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Radioautographic Study on the Obliteration of the Ductus arteriosus Botalli

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Autoradiographische Untersuchungen zur Obliteration des Ductus arteriosus Botalli

Zusammenfassung. Durch autoradiographische Untersuchungen wurde gezeigt, daß die Obliteration des Ductus arteriosus Botalli, welche während einer kritischen Zeit abläuft, nicht nur durch die Wandzellen des Ductus sondern auch Elemente des strömenden Blutes getragen wird. Während der Phase der Proliferation besteht eine besondere Störanfälligkeit der Zellen, insbesondere gegenüber Sauerstoffmangel. Es scheint, daß die Suszeptibilität genisch verankert ist.

Summary. From the findings obtained from the radioautographic study on the obliteration of the ductus arteriosus, the authors confirmed that the obliteration was not caused only by the proliferation of the cells in the intimal layer of the ductus, but rather by the migration of the cells into the lumen. When the embryos reached the final stage of fetal life, their ductus arteriosus possessed a specific property to obliterate after the blood flow ceased. Some discussions are devoted to the etiology of the patent ductus arteriosus.

Theories regarding the obliteration of the ductus arteriosus were postulated by many researchers. However, according to the critical review (Sciacca and Condorelli, 1960), there were many discrepancies among them. Some researchers believed that the obliteration of the ductus started only after birth. The others were of the opinion that the obliteration process took place preceding the onset of the respiration. The mechanism underlying the closure of the ductus arteriosus is a problem not yet entirely dissolved.

However, there is a general agreement that the obliteration of the ductus arteriosus is composed of two phases; functional and anatomical closure, and the essential event of the anatomical closure consists in the proliferation of intimal cells and connective tissue, accompanied with atrophy of the muscular portion. Especially, the contributions of proliferation of intimal cells and increase of elastic fibers to the obliteration have been strongly emphasized without any experimental evidences in the conventional theories.

In our previous report (Mato and Aikawa, 1968), the authors proposed that the proliferation of intimal cells in the ductus arteriosus did not take a principal role, but the sliding or migration of the cells composing the vascular wall into its lumen (owing to the disruption of elastic layers) seemed to be an essential event for its obliteration, and further the authors demonstrated a specific change of the vascular structures at the intrauterine life.

In order to get a conclusive evidence for this hypothesis, it was required to survey sites of cell-proliferation and cell-migration of the vascular wall after or before birth. Therefore, the authors employed radioautographic method using ^{3}H-thymidine under various conditions to dissolve the problem.

Materials and Methods

Through this experiment, rats belonging to *Wistar* strain were used. Specimens including ductus arteriosus and thoracic aorta were removed from the animals and fixed in Bouin's fluid. The animals used for the experiment were classified into A—D groups.

A. Pregnant rats were injected with 4 μc per gram of body weight of ^{3}H-thymidine at the 21st day of pregnancy. Younglings at the 2nd day after birth were employed for the examination of the ductus and the thoracic aorta.

B. Pregnant rats were injected with 4 μc per gram of body weight of ^{3}H-thymidine at the 20th day of pregnancy. 10 hours later, the embryos were obtained by laparotomy. The specimens were removed from these embryos.

C. Each younglings at birth day was injected with 4 μc per gram of body weight of ^{3}H-thymidine, and sacrificed at 24 hours after the injection. Specimens were got from these younglings.

D. Pregnant rats were treated with human gonadotropin (50 units) (Teikokuzoki) at the 21st day of pregnancy, and at the following day injected with 2 μc per gram of body weight of ^{3}H-thymidine.

The embryos were obtained by laparotomy from the pregnant rats after 24 hours. Some of specimens were fixed with Bouin's fluid just after excision and the others were incubated in rats' blood containing anticoagulant at 38° C for 120 minutes. Then, the specimens were immersed in the fixative.

For age determination of embryos, the vaginal plug method was employed. After fixation for 24 hours, the specimens were embedded in wax by the routine procedures. The cross sections at 4 μ were performed with a microtome. After deparaffin, they were covered with NR-M2 emulsion (Sakura) by coating technic of radioautography according to Messier and Leblond (1957). Exposure time varied from 25 to 30 days. Then, the tissues were weakly stained with Hansen's haematoxylin-eosin. The percentage of mitosis (radioactive nuclei) was determined by counting labeled and unlabeled nuclei. This was done under oil immersion separating inner and outer regions of the ductus. As a control, the cell labelling frequency was examined in thoracic aorta. Nuclei showing more than three grains were considered as labeled. Each index in Table represents account of at least 500 nuclei.

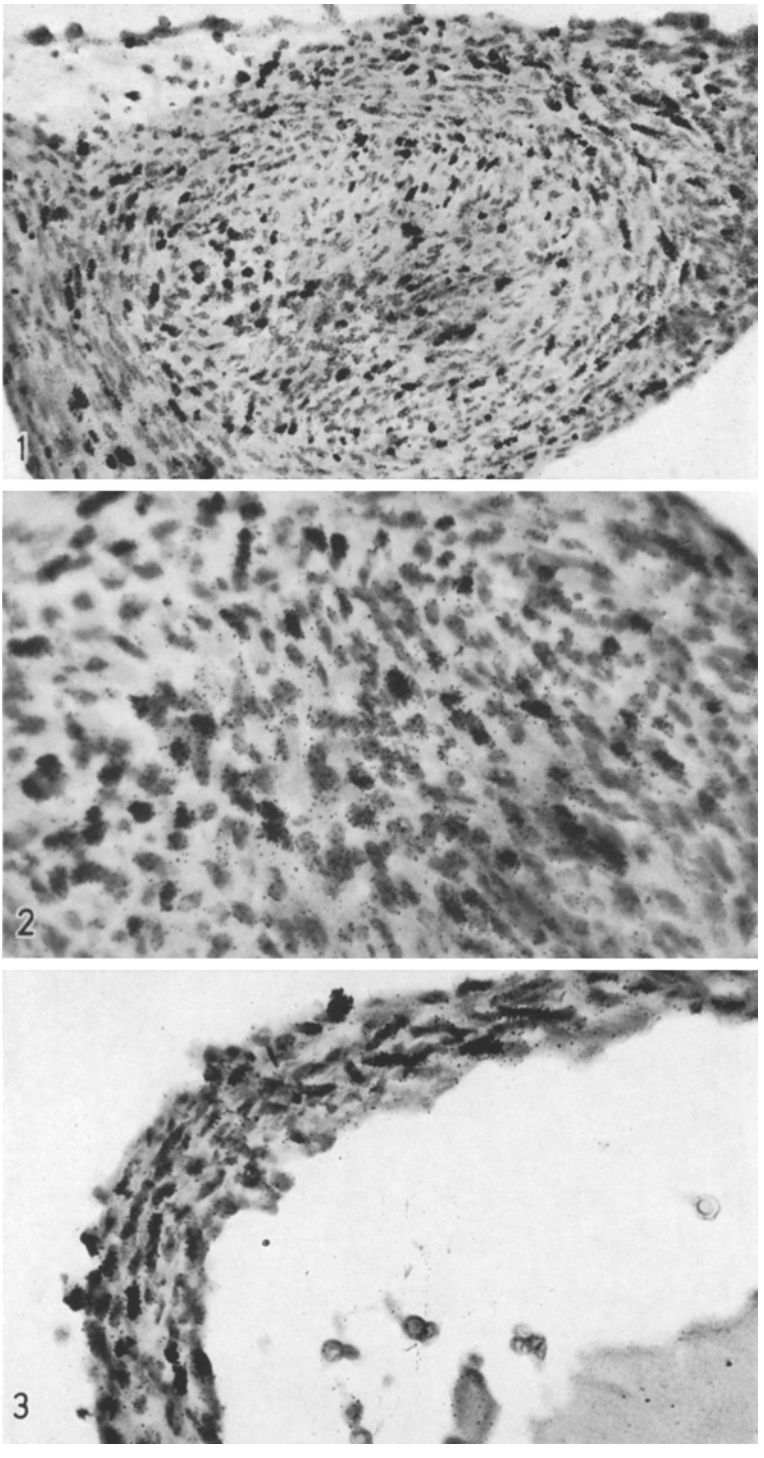
Results

The authors' attention was focused on the change of the sites of labeled nuclei accompanied with the obliterating process. If ^{3}H-thymidine was incorporated in dividing cells within several hours after the injection, the percentage of labeled nuclei seemed to indicate the mitotic activity of the cells in the vascular wall at the time. If the obliteration of the ductus arteriosus (d. a.) was due to a violent proliferation of intimal cells, lebeled nuclei should be seen in the inner portion of the ductus more frequently than in the outer portion.

Figs. 1 and 2 were photographed from specimens belonging to group A. In this stage, the ductus was obliterated completely and composed of the inner disarranged cell-mass and of the outer circular layers of myocytes (Fig. 1). The cells located in the inner region were oval or cuboidal in shape, while the cells in the outer region had slender profiles. The thoracic aorta of the same embryos took typical vascular structures.

The labeled nuclei in the ductus, as shown in Fig. 2, scattered considerably over whole of transverse sections of the d. a. and did not concentrate in any definite regions, and in the thoracic aorta a certain amount of the myocytes seemed to be labeled (Fig. 3).

Radioactive index of the cells in the obliterated ductus was similar to that of thoracic aorta treated with the same procedures (Table).



Figs. 1—3

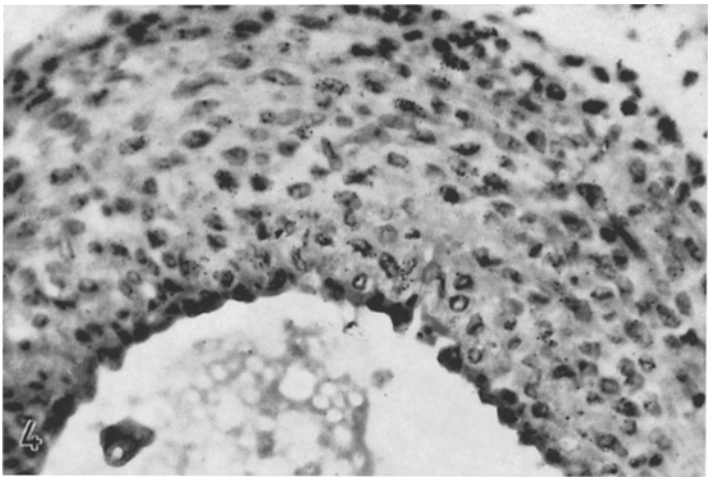


Fig. 4. 10-hour interval. The ductus excised from embryos which are at the 21st day in gestation. The distribution of the nuclei containing silver grains do not show any definite regions of the ductus

Table. *Labeling frequency of the nuclei in the ductus arteriosus and the thoracic aorta*

Group	Organ		
	Ductus arteriosus		Thoracic aorta (%)
	inner region (%)	outer region (%)	
A	36.5	38.0	39.5
B	27.2	26.5	—
C	7.8	11.0	18.0
D	12.2	10.8	12.7

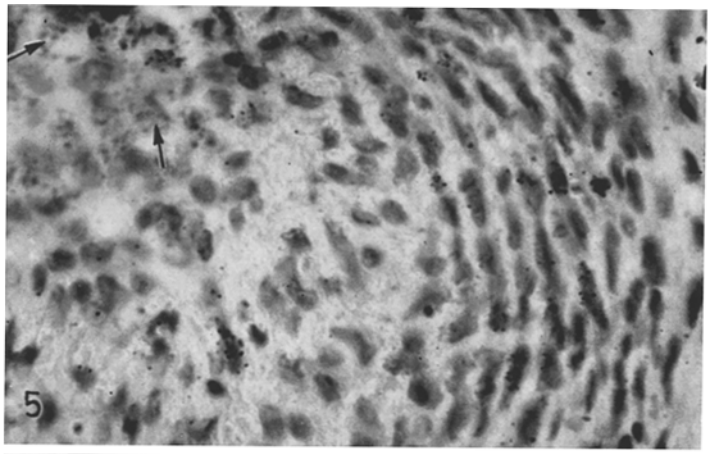
These evidences seemed to indicate that the d. a. and thoracic aorta maintained the same potency of mitosis till a certain stage of the fetal life, and a proliferation of the cells brought about everywhere and did not occur in the inner portion of the d. a. selectively.

Fig. 4 was obtained from the embryos belonging to the group B. At this stage, which the embryos of the group B reached, the obliteration process already started in the ductus according to the previous report (Mato and Aikawa, 1968). As shown in Fig. 4, endothelial cells were swollen and cell arrangement in the intimal layer were somewhat out of order. However, at this stage the characteristic increase of

Fig. 1. This figure shows the general picture of obliterated ductus arteriosus of the youngling of group A. Inner region of it is composed of disarranged cells and surrounded with some circular layers of myocytes. Labelled nuclei distributed evenly over whole area

Fig. 2. High magnification of the inner region of Fig. 1. Silver grains in the nuclei are various in number

Fig. 3. The thoracic aorta removed from the youngling under the same conditions of Fig. 1. Labeled nuclei are located mainly in circular arranged myocytes



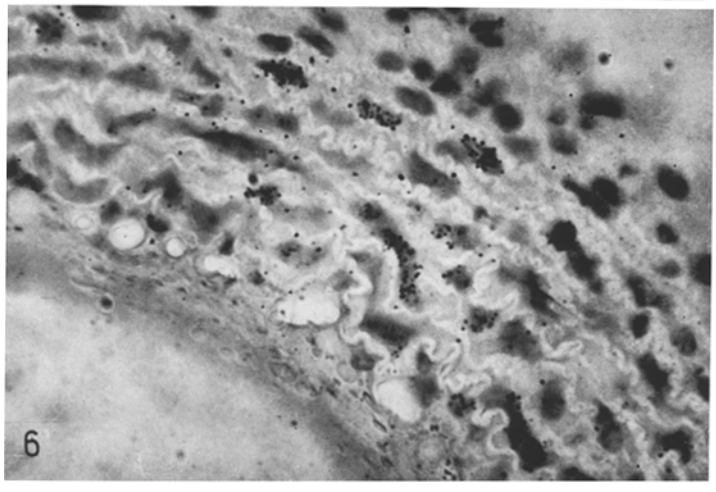


Fig. 5. 24-hour interval. The ductus is obtained from the younglings at the 2nd day after birth. Radioactive nuclei may be rather seen in outer layer of the ductus. The cells in the central part are disarranged and some of them show a positive reaction. Some destroyed nuclei are shown by the arrow

Fig. 6. The thoracic aorta in the same condition. The nuclei located in the muscular layers show a reaction

cell-mitosis was seen neither in the intimal layer nor in the other definite regions (Fig. 4).

The next experiment was dedicated to clarify whether cell-mitosis in the obliterating ductus continued to occur at the same level after birth or not.

Figs. 5 and 6 were photographed from the younglings belonging to the group C. Some high concentration of silver grains in labeled cells was found evenly in an obliterating ductus as shown in Fig. 5. As described in the previous report, in the central part of the ductus at this stage, the degenerating cells scattered mixing with intact cells randomly and disarrangement of the cells was discernible. And in the outer zone, myocytes sometimes including lysosomes were arranged circularly.

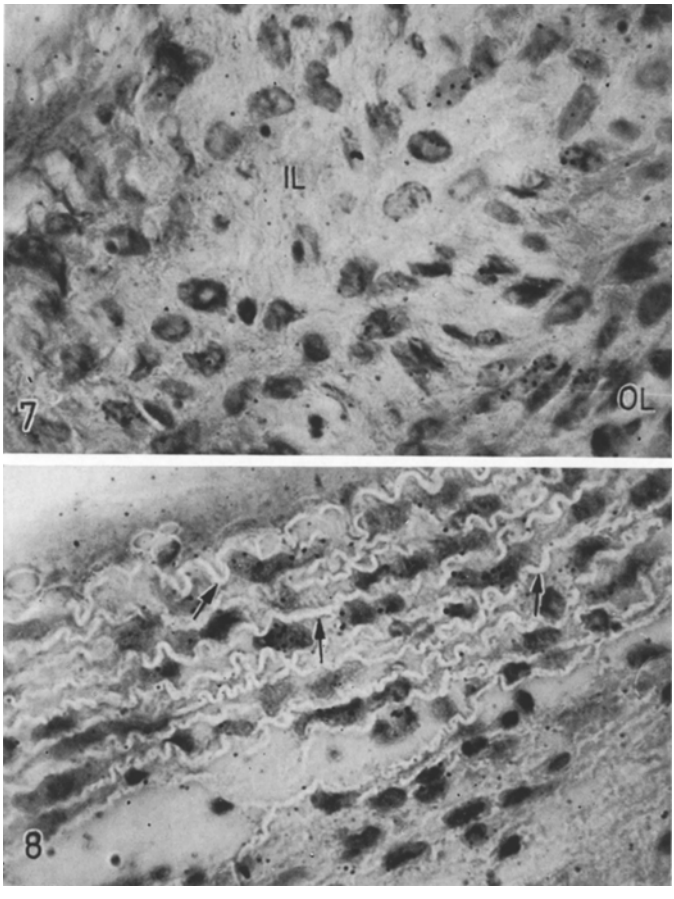
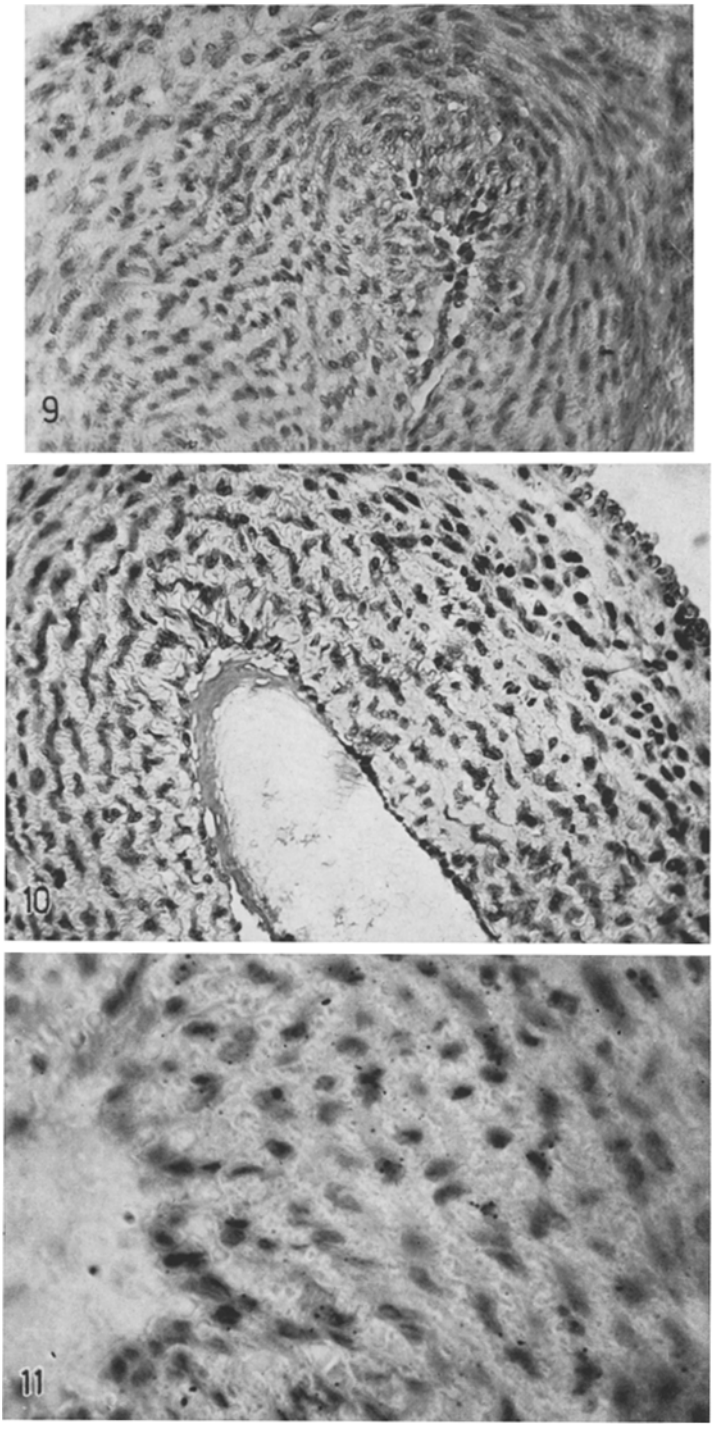


Fig. 7. 24-hour interval. The ductus of embryos treated with human gonadotropin. The ductus is composed of inner and outer layers. The nuclei having a relatively small quantity of silver grains are not always seen more frequently in the intimal layers. inner layer, *IL*; outer layer, *OL*

Fig. 8. The thoracic aorta under the same condition. Labeled nuclei are present in the smooth muscle cells. Among them, elastic membranes are evident (arrow)

In fact in Fig. 5, pyknotic and destroyed nuclei were obvious in some portions of the central region. Frequency of labeled nuclei in this obliterated ductus was relatively low compared with that of thoracic aorta excised from the same embryo (Figs. 5 and 6, Table).

The decrease in number of labeled nuclei in this case was considered to depend partially on an arrest of blood flow containing ^{3}H-thymidine and on an occurrence of cell-death in it. Here, it was noticeable that even in this condition the specific distribution of labeled nuclei was not found as shown in Fig. 5. The findings obtained from Figs. 2 and 5 suggested that most of labeled cells in Fig. 2 became labeled at the beginning of obliterating process of the d. a., and the labeled cells migrated into the inner region of the obliterated ductus. Of course, some of cells took up ^{3}H-thymidine after its obliteration.



Figs. 9—11

The aim of the following experiment was to show more clearly that the obliterating process of the d. a. already began in the intrauterine life and reached at a certain advanced stage in the end of fetal life, and to show a frequency of cell-mitosis and distribution pattern of labeled nuclei in the vessels.

In order to get a certain evidence for this problem, postmatured embryos were required. To get postmatured embryos, the injection of human gonadotropin (HCG) was employed. Owing to the injection, delivery of pregnent rats was retarded. Thus, by laparotomy, the embryos were removed according to the schedule. Under the condition, the d. a. of the embryos kept a relatively wide lumen even at the 22nd day of fetal life, though in the vessels a protrusion of the vascular wall into the lumen was discernible and inner and outer zones were distinguishable obviously. The cells in the inner layer disarranged considerably and contained relatively large nuclei, and further the marked increase of disarranged cells was clearly demonstrated. These findings suggested that the obliterating process developed remarkably in the d. a. of the postmatured embryos.

On the other hand, as shown in Fig. 8, thoracic aorta under the same condition kept a normal vascular structure. The labeling frequency in the ductus and the thoracic aorta decreased considerably in comparison with that of untreated ones with HCG, though the reason remained undissolved (Figs. 7 and 8, Table). Distribution of labeled nuclei in such condition did not show any characteristic pattern in the transverse sections of the obliterating ductus and the thoracic aorta as shown in Figs. 7 and 8.

To ascertain the change of physiological property of the ductus wall at the final stage of the fetal life, the other ductus treated with the same manner was incubated in adult rat blood for 120 minutes after the excision. As a result, it was clarified that the ductus constricted markedly, while the thoracic aorta did not show the considerable decrease in the diameter as different from the ductus (Figs. 9 and 10). Intimal cells in the ductus after the incubation had a tendency to migrate into the inner part of the ductus. Labeled cells located in the inner region seemed to translocate into the central region concurrently (Fig. 11).

Discussion

The theories regarding the closure of the ductus have been discussed for a long time, and most of researchers engaging in the problem coincided in the interpretation of the phenomenon, being indifferent to passive or active processes, as follows; the closure of the ductus arteriosus was caused with the muscular contraction and followed by the intimal proliferation of the vessels before or after birth.

Noticeable findings as to it in the past were obtained by several workers; Schaeffer (1914) and Melka (1926) attached great importance to the proliferation of elastic fibers for the obliteration; Variot and Cailliau (1920) reported the appearance of mucoid substance in the intima at the first stage of the obliteration; Melka (1926) observed the characteristic change in the ductus for the obliteration at the

Fig. 9. This figure shows the constriction of the ductus after the incubation for 2 hours in rat blood

Fig. 10. The thoracic aorta under the same condition. The vascular lumen remains relatively wide after the incubation for 2 hours

Fig. 11. This figure shows labeled nuclei under the same condition as in Fig. 9. The cells located in the intimal layer do not indicate more frequent labeling

intra-uterine life; Sciacca and Condorelli (1960) also emphasized the beginning of the obliteration in the ductus at the final stage of fetal life; and Meyer and Simon (1960) demonstrated a preparatory angiomalacia in the ductus arteriosus of the matured stillborn child and a dissociation of its inner layer from the outer layer of the obliterating ductus. Further, Doerr (1960) pointed out the presence of "critical period" for the obliteration after birth. Hoffmann (1964) described the appearance of localized mesenchymal cell-reaction in the ductus' ends during the obliteration.

In the previous report (Mato and Aikawa, 1968), the authors suggested that the obliteration of the ductus was not caused only by the proliferation of the intimal cells, but rather by the migration of the cells owing to a disruption of the elastic layers under hypoxia.

However, the precise data concerning the change of cell-population in the vessels during the obliteration have not been known up to the present.

According to Messier and Leblond (1957), the radioautographic method seemed to be at present the most convenient tool to caluculate the population of cell-mitosis, though ^{3}H-thymidine method was not necessarily decisive method for detecting proliferation and migration of the cells.

In order to confirm the authors' hypothesis mentioned above, radioautographic method was employed in this experiment.

From this experiment, the followings were established; (1) in a course of closure there were no specific portions showing any high concentration of labeling cells in the obliterating ductus; (2) in an early phase of the obliterating process, the frequency of cell-mitosis of the ductus was similar to that of thoracic aorta; (3) in the final stage, the cells in the ductus arteriosus showed a weak uptake of ^{3}H-thymidine as different from thoracic aorta; (4) further, the ductus became to have a strong tendency to be closed after the arrest of blood stream.

These findings could not necessarily be explained by the hypotheses proposed by the other authors. In other words, if the ductus was obstructed by the intimal proliferation, labeled nuclei might be frequently found in the intima of the ductus at the beginning and midstage of closure, and at the final stage, most of centrally located cells might become labeled as different from peripheral regions. However, the results obtained from this experiment were not in accordance with these expectations as mentioned above.

Therefore, it was likely that most of the cells migrated into the lumen during the obliteration, and that some of the cells during the migration had yet a mitotic activity as well as in a vascular wall at an early stage. In this point, the authors' hypothesis in the previous paper (Mato and Aikawa, 1968) which a specific cell-mitosis did not occur in intimal cell-layer seemed to be more preferable.

Further, in order to study the mechanism of the obliteration, the anatomical closure has been investigated separately from the functional closure for a long time. For the anatomical closure, necessity of the proliferation of the fibrocytes may be clear without doubt. However, for the functional closure, nerve reflex via recurrent nerve of vagus nerve, translocation of mediastinal organs, and change of oxygen content in the blood flow through the ductus had required great importance.

But, as stated in the previous report (Mato and Aikawa, 1968), nerve ending in the ductus of rats was rarely seen in number. The similar finding was already

obtained by Harms' (1967) pharmacological experiment using *gallus domesticus*. Therefore, the role of nervous reflex via vagus nerve seemed not to be essential for the obliteration.

However, it seems to be reasonable that obliterating process of the ductus is initiated with hypoxia caused by the decrease or arrest of blood flow through the ductus, and under *the condition*, cell-death in the vascular wall and a disruption of elastic layers give rise subsequently. These events are expected to produce considerable changes in vascular architecture, and to take off physiological vascular properties from the obliterating ductus. Therefore, quantity of blood flow through the ductus was the most significant factor for the initiation of the closure. The "critical period" by Doerr (1960) was highly estimted from this point of view.

From the experiment of the D group, it was clear that physiological elasticity of the ductus decreased remarkably and the ductus in the later stage had a tendency to be closed after the excision as expected above. The result was considered to be intimately related to the disruption of the elastic layers and to the modification of the architectures in the ductus as described in the previous report (Mato and Aikawa, 1968). Excellent work by Meyer and Simon (1960) which was concerned to angiomalacia in the obliterating ductus afforded the agreement of such observation.

The ductus in the final stage of fetal life might be in expanded stage only by blood pressure. So that, the complete arrest of blood stream might induce easily a complete involution of the ductus, and with time the ductus gradually transformed into the string-like structure owing to activation of mesenchymal cells. At that time, anatomical closure seemed to begin.

If these sequences failed to precede favorably in the vascular wall, for example, when disruption of elastic fibers and cell-death, under hypoxia, did not occur at "critical period", or when the "critical period" was too short to produce each change, the occurrence of some defect in the closure of the ductus would be expected to appear. Namely, in such abnormal specimens, the cells composing the ductus might remain alive even after suffering from the decrease or arrest of the blood stream. Such ductus arteriosus would remain patent after birth.

Though there may be individual difference about the degree of modification in vascular architecture under hypoxia, it is generally accepted that patent ductus arteriosus occurs more commonly in females (Beeson and McDermott, 1967).

Further, cardiosurgically, it is well known that in a case of patent ductus arteriosus treated with ligature, recanalization of the ductus sometimes takes place at some time after operation (Ross, Feder and Spencer, 1961). That is, the temporary arrest of the blood flow through the ductus do not always induce the change of the vascular architecture.

From these events, it might be reasonable to consider that during the closure of the ductus, the reactivity of the cells against oxygen defficiency is one of the significant elements in the obliteration of the ductus arteriosus, and that the reactivity of the cells in the ductus seemed to be genetical as stated by Buchanan (1968).

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