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CD44 is highly expressed in stem/progenitor cells originating the intervertebral discs in the human notochord

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Abstract. – OBJECTIVE: The notochord acts as a patterning structure, playing a key role in the formation of the vertebral column, both indirectly by inducing sclerotome cell differentiation and directly by forming the nucleus pulposus of intervertebral discs. The abnormal development of the notochord results in an easy equation with a variety of birth defects. Therefore, we focused our attention on the analysis of the early stages of human notochord development by highlighting the role of progenitor stem cells involved in the origin of intervertebral discs (IVDs).

MATERIALS AND METHODS: Eight human fetuses, ranging from 8 up to 21 weeks of gestational age, were obtained from spontaneous abortion or voluntary interruption of gestation. Samples were 10% formalin-fixed, routinely processed, and paraffin-embedded. Five micron-tick paraffin sections were obtained from each sample. Sections were stained with hematoxylin-eosin and PAS stain for a morphological examination. Tissue samples were immunostained with a commercial anti-human CD44 rabbit monoclonal antibody at 1:100 dilution.

RESULTS: Immunoreactivity for CD44 was detected in six out of eight notochords examined in this study. Reactivity for CD44 was restricted to progenitor cells giving rise to the nucleus pulposus (NP) of the developing IVDs. Positive cells showed a membranous and/or cytoplasmic immunostaining, no reactivity was observed in the nuclear compartment. CD44 expression was always restricted to IVD precursor cells, whereas cartilage precursors were devoid of labelling.

CONCLUSIONS: Our study shows, for the first time, that the stem cell marker CD44 selectively marks intervertebral disc progenitor cells, paralleling their differentiation toward a discogenic phenotype. Therefore, our results suggest that CD44 plays a key role in IVD development, allowing its differentiation from surrounding undifferentiated notochordal cells toward a IVD phenotype. Given the role of CD44 in IVD development, we may hypothesize that low CD44 levels might be associated with changes in IVD development and with susceptibility to develop back pain later in life.

Key Words:
Notochord, Immunohistochemistry, Intervertebral disc, CD44.

Introduction

Intervertebral discs (IVDs) are fibrocartilaginous joints located between the bony vertebrae, that provide flexibility and load transmission throughout the spinal column. IVDs derive from the embryonic notochord, a flexible boxy derived from the mesoderm that originates the axial skeleton[1]. The notochord gives rise to the multiple cell types detectable inside the adult nuclei pulposi[2,3]. IVDs are composed of different interrelated tissues, including the central highly hydrated nucleus pulposus (NP), the surrounding elastic and fibrous annulus fibrosus (AF), and the cartilaginous endplate (CEP), which provides the connection to the vertebral bodies[4]. Each of these tissues consists of a specific matrix structure that is maintained by a cell population with distinct phenotype[5]. Few data are available, at the best of our knowledge, on the mechanisms regulating the emergence of the IVD from the human embryonic notochord.

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In humans, the formation of the notochord starts in the middle of the embryo at Carneige stage 8 to 15 (17-41 days of gestation) and proceeds in both cranial and caudal directions[6]. During development, the notochord acts as a patterning structure, playing a major role in the formation of the vertebral column, by inducing sclerotic cell differentiation and forming the nucleus pulposus of IVDs. The notochord thus has indirect roles in the formation of vertebrae and annuli. Simultaneously, it undergoes profound changes. Notochord cells located in vertebral bodies are removed and probably relocated into intervertebral regions[7]. After birth, embryonic notochordal cells are replaced by fibrocartilage[6]. In preview studies, immunohistochemistry for beta-catenin, Wnt, axin2, cyclin D1, and c-myc have been proposed as markers for identifying notochordal cells[8]. The aim of this study is to provide additional data regarding the initial phases of development of the human notochord, focusing on the identification of the multiple stem/progenitor cells involved in the origin of IVDs, which are crucial for both IVD development and, probably, for postnatal disc maintenance.

Materials and Methods

Eight human fetuses, ranging from 8 up to 21 weeks of gestational age were analyzed in this study. All fetuses were obtained from the archives of the Institute of Pathology of the University of Cagliari. Spontaneous abortion or voluntary interruption of gestation were the cause of death. All procedures were approved by the Ethic Human Studies Committee of University Medical Centre of Cagliari (n° PG/2020/10914 codice protocollo EMIFU Riunione del 27/05/2020), according to the instructions of the Declaration of Helsinki. Only cases with clear evidence of the notochord or the vertebral column were selected. A total of eight cases were utilized for the immunohistochemical study. Gestational age ranged from the 8th to the 21st week. Samples were 10% formalin-fixed, routinely processed, and paraffin-embedded. Five micron-tick paraffin sections were obtained from each sample. Sections were stained with hematoxylin-eosin and PAS stain for a morphological examination.

Immunohistochemistry

The streptavidin-biotin-peroxidase detection system was employed for immunohistochemistry. In brief, paraffin sections were mounted on aminopropyl-triethoxysilane-coated glass slides, then deparaffinized in xylene (2 X 5 min) and rehydrated in graded solutions of ethanol (100, 90 and 70%, 3 min each). To block endogenous peroxidase, sections were immersed in 3% hydrogen peroxide for 10 min and then left in running water for 2 min. For antigen retrieval, sections underwent microwave treatment and then they were incubated with the primary antibody against CD44 (Source: Ventana; Clone: SP37; Working dilution: 1:600; Incubation time: 20'). Sections were incubated with the secondary antibody, biotinylated rabbit anti-mouse IgG (1:50 dilution in phosphate buffered-saline, PBS; Roche, Basel, Switzerland) for 30 min and washed in PBS (3 X 5 min). The streptavidin-biotin-horseradish peroxidase complex (Roche) was then added to the sections (1:500 dilution in Tris-buffered saline) for 30 min, followed by a wash in PBS (3 min).

Sections were counterstained with Mayer hematoxylin, rehydrated in graded ethanol rinses, cleared with xylene and mounted in DPX. For negative controls, the primary antibody was replaced by PBS.

Results

Immunoreactivity for CD44 was detected in six out of eight cases. In all positive cases, CD44 was expressed in the cytoplasm and along the cell membrane. No nuclear reactivity was observed. CD44 expression was restricted, in all cases, to IVD precursor cells whereas no reactivity was observed in cartilage precursors. Given the marked differences regarding CD44 expression from one case to the next, the pattern of CD44 reactivity is reported for each gestational age.

8 Weeks

No reactivity for CD44 was detected in the notochord at this gestational age. Developing IVDs, at this age, were evident in H&E-stained sections, appearing as hypercellular zones intermingled between clear zones, representing the developing cartilage (Figure 1).

9 Weeks

At this gestational age, at low power we observed the appearance of immunostaining for CD44 in the progenitor cells giving rise to the

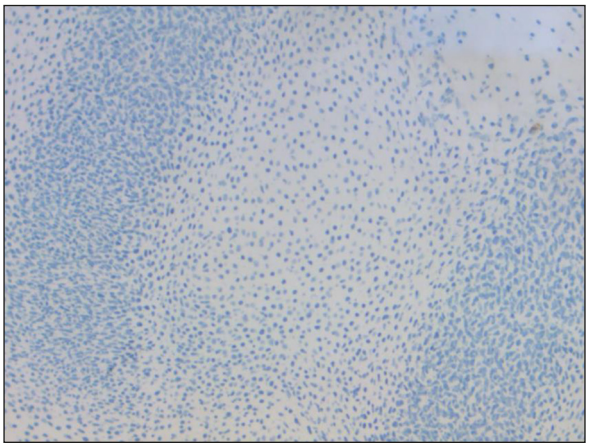

Figure 1. Gestational age eight weeks, no reactivity for CD44 was found.

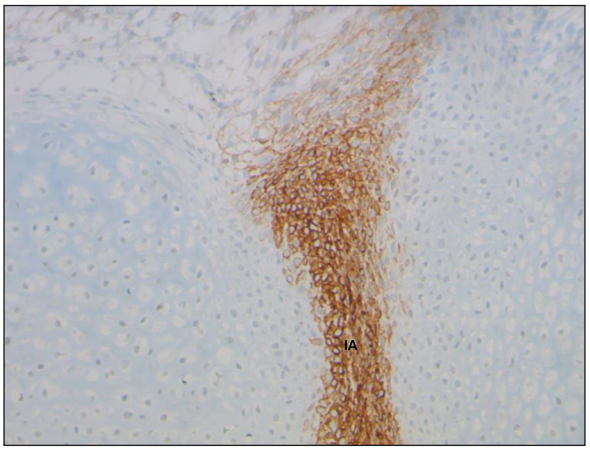

Figure 3. Gestational age ten weeks, strongly marked for CD44 was found around the cartilaginous inner anulus (IA).

intervertebral discs. At higher magnification, reactivity for CD44 was found to be restricted to the cell membrane of stem/progenitors of the intervertebral discs and in cartilaginous inner anulus (IA) (Figure 2). Immunoreactivity for CD44 changed from one intervertebral disc to the next. We found a decreasing reactivity for CD44 from the cranial to the caudal IVDs.

10 Weeks

Immunoreactivity for CD44 appeared, at this age, stronger and more diffuse as compared with fetuses of 9 weeks of gestation. The highest levels of immunostaining for CD44 were found around the cartilaginous inner anulus (IA), allowing a clear identification of the developing intervertebral disks (Figure 3).

11 Weeks

A decrease in CD44 reactivity was observed at this age (Figure 4). A slight reactivity was detected only in precursor cells located in the centre of the nucleus polposus (NP).

12 Weeks

The expression of CD44 in the developing IVDs was appeared markedly decreased at this gestational age. CD44 was expressed in a minority of IVDs precursor. Moreover, the expression of CD44 was mainly cytoplasmatic in the absence of any significant membranous immunostaining (Figure 5).

13 Weeks

Immunoreactivity for CD44 was like that detected at 12 weeks, with only a minority of IVD

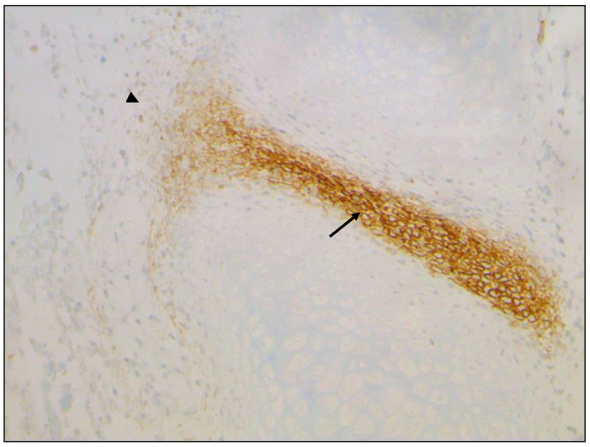

Figure 2. Gestational age nine weeks, Intervertebral discs (*arrowhead*) strong reactivity for CD44 was found in the cell membrane of inner anulus (IA).

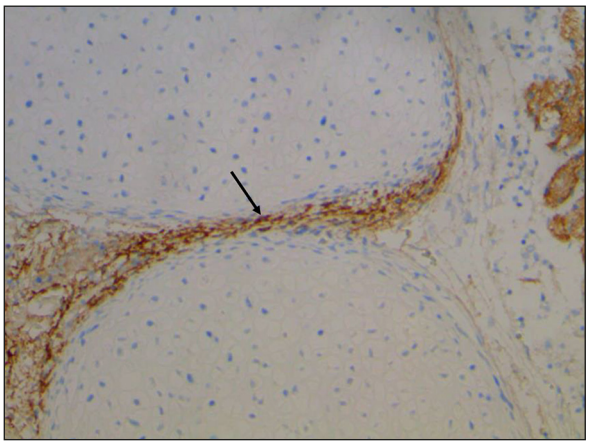

Figure 4. Gestational age eleven weeks, slight positivity for CD44 A in the centre of the nucleus pulposus (NP) (*arrow*).

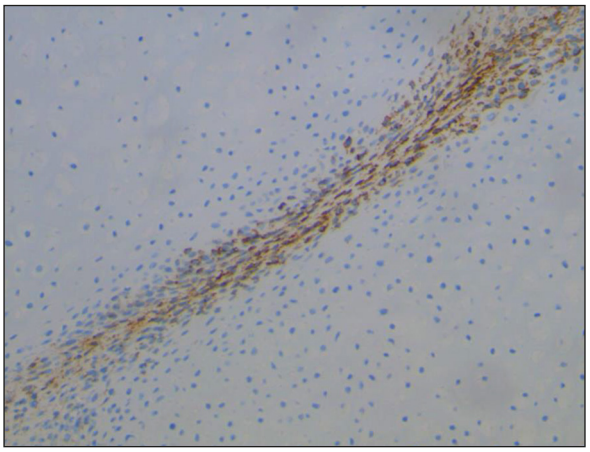

Figure 5. Gestational age twelve weeks, CD44 positivity decreases even more.

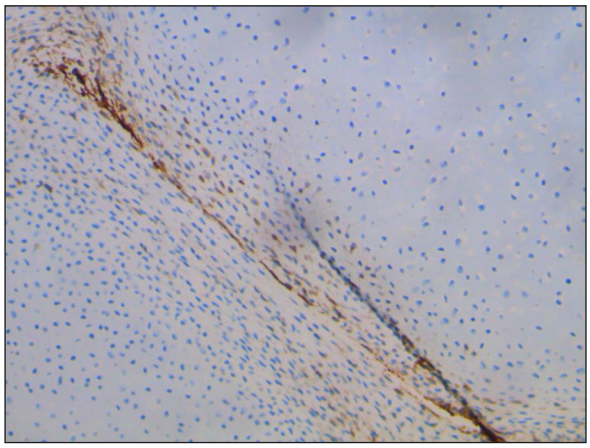

Figure 7. Gestational age fifteen weeks, few, and even rarest staining for CD44.

precursors immunostained (Figure 6). Even at this age, immunoreactivity for CD44 was cytoplasmic and strong.

15 Weeks

Only few scattered cells, mainly located in the central area of the IVDs showed weak reactivity for CD44 of this gestational age (Figure 7).

21 Weeks

No immunoreactivity for CD44 was found at this gestational in the age IVD precursor cells (Figure 8).

Discussion

Back pain affects more than half of people over the age of 65 around the world, and it is mainly due to age-related changes in the intervertebral discs. These are complex structures located between the vertebrae, that may deteriorate in older subjects and, less frequently, in young people. Reactivation of developmental programs occurring in the human intervertebral discs during fetal life represents a fascinating and promising strategy for treating intervertebral disc degeneration occurring in adulthood[9]. The objective of our study was to add some data on the immunohistochemical profile of progenitor cells originating the IVDs during embryonic development. Pilot studies on human IVDs have identified cytokeratin CK-8, -18 and -19 as well as Galectin-3 as potential notochordal cell markers[10]. Moreover, the stromal cell-derived factor 1 alpha (SDF-1-alpha) has been shown to enhance recruitment and differentiation of nucleus pulposus stem cells (NPSCs), by activating its receptor

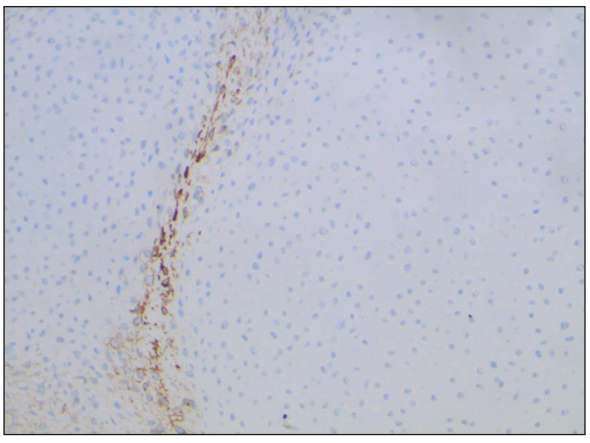

Figure 6. Gestational age thirteen weeks, CD44 positivity decreases even more.

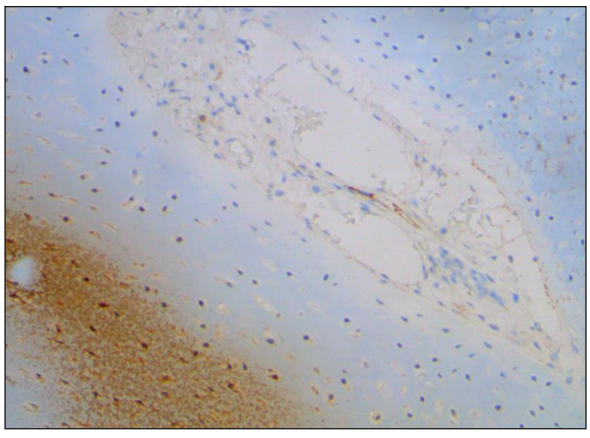

Figure 8. Gestational age twenty-one weeks, no reactivity for CD44 was found (*like eight weeks*).

C-X-C chemokine receptor type 4 (CXCR4)[11]. Sox5 and Sox6 have been reported to be required for notochord development and for originating nucleus pulposus of IVDs[12]. The following markers have been indicated as notochordal markers: OCT4, Sox9, Stromal cell-derived factor 1alpha (SDF-1alpha) CXCR4, Sonic Hedgehog, versican, brachyury, CD24, PAX1 and FoxF1[13,14]. Evidence from studies carried out in experimental models showed that CD44, a transmembrane protein functioning during development as Hyaluronal receptor[15], is expressed in the rat intervertebral discs during development[16]. Our data evidence that CD44 is highly expressed in progenitor cells giving rice to the intervertebral discs inside the human notochord, confirming previous data on the rat intervertebral discs. Interestingly, in this study CD44 expression was restricted to short periods during embryonic fetal life, starting from the 9th week of gestation and ending at about the 21st week.

In this report, for the first time we describe that the immunoreactivity for CD44 was restricted to progenitor cells of the intervertebral disc, paralleling their differentiation toward a discogenic phenotype. Our findings suggest that CD44 plays a key role in IVD development, allowing its differentiation from the surrounding notochordal cells which undergo. differentiation towards vertebral bodies. In our study, CD44 was not detected in notochordal cells in the very initial stage of disc formation, at the 8th week of gestation, confirming previous data in rats[16]. CD44 positivity was also present in milk mesenchymal staminal cell[17,18]. Therefore, a possible role of these cells in the notochord evolution could be proposed also in the neonatal period. Regarding the significance of CD44 expression in the IVD precursors during development, CD44 might act as a receptor of Glycosaminoglican Hyaluronan (HA), representing one of the principal surface receptors for HA[19]. Through CD44 interaction, HA might activate multiple intracellular signaling systems involved in differentiation, proliferation, and cell motility[20]. The expression of CD44 at the cell membrane of the notochordal cells differentiating toward a discogenic phenotype may play a regulatory role in extracellular matrix expansion like that reported during the formation of human synovial joints[21] and in the tibia of the developing rat[22]. According with this hypothesis, CD44 expression might be finalized to the formation of a hyaluronan-rich pericellular matrix useful for the incorporation of other molecules, and for the generation of a peculiar extracellular matrix of the nucleus pulposus. Recently, a major role has been assigned to the extracellular matrix in the promotion of cell differentiation toward a discogenic phenotype, maintaining the viability of IVD cells and positively affecting the expression of critical regulators of their homeostasis[23].

All these results support the hypothesis that fetal and perinatal programming may increase our susceptibility to certain diseases in childhood and adulthood[24].

Conclusions

Our findings clearly indicate that CD44 plays a role in the development of intervertebral discs in the human notochord. The expression of CD44 in the developing notochord was restricted, in this study, to fetuses of gestational age ranging from 9 to 15 weeks. During this period of intrauterine life, CD44 expression paralleled the initial phase of differentiation of cells undergoing to acquire the IVD phenotype. Further studies are needed in order verify a possible interindividual variability regarding CD44 expression in the human notochord. Given the role of CD44 in IVD development, we may hypothesize that low CD44 levels might be associated with IVD development and with susceptibility to develop back pain later in life.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Funding

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Ethics Approval

All procedures were approved by the Ethic Human Studies Committee of University Medical Centre of Cagliari (n° PG/2020/10914 codice protocollo EMIFU Riunione del 27/05/2020), according to the instructions of the Declaration of Helsinki.

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