Sclerotom al origin of the ribs

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SUMMARY

The somites of vertebrate embryos give rise to sclerotomes and dermomyotomes. The sclerotomes form the axial skeleton, whereas the dermomyotomes give rise to all trunk muscles and the dermis of the back. The ribs were thought to be ventral processes of the axial skeleton and therefore to be derived from the sclerotomes; however, recently a dermomyotomal origin of the distal rib (the costal shaft) was suggested, with only the proximal parts (head and neck of the rib) being of sclerotomal origin. We have re-investigated the development of the ribs in quail-chick chimeras and carried out three experimental series. (1) Single dermomyotomes and (2) single sclerotomes were grafted homotopically, and (3) the ectoderm overlying the unsegmented paraxial mesoderm was removed in the prospective thoracic region. We found that the cells of the dermomyotome gave rise to epaxial and hypaxial trunk muscles, dermis of the back and endothelial cells, but not to ribs. Cells of the sclerotome formed the axial skeleton and all parts of the ribs. Ablation of the ectoderm, which affects dermomyotome development, results in severe malformations of the ribs, probably due to disturbed interactions between dermomyotome and sclerotome. Our results strongly confirm the traditional view of the sclerotomal origin of the ribs.

Key words: Rib, Somite, Sclerotome, Dermomyotome, Quail-chick chimera

INTRODUCTION

In higher vertebrates, the thorax consists of paired ribs spanning from the vertebral column to the sternum. The spaces between neighbouring ribs are bridged by the intercostal muscles. Each rib is made up of the costal head articulating with two adjacent thoracic vertebral bodies and the intervertebral disc, the costal neck and tubercle articulating with the transverse process of the corresponding vertebra, and the costal shaft. The costal head, neck and tubercle can be referred to as the proximal parts of the rib, the costal shaft as the distal part. Furthermore, the costal shaft can be subdivided into a vertebral portion and a sternal portion, the latter being in contact with the sternum.

The origin of the ribs is controversial. The first experimental analysis was performed using a carbon particle labelling technique and ectopic grafting (Straus and Rawles, 1953). The authors concluded that the head, neck and vertebral portion of the shaft of avian ribs are of somitic origin whereas the sternal portion of the shaft originates from somatic mesoderm. In contrast, based on slightly modified techniques, Seno (1961) described that the somites give rise to all parts of ribs. Subsequent studies using coelomic grafting and localized X-ray irradiation (Pinot, 1969) and tantalum foil barriers (Sweeney and Watterson, 1969) confirmed Seno’s conclusions. The introduction of the quail-chick chimera technique (Le Douarin, 1969, 1973) opened up the possibility of tracing the somitic derivatives even after long reincubation periods. Using this cell-lineage labelling technique, Christ et al. (1974) showed that both the entire ribs and the intercostal muscles are derived from the somites, whereas the connective tissue in the ventrolateral thoracic wall is of somatopleural origin. The rib-forming potency of the thoracic somites is already determined in the unsegmented paraxial mesoderm, as was shown by heterotopic grafting (Kieny et al., 1972; Jacob et al., 1975). Positional information determining cranio-caudal positional identity of the somites is controlled by genes that establish the so-called Hox-code (Kessel and Gruss, 1991).

Nevertheless, the question remains from which compartment of the somites the ribs are formed. The early somite consists of an epithelial wall enclosing the mesenchymal somitocoel cells. The ventral part of the epithelial wall forms the mesenchymal somitocoel cells. The ventral part of the epithelial wall and the somitocoel cells, which express Pax-1 and Pax-9, form the sclerotome, while the dorsal, Pax-3-positive part, retains its epithelial structure and is called dermomyotome (Christ and Ordahl, 1995). The general view is that the ribs are derived from the sclerotomes and the intercostal muscles from the dermomyotomes (Christ and Wilting, 1992). In previous studies we have shown that the somitocoel cells form the proximal parts of the ribs and also contribute to the distal ones (Huang et al., 1994, 1996). However, Kato and Aoyama (1998) have recently postulated that the distal parts of the ribs originate from the dermomyotomes. Their data are based on experimental dermomyotome ablation and interspecific grafting of three adjacent dermomyotomes.

To clarify the controversial view concerning the origin of the
ribs, we carried out three experimental series in avian embryos. First we transplanted a single thoracic dermomyotome homotopically from quail into chick. In this way, the dermomyotome can be grafted without any adhering sclerotomal cells. The results show that quail-derived nuclei are located in the intercostal muscles, but not in the ribs. In the second series, the sclerotomal mesenchyme of a single somite was grafted interspecifically. The results show that both proximal and distal parts of the ribs consist of graft-derived cells. In the third experimental series, we removed the ectoderm covering the unsegmented paraxial mesoderm at the prospective thoracic level. As shown previously, the dermomyotome fails to be formed in the absence of the surface ectoderm (Sosic et al., 1997; Schmidt et al., 1998). We observed that the ribs were misshapen, shortened or missing on the operated side as a consequence, meaning that the formation of the ribs depends on interactions between dermomyotome and sclerotome, while the sternum was not affected.

MATERIALS AND METHODS

Embryos
Fertilized eggs of the White Leghorn chicken (Gallus gallus domesticus) and the Japanese quail (Coturnix coturnix japonica) were obtained from a local hatchery and incubated at 37.8°C and 80% relative humidity. Embryos were staged according to Hamburger and Hamilton (1951).

Dermomyotome grafting
Transplantations of the dermomyotome were carried out on stage 16-17 HH quail and chick embryos (Fig. 1). A single quail dermomyotome with overlying ectoderm at the prospective thoracic level (somites 19-26) was cut out with an electrolytically sharpened tungsten needle. The dermomyotome was carefully separated from the sclerotome. In the stereomicroscope, the dermomyotome can be recognized as an epithelial structure, which is separated from the sclerotome by a basement membrane (Mestres and Hinrichsen, 1976). The isolated dermomyotome was transferred to the chick host by means of a Spemann pipette. It was then adjusted in the corresponding position of the chick host after the dermomyotome of the host had been removed. The surface ectoderm overlying the grafted dermomyotome was helpful for the positioning of the transplant. The chick hosts were reincubated for 5 hours and 4-8 days. Further details of the grafting procedure were described previously (Huang et al., 1997).

Sclerotome grafting
For the transplantation of the sclerotomal mesenchyme, quail and chick embryos of stage 16-17 HH were used (Fig. 2). The microsurgery was performed at the level of somites 19-26. In the chick host, the lateral, anterior and posterior boundaries of the dermomyotome of one thoracic somite, together with the adjacent ectoderm, were dissected with an electrolytically sharpened tungsten needle. The dermomyotome was then flapped medially and the sclerotomal mesenchyme was sucked out with a micropipette. In the quail donor, the dermomyotome of one thoracic somite was removed and the sclerotomal mesenchyme was isolated as a cell block. This block of cells was then transferred to the chick host and implanted into the space, where the chick sclerotome had previously been removed. At the end, the dermomyotome of the host was flapped back. The chimeras were reincubated for 4 hours and for 6-8 days.

Removal of the surface ectoderm
Chick embryos of stage 10-12 HH were used. The surface ectoderm covering the unsegmented paraxial mesoderm and the adjacent lateral plate at the prospective thoracic level was removed with a tungsten needle (Fig. 3). The operated embryos were reincubated for 10-12 days. Subsequently, skeletal preparations were performed as described below.

Skeletal preparation
The embryos were stained according to a modification of the Alcian blue and the Alizarin red staining procedure described by Wallin et al. (1994). Embryos were first deskinned and then fixed in 100% ethanol for 24 hours, followed by 100% acetic acid for another 24 hours. Incubation in the staining solution, containing 0.3% Alcian blue in 70% ethanol, 0.1% Alizarin red S in 96% ethanol, glacial acetic acid, 70% ethanol, diluted 1:1:1:17 (v:v), was performed for 24 hours at room temperature. The specimens were then rinsed in water and transferred into 20% glycerol with 1% potassium hydroxide, then transferred into 50% and 80% glycerol, and finally stored in 100% glycerol.

Immunohistochemistry
The chimeras were fixed in Serra’s fixative (Serra, 1946), dehydrated, embedded in paraffin and sectioned (8 μm thick) serially in coronal or sagittal planes. Quail cells were detected by an anti-quail antibody (QCPN) (diluted 1:500; Developmental Studies Hybridoma Bank, Iowa City, USA).
Iowa City, IA, USA) as a primary antibody and an alkaline phosphatase-conjugated goat-anti-mouse antibody (diluted 1:400; DAKO, Hamburg, Germany) as a secondary antibody. Nitroblue tetrazolium (NBT) and X-phosphate (Boehringer, Mannheim, Germany) were used as chromogens to reveal a blue signal. Muscle cells were identified by a polyclonal anti-desmin antibody (diluted 1:400; Sigma, Deisenhofen, Germany) as a primary antibody and peroxidase-conjugated goat-anti-rabbit antibody (diluted 1:300; Sigma) as a secondary antibody. 3,3'-diaminobenzidine tetrahydrochloride (DAB) was used as a chromogen, yielding a brown signal.

For double labelling, 0.3% H₂O₂ in methanol was used to block endogenous peroxidase activity. After blocking of non-specific binding with 10% horse serum (Gibco, Karlsruhe, Germany) and 2% bovine serum albumin (BSA; Serva, Heidelberg, Germany), the sections were incubated with the primary antibodies (anti-quail and anti-desmin). After repeated blocking with horse serum and BSA and rinsing with PBS, the sections were incubated with the secondary antibodies (alkaline phosphatase- and peroxidase-conjugated antibodies). The DAB reaction was performed first, and the NBT and X-phosphate staining was carried out subsequently. Finally the sections were counterstained with Nuclear Fast Red (Chroma, Germany).

RESULTS

Dermomyotome grafting
A total of 16 transplantations were performed and 11 embryos that developed normally were subsequently evaluated after various reincubation periods. In order to control the quality of the grafting procedure, three embryos were fixed after 5 hours of reincubation and sectioned transversally. The grafted dermomyotome was made up exclusively of quail (donor) cells (Fig. 4). No quail cells could be found in the sclerotome. The surface ectoderm overlying the grafted dermomyotome was also of quail origin. After a reincubation period of 4 days, the ribs and the intercostal muscles were forming. In the operated segment, quail nuclei were found in the myotubes of the intercostal muscles, but not in the rib blastemas (Fig. 5). Most of the connective tissue in the musculature was of chick origin, but some quail cells were also present. This result was confirmed in embryos that were reincubated for 8 days. In the back, quail cells were located in both the intrinsic segmental muscles and in the superficial back muscles. The dermis of the back was also made up of quail cells (data not shown).

Sclerotome grafting
A total of 12 transplantations were performed and 10 embryos were evaluated at the end of the reincubation period. Three embryos were fixed after 4 hours of reincubation to study the quality of the grafting procedure. The major portion of the sclerotome was of quail (donor) origin (Fig. 6). Only medially, a few cells adjacent to the aorta and the ventral part of the neural tube were chick cells. The dermomyotome was of chick origin.
origin. After 6-8 days of reincubation, the costal head and neck, the costal tubercle and most of the costal part of the shaft were formed. The costal head, which was exclusively made up of quail cells, formed a joint with the two adjacent vertebral bodies and the intervening tissue (intervertebral disc homologue). This intervening tissue and the neighbouring halves of the two adjacent vertebral bodies were made up of quail cells. The costal neck and tubercle were also derived from the grafted sclerotome (data not shown). In the distal part of the rib (the shaft) quail sclerotome-derived cells were located in the caudal half of one rib and the cranial half of the next caudal rib, which is in line with previous findings showing resegmentation of both the vertebral column and the ribs (Huang et al., 1996). The connective tissue surrounding the ribs was also of quail origin (Fig. 7), whereas the intercostal muscles were devoid of any quail nuclei.

Removal of surface ectoderm

The chick has seven ribs (Fig. 8). The first two ribs are not in contact with the sternum and therefore lack the sternal part of the shaft (Costae fluctuantes). The third to the sixth rib reach the sternum with their sternal parts. The seventh rib can either have a connection to the sternum or may have a free end (Nickel et al., 1992). The surface ectoderm overlying the unsegmented paraxial mesoderm was removed at the prospective thoracic level of 20 chick embryos. 11 embryos survived until day 10-12 of reincubation. The ribs of the control side (unoperated side) displayed normal development in most of the operated embryos. However, in one embryo, the third rib had an abnormal fissure in its distal part. In another embryo, the distal parts of the first and the second ribs were fused. The abnormalities of the ribs of the operated side could be summarized into three types. They were either shortened, missing or misshapen (Fig. 8). The first one or two ribs were absent in two cases and shortened in four cases. In two embryos, the third rib did not have a sternal part of the shaft. In one embryo, the fourth to the sixth rib contained only the vertebral but no sternal part of the shaft. In three cases, the first three ribs were misshapen. In most embryos, the processus uncinati, which connect and stabilize two adjacent ribs, were missing on the operated side, and a bifid spine was also observed (Fig. 8).

DISCUSSION

The entire vertebral column is derived from the somitic sclerotomes (Christ et al., 1974). Concerning the origin of the...
ribs, which can be regarded as processes of the vertebral column, most studies have indicated that they are formed by condensations of sclerotomal cells, whereas the intercostal muscles are derived from the dermomyotomes (Verbout, 1985; Christ and Wilting, 1992). However, recent findings by Kato and Aoyama (1998) postulated that the distal part of the ribs originate from the dermomyotome. This conclusion was based on the removal and the transplantation of the dermomyotomes in avian embryos. Our results are in conflict with the conclusions of Kato and Aoyama (1998), because our grafting experiments show clearly that both the proximal and the distal parts of the ribs are formed by the sclerotomal mesenchyme. The dermomyotome gives rise only to trunk muscles in the thoracic wall, including the intercostal muscles (Fig. 9). The interpretation by Kato and Aoyama (1998) was possibly due to the grafting technique in which three consecutive thoracic dermomyotomes with adjacent lateral plate mesoderm were grafted. The authors admit that the dermomyotomes were grafted together with adhering sclerotomal cells. If there were as few as 10 sclerotomal cells adhering to each dermomyotome, the small number of sclerotomal cells at the beginning of the experiment would have resulted in millions of cells after 7 days of reincubation, assuming a cell cycle time of 8 hours (Langman and Nelson, 1968). These might be the cells that gave rise to the distal part of the ribs in the above-mentioned experiments. To avoid inclusion of sclerotomal cells into the graft we isolated a single dermomyotome only. Only in this way can the dermomyotome be cleanly separated from the sclerotome.

It has been shown that Pax-1 protein is required for the formation of the proximal parts of the ribs (Wallin et al., 1994). The ability to form ribs is determined before somitogenesis (Kiény et al., 1972; Jacob et al., 1975) but local interactions are also involved, especially in the morphogenesis of the distal parts of the ribs. This is suggested by the fact that the distal parts are missing in Myf5 and Pax-3 deficient mice (Braun et al., 1992; Henderson et al., 1999; Dickman et al., 1999). On the other hand, the proximal parts of the ribs are missing in Pax-1 deleted mice (Wallin et al., 1994). This suggests that the formation of the distal part of the ribs depends on a cross-talk between sclerotome and myotome. Further investigations have shown that in the Myf-5 mutant mice the myotomes do not express FGF-4 and FGF-6, which together with TGFβ-2 induce chondrogenic nodule formation in the cultures of somitic cells (Grass et al., 1996). It has been proposed that the Myf-5-expressing cells in the myotome or dermomyotome normally secrete these growth factors, providing growth signals for the sclerotomal cells that form the distal part of the ribs.

The removal of surface ectoderm was performed to study the relationship between the dermomyotome and rib development. As shown in previous studies, the formation of the dermomyotome depends on signals from the surface ectoderm overlying the paraxial mesoderm, since removal of this tissue leads to the absence of the dermomyotomes (Sosie et al., 1997; Dietrich et al., 1997). After removal of the surface ectoderm, ribs can be missing, shortened or misshapen. This indicates that the normal development of the ribs is dependent on signalling from the dermomyotome or its derivative, the myotome. Thus, it is not surprising that the extirpation of the dermomyotome results in defects of the distal rib (Kato and Aoyama, 1998). This, however, is an indirect effect.

Furthermore, our results again confirm the resegmentation theory of the vertebrae and the ribs (Remak, 1855; Bagnall et al., 1988; Huang et al., 1996). Sclerotomal cells from one somite form the neighbouring part of two adjacent vertebral bodies and the intervertebral tissue (disc-homologues). Sclerotomal cells of one somite also form the caudal part of one rib and the cranial part of the next caudal rib. The dermomyotome derived from one somite contributes to the intercostal muscle, including its origin and insertion. A corresponding principle has also been found in the organisation of the craniofacial region (Köntges and Lumsden, 1996).

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