Role of Apoptosis in the Closure of Neonatal Ductus Arteriosus

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Apoptosis regulates the remodeling of tissue during embryonic development by eliminating unwanted cells and structures. The present study investigated smooth muscle cell (SMC) proliferation and apoptosis in the neonatal ductus arteriosus (DA) during closure. In the DA of 39 swine neonates and 5 autopsy human neonates, apoptosis was detected using in situ end-labeling and electron microscopy, and proliferation was evaluated using proliferating cell nuclear antigen. In swine, apoptosis of SMC was first observed at 24 h after birth. After 48 h, both apoptosis and proliferation quickly increased and became most prominent at 3 days, mainly in the intima and inner media. From 5 days, both apoptosis and proliferation quickly disappeared, and were present to a minor extent at the 2 weeks after birth. During these processes, there was no sign of inflammation or necrosis. In humans, apoptosis was found in tissue specimens obtained from 2 term neonates who died at 1 and 5 days after birth. These findings suggest that SMC contribute to the functional closure of the DA by active constriction, and soon after, they switch to proliferation and apoptosis, which may contribute to the anatomical closure of the DA. (*Jpn Circ J* 2000; 64: 684–688)

Key Words: Apoptosis; Ductus arteriosus; Neonate; Smooth muscle cells

The ductus arteriosus (DA) first closes functionally soon after birth as a result of strong constrictions of smooth muscle cells (SMC). Subsequently, the permanent anatomical closure of the DA progresses; proliferation and migration of medial SMC into the subendothelial region initiates the thickening of intimal cushions, which finally fuses the intimal cushions and thus completes the process of ductal closure¹.

During this process, many SMC are used for both the functional and anatomical closure and this increased number of SMC must be depleted in order to complete the process of closure and for the DA to become the ductus ligamentum. Previous studies have shown that cytolytic necrosis^{2,3} and degeneration¹ in the media take place during some phases of closure of the DA, and recent animal studies demonstrated that apoptosis regulates the remodeling of tissue during embryonic development by eliminating unwanted cells^{4–8}. Unlike necrosis, apoptosis does not have an inflammatory component^{4,5,7}.

A more recent study found apoptosis in an area of cytolytic necrosis in the DA^{9,10}, so the present study investigated proliferation and apoptosis in the wall of the DA during the process of closure in order to elucidate the mechanism of DA recession.

Methods

Materials

Specimens of the DA were obtained from 39 neonatal swine and 5 autopsy cases of human neonates (Table 1).

(Received January 7, 2000; revised manuscript received May 24, 2000; accepted May 25, 2000)

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Three swine neonates were killed under general anesthesia by inhalation of chloroform at each of the following 13 time points: 0 and 15 min; 1, 3, 6, and 12 h; and 1, 2, 3, 5, 7, 10, and 14 days after birth.

Sample Preparation

The resected DA samples from both swine and humans were fixed in 5% buffered formalin for 10 h, embedded in paraffin, and cut into serial, 4- μ m-thick cross-sections. After being deparaffinized by passage through xylene and a descending series of alcohol, these sections were used for light microscopic examination, histochemical staining and in situ end-labeling.

Light Microscopy

For light microscopic examination, routine hematoxylin-eosin, elastica van Gieson, and Azan staining was performed for each sample.

Histochemical Staining

To evaluate cell proliferation, the presence of proliferating cell nuclear antigen (PCNA) was evaluated by immunohistochemical stain^{11,12}. After deparaffinization and dehydration of ultra-thin sections of DA, endogenous peroxidase activity was blocked by treatment with 3% H₂O₂ in methanol for 15 min. These sections were then immersed in a 1% zinc-sulfate solution, heated in a microwave oven (MWF-2, Nisshin EM, Japan) for 7.5 min, and cooled down to room temperature. They were incubated with anti-PCNA monoclonal antibody (Novocastra Laboratories Ltd, UK), followed by incubation with biotinylated secondary antibody and peroxidase-labeled streptavidin (Dako, Denmark). Finally, the sections were incubated with diaminobenzene solution containing nickel chloride¹³.

In Situ End-Labeling

In situ end-labeling was performed in order to demon-

Table 1 Clinical Data of the Human Neonates

Case no.	GA (weeks)	Birth weight (g)	Diagnosis	Time of death (day after birth)	TUNEL
1	39	1,960	Potter syndrome	0	–
2	41	2,464	CHD	0	–
3	40	2,412	Potter syndrome	1	+
4	42	2,652	CHD	1	+
5	40	3,160	Sudden death	5	+

GA, gestational age; TUNEL, TdT-mediated dUTP-biotin nick end-labeling; CHD, congenital heart disease.
Case 5: sudden death by rupture of hemangioma of the liver.

strate the appearance of apoptotic cells!⁴ Briefly, ultra-thin sections were digested with 20 mg/ml of proteinase K (Boehringer Mannheim, Germany) in 100 mmol/L Tris-HCL buffer [pH 7.4], and were then incubated at 37°C for 90 min in a reaction mixture containing 0.15 units/ μ l of terminal deoxynucleotidyl transferase (Gibco BRL, UK), 2 nmol/ml of biotin-dUTP (Boehringer Mannheim) in terminal deoxynucleotidyltransferase buffer (Gibco BRL). Next, these sections were washed at room temperature with a solution containing 3 mmol/L sodium citrate and 30 mmol/L sodium chloride for 30 min. Then, they were incubated with peroxidase-conjugated avidin (Dako), followed by incubation with diaminobenzidine solution containing nickel chloride!³ The endogenous peroxidase activity was blocked by pretreatment of the sections with H₂O₂.

Electron Microscopy

For electron microscopy, tissue samples were fixed in phosphate-buffered 2.5% glutaraldehyde, post-fixed in phosphate-buffered 1% osmium tetroxide, and embedded in Epon 812 (TAAB, UK). Ultra-thin sections, stained by uranyl acetate and lead citrate, were observed under an electron microscope (1200EX, Joel, Japan).

Results

Light Microscopy

Light microscopic examination of specimens from swine aged from 0 min to 14 days after birth revealed serial histological changes in the DA. The lumen of the DA quickly disappeared immediately after birth, and functional closure seemed to be completed at 12 h. Well-developed media and intimal cushions were first observed at 24 h, and the media became thick at 3 days after birth. At 7 days, this thickened inner media further narrowed the ductal lumen to a slit, whereas the intimal cushions were less pronounced. At 2 weeks after birth, the lumen of the DA became indistinct because of fusion of the intimal cushions, and the DA was apparently anatomically closed. At this time, the inner media and intimal cushions were inconspicuous because of reduced cellular elements and their replacement by hyalinized tissue. During ductal closure, there was no evidence of any oxidative inflammatory process at any site along the ductal wall.

In the DA sections from a human neonate who died on the first day after birth, the media were well developed and the intimal cushions were more pronounced than those of the swine samples from the first day after birth. In addition, the human intimal cushion was well developed, but only at certain portions of the ductal wall. In another neonate who died 5 days after birth, the thickening of the intimal cushions was more pronounced than that found in the 5-day-old swine.

Fig 1. Immunohistochemical examination of the ductus arteriosus in swine at 2 days after birth. Nuclei of some smooth muscle cells in the intima and inner media are PCNA-positive. Bar=0.1 mm.

Immunohistochemical Examination

Serial immunohistochemical examination of the DA in swine demonstrated serial changes in the site of proliferative cells, which had PCNA-positive nuclei (Fig 1). Some SMC and fibroblastic cells in the outer media and the adventitia were already PCNA-positive at 0 min after birth, and the number of positive cells gradually increased until 3 days after birth. Proliferative cells were first observed in the inner media and intima at 2 days after birth (Fig 1). The greatest number of PCNA-positive cells was observed at 3 days after birth, mainly in the thickened intimal cushions and the protruding inner media. At 5 days after birth, proliferative cells had rapidly disappeared from the intima and inner media; proliferation in the outer media and adventitia was less pronounced than that observed at 3 days. After 2 weeks, when the DA lumen had completely closed, only a few positive PCNA cells were left and these were observed only at the outer layer.

In the human sections, proliferative cells were observed in all 5 cases. The time course of proliferation appeared to be similar in both human and swine samples. The PCNA-positive cells were most pronounced in the human specimen obtained 5 days after birth.

In Situ End-Labeling

Serial histochemical examination in swine DA detected serial changes in the site of apoptotic cells, most of which were SMC (Fig 2). No apoptotic cells were found during the first 12 h after birth. At 24 h, apoptotic SMC cells were first observed at all layers of the DA wall, especially the outer media, and some apoptotic fibroblastic cells were also found in the adventitia. At 3 days after birth, apoptosis of

Fig 2. In situ end-labeling examination of the ductus arteriosus in swine. Stained nuclei represent apoptotic cells. (A) At 24 h, apoptotic smooth muscle cells were first observed in all layers of the DA wall, especially the outer media, and some apoptotic fibroblastic cells were also found in the adventitia. (B) At 3 days after birth, apoptosis of smooth muscle cells is very prominent during all process of closure. Many apoptotic smooth muscle cells are found in the intima and inner media. Of note, there is a clear border between the inner media with apoptosis and the outer media without apoptosis, even though it can not be distinguished by routine hematoxylin-eosin, elastica van Gieson or Azan staining. (C) Ten days after birth, only minor apoptosis is detected in the intimal cushions and media. Bars=0.1 mm.

Fig 3. Sections of the ductus arteriosus from a human neonate who died 5 days after birth. Stained nuclei represent apoptotic cells. Compared with the findings in 5-day-old swine, the intimal cushions is more focally thickened. Marked apoptosis of the smooth muscle cells is observed in the inner media just under the thickened intima. Bar=0.5 mm.

SMC was most active during all process of closure. Many apoptotic SMC were found in the intima and inner media, and only a few apoptotic cells in the outer media. Starting at 5 days, the number of apoptotic SMC gradually decreased until after 2 weeks, very little apoptosis was detected in the intimal cushions and media. In a long-axis slice of DA, it was discovered that apoptotic SMC were most active in the central portion of the DA at 3 days after birth. From 5 days, this apoptosis declined, but extended to both the pulmonary arterial and the aortic ends of the DA.

In the human specimen, the results approximately paralleled those of swine. No apoptosis was found in the DA specimens from the neonates who died on the first day of life. In the DA from the 2 cases that died 1 day after birth, a few SMC were positive for apoptosis; this phenomenon was observed throughout the ductal wall. In the specimen from the neonate who died at 5 days after birth, SMC with apoptosis were detected, mostly in the inner media and just

Fig 4. Electron microscopic findings of the ductus arteriosus in a swine at 3 days, and a human neonate at 5 days after birth. The dead cells have the characteristic features of apoptosis: the nuclei have degenerative changes, such as peripherally aggregated chromatin, nuclear blebbing and nuclear fragmentation, but the membrane system is completely preserved. Bar=2 μ m.

under the thickened intima (Fig 3).

Electron Microscopy

Electron microscopy showed the presence of SMC apoptosis in the ductal wall. Fig 4 shows the characteristic features of SMC apoptosis in both the swine sections at 3

Fig 5. Schematic time course of the active sites of apoptosis (closed circles) and proliferation (open circles) in the ductus arteriosus after birth. Immediately after birth, there are some proliferative cells in the outer media and adventitia, but no apoptosis. At 24h, apoptosis of smooth muscle cells and fibroblastic cells starts in all layers. Proliferation in the inner media and intima gradually begins from 2 days after birth. At 3 days after birth, both apoptosis and proliferation are most active during all processes of DA closure. Most of the apoptotic smooth muscle cells are found in the intima and inner media. At 5 days after birth, proliferative cells quickly disappeared from the intima and inner media, but apoptosis is still most prominent in the inner media. After 2 weeks, only a few proliferative cells remain in the outer layer, and very little apoptosis is detected in the intimal cushions and media.

days after birth and in the human sections at 5 days after birth. The nuclei showed degenerative changes, such as peripherally aggregated chromatin, nuclear blebbing, and nuclear fragmentation, whereas the membrane systems of these nuclei were completely preserved. These electron microscopic feature of apoptosis was demonstrated in all slides that were positive for *in situ* end-labeling.

Discussion

Our results, observed in both swine and humans, demonstrated that proliferation and apoptosis take place simultaneously and at the same site in the wall of the DA shortly after birth (Fig 5). The location of most of the proliferative cells seems to be the same as that which previous studies have demonstrated as being where proliferation and migration of medial SMC into the subendothelial region initiates the thickening of intimal cushions. In addition, many SMC were rapidly disposed of by apoptosis during a short period. These results imply that through proliferation and apoptosis, SMC play an important role in both the functional and anatomical closure of the DA.

Apoptosis in the DA

Our observations revealed that apoptosis of the SMC contributes to the anatomical closure of DA, which is analogous to the normal embryonic development of various organs.⁴⁻⁷ Electrosopic examination clearly demonstrated nuclear death concomitant with preserved cell membranes. Apoptosis is a type of cytotoxicity with characteristic features, which are apparent upon morphological and molecular assessment. One of the most striking features is that cell death occurs without inflammation, which is quite different from necrosis; it presents a clear biological advantage because it prevents scar formation. Hence, apoptosis is preferable to necrosis for cell deletion, which appears to be necessary for the process of DA closure.

Our results are compatible those of Slomp et al who found extensive apoptosis in the area of cytolytic necrosis in the human DA.⁹ Our results also support the findings of

Imamura et al who used a rabbit model and found apoptotic cells in the DA wall, mainly the inner layer of the media, from 24h to 9 days after birth.¹⁰ Our observations add further information about the serial time course of the location and extent of apoptosis in the DA during the closing process with the relation to cell proliferation.

Interestingly, SMC in the inner and outer media have different properties with regard to apoptosis. Apoptosis is more prominent in the outer media at the beginning, and then the main site of apoptosis quickly changes to the inner media. In addition, the border of the inner media with apoptosis and the outer media without apoptosis can be clearly differentiated, even though it can not be distinguished by routine hematoxylin-eosin, elastica van Gieson or Azan staining. Imamura et al also found apoptotic cells in the inner layer of the SMC.¹⁰ Although the implication of the different features of the inner and outer SMC layers is uncertain, apoptosis of either site is clearly regulated by different biochemical and molecular mechanisms.

In the human subject at 5 days after birth, apoptosis of SMC in the inner media was observed focally, which was not found in the swine in any stage of their DA closure. It is interesting that this focal apoptosis was mainly observed just below the focally thickened intima. In the swine, the intima was diffuse along the lumen of the DA, and apoptosis was also diffuse all around the DA wall. In contrast, in the human DA the intima was thickened focally, and apoptosis was very active just below this thickened intima. These findings suggest that there are some regulatory mechanisms between the thickened intima and apoptosis of the SMC in the inner media during DA closure. Further studies using larger numbers of human subjects are required to elucidate this relationship.

Mechanism of Closure of the DA

Our serial observations of the closure of the DA were made by simultaneous use of light microscopic, immunohistochemical and *in situ* end-labeling examinations. After active constriction of the DA, the SMC also seem to contribute to the anatomical closure by proliferation and

apoptosis (Fig 5).

In the specimens from swine DA, strong constriction by the SMC completed functional closure by 12h after birth. At that time, proliferation of the SMC started to gradually produce the intimal cushions;^{2,16-18} however, no apoptosis was observed then, but it began as early as 24h after birth, probably to delete the already constricted SMC in order to render the changes permanent. After apoptosis set in, proliferation of the SMC progressed until day 3, thickening the media and intima and thus completing fusion of the intima. Interestingly, apoptosis of the SMC in the inner media was also observed to be most prominent at day 3. When proliferation of the SMC was most active, SMC had already started to be deleted, probably in preparation for the next stage of reducing the size of the DA by eliminating excessive cells and replacing hyalinized tissue, thus completing anatomical closure. Five days following birth, there was some apoptosis in the inner site of the DA, but the main site of proliferation had changed to the outer layer of the DA. These simultaneous phenomena, apoptosis in the inner media and proliferation in the outer media, may be involved in reducing the diameter and tightening the DA in order to prevent re-opening of the DA. The proliferation in the outer layer was sustained for 2 weeks, which may aid in the process of anatomical closure of the DA by further tightening the lumen externally.

Study Limitation

The differences in animal (swine) and human DA are a clear limitation in our study. Neonatal swine were used in order to demonstrate serial changes in the DA after birth. In contrast, human neonates may display different closure mechanisms. In fact, the shape of the intimal cushion and the thickness of the media were slightly different between the 2 tissues sampled in this study. In addition, although the intimal cushion is already formed before birth in humans, it was first observed 12h after birth. However, the morphological changes in functional and anatomical closure of the DA after birth have been reported to be quite similar in several species (ie, humans^{2,3,17} rabbits^{10,15,18} and sheep^{16,19}). We clearly demonstrated that both apoptosis and proliferation of the SMC take place in human neonates. Nevertheless, the results from the swine study may not directly apply to humans. Therefore, further observation of human DA is necessary.

We did not prove whether the PCNA-positive cells in the intimal cushion originated from SMC or not. Because several studies have demonstrated that the SMC migrate to the intima and produce the thick intimal cushion during the process of DA closure, we assumed that the principal cells proliferating in the intima were of SMC origin. However, these proliferating cells could be non-muscle cells, such as a type of blast cell or endothelial cell. In addition, we do not know from where these proliferating cells migrate during the process of ductal closure.

Conclusions

Proliferation and apoptosis of SMC are opposing cellular

processes that take place in the wall of the DA shortly after birth, which suggests that SMC contribute to several phases of DA closure. Further investigation is necessary in order to elucidate the mechanisms that regulate cell proliferation and apoptosis during DA closure.

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