ABSTRACT

Objective
Previous research in fetuses with increased nuchal translucency demonstrated abnormal lymphatic endothelial differentiation characteristics, including increased vascular endothelial growth factor (VEGF)-A expression, and aberrant smooth muscle cells (SMCs) surrounding enlarged jugular lymphatic sac (JLS). We hypothesized that abnormal Sonic hedgehog (Shh) expression would result in altered VEGF-A signaling in the lymphatic endothelial cells of the JLS and that aberrant acquisition of SMCs could be caused by downregulation of forkhead transcription factor FOXC2 and upregulation of platelet-derived growth factor (PDGF)-B in the lymphatic endothelial cells of the JLS.

Method
Five trisomy 21 fetuses and four controls were investigated using immunohistochemistry for Shh, VEGF-A, FOXC2 and PDGF-B expression in the lymphatic endothelial cells of the JLS.

Results
An increased Shh, VEGF-A and PDGF-B expression, and decreased FOXC2 expression were shown in the lymphatic endothelial cells of the JLS of the trisomic fetuses.

Conclusion
Increased Shh and VEGF-A expression is correlated with an aberrant lymphatic endothelial differentiation in trisomy 21 fetuses. The SMCs surrounding the JLS can possibly be explained by increase of PDGF-B induced SMC recruitment and / or differentiation. This underscores earlier findings that indicate the loss of lymphatic identity in trisomy 21 fetuses and a shift towards a blood vessel wall phenotype.
INTRODUCTION

Nuchal translucency (NT) is a translucent area in the neck region of the human fetus that can be measured using ultrasound between 10 and 14 weeks of gestation and is used as part of a screening method for trisomy 21. Increased NT is also associated with other chromosomal abnormalities like trisomy 18 and 13, structural anomalies such as cardiac defects and several genetic syndromes. Recent studies implicate disturbed lymphatic development as a likely explanation for the origin of increased NT. It has been demonstrated that fetuses with increased NT morphologically show nuchal edema (NE), accompanied by distended jugular lymphatic sacs (JLS). This has been described in both human fetuses and trisomy 16 mouse embryos, a mouse model for human trisomy 21.

The JLS is the first part of the lymphatic system to develop. Bilaterally, small buds of lymphatic endothelial cells arise from the internal jugular veins, which fuse and form the JLS. The peripheral lymphatic system is formed by sprouting from these sacs (i.e. lymphangiogenesis) and from lymphvasculogenesis from local (mesenchymal) lymphangioblasts. During these stages of development, the JLS serve as a primitive lymphatic drainage site. The JLS in human fetuses normally reorganize into lymphatic nodes after 10 weeks of gestation. The formation of the lymphatic system in humans is completed by the ingrowth of the right thoracic duct into the left JLS, thereby connecting several lymphatic vessels, and forming the main drainage-site of lymphatic fluid into the systemic circulation after the reorganization of the JLS.

Our group previously described alterations in endothelial differentiation of the JLS in mouse embryos and human fetuses with aneuploidy, which coincided with NE and enlarged JLS. Diminished expression of lymphatic markers Prox-1 and Podoplanin and the presence of blood vessel characteristics, including presence of Vascular Endothelial Growth Factor (VEGF)-A and Neuropilin (NP)-1, were found in the lymphatic endothelial cells of the JLS. Previous research showed that the morphogen Sonic hedgehog (Shh) acts upstream of VEGF. Zebrafish embryos mutant for Shh show diminished arterial endothelial differentiation due to impaired endothelial VEGF signaling. In the current research, we hypothesize that an abnormal endothelial Shh expression might result in an altered VEGF-A signaling (as exemplified by increased immunohistochemical VEGF activity) in the lymphatic endothelial cells of the JLS.

Besides the altered endothelial differentiation found in the lymphatic endothelial cells, smooth muscle cells (SMCs) surrounded the JLS which normally only line arteries, veins and large collecting lymph vessels. Interestingly, previous research in Foxc2 -/- mice also showed an abnormal amount of SMCs surrounding enlarged lymph vessels. Mutations in the human FOXC2 gene are associated with congenital lymphedema and defective lymph valves. FOXC2 normally suppresses the expression of platelet-derived growth factor (PDGF)-B in lymphatic endothelial cells, thereby inhibiting SMC attraction and proliferation, as these processes are positively influenced by PDGF-B. We hypothesize that in trisomy 21 fetuses with NE and enlarged JLS, recruitment towards and/or differentiation of SMCs surrounding the JLS is caused...
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by downregulation of FOXC2 in the lymphatic endothelial cells. The subsequent increase in PDGF-B expression in lymphatic endothelial cells would therefore result in recruitment of SMCs. To further establish these hypotheses, we evaluated the lymphatic endothelial cells and blood endothelial cell-expression of Shh, FOXC2 and PDGF-B in trisomy 21 fetuses with NE and enlarged JLS in the vasculature of the neck.

MATERIALS AND METHODS

Fetal tissue

The study was approved by the medical ethical committee of the VU University Medical Center. All patients received information and gave written informed consent. Fetal tissue was obtained after termination of pregnancy either by spontaneous abortion (due to cervical insufficiency) or abdominal hysterectomy (for oncological reasons) in the case of the control fetuses or by misoprostol induction in the case of aneuploidy. All fetuses were prenatally examined by ultrasound and diagnosed with normal or increased NT. Karyotyping of the fetuses was performed, using chorion villus sampling or amniocentesis. Termination of the pregnancy was performed at request of the parents for medical or psychosocial reasons, either by an operative or a misoprostol procedure. Post mortem examination of the whole fetus was executed in all cases. The fetal neck was removed from below the eye level to the clavicle level. The tissue was fixed in 4% paraformaldehyde, embedded in paraffin, and transversely sectioned into serial sections of 8 μm. Characteristics of the human fetuses included in the study are listed in table 1.

<table>
<thead>
<tr>
<th>Human fetus</th>
<th>GA (weeks, days)</th>
<th>Nuchal translucency (mm)</th>
<th>Cardiovascular defects</th>
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<td>Control (n=4)</td>
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<td>13,5</td>
<td>4.9</td>
<td>Complete AVSD, hypoplastic aortic arch</td>
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<td>14,0</td>
<td>3.8</td>
<td>ASD, aberrant subclavian artery</td>
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</tr>
<tr>
<td>18,2</td>
<td>3.6</td>
<td>Complete AVSD, hypoplastic aortic arch</td>
<td></td>
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</table>

GA, gestational age; ASD, atrial septal defect; AVSD, atrioventricular septal defect.
Immunohistochemistry

We used antibodies against LYVE-1 (biotinylated, ReliaTech, Braunschweig, Germany), Prox-1 (ReliaTech), NP-1 (Santa Cruz Biotechnology, Santa Cruz, USA), VEGF-A (Santa Cruz Biotechnology), SMA (Sigma-Aldrich, St Louis, USA), FOXC2 (Abcam, Cambridge, UK), PDGF-B (Affinity Bio Reagents, Golden, USA) and Shh (Santa Cruz Biotechnology). Sections were deparaffinised and rehydrated. In the case of Prox-1, FOXC2, PDGF-B and Shh, the sections were incubated for 12 minutes in 0.01 M citric buffer of pH6.0 in the microwave at a temperature of 97°C to recruit epitopes. Inhibition of endogenous peroxidase was performed by incubating the sections for 20 minutes in a solution of 0.3% H₂O₂. After this, all sections were rinsed twice in PBS and once in PBS/0.05% Tween. Subsequently, they were incubated overnight with the first specific antibody. On the next day, rinsing was performed twice in PBS and once in PBS/0.05% Tween followed by incubation of the sections with the second antibody which differed per staining procedure. For LYVE-1, no second antibody was required as the primary antibody was labeled with biotin. For Prox-1, VEGF-A and PDGF-B, the second antibody was a biotinylated goat-anti-rabbit antibody (Vector Laboratories, Burlinghame, USA); for NP-1, FOXC2 and Shh a biotinylated horse-anti-goat antibody (Vector Laboratories) and for SMA, a horseradish peroxidise-conjugated rabbit-anti-mouse antibody (Dako, Glostrup, Denmark). Thereafter, rinsing twice in PBS and once in PBS/0.05% Tween took place. The sections, except those following the staining procedures for SMA, were incubated with ABC-reagents (Vector Laboratories) for 40 minutes. All sections were rinsed twice with PBS and once with Tris/maleate pH 7.6, followed by a 10-minute incubation period with 3,3’-diaminobenzidin tetrahydrochlorid (DAB; Sigma-Aldrich) for visualization. Finally, all sections were counterstained with haematoxylin (Merck, Darmstadt, Germany), rinsed in tap water, and dehydrated to xylene. The sections were mounted with Entellan (Merck).

Morphometry

Morphometry of the lymph node tissue lining the JLS and the size of the JLS of both control fetuses and trisomy 21 fetuses was performed based on Cavalieri’s method as described previously. In summary, regularly spaced points (100 mm² grid) were randomly positioned on the lymph node tissue and JLS. The distance between the subsequent sections of the slides was 0.16mm. The volume measurement was done using the HB2 Olympus microscope with a x 40 “final magnification”.
**RESULTS**

**General nuchal and cardiac morphology**

Trisomy 21 fetuses with NE (n = 5) were compared with controls (n = 4) (Table 1) with a normal nuchal skin. The four control fetuses all showed no cardiac defects. Four of the five fetuses with NE were diagnosed with cardiac abnormalities varying from an atrial septal defect (ASD) in one case to a complete atrioventricular septal defect (AVSD) in three cases. Furthermore, in two cases a hypoplastic aortic arch and in one case an aberrant subclavian artery was found (Table 1). When compared with controls, the fetuses with NE all showed enlarged JLS (Figure 1a-b). Reorganization of the JLS into lymph nodes was in progress in the control fetus with a gestational age (GA) of 14 weeks (Figure 1a). Conversely, in the trisomy 21 fetuses the JLS was larger at GA 14 weeks and still present at GA 18 weeks. Morphometry of the lymph node tissue of control (n = 2; GA 12.1 weeks, GA 14 weeks) and trisomy 21 fetuses (n = 3; GA 11.2 weeks, GA 11.5 weeks, GA 12.2 weeks) was performed. Compared to the size of the jugular lymphatic sac there was relatively less lymph node formation in the trisomy 21 fetuses. The absolute amount of lymph node tissue was comparable with the control fetuses. In four out of five NE fetuses, erythrocytes were demonstrated in the JLS (arrow in Figure 1b). These results are in agreement with previously published data.9

*Endothelial expression of lymphatic markers*

The lymphatic endothelial cells of the JLS stained positive for LYVE-1 in all fetuses and showed a similar staining pattern between karyotypes (Figure 2 c-d). A positive LYVE-1 staining was also found in several scattered mesenchymal cells of both the control and trisomy 21 fetuses (arrows in Figure 2 c-d). Nuclear Prox-1 expression, normally high in lymphatic endothelial cells, was diminished in the lymphatic endothelial cells of the JLS of the NE fetuses. The endothelial cells of the carotid artery and internal jugular vein stained negative for Prox-1 and LYVE-1 in both control and trisomy 21 fetuses. The changes in protein expression were consistent in all samples we have examined. Differences between karyotypes are shown in table 2.

*Expression of Shh and (arterial) blood vessel markers*

Shh-expression was increased in the lymphatic endothelial cells of the JLS compared with the controls (Figure 1c-d). Also, a clear increase of Shh was shown in the subendothelial mesenchym, which was absent in the control fetuses (Figure 1c-d). The endothelial cells of the carotid artery stained positive for Shh in both control and trisomy 21 fetuses and showed a similar staining pattern between karyotypes (data not shown). Both NE fetuses and controls showed substantial expression of Shh in the endothelial cells lining the internal jugular vein (data not shown). An increased VEGF-A expression in both the nuclei and cytoplasm was shown in the lymphatic endothelial cells of the trisomy 21 fetuses compared with controls, concomitant with
previous research (Figure 1 e-f).

The endothelial cells of the carotid artery and internal jugular vein stained negative for VEGF-A in both control and trisomy 21 fetuses (data not shown). The lymphatic endothelial cells of the JLS of the trisomy 21 fetuses showed an increased NP-1 expression compared with controls (data not shown). NP-1 staining was positive in the endothelial cells of the arteries and negative in the endothelial cells of the veins. No difference in the NP-1 staining intensity was found in the blood endothelial cells between control and trisomy 21 fetuses (data not shown). The changes in protein expression were consistent in all examined samples. These data are summarized in table 2.

**Figure 1** Immunohistochemical analysis of JLS

a) Transverse section of the neck of a human fetus with normal nuchal skin (GA of 14 weeks, control) and JLS. LN tissue is visible in the JLS. b) Transverse section of the neck of a human trisomy 21 fetus with nuchal edema at GA 14 weeks. The JLS is enlarged and filled with erythrocytes (arrow). LN tissue is visible in the wall of the JLS. Magnification of consecutive sections of the boxed areas in (a) and (b) show the staining results of Shh and VEGF-A. c) Increased staining of Shh in the lymphatic endothelial cells and scattered cells in the mesenchym compared with the control fetus (c). d) Increased VEGF-A staining compared with the control fetus (f). Scale bars (a, b) 600 μm; c-f 20 μm.

A, carotid artery; GA, gestational age; JLS, jugular lymphatic sac; LN, lymph node; NX, vagal nerve; V, internal jugular vein; Tris 21, trisomy 21.
Shh and FOXC2 expression in fetuses with increased nuchal translucency

Figure 2 Immunohistochemical analysis of JLS and CA

a) Magnification of the JLS in the neck of a human fetus with normal nuchal skin demonstrating a negative subendothelial expression for SMA (GA of 14 weeks, control). b) Magnification of the JLS in the neck of a human trisomy 21 fetus with nuchal edema demonstrating a positive subendothelial expression for SMA (GA of 14 weeks). A positive LYVE-1 staining with a similar staining pattern in both the control (c) and trisomy 21 fetus (d) is observed. A positive LYVE-1 staining is also found in scattered mesenchymal cells of both the control and trisomy 21 fetus (arrows, c-d). The staining results for FOXC2 and PDGF-B expression in the JLS are shown (e-j). Decreased expression of FOXC2 (f) compared to (e) and increased expression of PDGF-B (h) compared to (g) is seen when comparing the trisomy 21 fetus with the control fetus. i) Magnification of the CA in the neck of a human fetus with normal nuchal skin (GA of 14 weeks, control). j) Magnification of the CA in the neck of a human trisomy 21 fetus with NE (GA of 14 weeks). Staining results of FOXC2-expression in the CA show a decreased expression of FOXC2 (j) in trisomy 21 fetus when compared with control (i). Scale bars (a-h) 20 μm.

CA, carotid artery; GA, gestational age; JLS, jugular lymphatic sac; Tris 21, trisomy 21.
Expression of SMA, FOXC2 and PDGF-B

SMA expression was observed subendothelially of the JLS of NE fetuses in contrast to the control fetuses, implying the presence of SMCs surrounding the JLS, concomitant with previous research (Figure 2 a-b). The lymphatic endothelial cells of the JLS showed a decreased expression of FOXC2 in trisomy 21 fetuses compared with the controls, where FOXC2 expression was evident (Figure 2 c-d). In contrast to the trisomy 21 fetuses, scattered cells in the mesenchymal surrounding of the controls showed a positive FOXC2 staining (arrows in Figure 2e). PDGF-B staining intensity was higher in the lymphatic endothelial cells of NE fetuses as compared with the lymphatic endothelial cells of the controls, which normally show low levels of PDGF-B (Figure 2e-f). A decreased staining intensity for FOXC2 was seen in the endothelial cells of the carotid artery in the NE fetuses compared with the relatively high levels in the endothelial cells of control fetuses.
Shh and FOXC2 expression in fetuses with increased nuchal translucency

fetuses (Figure 2 g-h). However, both NE and control fetuses showed only little PDGF-B staining in the endothelial cells of the carotid artery (data not shown). In addition, both NE fetuses and controls showed substantial expression of FOXC2 and PDGF-B in the endothelial cells of the internal jugular vein (data not shown). The changes in protein expression were consistent in all examined samples. These data are summarized in table 3.

DISCUSSION

In this study, the expression of Shh in the lymphatic endothelial cells and blood endothelial cells of trisomy 21 human fetuses with nuchal edema and increased JLS was evaluated. Previous research showed increased expression of blood vessel markers, including VEGF-A, in the enlarged JLS.9 We hypothesised that an increased VEGF-A expression might be the result of an altered Shh signalling as Shh acts upstream of VEGF.15;16 We indeed found an increased Shh and VEGF-A expression in the lymphatic endothelial cells of the JLS of the trisomy 21 fetuses compared to the control fetuses. The increased Shh was present in both endothelial cells and underlying mesenchym. This most likely reflects broad expression of Shh, because also nuclei of the mesenchymal cells of the trisomy 21 fetuses stained positive. However, diffusion of endothelial-derived Shh cannot be excluded. The increased VEGF-A expression was shown in both the nuclei and cytoplasm of the lymphatic endothelial cells. This was also presented in a previous study of our group.9 It was hypothesized that the VEGF-A overexpression results in an increased permeability and decreased cell adhesion with NE as a consequence.

Besides the increased Shh and VEGF-A expression, an increased NP-1 expression was found in the lymphatic endothelial cells of the JLS of the trisomy 21 fetuses compared with the control fetuses. NP-1 is a co-receptor for VEGF-A, which increases VEGFR-2 activity.21 Shh and VEGF-signalling have been shown to act upstream of the Notch pathway.15 This pathway plays an important role in the expression of artery-specific genes and repression of venous markers within the developing vasculature.22 This might be a mechanism through which the disturbance of endothelial differentiation in the trisomy 21 fetuses can be explained. However, to make sure this pathway is involved, further research is necessary.

We further established aberrant differentiation of the lymphatic endothelial cells of the JLS by showing low FOXC2 and high PDGF-B-expression in the lymphatic endothelial cells, together with the presence of SMCs around these cells. The lymphatic endothelial cells additionally showed diminished expression of the lymphatic marker Prox-1, which is known to regulate lymphatic endothelial cell differentiation. Decreased Prox-1 expression could result in the diminished FOXC2 expression, as FOXC2 acts downstream of Prox-1.17 Subsequently, the decrease in FOXC2 expression could lead to the increase in lymphatic endothelial PDGF-B expression and thus the formation of SMCs surrounding the JLS as we observed in the current study.
In addition to FOXC2 and PDGF-B expression in the lymphatic endothelial cells, we evaluated their expression in the carotid artery and the internal jugular vein and found a decreased staining of FOXC2 in the endothelial cells of the carotid artery in the trisomy 21 fetuses compared with the control fetuses. Interestingly, FOXC2 is, besides in lymphangiogenesis, important for cardiovascular development as *Foxc2* -/- mice have cardiovascular abnormalities, including ventricular septal defects (VSD) and abnormalities in the aortic arch such as interruption or coarctation.\(^{23,24}\) In four of the five trisomy 21 fetuses we analyzed, cardiac abnormalities like an AVSD, but also aortic arch anomalies such as an aberrant subclavian artery were found. These kind of malformations also have been described in 12-36% of the postnatal individuals with trisomy 21.\(^{25-27}\) Interestingly, Tbx1 has been found as a direct target of Foxc2.\(^{28}\) Earlier research suggests that Tbx1 may regulate fibroblast growth factor 8 (Fgf8) in the endoderm, which possibly signals to adjacent neural crest cells and in this way regulates aortic arch development.\(^{29-31}\) Abnormal cross talk of the Tbx1 population and / or deficient differentiation of neural crest cells is a mechanism that might explain an abnormal development of the aortic arch (type B interruption) in trisomy 16 and other mouse models.\(^{32-37}\) Shh also regulates Foxc2 expression and is required for neural crest cell development.\(^{28,38}\) However, in the current research no altered Shh expression was found in the carotid artery of the trisomy 21 fetuses compared to the controls. This might be explained by the fact that in the age category of fetuses we evaluated, the remodelling of the aortic arch already had been completed.

In conclusion, our current study shows that a locally increased Shh and VEGF-A expression in human trisomy 21 fetuses with enlarged JLS is correlated with an aberrant lymphatic differentiation. The abnormal SMC investment surrounding the enlarged JLS can possibly be explained by an increase of PDGF-B-induced SMC recruitment and / or differentiation. This further underscores the loss of lymphatic identity in trisomy 21 fetuses and the shift towards a blood vessel wall phenotype.

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