ORIGINAL ARTICLE

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Cytokeratin expression in human arteries pertinent to intimal thickening formation in the ductus arteriosus

Abstract Expression of epithelial cytokeratins type 8, 18 and 19 can be used to study smooth muscle cell differentiation during development. We studied the differentiation of smooth muscle cells in the ductus arteriosus before and during intimal thickening and compared the changes occurring in this vessel with the adjoining elastic ascending and descending aorta and the pulmonary trunk. The ductus arteriosus, a vessel connecting the pulmonary trunk and the aorta during fetal life, constricts shorty after birth and eventually closes. Effective closure occurs only in the case of well developed intimal thickening. Cytokeratin expression during fetal development was greatest in the media of the ascending aorta and pulmonary artery, while in the ductus and descending aorta cytokeratin staining was slight. These results suggest that ductus smooth muscle cells and the smooth muscle cells of the descending aorta show a more advanced differentiation as compared to the ascending aorta and pulmonary artery. At neonatal stages cytokeratin expression in the descending aorta, pulmonary artery and the ascending aorta had disappeared as was expected with increased differentiation. In the neonatal ductus arteriosus reexpression of cytokeratins was found in cell clusters in the hyaluronic acid rich environment of the intimal thickening and in the inner media. Reexpression of cytokeratins, especially when organized in clusters, may reflect changes in gene regulation. Therefore the clusters of cytokeratin positive cells in the ductus may be indicative of extensive changes, occurring during closure of this vessel in the neonatal period, in which inner media and intima are especially involved.

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Introduction

Differentiation of vascular smooth muscle cells (SMC) can be characterized by the up and down regulation of several structural proteins. The amount of contractile proteins increases as the SMC become more differentiated [13], whereas the expression of several splice variants of fibronectin [14] and cytokeratins [15] is reported to decrease during development. Cytokeratins are an intermediate filament protein family, normally expressed in epithelial cells and epithelial neoplastic tissues [24]. Expression of the cytokeratins of the simple epithelium type is not restricted to epithelial cells, however, and these cytokeratins are also found in certain cells of mesenchymal origin [36, 37]. For example cytokeratins 8, 18 and 19 have been reported in a number of SMC tumors [4, 16, 26, 31], in vascular SMC during fetal development [15, 36] and in pathologic intimal thickening [15, 17, 18]. In vascular SMC cytokeratins 8, 18 and 19 are associated with the undifferentiated phenotype during development [15] and the dedifferentiated phenotype in pathologic intimal thickening [18]. In certain SMC it is remarkable that both desmin and vimentin can be co-expressed with the cytokeratins [1], whereas a hyaluronic acid rich environment [19] also seems to be favoured. The latter is a typical phenomenon for the neonatal DA [8, 34].

In the present study we have examined the expression of cytokeratins 8, 18 and 19 in the human arterial great vessels and focused on the ductus arteriosus (DA) as this vessel has an advanced stage of differentiation of the contractile apparatus during fetal development as compared to the ascending and descending aorta and pulmonary trunk [20, 35]. The DA is muscular artery connecting the aorta and pulmonary trunk during fetal life. The closure of this vessel shortly after birth is preceded during fetal gestation by the formation of intimal thickening that is essential for effective closure [9]. This physiological intimal thickening resembles the intimal thickening found in arteriosclerosis [8, 34]. In this way cytokeratin expression can be linked to the various stages of SMC
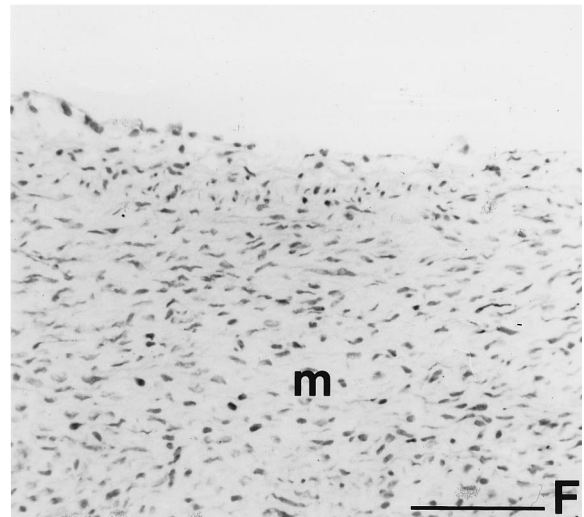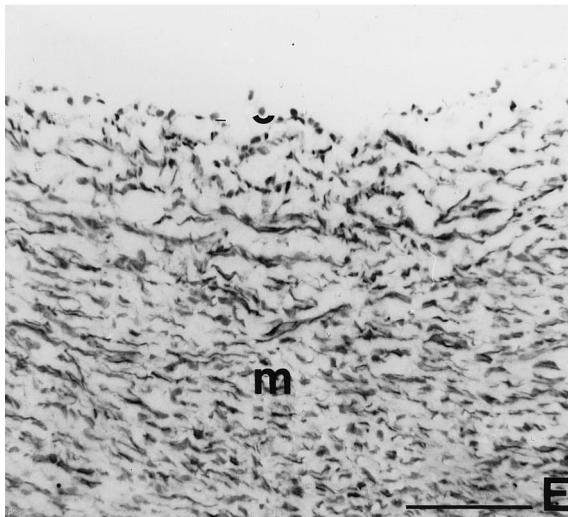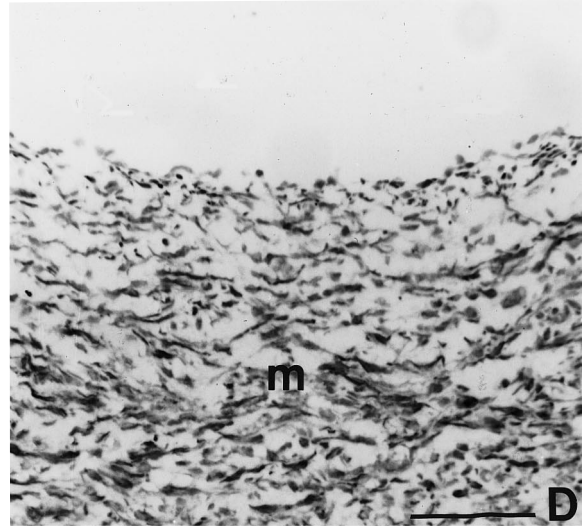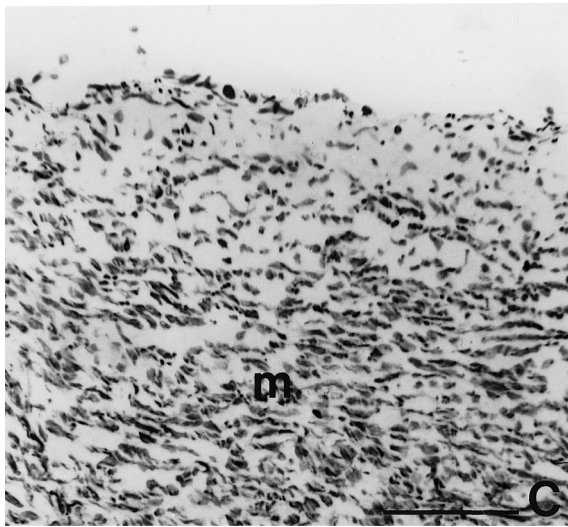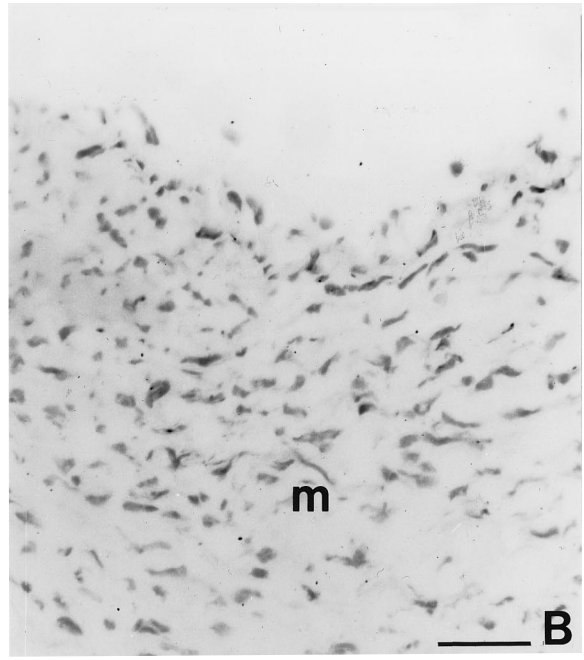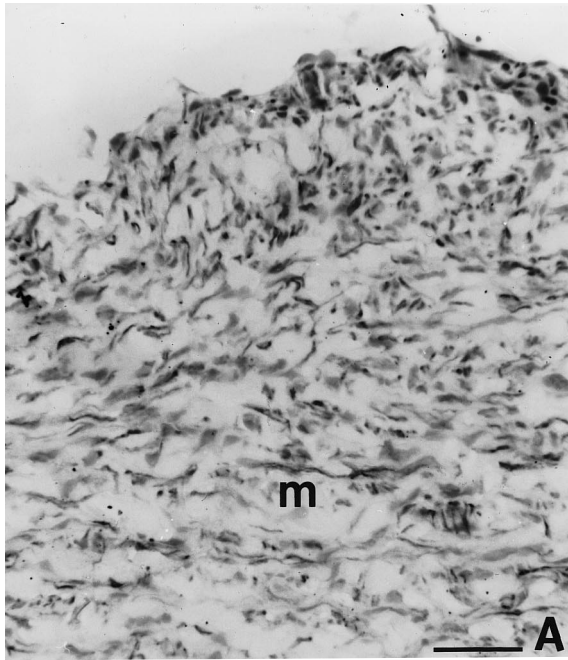
differentiation that are present in the DA and adjoining great vessels during development, including the formation of intimal thickening.

Methods

Tissue samples

Fetal DA, aorta and pulmonary trunk were obtained from five human fetuses between 13 and 19 weeks of amenorrhea. Two DA did not show signs of intimal thickening (13–15 weeks gestation) and three DA were showing intimal changes (17–19 weeks). The fetuses studied did not show any macroscopically detectable abnormalities. Five normal neonatal DA were resection specimens of the middle part of the DA obtained during cardiac surgery from children between 1 and 3 weeks of age. These children had been treated with prostaglandin E_1 to maintain ductal patency. Previous studies have shown that despite the various cardiac malformations the DA of these patients is normal with respect to functional and morphological characteristics [10, 22, 34]. Aortic and pulmonary vessel wall of three similarly treated patients between 1 and 3 weeks of age were used as well as an aorta from a 21-month-old child. Specimens were obtained with approval of the Institutional Review Board of the Dijkzigt University Hospital, the Netherlands. All specimens were rinsed in phosphate buffered saline (PBS) containing 6.8% sucrose and were submerged in OCT compound (Tissue Tek, Miles Laboratories, Naperville, USA). Specimens were quickly frozen in liquid-nitrogen-chilled isopentane and stored at $20°$ C. Sections (5 μm thick) were cut at $-12°$ C and stored at $-20°$ C until immunohistochemistry was performed. To study the morphology of the arteries we used hematoxylin-eosin (HE) and resorcin-fuchsin (RF) staining.

Monoclonal antibodies

For the detection of cytokeratins 8, 18 and 19 several monoclonal antibodies were used. The monoclonal antibodies *CAM 5.2* [23] and *M2O* [32, 36] are specific for cytokeratin 8. *RGE 53* [29] and *RCK 106* [30] recognized cytokeratin 18 and *RCK 108* [33] recognized cytokeratin 19.

Immunohistochemistry

Both the immunofluorescence and the immunoperoxidase method were used. Immediately before use the sections were fixed in cold methanol ($-20°$ C), followed by a 1-min acetone fixation at room temperature and were air dried for 1 h. The sections were hydrated and rinsed in PBS. For the immunoperoxidase method endogenous peroxidase activity was removed by 15 min of incubation in PBS containing 0.3% H_2O_2.

The primary antibody was diluted 1:5 in PBS containing 0.1% bovine serum albumin and 0.05% Tween-20. After an overnight incubation in a moist chamber, three washing steps in PBS were performed. For the immunofluorescent detection method we incubated the sections with a TRITC-conjugated rabbit antibody to mouse immunoglobulin (1:50, DAKO A/S, Glostrup, Denmark), washed three times in PBS and mounted in a mixture of 80% glycerol and 20% PBS, pH 8.0, containing 1 mg/ml p-phenylenediamide as anti-fading reagent. For the peroxidase method we used a horseradish peroxidase conjugated rabbit antibody to mouse immunoglobulin (1:300, DAKO A/S) as a secondary antibody. After three washing steps in PBS, the sections were exposed to 0.04% diaminobenzidine tetrahydrochloride (DAB) in 0.05 *M* Tris-maleate buffer (pH 7.6) with 0.006% H_2O_2 for 8 min. The reaction was stopped in PBS and sections were counterstained with hematoxylin. The sections were dehydrated in graded ethanol and mounted in Entellan (Merck, Darmstadt, FRG).

Immunohistochemical staining results were studied by light microscopy and scored by at least two independent researchers.

Results

Fetal Arteries

The two fetal DA between 13 and 15 weeks showed no signs of intimal thickening yet. The three fetal DA between 17 and 19 weeks displayed fragmentation of the internal elastic lamina and concomitant lifting of endothelial cells, i.e. the initial signs of intimal thickening formation. In the ascending and descending aorta and the pulmonary trunk no intimal thickening was observed.

The ascending aorta (Fig. 1A, C) and pulmonary trunk (Fig. 1D) of young fetuses between 13 and 15 weeks showed strong expression of cytokeratins 8, 18 and 19. Expression of cytokeratins in the DA (Fig. 1B) and the descending aorta was weak or even absent. In fetuses between 17 and 19 weeks, the expression in the ascending aorta was slightly diminished (Fig. 1E), whereas cytokeratin expression in the pulmonary trunk had decreased (Fig. 1F) to become weaker than the expression in the ascending aorta. Cytokeratin 8 expression in the DA showed a slight increase, while cytokeratin 18 and 19 expression had remained similar to the younger stages. Results are summarized in Table 1.

Neonatal arteries

The neonatal DA showed well developed intimal thickening. Two of the DA studied had areas of cytolytic necrosis in the media. Cytolytic necrosis is characterized by loss of nuclei without an inflammatory response [9, 11]. The aorta and pulmonary trunk specimens that were studied showed no signs of intimal thickening.

Neither the neonatal ascending aorta, and pulmonary trunk, nor the ascending aorta and pulmonary trunk from a 21-month-old child, showed any expression of cytokeratin 8 (Fig. 2D), 18 or 19. Results for the descending aorta are described by Glukhova and colleagues [15] and correspond with our data on the ascending aorta and pulmonary artery. The neonatal DA did express cytokeratins 8, 18 and 19. There was, however, a variation in expression between the various DA studied. Expression of cytokeratin 8 was the most pronounced, in some DA al-

◄ **Fig. 1A, B** Immunohistochemical staining for cytokeratin 8 of the ascending aorta (**A**) and ductus arteriosus (**B**) of a 13-week-old fetus. Staining of the smooth muscle cells of the ascending aorta is stronger than of the smooth muscle cells of the ductus, indicating an advanced differentiation of the smooth muscle cells of the ductus arteriosus. **C, D** Cytokeratin 8 expression of the ascending aorta (**C**) and pulmonary artery (**D**) of a 15-week-old fetus showing an almost similar staining of the smooth muscle cells. **E, F** In an older fetus (18 weeks) the ascending aorta (**E**) and pulmonary artery (**F**) show a difference in that the smooth muscle cells of the pulmonary artery are now almost negative, indicating advanced differentiation of these cells as compared to the smooth muscle cells of the ascending aorta. *m* media, **A** and **B**; *bar* 50 μm, **C**–**E**. In **F**, *bar* 100 μm

Table 1 Results of the immunohistochemical cytokeratin staining reactions in the fetal and neonatal arteries are summarized. The percentage of positive cells was scored from +++ (all cells) to − (no cells) using the following catagories: +++>++>+>+/−>+/=>−.

Except for the neonatal DA (see Results) the interindividual variability of the staining results was low. DA, ductus arteriosus; DAo, descending aorta; AAo, ascending aorta; PT, pulmonary trunk; wk, weeks

Antigen	Fetal (13–15 wk)				Fetal (17–19 wk)			Neonatal (1–3 wk)			1.2 year	
	DA	DAo	AAo	PT	DA	AAo	PT	DA	AAo	PT	AAo	PT
Cytokeratin 8	+/=	+/=	+++	+++	+	++	+	++[a]	−	−	−	−
Cytokeratin 18	+/=	+/=	++	++	+/=	+	+/−	+[a]	−	−	−	−
Cytokeratin 19	−	−	++	++	−	+	+/−	+[a]	−	−	−	−

[a] Staining localized in cell clusters

Fig. 2A–D Neonatal ductus arteriosus stained for cytokeratin 8. **A** Expression of cytokeratin 8 was found in the intima (*i*) and inner media (*im*), the outer media being negative. **B** Higher magnification of a positive area, in which cytokeratin expression was found in almost every cell. **C** Cluster of cytokeratin positive cells (*arrows*) in the intima in another neonatal ductus (**C**). **D** The media (*m*) of the neonatal ascending aorta was negative for cytokeratin 8; *bar* 100 μm
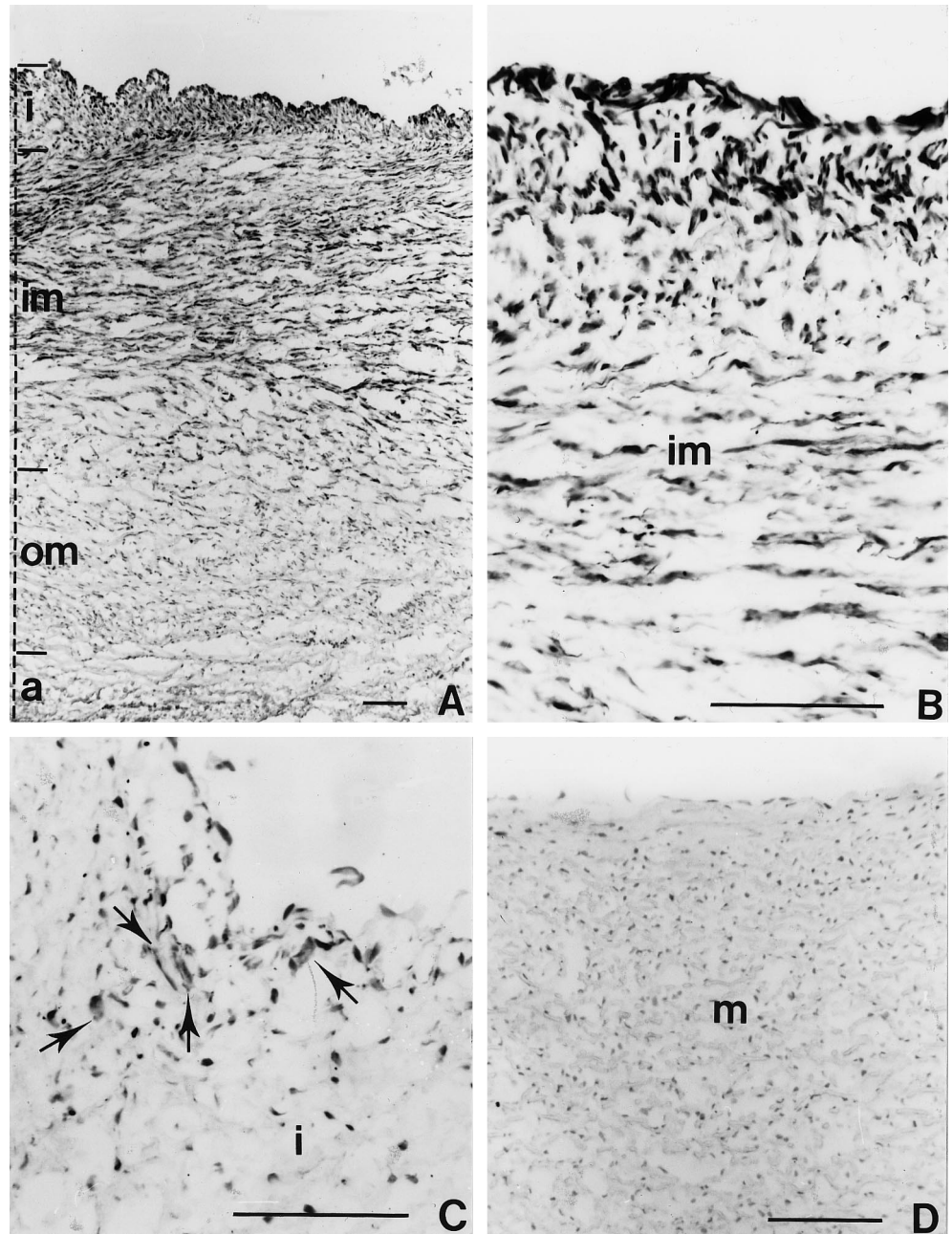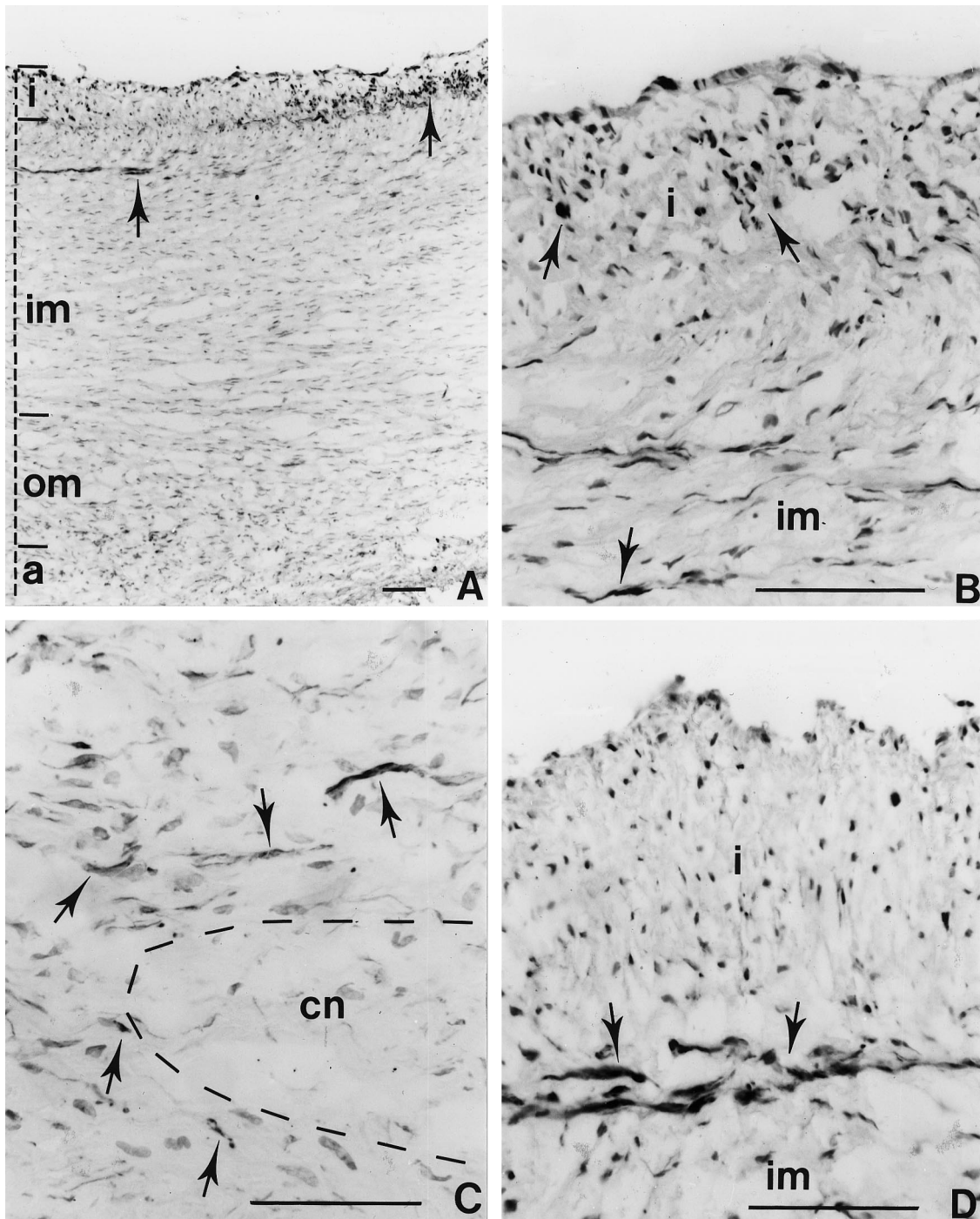
Fig. 3 A Cytokeratin 18 expression was found in cell clusters (*arrows*) present in all parts of the vessel wall of the ductus arteriosus except in the outer media. **B**, **C** Detail of cytokeratin-positive cell clusters (*arrows*) in the intima (*i*) and inner media (*im*) (**B**) and surrounding the areas with cytolytic necrosis (*cn*) (**C**). **D** Detail of a cytokeratin-19-positive cluster on the border of the intima and inner media (*im*); *bar*, 100 μm

cytokeratin-positive cells were found around areas of cytolytic necrosis (Fig. 3C) and in areas showing onset of cytolytic necrosis, but we also encountered randomly localized clusters of positive cells. No clusters were found in the outer media. The results are summarized in Table 1.

most every SMC was stained except in the outer media (Fig. 2A, B). Other DA showed clusters of cytokeratin-8-positive cells located in the intima (Fig. 2C) and inner media. Expression of cytokeratin 18 (Fig. 3A–C) and 19 (Fig. 3D) was also found in cell clusters. Clusters of

Discussion

In this study we have examined the expression of cytokeratins 8, 18 and 19 in SMC of the arterial great vessels

during development. In the adult cytokeratins are generally known as markers of epithelial differentiation and belong to the family of intermediate filaments [24]. These intermediate filaments consist of six subtypes, classifying different tissue types. Type I are the small acidic cytokeratins (9–20) and type II are the large neutral to basic cytokeratins (1–8). The third class, type III of the intermediate filaments, is formed by the mesenchymal protein vimentin, the muscle protein desmin, the glial fibrillary acidic protein expressed in glial cells and astrocytes, and peripherin, specific for neuronal cells. Neurofilaments are type IV, nuclear laminins A and B type V, and nestin, an intermediate filament specific to central nervous system stem cells, is type VI. In pathological situations and in tumors, these cell lineage markers are generally retained, although switches in expression have been described [24]. Coexpression of two or three intermediate filaments in one single cell has been reported. SMC coexpress vimentin, desmin and cytokeratins under certain conditions, such as during fetal development [36] and SMC oncogenesis [16]. Furthermore, the cytokeratins can be used as markers of SMC differentiation, as expression of cytokeratins in vascular SMC is observed during early fetal development, whereas normal adult SMC do not express cytokeratins [15]. In a 10-week-old embryo SMC of the descending aorta contained cytokeratin 8 [15]. In the present study we found that cytokeratin expression in fetuses between 13 and 15 weeks was very low in the descending aorta and DA. In contrast the ascending aorta and pulmonary trunk show a high expression, suggesting that SMC of the DA and descending aorta are already more differentiated than those of the pulmonary trunk and the ascending aorta. The special status of the ascending aorta and pulmonary trunk with regard to SMC differentiation has also been described on the basis of actin expression [3, 12]. In the avian and mouse embryo this region shows transient loss of actin expression. We do not know whether differences in embryonic origin, mesenchymal versus neural crest, are related to these phenomena [3, 12, 25]. Differences in differentiation between the SMC of the ascending and descending aorta were encountered in this study with the cytokeratins, and in a previous study [35] with EDA and EDB splice variants of fibronectin, all markers of early SMC differentiation. Since the SMC of the ascending and descending aorta show various differences in phenotype, varying results can be expected in studies using aortic tissue from different origins. A difference in differentiation between SMC of the ascending and descending aorta was not encountered in studies [20, 35] using SMC contractile proteins, being markers of late SMC differentiation. These studies showed that the SMC of both the ascending and descending aorta were less differentiated than SMC of the DA. The early appearance of the contactile proteins in the DA may be related to the intense effective contraction of the SMC when the DA closes shortly after birth. We could also show, as reported [1, 16, 36] that the SMC of the DA express desmin, vimentin [35] and cytokeratin. It is re-

marked in this respect that the DA has a large amount of hyaluronic acid in both the intima and inner media [8, 30], a correlation that was reported by Kasper and colleagues [19].

Cytokeratin re-expression in the DA was observed in clusters of positive cells at neonatal stages. There was also an overall variation in cytokeratin expression between the neonatal DA studied. This variation may have been due to different stages of maturation and closure of the DA in the neonate [9]. Our study shows that the clusters of cytokeratin positive cells did not colocalize with the dedifferentiated areas defined by the absence of cytoskeletal and contractile protein differentiation markers and the presence of EDA: Extra Domain A and EDB: Extra Domain B fibronectin. These were found in the inner intima and areas of cytolytic necrosis containing apoptotic cells [35]. These apoptotic areas are widely extended during the anatomical closure of the ductus, but were not observed in the outer media (unpublished results). We have no explanation for the special behaviour of SMC in the outer media, as this area in the DA is cytokeratin-negative, thus resembling the adjoining elastic vessels. Recent published data [7] show that the outer media has specific SMC differentiation indicated by the expression of a retinoic acid responsive SMC phenotype. The intima and inner media of the DA are actively involved in the process of intimal thickening in the DA [34] and undergo extensive changes during closure of the DA [9, 35]. The outer media does not take part in these processes. Restriction of the location of cytokeratin positive cells in the neonatal DA may therefore be related to these phenomena. A direct link with proliferation of SMC could not be made (unpublished results). Reexpression of cytokeratins has also been described in pathologic intimal thickening [15, 17, 18]. In fibromuscular lesions, particularly those found in vein grafts, cytokeratin-positive cells are also found in clusters [18]. The presence of clusters might relate to a number of phenomena. It is possible that cells in the vessel wall produce a diffusible factor that is involved in the induction of cytokeratin expression [18]. It may also be that these cells are monoclonal, as was found for large proportions of the atherosclerotic plaques [2]. Another explanation for the presence of clusters of cytokeratin-positive cells may be that these cells are intrinsically unstable in their expression of genes encoding cytokeratins 8 and 18, as has been observed for clusters of cytokeratin-positive cells in transformed non-epithelial cells [21]. Finally, reexpression of cytokeratins may reflect rearrangement of the regulation of gene expression [15], as the expression of cytokeratin 8 and 18 is dependent on the extent of DNA methylation [27] and requires the coexpression of c-jun and c-fos proto-oncogene transcription factors [28].

The latter explanation may be the most suitable for the situation in the neonatal ductus, as this vessel is undergoing many changes particularly in the intima and inner media where the cytokeratin clusters are found during postnatal closure [5, 6].

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