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Hyaluronic Acid Accumulation and Endothelial Cell Detachment in Intimal Thickening of the Vessel Wall

The Normal and Genetically Defective Ductus Arteriosus

E. G. DE REEDER, MSc,* N. GIRARD, PhD,†
R. E. POELMANN, PhD,*
J. C. VAN MUNSTEREN, BSc,*
D. F. PATTERSON, DVM, DSc,‡ and
A. C. GITTENBERGER-DE GROOT, PhD*

From the Department of Anatomy and Embryology, University of Leiden, The Netherlands, the Laboratory of Immunohistochemistry, Centre Henri Becquerel, Rouen, France,† and the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania‡*

The closing ductus arteriosus (DA) was studied as a model for the development of intimal thickening of vessel walls using ultrastructural and immunohistochemical techniques. The material consisted of DA from neonatal dogs of three types: normal beagles, DA-defective pups from a line of mixed poodles with a genetic defect in the closure of the DA leading to persistent ductus arteriosus (PDA line), and normal litter-mates of DA-defective pups in the PDA line. The DA of the normal litter-mates of DA-defective pups did not differ from those of normal beagles. In the DA of normal beagles and normal PDA-line pups, closure is preceeded by intimal thickening characterized by formation of a widened subendothelial region (SR), detachment of endothelial cells, invagination of endothelial cells, and migration of smooth muscle cells into the SR. It was observed that immediately before and after endothelial cell detachment, there was an increase in hyaluronic acid (HA) in the SR and inner media. In the DA-defective pups, the increase in hyaluronic acid failed to occur and there was no intimal thickening. The SR failed to expand, endothelium remained attached to the internal elastic membrane, and there was no invagination of endothelium or migration of smooth muscle cells. It is hypothesized that the increased synthesis of HA is an important early event leading to intimal thickening in the normal DA and perhaps to abnormal intimal thickening of other vessels. By its hygroscopic properties, HA may be directly involved in the formation of a wide SR, inducing endothelial cell detachment and favoring smooth muscle cell migration. In affected pups of the PDA line, there is a genetically-determined "block" in the normal process of intimal thickening at or before the initiation of increased HA synthesis. (Am J Pathol 1988, 132:574–585)

INTIMAL THICKENING OF the vessel wall is considered to be an aspecific process that can be influenced by various endogenic or exogenic factors.[1] Studies on the influence of hypertension on the morphology of the vessel wall in experimental rat models have shown the development of intimal thickening, with the formation of a wide subendothelial layer.[2,3] The morphologic changes of the intima have been studied with ultrastructural methods in the normal ductus arteriosus (DA) of the dog,[4] in which intimal thickening is a physiologic process preceding normal closure, and in a hereditary form of persistent ductus arteriosus (PDA) in the dog, a defect in which the normal process of intimal thickening is impaired.[4–6]

During the process of intimal thickening in the DA, invagination of endothelial cells and migration of

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Address reprint requests to A. C. Gittenberger-de Groot, Dept. of Anatomy and Embryology, Wassenaarseweg 62, Postbus 9602, 2300 RC Leiden, The Netherlands.

smooth muscle cells takes place. The formation of endothelial cell strands in the SR is suggestive of capillary formation.[4] In the present study, immunohistochemical methods were used to trace the undetached, detached, and invaginated endothelial cells of both DA and PDA. Detailed characterization of extracellular matrix changes in the SR during intimal thickening is still lacking.[2,3] The possible role of hyaluronic acid in the formation of the SR was examined specifically. Hyaluronic acid has been detected next to other glycosaminoglycans, such as chondroitin sulfate, heparan sulfate, and dermatan sulfate in the extracellular matrix of the human and the bovine aorta.[7-10] Hyaluronic acid can exert high osmotic pressure on surrounding tissues by binding considerable amounts of water, which, after degration of the normal connections between cells or cell layers, causes swelling of tissue.[11] In this way it might influence the detachment of endothelial cells and the formation of the SR as has been described during intimal thickening.[4] It might also influence subsequent cell migration, proliferation, and cell–cell and or cell–matrix interactions.[11-16]

The distribution of hyaluronic acid was demonstrated by using a hyaluronectin-antibody complex. Hyaluronectin is a protein that binds specifically to hyaluronic acid.[17] This method offers an advantage over previously used indirect methods using Alcian blue staining, which also tends to stain the other glycosaminoglycans.[18]

Material and Methods

Material

The DA studied (Table 1) were obtained from newborn dogs immediately following euthanasia with anesthetic overdose (ether). The pups were of three types: 1) Offspring of normal beagles, a breed of dogs known by epidemiologic and family studies to have a low incidence of persistent ductus arteriosus.[19] 2) DA-defective pups from a line of mixed poodles with a hereditary form of persistent ductus arteriosus (PDA).[6] Matings in which one or both parents of the PDA-line have a PDA produce a high proportion of offspring with PDA as well as some normal pups. Failure of ductal closure in the DA-defective pups is associated with a defect in the histogenesis of the ductal wall. This defect is identifiable histologically before birth[5] and is characterized by an abnormal ductal media and failure of intimal thickening.[4] 3) Normal litter-mates of DA-defective pups. In these pups, the ductal wall structure is indistinguishable from that of normal beagles and ductal closure occurs normally.

The normal beagle pups were obtained from the department of Obstetrics and Gynaecology of the Veterinary Hospital of the University of Utrecht, The Netherlands. The PDA-line is maintained at the University of Pennsylvania in a closed colony according to guidelines set forth in the Guide to the Care and Use of Laboratory Animals.[20]

In this study, the DA from beagle pups served to define the normal histologic and histochemical changes occurring preceding and during intimal thickening. The normal DA from the PDA-line served as a "control" in which these changes failed to occur. The normal litter-mates of DA-defective pups provided assurance that the differences in histology and histochemistry observed in these studies were associated with a specific genetic defect in the DA of individual PDA-line pups rather than some more general difference between beagles and PDA-line dogs. Another feature of the DA in the PDA-line was of particular importance in these studies. Among the DA-defective animals, the histologic defect of the DA occurs in varying degrees.[4] In mildly affected pups, the pulmonary arterial end of the DA has a normal architecture and ductal closure takes place normally in this localized segment, while the aortic end of the DA is abnormal and does not close, resulting in a ductus diverticulum.[6] In such ductuses, termed the "mixed type," intimal thickening occurs in the normal segment but not in the abnormal portion, providing the opportunity to observe the normal and abnormal process within the same vessel. In each pup studied, the aortic wall was used as an additional control.

Methods

Ultrastructural Study

Twelve DA specimens were obtained (Table 1) and an ultrastructural study was performed according to the procedure described by Gittenberger-de Groot et al.[4] To improve staining of elastin, some specimens were fixed in half strength Karnovsky[21] containing 1% tannic acid (Sigma).

To get comparable results, each specimen was cut into three parts (Figure 1). Parts A and C were sectioned sagittally and part B was sectioned transversely, at various indicated levels.

Immunohistochemical Study

Dogs were anesthesized with ether and perfused via the left ventricle with physiologic saline containing 10

Table 1—Normal and Genetically Defective Ductus Arteriosus (DA)

Ultrastructural study				Immunohistochemical study					
No.	Age	Strain	Fixation	No.	Age	Strain	Fixation	EC localization	HA distribution
1	12 hr	beagle	½ Karnovsky	13	1 d	beagle	frozen	x	-
2	1 d	beagle	½ Karnovsky	14	1 d	beagle	frozen	x	-
3	1 d	beagle	½ Karnovsky	15	1 d	beagle	frozen	x	-
4	1 d	beagle	½ Karnovsky	16	3 d	beagle	frozen	x	-
5	2 d	poodle*	½ Karnovsky	17	1 d	beagle	ethanol-acetic acid	-	x
6	2 d	poodle*	½ Karnovsky	18	2 d	poodle†	frozen	x	x
7	4 d	poodle*	½ Karnovsky	19	4 d	poodle†	frozen	x	-
8	4 d	poodle†	½ Karnovsky	20	4 d	poodle‡	frozen	x	
9	4 d	poodle†	½ Karnovsky	21	20 d	poodle‡	frozen	x	
10	7 d	poodle‡	½ Karnovsky						
11	27 d	poodle‡	½ Karnovsky						
12	27 d	poodle‡	½ Karnovsky						

* Histology of a normal DA.
† Mixed type: histology of both normal and persistent DA.
‡ Histology of persistent DA.
EC, endothelial cell.
HA, hyaluronic acid.

UI/ml heparin (Organon Teknika) and 0.001% $NaNO_2$ before removing the ductus arteriosus. To achieve frozen specimens the ductus arteriosus was rinsed in PBS, containing 6.8% sucrose, put in Tissue tek (Miles Laboratories Inc.) and quickly frozen in chilled isopentanol (−196 C). Sections (6 μ) were cut at −12 C and fixed in cold acetone (−12 C) for 10 minutes and stored at −20 C. Just before use sections were fixed again in cold acetone for 10 minutes and air-dried for 1 hour at room temperature. For ethanol-acetic acid fixation, the DA was rinsed in PBS and fixed in 2% acetic acid in ethanol at 4 C for 24 hours and thereafter embedded in paraffin and cut into 6-μ serial sections.

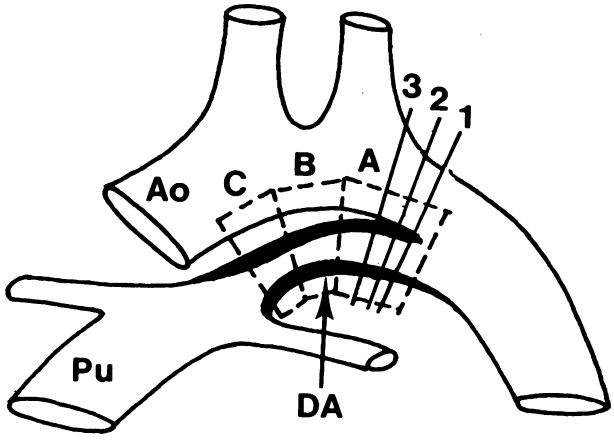

Figure 1—Schematic representation of the ductus arteriosus and the adjoining great arteries. DA, ductus arteriosus; Ao, aorta, Pu, pulmonary trunk. Section level 1, 2 3 are indicated in block A.

Detection of Endothelial Cells with Monoclonal Antibodies

For localization of endothelial cells with monoclonal antibodies four normal DA, two persistent DA, and two persistent DA (mixed type) were studied (Table 1). Sections were preincubated in PBS for 10 minutes at room temperature and incubated overnight with monoclonal antibodies directed against endothelial cells. Two different monoclonal antibodies were used: anti-Von Willebrandt Factor (VWF) antibody (RAG20)[22] directed against FVIII dilution 1:50.000 in PBS containing 1% BSA (Sigma A 9647) and 0.05% Tween 20 (Sigma); and Pal-E directed against venous and capillary endothelial cells,[23] dilution 1:1000 in PBS.

After incubation for 15 hours, sections were washed twice in PBS and once in PBS containing 0.05% Tween 20. To detect bound antibodies 40 μl of rabbit anti-mouse IgG/PO (DAKO) diluted 1:300 in PBS containing 1% BSA and 0.05% Tween 20 were added and sections were incubated for 60 minutes at room temperature. Sections were washed again as described above and stained using diaminobenzidine (0.4 mg/ml, Sigma), dissolved in Tris-maleate buffer (pH 7.6) containing 0.02% H_2O_2 for 8 minutes at room temperature. Staining was stopped by washing the sections in tap water. Sections were counterstained with hematoxylin, dehydrated in graded ethanol, and mounted in Entellan (Merck).

Controls

Control sections were incubated with PBS, containing 1% BSA and 0.05% Tween 20 instead of the specific anti-endothelial antibody.

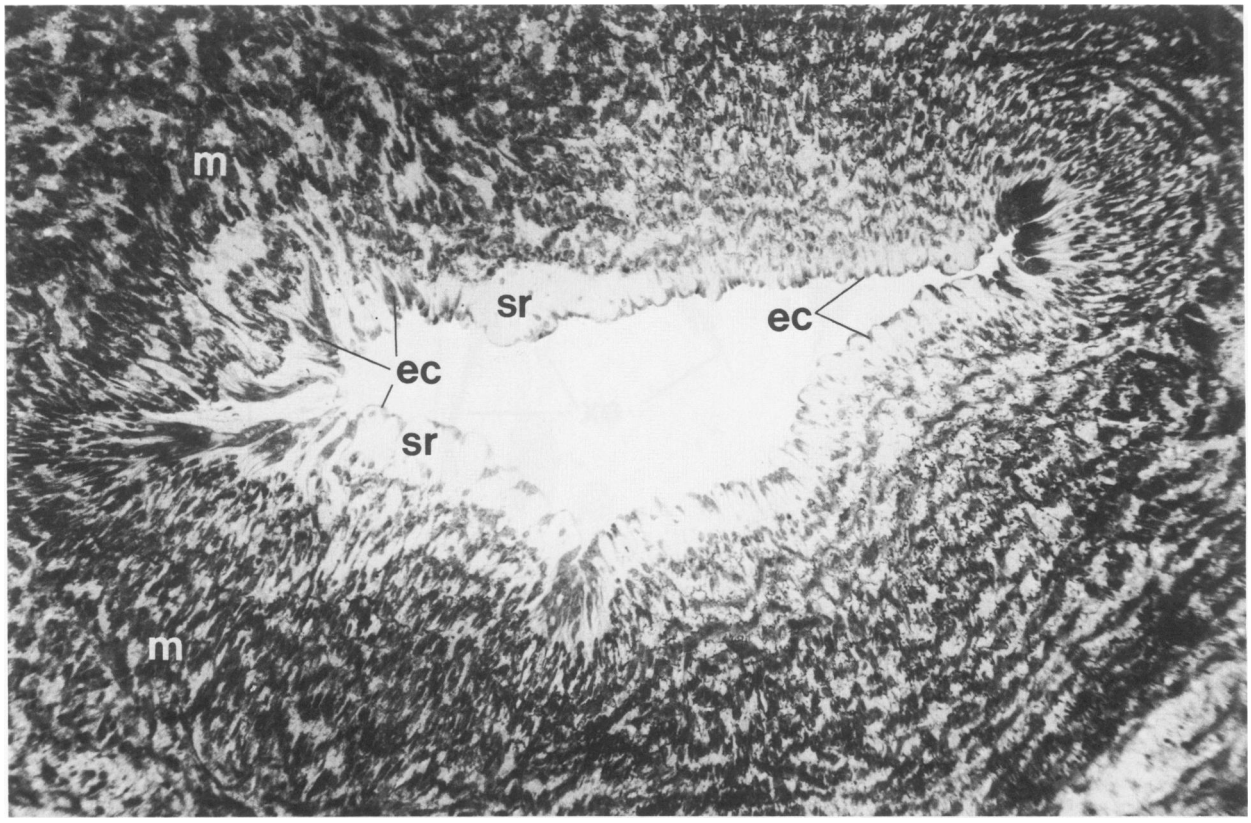

Figure 2—Transverse section of a normal DA of a 2-day-old poodle (case 6). Endothelial cells (ec) are separated from the media (m) by a wide subendothelial region (sr). toluidin blue tissue stain. (×151)

Detection of Hyaluronic Acid

One normal DA and one persistent DA (mixed type) were used to study hyaluronic acid distribution (Table 1). Paraffin was extracted and endogenous peroxidase activity was inhibited with 3% H_2O_2 for 20 minutes. Sections were preincubated with goat serum (1:50 in PBS) for 20 minutes and incubated with immune complex hyaluronectin-antihyaluronectin HN/aHN as described by Girard.[24] Bound HN/aHN immune complexes were detected using avidin-biotin conjugated antibodies (ABC kit Vectastain, Vector Laboratories, Burlingame, California). Sections were stained with 0.5 ml aminoethylcarbazol (10 mg/ml) in 4.5 ml buffer (pH 5.0) to which 25 μl H_2O_2 10% was added.

Controls

The specific controls were made by blocking immune complexes with hyaluronic acid-derived oligosaccharides,[25] or omitting the hyaluronectin anti-hyaluronectin complex.

Results

Morphology of the DA and PDA

In the normal DA (Table 1, cases 1 to 7) the process of intimal thickening starts at the pulmonary end of the DA and proceeds towards the aortic end. The first visible sign of intimal thickening is widening of the SR. The endothelial cells are separated from the internal elastic lamina by a clear space (Figures 2 and 3). Endothelial cell extensions are seen running from the just-detached endothelial cells toward the media. In time the SR enlarges and is filled by migrating smooth muscle cells and invaginating endothelial cells. Invaginated endothelial cells have a swollen appearance and lie in clumps or cell strands. The endothelial cell strands do not possess a lumen that would be expected in a capillary. Isolated cells resembling monocytes were seen occasionally in the subendothelial region just underneath the endothelial cells lining the lumen.

In the PDA (Table 1, cases 10 to 12) endothelial cells in general remained attached to the internal elastic lamina. At some sites minor detachment was observed but the SR remained small, and no endothelial

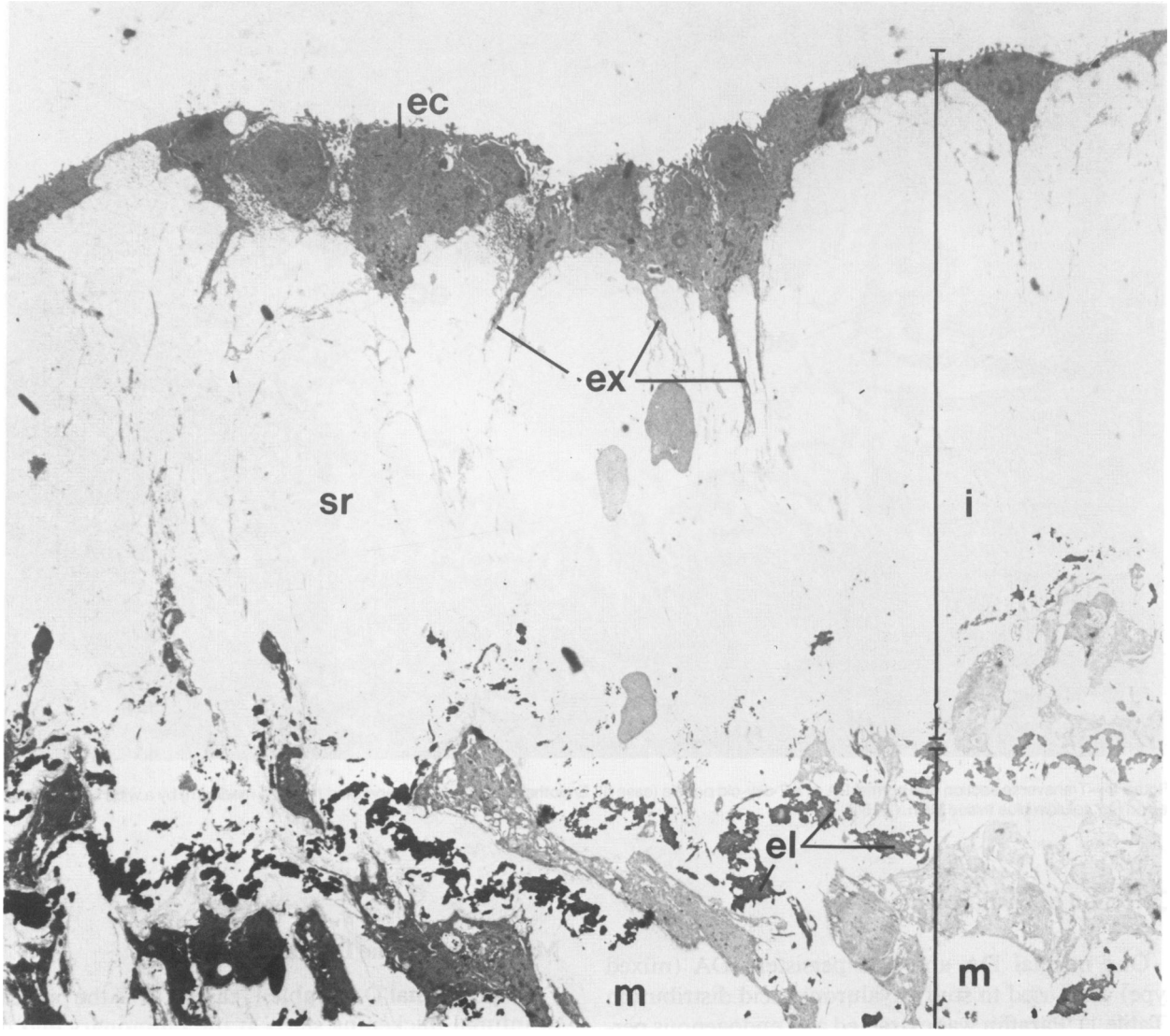

Figure 3—Electron micrograph of the intima (i) of a normal DA of a 1-day-old beagle (case 2). Endothelial cells (ec) showing cell extensions (ex) are separated from the media (m) by a wide subendothelial region (sr). el, elastin. (×2240)

cell invagination was seen. PDA (mixed type, Table 1, cases 8 and 9) showed intimal thickening at one side while at the other side, adjacent to the aorta, endothelial cells remained adhered (Figure 4).

Immunohistochemical Properties of Undetached, Detached and Invaginated Endothelial Cells

The anti-VWF antibody stained all endothelial cells of vasa vasorum, aorta, DA, and PDA. No difference in staining distribution and intensity was observed between undetached, detached, and invaginated endothelial cells. The endothelial cells were found only in the SR and not in the media (Figure 5).

As expected, Pal-E stained venous and capillary endothelial cells from the vasa vasorum only but not the small arteriolae. The results for the endothelial cells of the aorta and DA were less consistent. In general, endothelial cells of aorta and DA were not or only lightly stained by Pal-E but staining intensity was variable locally. More strongly stained endothelial cells were found occasionally among the endothelial cells bordering the lumen of aorta and DA. This was also the case for the invaginated endothelial cells located in the SR. A specific distribution of the more strongly stained endothelial cells could not be determined in this region.

Controls

No staining was observed in control sections.

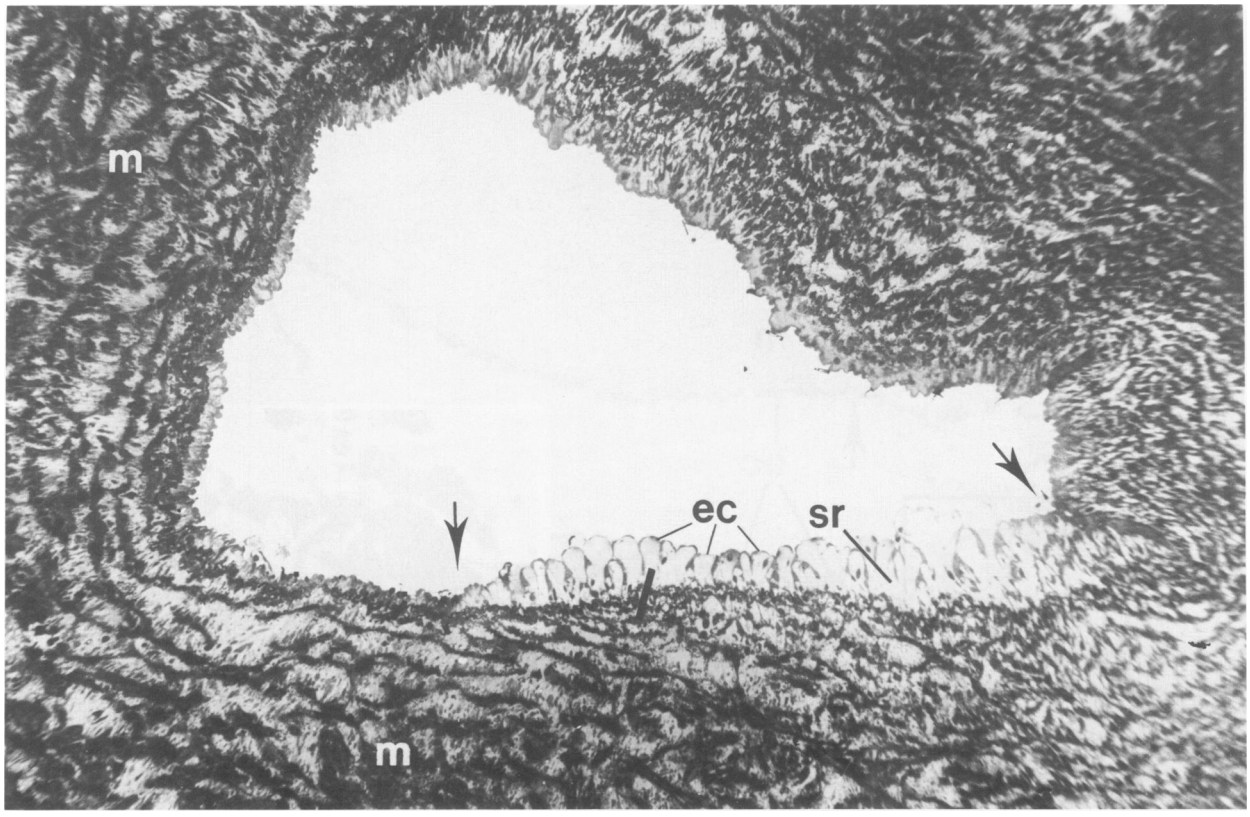

Figure 4—Transverse section of a mixed type PDA of a 4-day-old poodle (case 8). Intimal thickening takes place at only one side of the vessel wall (between arrows). ec, endothelial cells; m, media. toluidin blue tissue stain, (×135)

Distribution of Hyaluronic Acid in the Aorta and Ductus Arteriosus

The distribution of hyaluronic acid in four series taken at different levels in part A (Figure 1) of both DA was examined. Each series consisted of nine successive sections. Level 1 was taken just after bifurcation of the DA from the aorta. No endothelial detachment was seen in the DA. Levels 2 and 3 were still taken from part A but more towards the pulmonary end of the DA. Sections from series showed detachment of an increasing number of endothelial cells as the pulmonary arterial end of the DA was approached.

Aorta

Staining was present throughout the complete vessel wall, and was strongest beneath the endothelial cells and minimal in the inner media.

Normal Ductus Arteriosus

The sections taken at level 1 did not show detachment of the endothelial cells. Distribution of hyaluronic acid was the same as in the aorta, though the inner media in general stained somewhat more strongly (Figure 6A).

The first signs of detachment of endothelial cells were observed at level 2. An intensely stained layer of hyaluronic acid was present underneath detached and undetached endothelial cells. Small extracellular spaces, lined by a strongly stained layer, were visible in the inner media (Figure 6B). The ductal media as a whole stained more intensely compared with the aortic media. Staining between the smooth muscle cell layers was continuous but variable, indicating locally higher concentration of hyaluronic acid.

The sections at level 3 showed more detached endothelial cells and increased development of the SR compared with the sections taken at level 2 (Figure 6C). Staining in the SR is concentrated at different sites. A thick, intensely stained layer was visible along the smooth muscle cells migrating into the SR. A thinner layer was localized underneath the invaginated and the detached endothelial cells.

Staining was also visible in the media, especially lining the small extracellular spaces in the inner media,

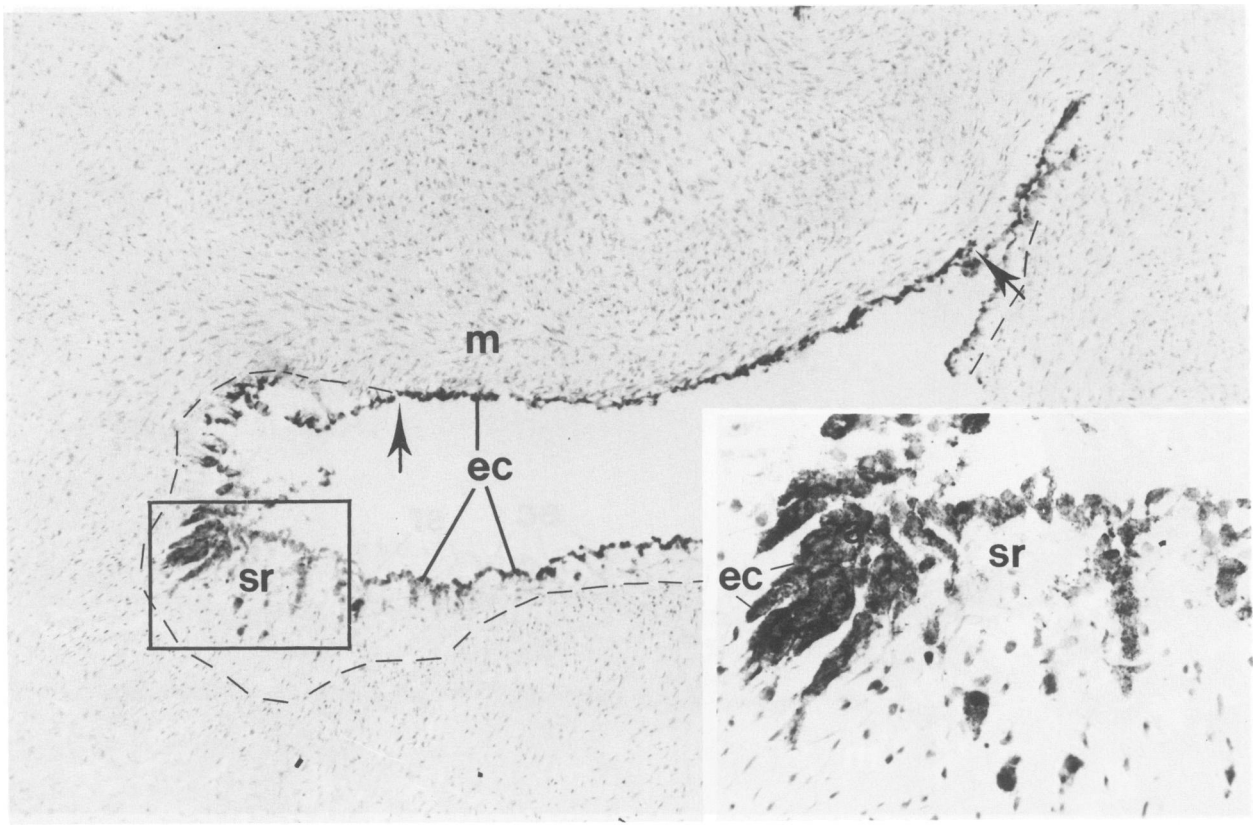

Figure 5—Transverse section of a mixed type PDA of a 4-day-old poodle (case 19). Endothelial cells (ec) are stained by a monoclonal anti-VWF antibody. The upper side (between arrows) does not show intimal thickening. The transition between the subendothelial region (sr) and media (m) is indicated by a dotted line. (×104) The inset shows a detail of detached and invaginated ec. (×250)

which were present in a greater number as compared with sections of level 2.

Persistent Ductus Arteriosus (Mixed Type)

The PDA showed endothelial detachment at one side while no separation of endothelial cells was seen at the side adjacent to the aorta. Though intensity of staining in frozen sections proved to be less intense as compared to acetic acid-fixed sections, there was no difference in staining pattern.

Beneath the endothelial cells from aorta and the side of the DA without detachment of endothelial cells, diffuse staining was visible (Figure 7A). Irregular staining was seen under undetached endothelial cells from the side showing separation of endothelial cells. This staining became stronger and more continuous towards the places with endothelial detachment (Figure 7A to C).

The inner media at the side with endothelial detachment showed irregular staining, particularly along the extracellular spaces. No extracellular spaces and only diffuse staining were visible in the media at the side without endothelial detachment. Staining at this side of the media was comparable with the aortic media.

Controls

No staining was observed after addition of hyaluronic acid derived oligosaccharides or if the hyaluronectin-anti-hyaluronectin complex was omitted.

Discussion

Intimal thickening of blood vessels is usually considered to be a pathologic process. In the "response to injury" hypothesis of Ross, it is postulated that the initial triggering event is endothelial cell injury.[1] Under this hypothesis, two different pathways leading to intimal thickening are described. In the first, endothelial injury is followed by monocyte migration to the subendothelial region. In the second, which is characteristic of the intimal thickening associated with arterial hypertension, monocyte migration does not occur.[1] As shown in previous studies in the dog, intimal thickening of the ductus arteriosus is a normal devel-

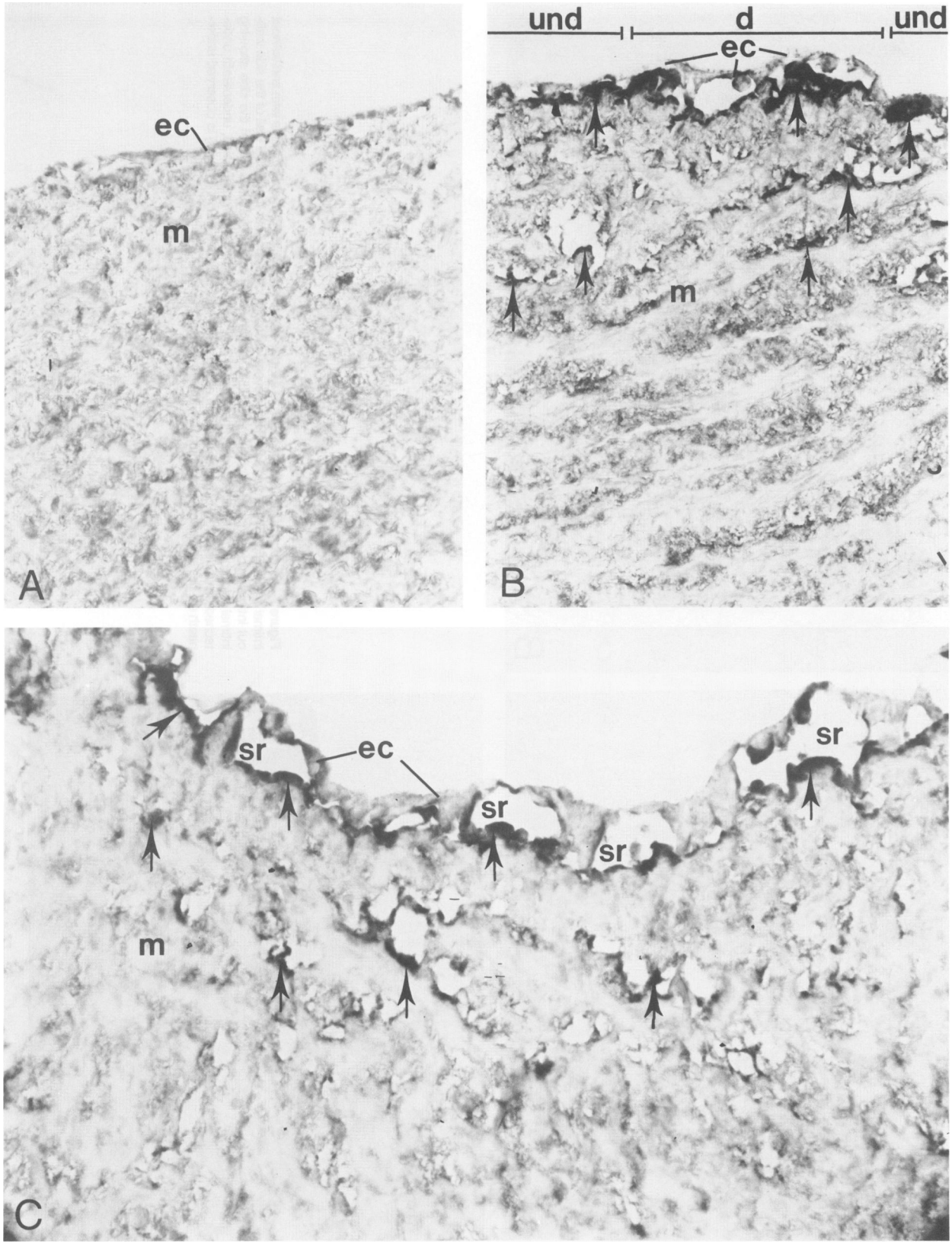

Figure 6—Series of sections from level 1 to level 3 of a DA of a 1-day-old beagle (case 17) showing the development of intimal thickening. Detachment of endothelial cells (ec) does not take place at the aortic side of the DA (**A**). At this level hyaluronic acid (HA) is evenly distributed throughout the vessel wall. (×375) At level 2 (**B**) the first indication of endothelial detachment is visible. Accumulation of HA (arrows) can be observed underneath both undetached (und) and detached (d) ec as well as in the inner media. (×375) Toward the pulmonary side of the DA (level 3) formation of the subendothelial region (sr) takes place (**C**). Accumulation of HA (arrows) is visible in the sr and the inner media. (×472)

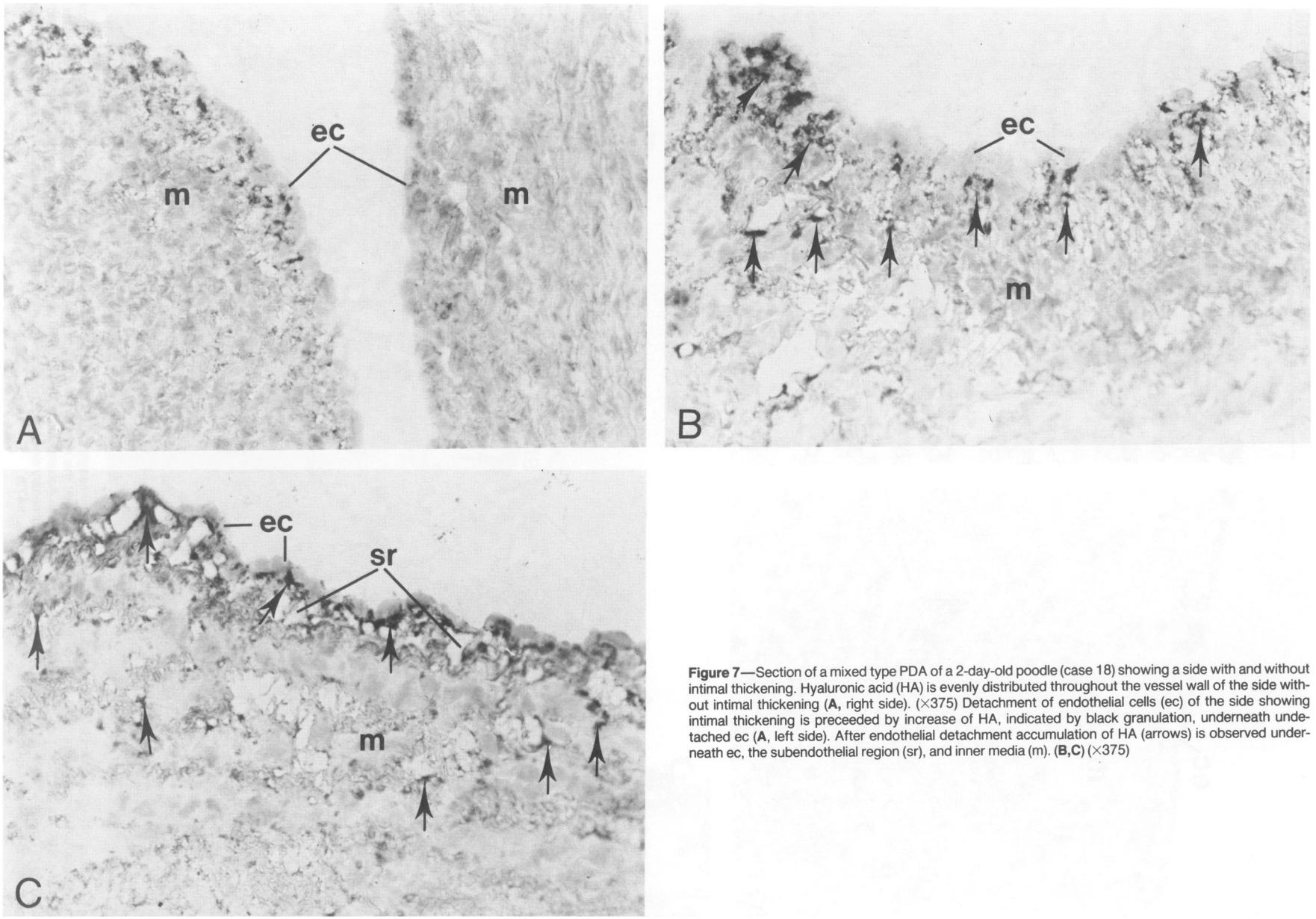

Figure 7—Section of a mixed type PDA of a 2-day-old poodle (case 18) showing a side with and without intimal thickening. Hyaluronic acid (HA) is evenly distributed throughout the vessel wall of the side without intimal thickening (**A,** right side). (×375) Detachment of endothelial cells (ec) of the side showing intimal thickening is preceeded by increase of HA, indicated by black granulation, underneath undetached ec (**A**, left side). After endothelial detachment accumulation of HA (arrows) is observed underneath ec, the subendothelial region (sr), and inner media (m). (**B,C**) (×375)

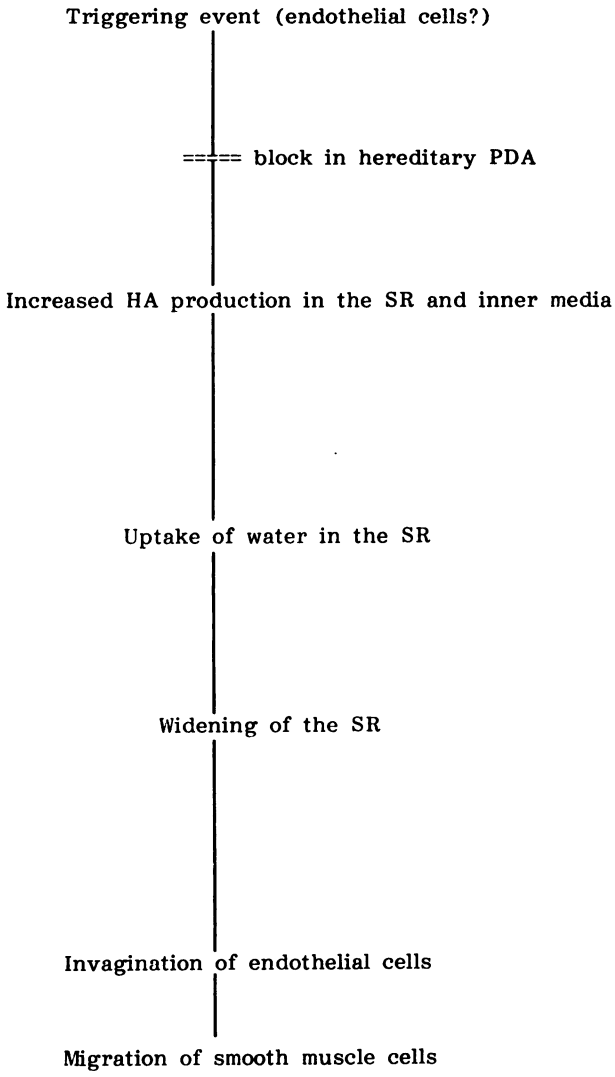

Figure 8—Schematic representation of the hypothesis explaining the sequence of events during intimal thickening of the DA, as well as an indication of the site where the sequence is blocked in case of a genetically defective PDA.

opmental process that begins before birth.[4] The morphologic changes accompanying intimal thickening in the normal DA resemble in detail those described in the aorta of the rat following the experimental induction of arterial hypertension.[2,3] Although triggering events of intimal thickening may differ between hypertension and normal ductal closure, the morphologic similarities in the two cases suggest that common mechanisms may be involved in subsequent stages of histogenesis.

The presence of cell strands in the SR after endothelial cell detachment suggested that the endothelial cells were capable of capillary formation. Because the ultrastructural studies were not convincing with regard to capillary formation, PAL-E was used as a discriminating antibody.[23] Anti-VWF was used as a general marker of endothelial cells. Anti-VWF stained all endothelial cells more or less irregularly, which seems to be related to the variable amount of VWF in the cytoplasm of the endothelial cells.[28–30] It demonstrated clearly the presence of endothelial cells isolated and in strands in the SR after endothelial cell detachment. Endothelial cells were never seen to cross the internal elastic lamina and enter the region of the smooth muscle cells in the media. The endothelial cell layer bordering the lumen did not show interruptions of the continuity. Pal-E not only stained ductus endothelial cells bordering the lumen and in the subendothelial region in a variable way, but also the aortic endothelial cells. Therefore, it could not be proven that the invaginating endothelial cells really indicated capillary formation. Further research is necessary for this aspect.

Widening of the SR is a major component of pathologic intimal thickening and has been described by a number of authors,[2,3,26,27] but its pathogenesis remains unexplained. The present study investigates the possible role of hyaluronic acid in the development of the expanded SR by comparing changes in the normal DA with those in a genetic defect of the DA in which intimal thickening fails to occur. Immunohistochemical studies of the normal DA showed a specific increase of hyaluronic acid subendothelially and in the inner media just before and after endothelial cell detachment, suggesting that HA is important in the widening of the SR. The hygroscopic properties of HA might very well cause an influx of water, thus widening the SR and creating an environment well suited for cell migration.[11,12,15] As degradation products have been shown to stimulate endothelial cell migration,[14] a direct effect on migration might also be assumed.

The importance of HA in the development of intimal thickening of the DA is further supported by the finding that, in DA-defective pups in which intimal thickening fails to occur, there is no histochemical evidence of an increase in hyaluronic acid. It is hypothesized from these observations that intimal thickening occurs through a cascade of events, an early and key event being the increased synthesis of HA (Figure 8). An underlying pathologic abnormality in hereditary canine PDA appears to be a "block" in the normal process of induction and progression of intimal thickening at or immediately before the stage of increased HA synthesis.

Though an increase in hyaluronic acid has been demonstrated clearly, it is not known exactly what cells are active in its production. Accumulation in the inner media probably is the result of production by

smooth muscle cells, but accumulation in the subendothelial region could be produced by endothelial cells, smooth muscle cells, or both. Data are scarce on the subject of hyaluronic acid production. An ultrastructural study[31] demonstrated the presence of hyaluronic acid at the tip of growing capillaries, suggesting that endothelial cells are capable of producing hyaluronic acid.

Moreover, it cannot be excluded that hyaluronic acid production by smooth muscle cells has been induced by the endothelial cells, which are able to stimulate synthesis of hyaluronic acid and sulfated glycosaminoglycans *in vitro*.[32]

In conclusion, the authors hypothesize that activated endothelial cells of the normal DA both produce hyaluronic acid and trigger smooth muscle cells to produce this substance as well. This increase induces the swelling of the SR with detachment and invagination of the endothelial cells. Migration of smooth muscle cells follows immediately. In this DA model, the morphologic changes during development of intimal thickening resemble in great detail the changes described in blood vessels exposed to experimentally-induced hypertension.[2,3,26,27] Here, injury to the endothelial cell seems to be the triggering event. This leaves the possibility that in pathologic intimal thickening, the endothelial cells synthesize the same factor and thereby induce abnormal intimal thickening. In the DA, the onset appears to be programmed, so that activation of the endothelial cell is a better term. In this model the endothelial cells from the aorta and genetically determined PDA lack the capacity to initiate intimal thickening, perhaps due to failure to synthesize a specific factor. A better understanding of the genetically determined activation process in the DA that fails in the PDA will be a step forward in the understanding of the phenomenon of intimal thickening in general.

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