclusions on basichondrocranium in human chondrodystrophies support the manner in which segmentation of the human hindbrain is established.

Histological analysis of the complete basichondrocranium and its surrounding tissues was essential to clarify subtle, but important differences that occur during abnormal formation of the skull base in human embryos and fetuses. The primary mesodermal insufficiency (Marin-Padilla 1979, 1991) or abnormalities of intersegmental arteries (Tanaka and Uhthoff 1981a, b) could influence such tissue development. Our investigation revealed that in humans neurodevelopmental abnormalities underlying skeletal defects of the basichondrocranium were always seen in embryos and fetuses accompanied by an abnormal notochord. These findings strongly suggest the role for the notochord during formation of cephalic structures in humans.

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