Angiogenic Gene Expression in Down Syndrome Fetal Hearts

Olga Sánchez, Carmen Domínguez, Aina Ruiz, Irene Ribera, Jaume Alijotas, Lluís Cabero, Elena Carreras, Elisa Llurba

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Angiogenic factors were similar in DS fetuses and CHD euploid fetuses with CHD. Conclusion: Abnormal angiogenesis was detected in the hearts of DS fetuses with and without CHD. Our results suggest that DS determines an intrinsically angiogenic impairment that may be present in the fetal heart.

Key Words
Down syndrome · Congenital heart defects · Angiogenesis · Hypoxia

Abstract
Introduction: Forty percent of Down syndrome (DS) fetuses have congenital heart defects (CHD). An abnormal angiogenic enviroment has been described in euploid fetuses with CHD. However, the underlying pathophysiologic pathway that contributes to CHD in DS remains unknown. The objective was to compare the expression of angiogenic factors and chronic hypoxia genes in heart tissue from DS and euploid fetuses with and without CHD. Methods: The gene expression profile was determined by real-time PCR quantification in heart tissue from 33 fetuses with DS, 23 euploid fetuses with CHD and 23 control fetuses. Results: Angiogenic factors mRNA expression was significantly increased in the DS group compared to the controls (soluble fms-like tyrosine kinase-1, 81%, p = 0.007; vascular endothelial growth factor A, 57%, p = 0.006, and placental growth factor, 32%, p = 0.0227). Significant increases in the transcript level of hypoxia-inducible factor-2α and heme oxygenase 1 were also observed in the DS group compared to the controls. The expression of angiogenic factors was similar in DS fetuses and CHD euploid fetuses with CHD. Conclusion: Abnormal angiogenesis was detected in the hearts of DS fetuses with and without CHD. Our results suggest that DS determines an intrinsically angiogenic impairment that may be present in the fetal heart.

Introduction

Down syndrome (DS) is a genetic disorder caused by trisomy of chromosome 21 (HSA21) and is characterized by a complex phenotype in which over 80 features occur with various degrees of expression and frequency [1]. DS is the most frequent autosomal aneuploidy, affecting 1 in 700 live births [2, 3].

The incidence of congenital heart defects (CHD) in the general population is 0.8% [4], rising to approximately 40–60% in DS [5]. Atrioventricular septal defects (AVSD) are the most common forms of CHD in DS [6, 7], followed by ventricular septal defects and Tetralogy of Fallot (TF) [7].

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Despite significant advances in the understanding of mechanisms determining heart formation, the causes of CHD remain unknown in the vast majority of cases [8] and probably depend on the interplay of multiple genetic and environmental factors [9]. In DS, it is postulated that variations in the gene dosage of chromosome 21, environmental factors and genetic modifications not linked to chromosome 21 contribute to the development of CHD [10]. The expression of some of these genes present in the extra copy increases 1.5-fold due to triplication of HSA21. This genetic imbalance will seriously disrupt one or more developmental pathways [11]; however, the increased gene dosage in itself is not sufficient to cause the defect.

Animal models have shown angiogenesis to be related to abnormal heart development. In mutant mouse embryos, a 2- to 3-fold overexpression of vascular endothelial growth factor (VEGF) resulted in severe abnormalities in cardiac development, including an attenuated compact layer of myocardium, overproduction of trabeculae, defective ventricular septation and remodeling of the outflow tract [12]. In zebrafish embryos, the blockage of VEGF receptors resulted in a functional and structural defect in cardiac valve development, thereby suggesting that these receptors are involved in heart valve formation [13]. We have recently reported that, in isolated major fetal heart defects, an antiangiogenic imbalance existed in maternal and cord blood, indicating impaired placental angiogenesis [14, 15]. Such differences were observed in cases with valvular and septal defects which, in turn, are the most common types of CHD observed in DS [6, 16]. Moreover, we related maternal first-trimester placental growth factor (PIGF) levels to abnormal nuchal translucency (NT) thickness, a surrogate marker of DS and CHD. More recently, an excess of deleterious variants in vascular endothelial growth factor A (VEGF-A) pathway genes was reported in DS-associated AVSD [17].

We hypothesize that, in DS, a dysregulation occurs in the angiogenesis pathways involved in heart development that may cause cardiac defects. The present study aimed to compare the expression of angiogenic factors and chronic hypoxia genes in heart tissue from DS and euploid fetuses with and without CHD.

**Material and Methods**

All samples were obtained from pregnancy termination products in women carrying a DS fetus. All patients provided their written consent for the storage and use of tissue samples for research purposes. All samples were obtained from the Fetal Tissue Bank of Vall d’Hebron University Hospital. At autopsy, heart tissue from the apical area of the left ventricle was stored according to a standardized sampling protocol. This area was chosen because it is the most likely to meet quality criteria, regardless of the type of heart disease or weeks of gestation. Information on congenital heart anomalies was obtained from medical records. Individuals with partial or mosaic trisomy 21 were not included. The cases were classified as DS with CHD, documented by echocardiogram and necropsy, and DS without CHD. The study samples were compared with the samples of euploid fetuses with CHD (CHD group) or without CHD (CTRL group). All euploid fetus samples stemmed from therapeutic pregnancy terminations at 17–22 weeks of gestation in otherwise healthy women.

The heart tissue samples were provided by the Fetal Tissue Bank of the Vall d’Hebron University Hospital with appropriate ethics committee approval.

**Table 1. Inventoried TaqMan gene expression assays from Applied Biosystems**

<table>
<thead>
<tr>
<th>Gene</th>
<th>TaqMan gene expression assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>Hs00153153_m1</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>Hs01026149_m1</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>Hs0990055_m1</td>
</tr>
<tr>
<td>PIGF</td>
<td>Hs00182176_m1</td>
</tr>
<tr>
<td>HO-1</td>
<td>Hs01110250_m1</td>
</tr>
<tr>
<td>SOD-1</td>
<td>Hs00166575_m1</td>
</tr>
</tbody>
</table>

**Extraction and Real-Time PCR (RT-PCR) Quantification of mRNA**

Fetal heart tissue from the apical area of the left ventricle was used for total RNA extraction. Frozen fetal heart tissue samples were excised (20–30 mg) using mortar and pestle, immersed in RNasy Fibrous Tissue RLT buffer (Qiagen, Venlo, The Netherlands) and thoroughly homogenized (20–40 s) with a homogenizer (ULTRA-TURRAX T 10 basic Disperser/Homogenizer; IKA Works, Staufen, Germany). Total RNA was extracted and treated with DNase using the RNasy Fibrous Tissue Mini Kit (Qiagen) according to the manufacturer’s protocol. The RNA integrity number (RIN) of all samples was obtained from the Agilent Bioanalyzer 2100 to assess the RNA quality of the samples [18]. Samples with a RNA integrity number (RIN) below 7 were excluded. RNA (500 ng/reaction) was used for complementary DNA synthesis with random hexamer primers (RevertAid H minus first strand complementary DNA synthesis kit; Fermentas, St. Leon-Rot, Germany). Inventoried (Table 1) and customized soluble fms-like tyrosine kinase (sFlt-1) TaqMan gene expression assays were obtained from Applied Biosystems (Waltham, Mass., USA). Sequences for sFlt-1 primers and probes were as follows: forward, 5′-GGGAAGAAATCTCCAGAAGAGAGA-3′; reverse, 5′-GAGATCGGAGAAACAAGCCTTT-3′; probe, 5′-AGTGCTCACCTCTGATTG-3′ [19]. mRNA expression levels were normalized to the levels of human DNA-directed RNA polymerase II (assay ID: Hs00172187).
m1), and a control sample was used as a calibrator. Relative expression was calculated by the double-delta Ct method, using Prism 7000 Sequence Detection System Software (version 1.2.3) from Applied Biosystems.

**Statistical Analysis**

Statistical analyses were made using GraphPad Prism software (version 5.0b; GraphPad, San Diego, Calif., USA). The results are presented as means with SEM. The D’Agostino-Pearson omnibus K2 tested the distribution of the variables for normality. When the assumption of normality was satisfied, the data were analyzed by the one-sided Student unpaired t test; otherwise, the nonparametric Mann-Whitney U test was used. The p-value threshold used for significant differences was <0.05.

### Results

Heart tissue was analyzed in 33 DS fetuses (DS), 23 euploid fetuses with CHD (CDH) and 23 euploid fetuses without CHD (CTRL). The demographic data and outcome of the population are presented in table 2. No differences were observed in gestational age of the tissue samples among the groups.

At autopsy, the phenotype of the DS fetuses was found to be consistent with the syndrome. Heart samples were divided into two groups as follows: tissues with (T21-CHD) and without heart defects (T21).

### Table 2. Demographic characteristics and perinatal outcome of the study groups

<table>
<thead>
<tr>
<th></th>
<th>DS (n = 33)</th>
<th>CHD (n = 23)</th>
<th>CTRL (n = 23)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age, years</td>
<td>35.36 (4.13) a, b</td>
<td>31.43 (4.21)</td>
<td>29.7 (6.57)</td>
</tr>
<tr>
<td>GA at sample, weeks</td>
<td>20.08 (1.35)</td>
<td>20.89 (1.43)</td>
<td>19.96 (2.05)</td>
</tr>
<tr>
<td>Gender, M/F</td>
<td>14/19</td>
<td>12/11</td>
<td>10/13</td>
</tr>
<tr>
<td>Birth weight, g</td>
<td>352 (136)c</td>
<td>446 (152)</td>
<td>390 (209)</td>
</tr>
<tr>
<td>Pregnancy termination</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CHD</td>
<td>18 (54%)</td>
<td>23 (100%)</td>
<td></td>
</tr>
<tr>
<td>T21</td>
<td>15 (46%)</td>
<td>10 (43%)</td>
<td></td>
</tr>
<tr>
<td>CMF</td>
<td></td>
<td>10 (43%)</td>
<td></td>
</tr>
<tr>
<td>GMF</td>
<td></td>
<td>13 (57%)</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as means (SD), unless otherwise indicated. Comparisons among groups were made by the Student unpaired t test or the nonparametric Mann-Whitney U test, as appropriate. GA = Gestational age; T21 = trisomy 21; CMF = cerebral malformations; GMF = gastrointestinal malformations. a p < 0.001, DS vs. CTRL group. b p < 0.01, DS vs. CHD group. c p < 0.05, DS vs. CHD group.

### Table 3. Types of heart defects in the study groups

<table>
<thead>
<tr>
<th></th>
<th>Euploid fetuses (CHD, n = 23)</th>
<th>Trisomic fetuses (T21-CHD, n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A-V</td>
<td>7 (30%)</td>
<td>17 (94%)</td>
</tr>
<tr>
<td>AVSD</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Interventricular communication</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Ebstein’s anomaly</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Tricuspid valve atresia</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Pulmonary stenosis or atresia</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>LVOT</td>
<td>10 (44%)</td>
<td>1 (6%)</td>
</tr>
<tr>
<td>Aortic valve stenosis</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Hypoplastic left heart syndrome</td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Conotruncal</td>
<td>6 (26%)</td>
<td></td>
</tr>
<tr>
<td>Transposition of the great arteries</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>TF</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Double outlet right ventricle</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as numbers, unless otherwise indicated. A-V = Atrioventricular valve; LVOT = left ventricular outflow tract obstruction; T21-CHD = trisomic fetuses with CHD.
The presence of CHD was detected by prenatal ultrasound and confirmed by autopsy. The types of heart disease in euploid and DS fetuses are listed in Table 3. The most frequent heart defect in the CHD group was left ven- tricle outflow tract (44%), while 94% of the CHD cases in DS fetuses were atrioventricular valve-type defect.

**Gene Expression Profile in Heart Tissue of Fetuses with DS Compared with Euploid Fetuses without CHD (CTRL)**

Genes differentially expressed in fetal heart tissue are shown in Figure 1. Expressions of sFlt-1, VEGF-A and PlGF mRNA were significantly higher in the DS group than in the controls (81%, p = 0.007; 57%, p = 0.006 and 32%, p = 0.0227, respectively). Significant increases in the transcript level of hypoxia-inducible factor-2α (HIF-2α) and heme oxygenase 1 (HO-1) were also observed in the DS group compared to the controls (21%, p = 0.039 and 68%, p = 0.014, respectively). The expression of superoxide dismutase 1 (SOD-1), the gene located in HSA21, was upregulated in all trisomic hearts (76%, p = 0.0001). In contrast, no significant changes in HIF-1α mRNA expression were observed between groups.

**Gene Expression Profile in Heart Tissue of Fetuses with DS Compared with Euploid Fetuses with CHD (CHD)**

Gene expression in heart tissue from euploid CHD and DS fetuses is presented in Figure 2. Proangiogenic factors, VEGF-A and PlGF as well as the antiangiogenic factor sFlt-1 were upregulated in both groups compared with the control group (CTRL) used as a reference (dotted line). The increase in HIF-2α expression was lower in DS than in CHD (1.2- vs. 1.4-fold, respectively; p = 0.0481), while SOD-1 expression was significantly higher in DS than in CHD (34%).

**Comparison between DS Heart Tissue of Fetuses with and without CHD**

The differential genetic expression profile between DS fetuses with and without heart defects is presented in Table 4. No differences were found between the groups in any of the genes analyzed.

**Discussion**

This study presents the first evidence of abnormal angiogenesis in heart tissue of human fetuses with DS. DS tissue showed increased VEGF-A and sFlt-1 expression and overproduction of proteins such as HIF-2α, HO-1 and SOD-1 compared with heart tissue from euploid fetuses.

Significant progress has been made in defining the regulatory pathways that control normal and abnormal heart valve development. VEGF-A expression is dynamic during embryonic heart development and is involved in both long-range and short-range signaling that influence vessel patterning. In mutant mouse embryos, 2- to 3-fold overexpression of VEGF-A resulted in severe heart de-
Table 4. Real-time-PCR quantification in heart tissue from DS fetuses

<table>
<thead>
<tr>
<th>Genes</th>
<th>T21-CHD/CTRL</th>
<th>T21/CTRL</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIF-1α</td>
<td>1.018</td>
<td>1.096</td>
<td>0.4492</td>
</tr>
<tr>
<td>HIF-2α</td>
<td>1.254</td>
<td>1.153</td>
<td>0.5288</td>
</tr>
<tr>
<td>VEGF-A</td>
<td>1.541</td>
<td>1.595</td>
<td>0.5032</td>
</tr>
<tr>
<td>PlGF</td>
<td>1.230</td>
<td>1.413</td>
<td>0.5516</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>1.461</td>
<td>2.168</td>
<td>0.3193</td>
</tr>
<tr>
<td>SOD-1</td>
<td>1.711</td>
<td>1.845</td>
<td>0.5385</td>
</tr>
<tr>
<td>HO-1</td>
<td>1.460</td>
<td>1.923</td>
<td>0.2213</td>
</tr>
</tbody>
</table>

Data represents the fold change in the expression of genes in DS fetuses compared to euploid fetuses without CHD (CTRL). Comparisons between DS with CHD (T21-CHD) and without heart defect (T21) were made by the Student t test or Mann-Whitney U test, as appropriate.

development abnormalities, including attenuated compact layer of myocardium, overproduction of trabeculae, defective ventricular septation and outflow tract remodeling [12]. An excess of deleterious variants in VEGF-A pathway genes in DS has been associated with AVSD [20]. In biopsies from adults with TF, DNA microarray analysis revealed altered expression patterns for 236 genes, including enhanced expression (1.5- to 2.2-fold) of angiogenic factors and their receptors [21]. TF, a subvalvular pulmonary stenosis combined with ventricular septal defect, was observed in 6% of the DS cases; thus, the results of that study suggest that normal angiogenic environment can also contribute to this type of cardiac anomaly in DS.

Interestingly, sFlt-1 expression was increased 2-fold in DS samples compared to controls. Flt-1 has also been implicated in the regulation of embryonic heart function and cardiac morphogenesis [22]. In addition, PlGF has emerged as a central mediator in the coordination of both cardiomyocyte growth and neoangiogenesis [23]. Genetic and pharmacologic studies identified PlGF as a novel cardioprotective factor [24]. Although PlGF was significantly increased in DS fetuses compared to controls, no differences were found in PlGF expression in DS cases compared to euploid fetuses with CHD. The slight expression of PlGF and overproduction of sFlt-1 and VEGF-A in both DS and euploid fetuses with CHD suggests that CHD might be caused by the combination of a heart-specific vascular defect and a placental antiangiogenic environment.

The dysregulation of angiogenesis in the placenta and maternal-fetal circulation has emerged as one of the main pathophysiological features in the development of placental insufficiency and its clinical consequences. Abnormal angiogenesis has also been related to other obstetric and fetal conditions such as peripartum cardiomyopathy and fetal cardiac defects, opening up new challenges for our understanding of angiogenic involvement in maternal cardiovascular function and fetal cardiac development [25]. Reduced maternal serum PlGF at 11–13 weeks has been reported in pregnancies that subsequently develop preeclampsia and intrauterine growth restriction [26, 27] and in those with fetal trisomy 21 [28, 29]. In the case of preeclampsia, the low PlGF is accompanied by a low PAPP-A and a high uterine artery pulsatility index, and these findings are attributed to impaired trophoblastic invasion of the maternal spiral arteries, leading to reduced placental perfusion and function. In trisomy 21, the low PlGF level is accompanied by a low PAPP-A but a normal uterine artery pulsatility index, suggesting impaired placental function in the presence of normal placental perfusion.

Moreover, a lower maternal PlGF was also observed in women carrying a fetus with CHD, and PlGF levels were inversely related to NT thickness, a surrogate marker of DS and CHD [14]. NT is defined as nuchal fluid accumulation visible on ultrasound. Increased expression of type IV collagen, leading to an accumulation of highly hyaluronic hyaluronan, has been considered in the pathogenesis of NT in trisomy 21 fetuses [30, 31]. It has also been proposed that abnormal lymphangiogenesis [32] and heart failure [33] contribute to the pathogenesis of nuchal thickness. VEGF genes also play a major role in the development of lymphatic endothelial cells from veins, and mutations in the VEGF-C allele cause lymph vessel dysfunction, severe systemic edema and lipid accumulation in bodily fluids [34]. Receptors for vascular endothelial growth factors (FLT4) are less abundant in lymphatic vessels of the skin of Turner fetuses indicating shortage of lymph capillaries [35], and VEGF-A expression has been correlated with aberrant lymphatic endothelial differentiation in trisomy 21 [36]. Interestingly, DS embryos have an increased NT whether or not they have heart anomalies.

This study also evaluated the presence of hypoxia in DS fetal hearts by analyzing the expression of HIF-1α, HIF-2α, HO-1 and SOD-1. As expected, the expression of SOD-1, whose gene is located on human chromosome 21 [37], was upregulated in DS heart tissue. Moreover, in the DS group, an overexpression of HIF-2α and HO-1 was observed, probably as a consequence of chronic hypoxia. Previous in vitro studies demonstrated that HIF-2α pref-
erentially activates VEGF expression [38]. Some experimental models also showed the importance of HO-1 in heart development [39]. In our study, the increased expression of hypoxia-inducible genes suggests that a certain degree of hypoxia might be present in DS hearts. Although chronic hypoxia might upregulate the expression of proangiogenic factors, an overall deregulation of angiogenesis with a net balance towards an antiangiogenic environment was observed in heart tissue from fetuses with DS, pointing to an intrinsic angiogenic impairment in these cases that lead to cardiac defect and heart hypoxia.

Increased expression of proangiogenic (VEGF-A and PlGF) and antiangiogenic factor (sFlt-1) genes was detected in DS, irrespective of the presence or absence of CHD. There are several explanations for these findings. First of all, changes could be unrelated to CHD; however, the expression of angiogenic factors was similar in DS and CHD euploid fetuses, pointing out that abnormal angiogenesis is one of the main pathogenic pathways leading to abnormal heart development in both DS and CHD. Alternatively, additional factors are required for the expression of the CHD phenotype in DS [40, 41]; however, abnormal angiogenesis may determine a certain degree of abnormal development, as indicated by recent studies showing that trisomy 21 fetuses have abnormal heart histological characteristics [42, 43] and abnormal heart function regardless of their cardiac anatomy [44]. We acknowledge that heart histology information might have offered important insights in relation to the interpretation of this potential association and that future studies including this information are warranted. Finally, gene expression changes were not causal but instead secondary to the DS ‘milieu’. However, as the evaluation of these factors was made in the second trimester of pregnancy and not during embryogenesis, we are unable to ascertain whether angiogenic imbalance leads to abnormal heart development or rather is a consequence of the heart defect.

To the best of our knowledge, this is the first study to evaluate the relationship between angiogenesis and CHD in DS. Abnormal angiogenesis was detected in the hearts of DS fetuses irrespective of CHD. Our results suggest that DS determines an intrinsically angiogenic impairment and chronic hypoxia that may be present in the fetal heart. Our findings are in agreement with those of animal models that consistently showed angiogenesis to be related to abnormal heart development. To what extent angiogenesis is involved in DS heart development remains to be elucidated.

Acknowledgements

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