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Congenital Heart Disease (Atrioventricular Septal Defect) in the Mouse with Trisomy 16

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= Abstract = **Failure of fusion between the superior and inferior cushions has usually been assumed to be the main morphogenetic event in producing hearts with deficient atrioventricular septation (atrioventricular septal, or endocardial cushion defects). Morphological studies on human autopsy specimens, however, showed that another consistent finding is the marked disproportion between the dimensions of the inlet and outlet of the left ventricle, which, until now, has no known developmental basis.**

We have studied the early formation of the hearts with atrioventricular septal defects, using a mouse model with trisomy 16. Animals were studied between the 10th and 19th days of gestation by stereomicroscopic examination, scanning electronmicroscopy and the in-vitro incorporation of thymidine.

The first detectable morphological abnormality of the heart in the trisomic mouse was observed on the 11th day, being a persistence of an infolding at the inferior atrioventricular junction. This infolding was present in both trisomic and eusomic animals on the 10th day. This morphology could be explained by a differential growth of the myocardium at the inferior atrioventricular junction, which was found to be a distinct zone with low incorporation of thymidine. On the 11th day, the inferior atrioventricular cushion was bigger in the trisomic hearts. The abnormalities of the cushions observed on the 12th day or later were deemed to be consequences of these primary defects.

Sectioning of the heart from the left lateral aspect convincingly showed morphological changes of the superior and inferior cushion or bridging leaflets in this animal model. Abnormal endocardial cushions and abnormality in the proliferation index of the myocardium at the inferior atrioventricular junction play more significant roles in the formation of the hearts with deficient atrioventricular septation, than the abnormalities found in the atrioventricular cushions.

Key words: *Atrioventricular canal malformation, Endocardial cushion defect, Down's syndrome, Myocardial development*

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INTRODUCTION

Hearts with deficient atrioventricular septation (atrioventricular canal malformations, endocardial cushion defects) have several characteristic morphological features (Anderson *et al.*,

1991). Although the most obvious is usually a hole at the site of the anticipated atrioventricular septal structures of the normal heart (Becker and Anderson 1982), deficient atrioventricular septation can be recognized even when the septal structures are intact (Silverman *et al.*, 1984; Ho *et al.*, 1990). This is because the entire ventricular mass is abnormal, showing a marked disproportion between the dimensions of the inlet and outlet of the left ventricle (Penkoske *et al.*, 1985). Wenink and Zevallos (1988) have shown that such abnormal morphology is recognizable in young human embryos, while measurements of fetal human specimens confirm that the typical ventricular disproportion is well established by mid-gestation (Cook *et al.*, 1991).

There is a well known association between Down's syndrome and atrioventricular septal defect. About 40% to 60% of patients with Down's syndrome have atrioventricular septal defect (Rowe and Uchida 1961; Ferencz *et al.*, 1989), and more than one third of those with atrioventricular septal defect have Down's syndrome (Emanuel *et al.*, 1968; Dickinson *et al.*, 1981). It is difficult in human material, however, to chart the development of the ventricular abnormalities and to relate them to the characteristic malformations of the atrioventricular valves. Such development can be more readily studied in mice with trisomy 16, known to be a model of Down's syndrome (trisomy 21) in humans (Miyabara *et al.*, 1982; Miyabara 1990). Mice with trisomy 16 have a more than 95% incidence of atrioventricular septal defect (Miyabara *et al.*, 1984; Miyabara 1990). The breeding protocol for producing trisomy 16 gives litters with both trisomic and eusomic fetuses so that the eusomic littermates serve as a perfect control with which to study the progress of atrioventricular septation. In this report we compare the early formation of the ventricular mass in trisomic and eusomic mice, highlighting those features which we believe to underscore the formation of an atrioventricular septal defect.

MATERIALS and METHODS

We examined a total of 433 implantations in 62 litters from the breeding program described below. Of these, 245 (56.6%) were shown to be normal and 68 (15.7%) were trisomic, this being determined either by karyotyping or by morphological observation. The chromosomal status in 17 animals (3.9%) could not be determined, while the remaining fetuses (103) were resorbed (23.8%).

The morphology of the hearts from trisomic embryos/fetuses and controls were studied by stereomicroscopy before and after fixation. The embryos were then sectioned in various planes, followed by stereomicroscopic examination and scanning electron microscopy. Fourteen hearts from trisomic animals, and 13 hearts from control mice, were studied at days 11 to 16 by scanning electron microscopy so as to determine the morphology in the developmental stages. Six hearts from trisomic mice, and 7 from control mice, taken over days 17 through 19, were studied by scanning electronmicroscopy.

So as to chart the early developmental stage, 52 embryos at the 11th day and 24 embryos at the 10th day were studied by stereomicroscopy. Of the embryos collected at the 11th day, 28 had been prepared for in-vitro culture from the tenth day to assess the incorporation of thymidine by the myocardial cells. They were processed either by serial histological sectioning and autoradiography or by cryosectioning and electronmicroscopy.

Production of mice with trisomy 16 and determination of chromosomal status

Crosses between two mouse strains each carrying a Robertsonian translocation [Rb (16,17) 7Bnr and Rb (11,16)2H] were made to generate doubly heterozygous males. These were mated overnight with normal females of the C57Bl/6 strain. When a vaginal plug was found the following morning, this was counted as the first day of pregnancy.

For embryos aged 12 days or younger, the brain and lower half of the body were minced and cultured for 1 hour in a medium containing Colcemid. Chromosomal preparations were made and stained with Giemsa. Presence of more than one complete set of two Robertsonian metacentric chromosomes and 41 chrom-

osome arms indicated the presence of trisomy 16. Embryos and fetuses with one or more chromosome sets of balanced translocation were used as normal controls. For fetuses aged from the 13th to the 15th day, only the liver was minced and subsequent steps were as described above. For fetuses at the 16th day of gestation or beyond, we karyotyped only in some selected cases. This was because it proved to be possible to recognize trisomic mice from the characteristic features of the eyelids and nose and the presence of edema. These criteria possess a high sensitivity (89%) and specificity (99.5%) (Epstein and Vekemans 1990) for recognition of trisomy 16.

Sectioning·and scanning electronmicroscopy

The embryos and fetuses were fixed by immersion in 2.5% glutaraldehyde in 0.2 M cacodylate buffer at pH 7.4. The heart was immersed for 10 minutes in cryosectioning medium, and was then frozen and sectioned, using a cryomicrotome, in coronal, frontal and sagittal planes. The slices were mounted on glass slides and examined by microscopy so as to control the angle and level of sectioning (Seo *et al.*, 1995). When sectioning was deemed satisfactory, the heart was thawed and washed in phosphate buffered saline. This process cleared the cryomedia and also removed the blood cells from the cardiac chambers. Hearts were then osmicated and dehydrated as for conventional scanning electron microscopy and dried, coated and examined.

Cell proliferation in the embryonic heart

Pregnant mice were sacrificed on day 10 and the embryos explanted from the uterus and checked for their developmental stages (the number of somites). Sets of three embryos were prepared in 50 ml bottles containing 3 ml of rat serum and 1 ml of minimal essential medium and gassed with 40% oxygen (or 95% oxygen for older embryos) and 5% carbon dioxide balanced with nitrogen. After culturing for 6 hours, 4 μCi of methyl-3H-thymidine was added to the bottles which were then cultured for an additional 12 hours. The embryos were then removed from the incubator and rinsed three times in Hank's balanced salt solution for 5 minutes each. During washing, the embryos were examined for their developmental stage and the presence of malformations. The embryos were then dissected and processed for chromosomal studies, scanning electronmicroscopy and autoradiography. For autoradiography, 5 μm sections were coated with Ilford K-5 emulsion and exposed for 5 days. The area of the sectional surface of the superior and inferior cushions in scanning electronmicrographs and histological sections were measured in 6 abnormal embryos and 9 controls. The values are expressed in mean ± standard error of measurements and the statistical significance was assessed by one-way analysis of variance.

RESULTS

1) Morphology of the embryonic atrioventricular cushions and the inferior atrioventricular junction

At the earliest stages examined (afternoon of day 10, 23-28 somites), the superior and inferior atrioventricular cushions were identified as plates covering not only the inlet part of the ventricle but also most of the atrial wall, as well as the atrioventricular junction. At this stage, both superior and inferior atrioventricular cushions were similar in size, but the inferior atrioventricular junction was moderately excavated. These findings were present regardless of the chromosomal status, it not being possible, by stereoscopic examination, to differentiate three trisomic embryos from 15 embryos with normal karyotype and six embryos with an undetermined karyotype. Serial sectioning of the embryos at this age failed to reveal any further differences, but scanning electronmicroscopy was not carried out.

By day 11, the morphology of the atrioventricular cushions and the inferior atrioventricular junction permitted recognition of two groups of hearts. In one group, both cushions remained of similar size and the atrioventricular junction had become straight (Fig. 1). In the other hearts, in contrast, the folding of junction, as seen in

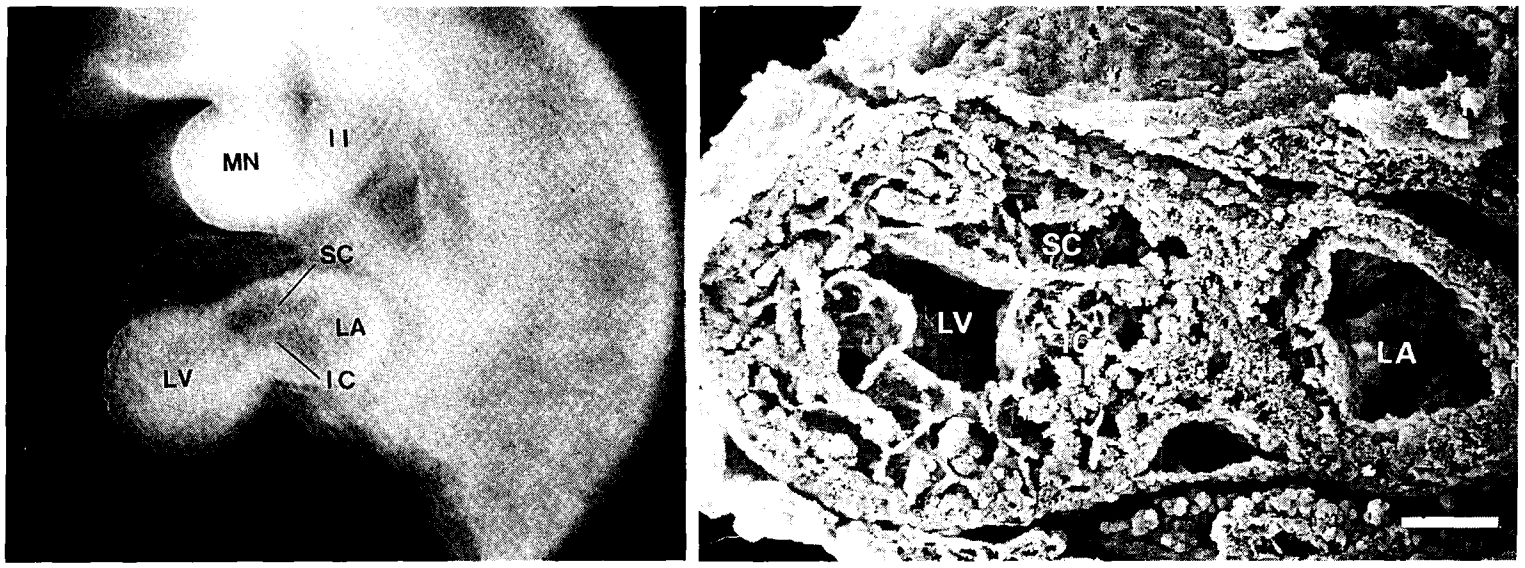
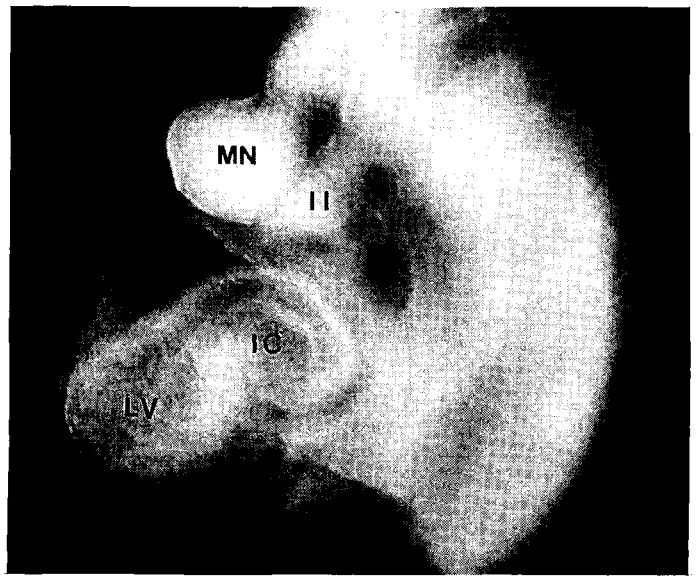
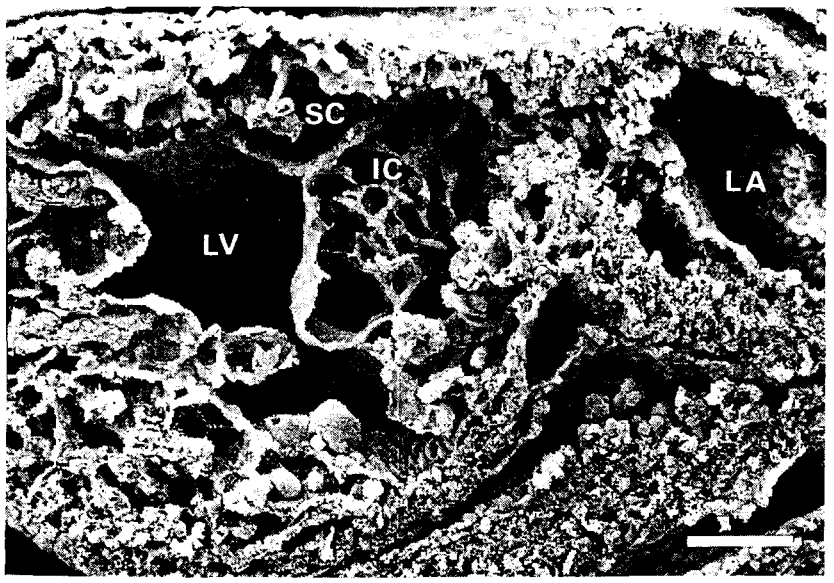


Fig. 1. (upper) Left lateral aspect of an embryo with a normal karyotype at the 11th day of gestation(32 somites). The embryonic right ventricle is more prominent than that of the embryo at the previous day. (right upper) A scanning electron micrograph of a heart of an embryo at the same day. The superior and inferior atrioventricular cushions(SC, IC) are of similar size and the inferior atrioventricular junction is less indented. The left lateral wall of the left ventricle(LV) and atrium(LA) are removed. Bar indicates 50 μm.(right) A histological section of the inferior atrioventricular junction was made in a similar plane to the scanning electronmicrograph after incorporation of tritiated thymidine. Grains of autoradiography are visible at the nuclei of ventricular and atrial myocardium and inferior endocardial cushion. There are a group of cells without grains at the atrioventricular junction.

the hearts studied on the 10th day, had persisted. The inferior cushion in those hearts was larger as compared to the superior one (Fig. 2). The absolute values of the size of the cushions were variable because of variable degrees of shrinkage in our preparations. The ratio between the sectional areas of the inferior and superior cushions, however, were significantly higher in the abnormal embryos (2.5 ± 0.3) than in the control embryos (1.2 ± 0.1). From our observations on the embryos in their 11th day, the persistence of a folded junction and a bigger inferior cushion was discovered in 8 of 10 cases (80%) with trisomy 16, but also in 6 of 33 cases (18%) which were eusomic. The number of somites of embryos with trisomy 16 was not different from those of eusomic embryos. The number of somites of eusomic embryos with abnormal morphology was, however, significantly lower than that of eusomic embryos with normal morphology (Table 1).





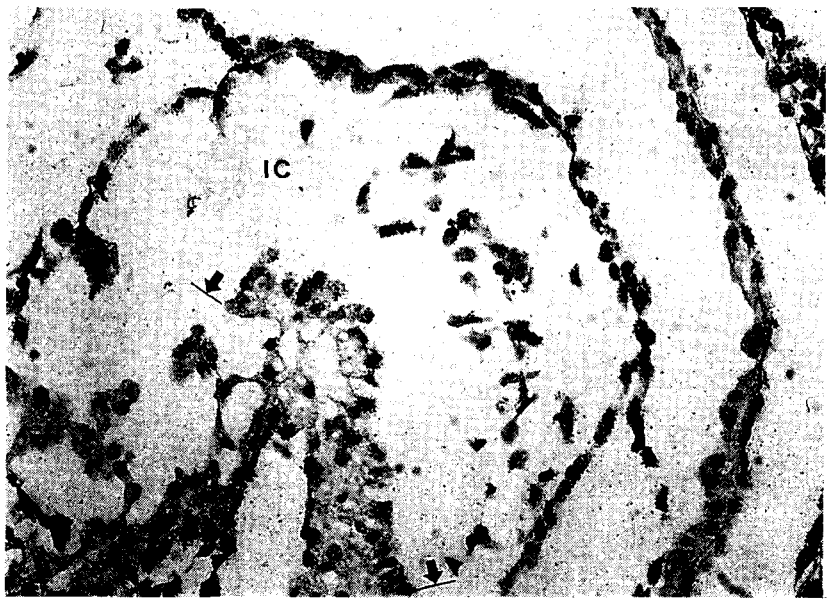


Fig. 2. (upper) Left lateral aspect of a trisomic embryo at the 11th day(32 somites). The inferior atrioventricular cushion is bigger in size than the superior one, and the inferior atrioventricular junction is still folded. (right upper) A scanning electronmicrograph of a heart of an embryo at the same day. The left lateral wall has been removed. Bar indicates 50 μm. (right) A section of the inferior atrioventricular junction of a mouse embryo with trisomy 16 was prepared in a similar way to the lower picture of Fig. 3. The cells without grains are shown at the atrioventricular junction, but they are smaller in number.

Table 1. Numbers of embryos at the 11th day according to the chromosomal status and morphology. The mean±SEM of numbers of the somites in each group is shown in parentheses.

	chromosome status			
morphology	eusomic	trisomic	undetermined	total
normal	27	2	5	34
	(32.9±0.7)	(38.0±2.8)	(33.4±0.7)	(33.5±0.8)
abnormal	6	8	4	18
	(28.2±0.7)	(30.4±0.5)	(28.7±0.3)	(29.1±0.5)
total	33	10	9	52
	(31.5±0.8)	(32.6±1.7)	(31.6±1.0)	(31.8±0.6)

2) Proliferation of the cells of the cushions and myocardium

No gross differences were found in the labelling by thymidine of the myocardium of the ventricle and atrium between the trisomic and eusomic mice. A marked difference in terms of labelling, however, was seen at the inferior atrioventricular junction. This junctional area was different from the rest of the myocardium in that the cells had a vacuolated cytoplasm and showed absence of labelling with thymidine. This "quiescent zone (Thompson *et al.*, 1990)" was 50 um long in the hearts of control embryos (Fig. 1C), but smaller, measuring only 20 um in length, in the trisomic embryos with persistence of the folded inferior junction (Fig. 2C). This difference was apparent in 3 trisomic mice when compared with 4 of their eusomic littermates.

3) Developmental morphology of the endocardial cushions and atrioventricular valves

As described above, the superior atrioventricular cushion was noted on the 11th day to be smaller than the inferior cushion in the hearts of trisomic mice (Figs. 1B, 2B). The inferior cushion of the trisomic mice was also noticeably edematous, its cells being swollen and rounded, and exhibited more interstitial space but less fibrillar material than in controls. The cells of the cushion in the eusomic mice, in contrast, were stellate and possessed more interstitial fibrils.

By the 13th day, the superior and inferior cushions were of similar size in the trisomic mice, but were condensed and smaller when compared with those of eusomic mice (Fig. 3). Magnification of the cushions revealed a homogeneous texture in the controls, but condensation of the margins of the cushions in the trisomic animals.

The cushions persisted until the 16th day as discrete structures in the trisomic mice, being evident as a thick covering on the fibrous layer of the ventricular aspect of the developing valvar leaflets (Fig. 4). In eusomic mice, in contrast, the valvar leaflets were thin, and the cushions could not be distinguished on the 16th day. The formation of tendinous cords by undermining of the superficial layer of the ventricular wall was first observed on the 14th day in the control animals, but only on the 15th or 16th days in the trisomic hearts.

4) Morphology of atrioventricular septal defect in fetal mice

A defect was visible at the expected site of atrioventricular septum in the abnormal hearts,

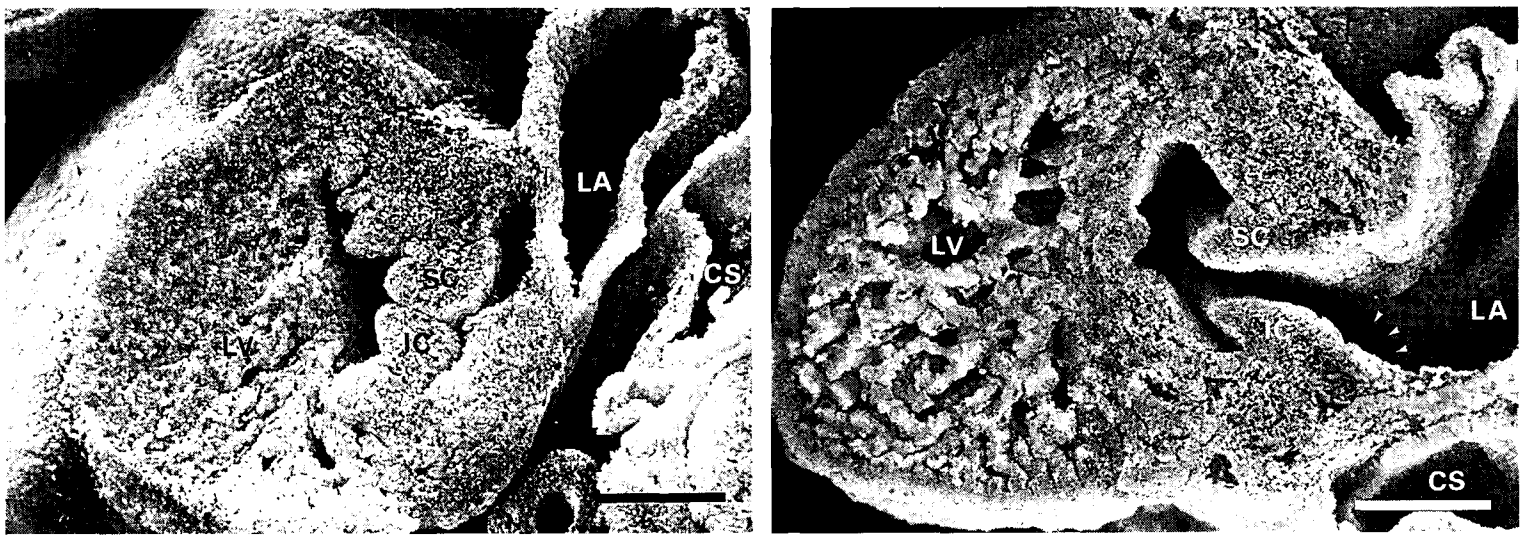


Fig. 3. (left) Left lateral view of the heart of a control fetus at the 13th day of gestation. Superior and inferior cushions(SC, IC) are well apposed but not yet fused. (right) Left lateral view of the heart of a trisomic fetus at the 13th day of gestation. The superior and inferior cushions (SC,IC) are smaller than those of the control heart and are not opposed. Arrow heads indicate the lower border of the atrial septum. (LV, left ventricle; LA, left atrium; CS, coronary sinus) Bar indicates 200 μm.

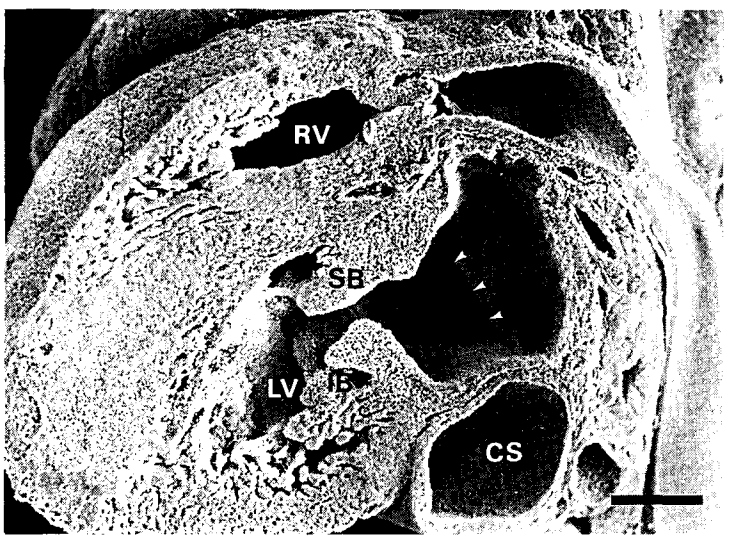


Fig. 4. Left anterior view of the heart of a fetus with trisomy 16 at the 16th day. The superior and inferior bridging leaflets (SB, IB) are seen astride the ventricular septum. The leaflets are thick and the cushion tissue remains at the atrial aspect of the leaflet. A big atrioventricular septal defect is shown anterior to the lower end of the atrial septum (arrow heads). (LV, left ventricle; RV, right ventricle; CS, coronary sinus) Bar indicates 200 μm.

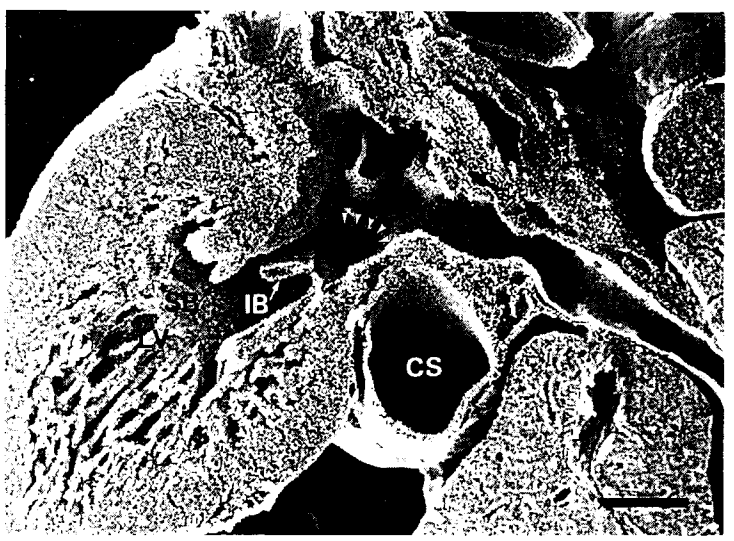


Fig. 5. Left anterior view of the heart of a fetus with trisomy 16 at the 18th day. The superior and inferior bridging leaflets (SB, IB) are thin and bridge the ventricular septum. The atrioventricular septal defect (bordered by arrow heads) is small in this case. The length of the inlet part of the left ventricle is short compared to the outlet part. (LV, left ventricle; CS, coronary sinus) Bar indicates 200 μm.

with bridging leaflets of a common valve straddling the ventricular septal crest (Fig. 5). The size of the defect was variable. The left ventricle was markedly shorter in its inlet part. The anteroposterior dimension of the heart in short axis was relatively longer than the long axis in those cases.

Sectioning from the left side of trisomic embryos with atrioventricular septal defect exposed the septal surfaces of the left ventricle and left atrium. When control hearts were cut in the same plane of section, in contrast, the ventricular septum and apex were cut away but the mitral valve and posterior wall of the left atrium remained intact. This suggested that the heart itself in the presence of deficient atrioventricular septation was rotated along its long axis in a counterclockwise direction as seen from the apex.

In those hearts with deficient atrioventricular septation, the atrial septum was developed normally at its anterosuperior end but less developed inferiorly, such that the interatrial communication was largely on the inferior aspect of the atrioventricular junction.

DISCUSSION

Miyabara and his colleagues (1982, 1990) have shown convincingly that, in terms of cardiac morphology, the mouse with trisomy 16 is an excellent model of Down's syndrome in the human (trisomy 21). Furthermore, other studies (Epstein *et al.*, 1991; Epstein 1991) have shown that the 16th chromosome in the mouse is syntenic with the 21st chromosome in the human. It is well established that atrioventricular septal defects (atrioventricular canal malformations, endocardial cushion defects) are the commonest cardiac malformations seen in Down's

syndrome (Epstein and Vekemans 1990), and the mice with trisomy 16 showed a comparable defect in almost every fetus (Miyabara *et al.*, 1984; Epstein *et al.*, 1991), although earlier studies had shown a lower incidence (56%) (Miyabara *et al.*, 1982) than we found. This model, in our hands, is an ideal one with which to study the morphologic mechanisms underscoring normal and abnormal atrioventricular septation.

Conventional wisdom holds that the atrioventricular septal structures, along with the leaflets of the atrioventricular valves, are derived from the substance of the atrioventricular endocardial cushions (Kurnit *et al.*, 1985; Van Mierop *et al.*, 1962). Thus, according to this hypothesis, the lower muscular portion of the atrial septum, along with the crest of the ventricular septum, take their origin from the cushions. It is then argued that it is failure of fusion of the cushions, and failure to contribute to these septal structures, which results in the characteristically abnormal septal morphology of atrioventricular septal defects (Kurnit *et al.*, 1985; De la Cruz *et al.*, 1983).

There is much circumstantial evidence to contradict this hypothesis, most notably the fact that, frequently, the atrial septum itself can be virtually normally formed in the presence of an atrioventricular septal defect (Allwork 1982), albeit without having contiguity with the ventricular septum. It was more likely that the endocardial cushions failed to act as a "glue" to hold together the centre of the developing atrioventricular junction. In consequence of this absence of "gluing", the abnormal heart with deficient atrioventricular septation was then able to grow with a "sprung" junction, explaining well the hallmarks of deficient septation, namely a common atrioventricular junction associated with an abnormally located subaortic outflow tract from the left ventricle (Anderson *et al.*, 1991). This hypothesis then received further support from the observations of the Leiden laboratory (Wenink and Zevallos 1988; Wenink and Gittenburg de Groot 1985; Wenink *et al.*, 1990), who had suggested that the atrioventricular endocardial cushions themselves made relatively scanty contributions to substance of the valvar leaflets, either in the normal heart or in the hearts with deficient atrioventricular septation. Our study confirms that the initial abnormalities leading towards deficient atrioventricular septation are to be found within the junctional myocardium, but further studies will be required to elucidate the exact contribution of the endocardial cushions to the valvar leaflets.

In terms of the initial abnormalities, trisomic and eusomic fetuses were identical until the tenth day of development. On the eleventh day, however, a group of hearts were identified in which there was persistence of the marked infolding of the inferior atrioventricular junction noted on the tenth day. In 27 of the 33 eusomic mice studied on the 11th day, this infolding had straightened, and the endocardial cushions were then of similar size. In 8 of 10 trisomic mice, but albeit in 6 of the 33 eusomic mice, this infolding had persisted. The eusomic mice with abnormal morphology had a smaller number of somites (Table 1), which suggests that their growth was retarded due to other reasons. We have also observed persistence of such an abnormal inferior atrioventricular junction in developing hearts from the iv/iv mouse (unpublished observations). Significantly, this is another animal model known to exhibit deficient atrioventricular septation (Icardo and Sanchez de Vega 1991; Seo *et al.*, 1992). Subsequent to this persistence of junctional infolding, marked differences were noted in our present study in the arrangement of the cushions between the trisomic and normal mice. The inferior cushion in the abnormal mice was already bigger than the superior cushion on the 11th day.

The cushions had become of similar size on the 13th day in trisomic mice, but both had now become smaller than in controls. The cushions then persisted longer as identifiable structures in the abnormal mice, still being identified on the 16th day. In the eusomic mice, in contrast, the cushions had been incorporated within the developing leaflets on the 14th day, and tendinous cords tethering the leaflets to the ventricular myocardium were already formed at this stage. Further differences were noted between the normal and abnormal mice in respect of the

structure of the cushions and the arrangement of the ventricular mass, but our studies with incorporation of thymidine confirmed an abnormality in the inferior atrioventricular junction as being the earliest identifiable change. Thus, these labelling experiments revealed a smaller quiescent zone in the infolded inferior atrioventricular junction of the trisomic mice.

We suggest, therefore, that not only the endocardial cushions but also the junctional myocardium between the left atrium and ventricle play important roles in the genesis of atrioventricular septal defect. Significantly a part of the equivalent area in the chick embryo has been shown to express Hox 8 m-RNA, whilst the cells of the endocardial cushions express Hox 7 m-RNA (Robert *et al.*, 1989). The embryonic atrioventricular myocardium is also the source of factors that induce the division and migration of the endocardial cells (Markwald *et al.*, 1990). It will be fruitful in future to study the distribution of growth factors and cellular adhesion molecules in these areas. Studies on other organs in the Down's syndrome or murine trisomy 16 suggest basic defects in the extracellular matrix in the formation of complex and systemic malformation in this chromosomal anomaly (Kurnit *et al.*, 1985; Kim *et al.*, 1994).

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