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Research article

Metric characterization of the aortic arch of early mouse fetuses and of a fetus featuring a double lumen aortic arch malformation

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SUMMARY

This study aimed at providing an objective metric characterization of the aortic arch of a mouse fetus featuring a double lumen aortic arch malformation. As a side effect it provides reference data defining the length and the diameters of the aortic arch segments of normally developed mouse fetuses at developmental stage 22 according to Theiler (TS22). We analyzed a total of 22 TS22 mouse fetuses of the Him:OF1 strain. We produced high-resolution three-dimensional (3D) computer models and measured the diameters and cross sectional areas of the aortic arch segments and of the ascending and descending aorta. In addition, we defined 3D skeletons of the arteries and measured the length of the aortic arch segments. We provide statistics on the measurements obtained from the normally developed TS22 fetuses and detailed characterizations of the double lumen aortic arch. Our data suggest that: firstly, in Him:OF1 fetuses of TS22, the formation of the aortic arch is not yet finished. The left subclavian artery still receives a significant amount of blood from the right ventricle. Secondly, persistence of the 5th pharyngeal arch artery does not affect remodeling of the arteries distal to the junction of 5th pharyngeal arch artery and dorsal aorta. Thirdly, hemodynamic forces define the dimensions of the aortic arch between the left common carotid and the left subclavian artery. Fourthly, the blood volume streaming through the 4th pharyngeal arch artery influences its enlargement between TS20 and TS22.

1. Introduction

The morphology and topology of the heart and blood vessels of mice closely resemble that of humans. Therefore the mouse is a very popular model for research on the mechanisms responsible for the genesis of cardiovascular pathologies and malformations, and for researching the genetic and epigenetic factors that drive cardiovascular morphogenesis.

Mice have an ascending aorta emerging from a left ventricle and a pulmonary trunk emerging from a right ventricle. They have a left sided aortic arch, which gives rise to a brachiocephalic trunk (innominate artery) that divides into a right common carotid artery and a right subclavian artery, a left common carotid artery, and a left subclavian artery. In the fetal period, a left sided ductus arteriosus (ductus Botalli) connects the pulmonary trunk and the proximal segment of the descending aorta.

According to a traditional view, mammals develop 6 pairs of pharyngeal arch arteries, with the 5th pair completely missing or appearing for a very short time span. The pharyngeal arch arteries appear in the second half of the embryonic period in a cranial to caudal sequence left and right to the pharynx. The arteries connect the aortic sac with an originally left and right dorsal aorta. In a complex remodeling process, the 3rd to 6th pharyngeal arch arteries and segments of the aortic sac and the dorsal aortae are remodeled to form the great intrathoracic arteries (Congdon, 1922; Kaufman, 1992; Williams et al., 1989). With the start of the fetal period, the definitive *intrauterine* pattern of the great intrathoracic arteries is established. In humans this is at Carnegie stage (CS) 23 (Congdon, 1922; O'Rahilly and Müller, 1999; Williams et al., 1989). In mice this is around developmental stage 22 according to Theiler (TS22) (Kaufman, 1992; Theiler, 1989; Weninger and Geyer, 2009).

Of special interest is the pair of 5th pharyngeal arch arteries and the malformations that are considered to result from their abnormal remodeling (Bernheimer et al., 2007; Gerlis et al., 1989; Khan and Nihill, 2006; Linhares et al., 2011; Van Praagh and Van Praagh, 1969). According to Tandler (1909), human embryos bilaterally show 5th pharyngeal arch arteries for a very short time span approximately at CS 14-15). Mouse and rat embryos show a vascular channel that connects the 4th and the 6th pharyngeal arch arteries (Geyer and Weninger, 2012; Bamforth et al., 2012; Tandler, 1902). Tandler considered these vessels to be homologous to the 5th pharyngeal arch artery of man (Tandler, 1902). Shortly after their appearance, they vanish without leaving derivatives.

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Higher vertebrates do not develop proper 5th and 6th pharyngeal arches. Consequently, the blood vessels of higher vertebrates that were originally termed as 5th and 6th pharyngeal arch arteries may not be homologous to the 5th and 6th pharyngeal arch arteries of lower vertebrates (De Ruiter et al., 1989). In fact, there is strong evidence that the termini 5th pharyngeal arch artery and 6th pharyngeal arch artery in higher vertebrates are misleading and should be replaced by proper names. For example, the terminus pulmonary arch artery was suggested to replace the terminus 6th pharyngeal arch artery (De Ruiter et al., 1989). We are sympathetic with the idea of changing the nomenclature. However the new nomenclature is not yet fully accepted. Thus, in order to avoid confusion we will use the traditional nomenclature throughout the text.

Double lumen aortic arch malformation is frequently explained by the abnormal persistence of the left 5th pharyngeal arch artery (Van Praagh and Van Praagh, 1969). In this malformation, the aortic arch segment between the origin of the left common carotid artery and the origin of the left subclavian artery is split into two channels. In humans this malformation is long known and well characterized (Bernheimer et al., 2007; Herrera et al., 1987; Khan and Nihill, 2006; Linhares et al., 2011; Marinho-da-Silva et al., 1998; Van Praagh and Van Praagh, 1969). In adult mice such a malformation has never been described. But recently, a double lumen aortic arch was found in a mouse fetus of TS22 (Geyer and Weninger, 2012). However, due to the lack of data defining the normal lengths and diameters of the aortic arch segments in fetuses at this developmental stage, a comprehensive characterization of the aortic arch of this fetus was not performed.

Our study aims at providing an objective metric characterization of the double lumen aortic arch seen in a mouse fetus of TS22 and at discussing the implications of this knowledge for understanding the mechanisms influencing the formation of the aortic arch. As a side effect it shall also provide reference data, which objectively define the lengths and the diameters of the aortic arch segments of normally developed TS22 mouse fetuses of the OF1 strain.

2. Material and methods

We analyzed the aortic arch of 22 mouse (*Mus musculus*) fetuses at developmental stage 22 according to Theiler (Theiler, 1989). In addition we calculated the diameter of the left 4th pharyngeal arch artery of 12 mouse embryos of TS 20. All embryos and fetuses were of the OF1 strain. None of them showed gross malformations, except for one fetus, which had a double lumen aortic arch malformation.

2.1. Animal preparation and embedding

Pregnant mice of the Him: OF1 Swiss strain were purchased from the animal laboratory Himberg (Austria). They were sacrificed by cervical dislocation on day 14 *post conception* (dpc) for collecting TS 22 fetuses, and on day 12 dpc for collecting TS 20 embryos. The embryos and fetuses were dissected from the uterus and transferred into phosphate buffered saline (PBS). In this solution they were staged according to Theiler (Theiler, 1989) with the aid of a dissection microscope (Wild-Heerbrugg M8). Only embryos staged as TS 20 and fetuses staged as TS 22 were processed further.

The specimens were divided into head, thorax and abdomen. The thoraces were fixed in 4% PBS-buffered paraformaldehyde (PFA) at 4 °C for 24–48 h and dehydrated in a series of increasing ethanols (70% 16 h; 80% 3 h; 90% 3 h, 96% 3 h). Then they were infiltrated with a solution comprising 100 ml JB-4 Plus solution A (JB-4 embedding kit, Polysciences), 1.25 g Benzoyl Peroxide, plasticized (catalyst), and 0.4 g Eosin spritlöslich (Waldeck GmbH) for 24 h (two changes). Finally the specimens were embedded in JB-4 resin (25 ml JB-4 Plus solution A, 0.3125 g Benzoyl Peroxide, plasticized (catalyst), and 1 ml JB-4 Plus solution B) containing eosin (0.4 g per 100 ml).

2.2. HREM data generation

The eosin dyed resin blocks were subjected to HREM data generation (Geyer et al., 2012a; Mohun and Weninger, 2011; Weninger et al., 2006). The blocks were mounted on a rotary microtome (Microtec CUT 4060E, microTec Laborgeräte GmbH) and physically sectioned. During the sectioning process an image of the block surface was captured after each sectioning cycle utilizing a magnifying optic (Leica DM LM) equipped with an YFP-filter (excitation filter 500/20 nm, emission filter 535/30 nm) and a digital camera (Leica DFC480). The resulting stacks of about 1000 single images were converted to a volume data set, which had a voxel size of 1.07 μm × 1.07 μm × 2 μm.

2.3. Data processing and analysis

The digital volume data sets were processed using the 3D analysis software Amira® 5.3.3 (Mercury Systems). The great intrathoracic arteries were segmented manually and the resulting binary data were used for generating surface rendered 3D computer models (Fig. 1). Following a recently published protocol (Weninger et al., 2009), the 3D-models were used to measure the perimeters and calculate the diameters (diameter d = perimeter/π) of the lumina of the aortic arch segments and of the ascending and descending aorta (Fig. 2). We calculated the cross sectional areas (area $A = (d/2)^2\pi$) from the measured diameters

During the reconstruction and measuring process, the borders of the blood vessel lumina must be defined by manual tracing. In order to minimize the negative influence of subjective interpretation of blood vessel borders we decided to provide the dimensions of the aortic arch segments also as ratios of the respective aortic arch segment and the ascending and descending aorta. We did these calculations using the Excel (Microsoft Office) software.

To provide comprehensive characterizations of the aortic arch segments of the TS 22 mouse fetuses, we determined the length of the aortic arch segments. To achieve this, we used the "skeletonization module" of the Amira software for producing skeletons of the surface rendered 3D models of the great intrathoracic arteries. In the skeletons, we then measured the length of the aortic arch segments, by using the measuring tool of the Amira software.

All statistics were performed with MS-Excel (Microsoft) and the software package SPSS 17.0 for Windows (SPSS Inc.).

3. Results

Our study provides reference data, which define the normal diameters and lengths of the aortic arch segments of mouse fetuses of TS22. It then provides reference data defining the luminal diameter of the left 4th pharyngeal arch artery of TS20 mouse embryos. Finally it provides a detailed metric characterization of the aortic arch of a mouse fetus featuring a double lumen aortic arch malformation.

3.1. Reference data (TS 22 mouse fetuses)

We provide information about the diameters and cross-sectional areas of the aortic arch segments of normally developed mouse fetuses of TS22 in Table 1. We also provide the lengths of the aortic arch segments, as well as the ratios between the diameters