Relation of the Distribution of Fibronectin to the Obliteration of the Arch Artery in the Chick

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Summary. Fibronectin has been believed to be involved in vasculogenesis. This study demonstrates by immunohistochemistry the distribution of fibronectin around the systemic arteries of chick embryos during the periods of aortic arch formation, and examines the possible relationship between the distribution of fibronectin and the obliteration of the arch artery.

In embryos at stage 23, no obliteration of arch arteries was recognized on sections. At this stage, intense staining with an anti-fibronectin antibody was seen in the developing tunica media of the carotid artery and dorsal aorta. Staining around the arch arteries was much weaker. At stage 26, the intensity of anti-fibronectin antibody staining was weaker around the left arch artery, although the diameter of the 4th arch artery was not different between the left and right. At stage 27, which is the early period for the obliteration of the left 4th arch artery, the intensity of the anti-fibronectin antibody staining for the left arch artery was much less than that for the right arch artery.

These results show that fibronectin does not accumulate around the left 4th arch artery during aortic arch formation and suggest the possibility that a smaller amount of fibronectin is responsible for the obliteration of the left 4th arch artery.

During formation of the left aortic arch in humans, the right dorsal aorta distal to the origin of the right subclavian artery is obliterated (BARRY, 1951). In contrast to the left aortic arch in mammals, the right aortic arch is formed in the chick by obliteration of the left 4th arch artery (BOCKMAN et al., 1987).

Neural crest cells migrate around the arch arteries (SUMIDA et al., 1989a) and participate in the tunica media formation of the aortic arch in the chick (LE LIÈVRE and LE DOUARIN, 1975). Failure of neural crest cells to migrate causes the malformation of the aortic arch in the chick (BOCKMAN et al., 1987, 1989).

In the past decade, molecules which mediate cell migration have been demonstrated, fibronectin being one such molecule mediating neural crest cell migration *in vitro* (GREENBERG et al., 1981; NEWGREEN et al., 1982; BILOZUR and HAY, 1988). Since fibronectin accumulates around the dorsal aorta during vasculogenesis in chick embryos (RISAU and LEMMON, 1988), it is thought to mediate neural crest cell migration around the arch arteries *in vivo*. We presumed that neural crest cells selectively migrate into the fibronectin-rich space, and that the developing vessels without fibronectin are obliterated.

On this presumption, we have examined by immunohistochemistry temporal changes in fibronectin distribution in the arch arteries and dorsal aorta of chick embryos during the period of aortic arch formation.

MATERIALS AND METHODS

Fertilized eggs, purchased from a local farm, were incubated at 38°C in about 90% relative humidity. On days 4, 4.5 or 5, embryos were fixed with 4% paraformaldehyde in PBS for 6 h at room temperature. After staging according to HAMBURGER and HAMILTON (1951), the specimens were dehydrated through a graded series of ethanol and cleared in xylene, and then embedded in paraffin.

Cross sections of the arch arteries were mounted on glass slides and deparaffinized. The sections were blocked with 3% H₂O₂ and washed in phosphate buffered saline (PBS). Then the sections were incubated with an anti-fibronectin antibody diluted 200 times with PBS for 1 h. The anti-fibronectin antibody, raised in rabbits, was against human plasma fibronectin (SUMIDA et al., 1989b). After washing with PBS, the sections were incubated with biotinylated anti-

rabbit-IgG followed by incubation with peroxidase labeled streptavidin (StrAvidin; BioGenex, USA). The sections were then developed with 0.03% diaminobenzidine, 0.006% H_2O_2 and counterstained with hematoxylin. Immunostaining procedure was performed at room temperature. The specimens were photographed with a Nikon IF440 interference filter for black and white film.

RESULTS

In embryos at stage 23, the 1st and 2nd arch arteries were already obliterated (BOCKMAN et al., 1987). Thus, the 3rd, 4th and 6th arch arteries could be observed in cross sections (Fig. 1a). The development of the tunica media of the arch arteries and the carotid artery was not clear. In contrast, developing tunica media of the dorsal aorta was recognized as an aggregation or condensation of mesenchymal cells around the endothelium (Fig. 1b). The anti-fibronectin antibody ubiquitously stained the extracellular matrix in embryos. However, intense staining was seen in the developing tunica media of the carotid artery and

dorsal aorta (Fig. 1a, b). Intense staining of the carotid artery and dorsal aorta was observed through stages 23 to 29. Sparsely located mesenchymal cells around the dorsal aorta also reacted to the antibody (Fig. 1b). Staining with the anti-fibronectin antibody was weaker around the arch arteries (Fig. 1a).

In embryos at stage 26, the 3rd and 4th arch arteries were observed in sections (Fig. 2). No differences between the right and left in the diameter of 3rd or 4th arch arteries were recognized. In the right 3rd and 4th arch arteries, the developing tunica media was stained much more intensely than in the left by the anti-fibronectin antibody. Both the right and left 6th arch arteries were stained weaker by the antibody than the right 3rd and 4th ones.

In embryos at stage 27, the left 4th arch artery was being obliterated (BOCKMAN et al., 1987). Thus on the left, only two arch arteries, the 3rd and 6th, could be seen, though on the right, three arch arteries, the 3rd, 4th and 6th, could be seen (Fig. 3). The left 4th arch artery was observed in more distal part of the arch artery of the same specimen (data not shown). Many more mesenchymal cells condensed around the endothelium of the arch arteries than at stage 23. At stage

Fig. 1. **a.** Anti-fibronectin staining around the arch arteries and carotid artery; **b.** the dorsal aorta at stage 23. Intense staining (*arrowheads*) was seen in the developing tunica media of the dorsal aorta, and the right and left carotid arteries (*RC*, *LC*) which are a continuation of the right and left dorsal aortae, respectively. The arch arteries are not so intensely stained by the antibody. The 1st and 2nd arch arteries have already been obliterated. *L3-6* left 3rd to 6th arch arteries, *R3-6* right 3rd to 6th arch arteries. Bars: 200 μ m on **a**, 100 μ m on **b**.

Fig. 2. **a.** Staining with the anti-fibronectin antibody of a stage 26 embryo. **b.** High magnification of the left 3rd (L3) and 4th (L4) arch arteries; **c** the right 3rd (R3) and 4th (R4) arch arteries. The left 4th arch artery is located more closely to the 3rd arch artery than the right 4th to the 3rd. Immunoreactivity of left arch arteries is weaker than that of the right. Bars: 200 μ m on **a**, 50 μ m on **b** and **c**.

Fig. 3. **a.** Staining with the anti-fibronectin antibody of a stage 27 embryo. **b.** High magnification of the left (*L3*) arch artery; **c** the right 3rd (*R3*) arch artery. The left 4th arch artery is obliterated at the level of this section. The left 4th arch artery is recognizable at a more distal part. Immunoreactivity of the left arch arteries is weak (**b**), while immunoreactivity is still intense in the developing tunica media of the right arch arteries (**c**). The configuration of the endothelial cells of the left 3rd arch artery was different from those of the right arch arteries, because the left arch artery was cut more obliquely than the right arch arteries on the section. *L6* left 6th arch artery, *R3-6* right 3rd to 6th arch arteries. Bars: 200 μ m on **a**, 50 μ m on **b** and **c**.

27, intense staining of the anti-fibronectin antibody was observed in the developing tunica media of the right 3rd and 4th arch arteries (Fig. 3). In the left 3rd arch artery, in contrast, much less staining was seen in the extracellular space of the developing tunica media. In the tunica media of the right and left 6th arch arteries, a medium intensity of staining was observed (Fig. 3a).

DISCUSSION

During formation of the aortic arch, definite parts of the embryonic arteries are obliterated. The pattern of obliteration, however, is different between mammals and birds. In rat embryos, the right dorsal aorta is obliterated (SUMIDA, 1988), while the left 4th arch artery is obliterated in chick embryos (BOCKMAN et al., 1987). In the present study, it was shown that the left 4th arch artery was present in the distal part but was not present in the proximal part at stage 27. This result indicates that the left 4th arch artery is gradually obliterated from proximal to distal.

Aspects of distribution of fibronectin in the arteries of the chick are also different from that of the rat. Fibronectin, an extracellular glycoprotein (reviewed by YAMADA, 1983), has been suggested to be involved in vasculogenesis by mediating mesenchymal cell migration (RISAU and LEMMON, 1988). In rat embryos, it was shown that fibronectin accumulates in the tunica media of the dorsal aorta (SUMIDA, 1988). No difference in distribution between the right and left dorsal aortae was marked in rat embryos. In the present study, however, we showed in the chick embryos that the tunica media of the right arch artery was stained more intensely than that of the left. In other words, in the chick, fibronectin is distributed more sparsely in the tunica media of the left arch artery than in the right arch arteries.

The tunica media of the arch arteries in the chick consists of ectomesenchymal cells derived from the neural crest (LE LIÈVRE and LE DOUARIN, 1975). Migrating neural crest cells do not produce fibronectin, whereas mesodermal mesenchymal cells migrating to the blood vessels do (FRENCH-CONSTANT and HYNES, 1988). In the present study, the carotid artery and dorsal aorta were intensely stained with the anti-fibronectin antibody. Cells forming the tunica media of the carotid artery are also derived from the neural crest (LE LIÈVRE and LE DOUARIN, 1975; NAKAMURA, 1982). Thus, different degrees of staining with the anti-fibronectin antibody between the arch arteries and carotid artery may not reflect differences in the origin of cells forming the tunica

media. Since fibronectin accumulates in the developing tunica media of the arch arteries and dorsal aorta in the rat, it may be involved in the tunica media formation of the arch arteries. However, the distribution of fibronectin was not different between the right and left dorsal aortae in the rat (SUMIDA, 1988), suggesting that fibronectin is not involved in the obliteration of the dorsal aorta in this rodent. Differences in the distribution of fibronectin between rat and chick embryos indicates that fibronectin plays different roles in vasculogenesis among different species. In other words, the mechanisms of obliteration may be different among species.

Ablation of premigratory neural crest cells (BOCKMAN et al., 1987) or bisdiamine which is suggested to perturb the neural crest cell migration (SUMIDA, 1988), damaged the morphogenesis of the aortic arch in the chick and rat. These results emphasize the role of neural crest cells in the formation of the arch arteries. BOCKMAN et al. (1989) showed that after ablation of premigratory neural crest cells, the number of cells was significantly reduced in the arch arteries, and the cells were not condensed in the tunica media. They clearly showed that the decreased number of cells derived from the neural crest around the arch arteries caused the abnormal formation of the arch arteries. Interaction between the cells and fibronectin seems important in tunica media formation. When cells are locomotory, fibronectin receptors are diffusely distributed, but once they become stationary, fibronectin receptors concentrate into the part of focal contact (DUBAND et al., 1988). This result indicates that fibronectin plays a role in cell migration and attachment. Thus, the fibronectin around the arch arteries may account for the aggregation of cells into the tunica media. The smaller amount of fibronectin in the tunica media of the left arch artery may result in the dispersion of cells from the tunica media and/or failure of the neural crest cells to migrate. As BOCKMAN et al. (1989) showed, a decreased number of the cells derived from the neural crest in the wall of the arch artery may cause the closure of arch arteries. Less fibronectin, therefore, is suggested to be one of causes of the obliteration of the left 4th arch artery in the chick embryo.

In the present study, the intensity of the staining of the left 3rd arch artery, which is not obliterated, was also weaker than that of the right. Reasons as to how the left 3rd arch artery persists with less fibronectin is unknown. The extracellular matrix might lead to more complex interactions among cells during vasculogenesis.

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