Cardiol Young 2005; 15: 26–30

ISSN 1047-9511

Original Article

Evidence of endothelial dysfunction in patients with functionally univentricular physiology before completion of the Fontan operation

Maria A. Binotto,[1] Nair Y. Maeda,[2] Antonio A. Lopes[1]

[1]Department of Paediatric Cardiology and Adult Congenital Heart Disease, Heart Institute, University of São Paulo Medical School; [2]Pró-Sangue Foundation, São Paulo, Brazil

Abstract *Introduction:* Postoperative thrombosis after a cavopulmonary connection has been widely described. Abnormalities in coagulation seem to occur early in the course of patients with functionally univentricular physiology, and may precede surgery. Endothelial abnormalities due to chronic hypoxia, and hyperviscosity, may contribute to this scene. The purpose of our study was to investigate if patients with a superior cavopulmonary connection have altered levels of endothelial and coagulative markers in the plasma. *Methods:* We compared findings in 10 patients, aged from 4 to 19 years, with 6 age-matched normal controls. We measured levels of von Willebrand factor antigen, thrombomodulin, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and D-dimer in the plasma using enzyme-linked immunosorbent assay. *Results:* We found increased levels of von Willebrand factor antigen ($p = 0.01$), tissue-type plasminogen activator ($p = 0.01$), and decreased levels of thrombomodulin ($p = 0.008$) in the patients when compared to controls, while levels of plasminogen activator inhibitor-1 were not different. Values of D-dimer were within the reference range. Levels of tissue-type plasminogen activator had a positive correlation with von Willebrand factor antigen ($r = 0.66$, $p = 0.008$). *Conclusions:* Altered levels of endothelial markers in the plasma, in the presence of normal levels of D-dimer, suggest that endothelial dysfunction may precede the occurrence of intravascular coagulation and thrombosis in patients with functionally univentricular physiology. These observations may have therapeutical implications.

Keywords: Functionally single ventricle; endothelium; cavopulmonary connection

THROMBOEMBOLISM IS A LONG-TERM complication of the Fontan circulation, and may decrease life expectancy and functional state of patients with functionally univentricular hearts. Several reports have also shown that thromboembolic events occur after a superior cavopulmonary connection.[1,2] A few studies have looked for factors associated with the late occurrence of thromboembolism in these settings, implicating local and hemodynamic conditions,[3–6] as well as haematological abnormalities, with multiple abnormalities in coagulation being found both before and after the various stages of the Fontan procedure.[5,7–13]

Some patients with a superior cavopulmonary connection experience different degrees of chronic hypoxemia. Chronic cyanosis, and secondary erythrocytosis, may induce a hypercoagulable state with increased risk of thrombosis. Vascular occlusion following thrombosis may result in long-term elevation of the pulmonary arterial resistance. It is well known that hypoxia shifts the endothelial phenotype towards a prothrombotic state.[14] We hypothesized that endothelial dysfunction might be present in these patients, leading to increased risk of thrombotic events on a long-term basis. The aim of our study, therefore, was to look for evidence of endothelial dysfunction late in

Correspondence to: Dr Maria A. Binotto, Av. Higienopolis 1048/86, CEP 01238-000, São Paulo, Brazil. Tel: +55 11 30695409; Fax: +55 11 36679728 E-mail: conangelica@incor.usp.br

Accepted for publication 10 September 2004

the postoperative course of patients undergoing a superior cavopulmonary connection. Several markers have been used as indexes of endothelial dysfunction. In this study, we measured levels of thrombomodulin, tissue-type plasminogen activator, plasminogen activator inhibitor-1 and von Willebrand factor antigen in the plasma, since these endothelial substrates are involved in coagulation, fibrinolysis, and adhesion of platelets. We also measured levels of D-dimer, as an attempt to look for an ongoing intravascular coagulative process.

Methods

Population studied. We enrolled patients who had previously undergone a superior cavopulmonary shunt, followed up in the outpatient clinic of the Department of Paediatric Cardiology, Heart Institute, São Paulo, Brazil. All except one of them had been considered unsuitable for completion of the Fontan procedure, either because of an increased mean pulmonary arterial pressure, or because of stenosed and/or hypoplastic pulmonary arteries. We used 6 aged-matched, healthy children and adolescents as controls. The patients, and their parents, were informed about the purpose of the collection of data, and gave their informed consent. The study was approved by the Scientific Committee of the Heart Institute.

Collection of blood. After a period of 15 minutes resting supine, we collected peripheral venous blood via a single, clean venepuncture in the antecubital fossa. We either avoided the use of a tourniquet, or limited it to less than one minute. The blood was collected in 1:10 volumes of 3.8 percent sodium citrate. Immediately after collection, the samples were centrifuged at 3,000 gravity for 20 minutes. Plasma was separated and stored at minus 80 degrees Celsius. Aliquots were thawed only once for use.

General laboratory determinations. A further sample was collected into ethylenediaminetetraacetic acid for determinations of the haematocrit, platelet count, and factor V. Saturations of oxygen were measured in room air using finger pulse oximetry.

Biochemical determinations. Levels of D-dimer, thrombomodulin, tissue-type plasminogen activator antigen, Plasminogen activator inhibitor-1 antigen and von Willebrand factor antigen were measured in the plasma using an enzyme-linked immunosorbent assay (Diagnostica Stago, Asnières, France). Samples were processed in duplicate. Results were obtained by comparison with a standard curve with reagents provided by the manufacturer. Results were expressed as nanograms per millilitre for D-dimer, thrombomodulin, plasminogen activator inhibitor-1 and tissue-type plasminogen activator, and as units per decilitre for von Willebrand factor antigen.

Statistical analysis. Results are expressed as mean plus or minus standard deviation, and median. Differences between patients and controls were tested using Student's t test, and the Mann–Whitney test was used for variables lacking a normal distribution. Correlations between variables were tested by calculating the coefficient of correlation. p values less than 0.05 were considered statistically significant.

Results

We enrolled 10 patients in the study, the diagnoses and characteristics being shown in Table 1. Their mean age was 10.7 years, with a range from 4 to 19 years. The mean age of the control group was 11.2 years, with a range from 9 to 16 years. The peripheral oxygen saturation was 82.2 plus or minus 7.6 percent. The haematocrit was 50.5 plus or minus 4.2 percent. The platelet count was within the reference range for all patients. Activity of factor V in the plasma was below normal in half of the patients.

Biochemical determinations are shown in Table 2. We found increased levels of von Willebrand factor antigen ($p = 0.01$), and tissue-type plasminogen activator ($p = 0.01$), in the plasma, but decreased levels of thrombomodulin ($p = 0.008$) when compared to controls. Levels of plasminogen activator inhibitor-1 did not differ from controls, while levels of D-dimer were within the reference range. Levels of tissue-type plasminogen activator correlated well with the levels of von Willebrand factor antigen ($r = 0.66$, $p = 0.03$, Fig. 1). We failed to demonstrate any kind of correlation between the degree of hypoxia as judged by peripheral saturations of oxygen and the various biochemical markers of endothelial dysfunction.

Discussion

Our results show that cyanotic patients with a superior cavopulmonary connection have biochemical evidence of endothelial cellular dysfunction, as demonstrated by high levels of tissue-type plasminogen activator and von Willebrand factor antigen in the plasma, with decreased levels of thrombomodulin, levels D-dimer being within the reference range. Our findings are similar to those found in adult cyanotic patients with Eisenmenger syndrome. Such patients have altered levels of endothelial markers in the plasma, associated with elevated levels of D-dimer, and a high prevalence of central pulmonary arterial thrombosis.[15]

Our results cannot be generalized to all patients with a superior cavopulmonary connection, since the majority of them are younger patients, mostly below two years of age. Thus, we assume that our patients are most likely representative of an atypical subset of individuals in whom the Fontan circulation cannot

https://doi.org/10.1017/S1047951105000065 Published online by Cambridge University Press

Table 1. Characteristics of patients and general laboratory determinations.

Patient	Gender	Age (years)	Age at surgery (years)	O_2 sat (%)	Ht (%)	Diagnosis	Surgical procedure
1	M	13	10	85	60	DILV, DORV, PS, previous MBTS	Bilateral SCPC
2	F	14	10	66	49	VSD, RV hypoplasia, left PA stenosis	Right SCPC, patch widening of left PA
3	F	13	9	86	50	TA, PS, left and right PA hypoplasia, previous MBTS	Right SCPC
4	F	6	5	86	48	TA, PS, left PA stenosis, previous MBTS	Right SCPC, patch widening of left PA
5	F	19	15	80	47	Absent left AV connection, discordant VA connections, previous banding of PT	Right SCPC
6	M	8	7	83	51	DILV, DORV, PS, left PA stenosis	Bilateral SCPC, patch widening of left PA
7	F	6	3	92	47	DILV, DORV, PS, left PA stenosis, moderate common valve regurgitation	Bilateral SCPC, patch widening of left PA
8	F	13	8	80	52	TGA, LV hypoplasia, PS, VSD	Right SCPC
9	M	4	3	80	50	PA, IVS, left PA stenosis, previous MBTS	Right SCPC, patch widening of left PA
10	F	11	7	72	57	DORV, LV hypoplasia, previous banding of PT	Bilateral SCPC

Abbreviations: M: male; F: female; DILV: double inlet left ventricle; DORV: double outlet right ventricle; PS: pulmonary valve stenosis; MBTS: modified Blalock–Taussig shunt; VSD: ventricular septal defect; RV: right ventricle; LV: left ventricle; PT: pulmonary trunk; TA: tricuspid atresia; AV: atrioventricular; VA: ventriculoarterial; TGA: transposition of great arteries; PA: pulmonary atresia; IVS: intact ventricular septum; SCPC: superior cavopulmonary connection; O_2 sat: peripheral oxygen saturation at rest; Ht: haematocrit

Table 2. Endothelial and coagulation markers in patients and controls.

Parameter	Patients	Controls	p value
vWF:Ag (U/dl)	118.4 ± 19.5 (120)	91.3 ± 18.7 (87.5)	0.01*
t-PA antigen (ng/ml)	7.6 ± 2.4 (7.5)	4.3 ± 1.6 (3.9)	0.01*
PAI-1 antigen (ng/ml)	18.4 ± 9.1 (15.9)	16.8 ± 6.0 (15.1)	0.70**
Thrombomodulin (ng/ml)	9.3 ± 5.4 (8.6)	31.3 ± 13.6 (29.2)	0.008**
D-dimer (ng/ml)	155.2 ± 58.2 (159.5)	200.8 ± 55.2 (201)	0.14**

Abbreviations: vWf:Ag: von Willebrand factor antigen; t-PA: tissue-type plasminogen activator; PAI-1: plasminogen activator inhibitor-1
Values given are mean ± SD and median (in parenthesis)
*Analyzed by Students *t*-test
**Analyzed by Mann–Whitney test

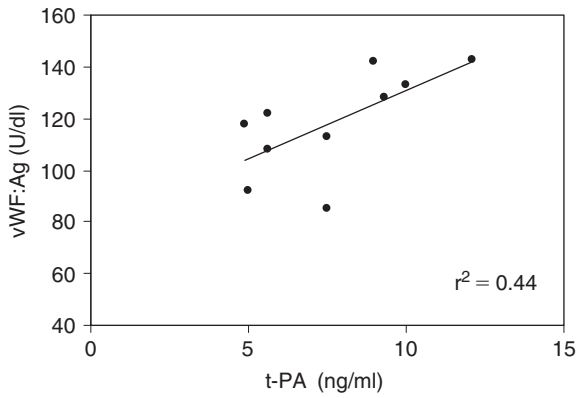

Figure 1.
Scatter plot showing a positive correlation between the levels of tissue-type plasminogen activator and von Willebrand factor antigen in the plasma.

be completed. The main reasons for not assigning our patients to a total cavopulmonary connection were stenotic or hypoplastic pulmonary arteries and increased pulmonary pressure. Patients with a superior cavopulmonary connection may have chronic systemic desaturation of oxygen and secondary erythrocytosis. Endothelial dysfunction due to chronic hypoxia and hyperviscosity may induce a hypercoagulable tendency, with increased risks of thrombosis.

Whereas the healthy endothelium exhibits anticoagulative properties, exposure to hypoxia and other stress factors can lead to a procoagulant phenotype. Endothelial cells exposed to hypoxia undergo time-dependent suppression of thrombomodulin, an endothelial membrane protein that accelerates the activation of protein C, which has potent anticoagulative properties.[14] Hypoxia also induces increased expression of tissue factor, an initiator of the extrinsic pathway of coagulation. Plasminogen activator inhibitor-1, a major endogenous inhibitor of fibrinolysis, is up-regulated during hypoxia and ischemia, and also contributes to a procoagulant state.[14]

https://doi.org/10.1017/S1047951105000065 Published online by Cambridge University Press

Increased levels of thrombomodulin have been reported in the plasma in several disorders associated with damage to endothelial cells. It is not clear, however, under which conditions thrombomodulin is actively or passively shed from the cell membrane, or is cleaved by the pathological process. This molecule is an important regulator of activated thrombin, converting thrombin from a procoagulant to an anticoagulant by altering its substrate specificity so that it activates protein C.[16] Decreased levels of thrombomodulin in the plasma is assumed to reflect impaired endothelial synthesis of this proteoglycan, and has been demonstrated, for example, in patients with Eisenmenger syndrome.[15] It is possible that chronic hypoxia may account for the reduction in levels of thrombomodulin in the plasma of cyanotic patients.[17] In fact, Ogawa et al.[18] have shown that cultured bovine endothelial cells exposed to low concentrations of oxygen expressed diminished thrombomodulin antigen and functional activity.

Hypoxia induces exocytosis of the Weibel-Palade bodies, which are storage sites of several proteins, including von Willebrand factor and tissue-type plasminogen.[19,20] von Willebrand factor is a glycoprotein synthesized mainly by endothelial cells. It is also a constituent of the platelet alpha granule. It is believed, however, that most, if not all, circulating von Willebrand factor is likely to originate from the endothelium. Among the various plasma markers of endothelial cellular dysfunction, von Willebrand factor is one of the best available, mainly because of its specificity.[21] Increased circulating levels are found in numerous clinical settings, such as inflammatory and atherosclerotic disease. In addition, increased levels have been demonstrated in cyanotic patients with Eisenmenger syndrome, particularly in a subgroup with a worse clinical condition.[15]

Tissue plasminogen activator, and its inhibitor-1, are proteins released by endothelial cells. Elevated levels of tissue-type plasminogen activator have been found in the settings of atherosclerosis, disseminated intravascular coagulation, and Eisenmenger syndrome.[15,22] The mechanism by which high levels of tissue-type plasminogen activator contribute to the prothrombotic condition is unclear, as elevated levels should theoretically increase fibrinolysis. Since in our study we did not measure the activity of tissue-type plasminogen in the plasma, we were unable to decide whether increased antigenic concentration was associated with an enhanced fibrinolytic capacity. Increased levels of tissue-type plasminogen activator, therefore, might be associated with activation of, or damage to, endothelial cells, and should not necessarily be viewed as a protective mechanism.

Plasminogen activator inhibitor-1 is up-regulated during hypoxia.[14] Despite the fact that plasminogen activator inhibitor-1 can also be produced by hepatocytes, platelets, mesothelial cells, monocytes and smooth muscle cells, and therefore is a less specific marker of endothelial cellular dysfunction, increased levels of this molecule have been described in arterial and thrombotic disease, and are considered a risk factor for myocardial infarction.[21] We failed to demonstrate differences in our group of patients in comparison to the controls.

The absence of intravascular coagulation in our patients was suggested by normal circulating levels of D-dimer. D-dimer has emerged as the most useful marker of ongoing fibrinolysis. It is very sensitive, although non-specific for the diagnosis of thrombosis and pulmonary embolism. Normal values, nonetheless, are very helpful in excluding the diagnosis of thrombosis.[23]

A few studies have addressed the role of endothelial dysfunction in patients with functionally univentricular physiology. It has been suggested that higher shear stress on the wall of the pulmonary vasculature may alter endothelial function, with vasoconstriction and an increased risk for formation of thrombus.[24] In fact, the Fontan operation leads to loss or great reduction of pulsatility in the pulmonary circulation. Pulsatile flow is important for shear stress mediated release of endothelial-derived nitric oxide, and reduction of pulmonary vascular resistance. Reduction of pulmonary vascular resistance following the administration of exogenous nitric oxide has been demonstrated in early and late postoperative states in those having the Fontan circulation.[25]

Mahle et al.[26] studied endothelial function in patients with the Fontan circulation using high-resolution ultrasonic interrogation of the brachial artery, and found an impaired flow-mediated vasodilation. In addition, they found increased levels of asymmetric dimethylarginine in the plasma, this being an endogenous competitive inhibitor of nitric oxide synthase, synthesized by endothelial cells and considered another circulating marker of endothelial dysfunction.

Considering the key role of hypoxia in the release of von Willebrand factor, thrombomodulin, and tissue-type plasminogen activator from the endothelium, we should anticipate the occurrence of a significant correlation between such markers and the degree of hypoxia. The lack of correlation found in our patients may reflect the small number of subjects studied. Alternatively, in patients with functionally univentricular physiology, other variables may influence the expression and secretion of these endothelial markers.

We have shown, therefore, that altered plasma levels of endothelial dysfunction markers may precede the occurrence of intravascular coagulation in chronically

https://doi.org/10.1017/S1047951105000065 Published online by Cambridge University Press

cyanotic patients with functionally univentricular physiology in whom a superior cavopulmonary connection has been constructed for definitive palliation. Our current policy for this particular subset of patients is to give warfarin for life, keeping their international normalized ratio between 2 and 3. It should be noted, however, that we did not address the relative contribution of pulmonary and systemic endothelial cells to the circulating levels of biochemical markers in this work. Further studies are necessary, involving a larger population of patients, including a subgroup with normal systemic saturations of oxygen. It is axiomatic that the better understanding of the risk factors for thromboembolic complications will help in the elaborations of rational recommendations for antithrombotic prophylaxis in this setting.

Acknowledgement

Supported by FAPESP.

References

1. Forbes TJ, Rosenthal GL, Reul Jr GR, Ott DA, Feltes TF. Risk factors for life-threatening cavopulmonary thrombosis in patients undergoing bidirectional superior cavopulmonary shunt: an exploratory study. Am Heart J 1997; 134: 865–871.
2. Hanséus K, Björkhem G, Jögi P, Sonesson S. Formation of thrombus and thromboembolism after the bidirectional Glenn anastomosis, total cavopulmonary connection and the Fontan operation. Cardiol Young 1998; 8: 211–216.
3. Day RW, Boyer RS, Tait VF, Ruttenberg HD. Factors associated with stroke following the Fontan procedure. Pediatr Cardiol 1995; 16: 270–275.
4. du Plessis AJ, Chang AC, Wessel DL, et al. Cerebrovascular accidents following the Fontan operation. Pediatr Neurol 1995; 12: 230–236.
5. Jahangiri M, Shore D, Kakkar V, Lincoln C, Shinebourne E. Coagulation factor abnormalities after the Fontan procedure and its modifications. J Thorac Cardiovasc Surg 1997; 113: 989–992.
6. Rosenthal DN, Friedman AH, Kleinman CS, et al. Thromboembolic complications after Fontan operations. Circulation 1995; 92: II287–II293.
7. Cromme-Dijkhuis AH, Henkens CM, Bijleveld CM, et al. Coagulation factor abnormalities as possible thrombotic risk factors after Fontan operations. Lancet 1990; 336: 1087–1090.
8. Jahangiri M, Kreutzer J, Zurakowski D, Bacha E, Jonas RA. Evaluation of hemostatic and coagulation factor abnormalities in patients undergoing the Fontan operation. J Thorac Cardiovasc Surg 2000; 120: 778–782.
9. Monagle P, Andrew M. Coagulation abnormalities after Fontan procedures. J Thorac Cardiovasc Surg 1998; 115: 732–733.
10. Odegard KC, McGowan Jr FX, DiNardo JA, et al. Coagulation abnormalities in patients with single-ventricle physiology precede the Fontan procedure. J Thorac Cardiovasc Surg 2002; 123: 459–465.
11. Odegard KC, McGowan Jr FX, Zurakowski D, et al. Coagulation factor abnormalities in patients with single-ventricle physiology immediately prior to the Fontan procedure. Ann Thorac Surg 2002; 73: 1770–1777.
12. Rauch R, Ries M, Hofbeck M, et al. Hemostatic changes following the modified Fontan operation (total cavopulmonary connection). Thromb Haemost 2000; 83: 678–682.
13. Ravn HB, Hjortdal VE, Stenbog EV, et al. Increased platelet reactivity and significant changes in coagulation markers after cavopulmonary connection. Heart 2001; 85: 61–65.
14. Ten VS, Pinsky DJ. Endothelial response to hypoxia: physiologic adaptation and pathologic dysfunction. Curr Opin Crit Care 2002; 8: 242–250.
15. Caramurú LH, Maeda NY, Lopes AA, Bydlowski SP. Age-dependent likelihood of in situ thrombosis in secondary pulmonary hypertension. Clin Appl Thrombosis/Hemostasis 2004; 10: 217–223.
16. Wu KK, Matijevic-Aleksic N. Thrombomodulin: a linker of coagulation and fibrinolysis and predictor of risk of arterial thrombosis. Ann Med 2000; 32: I73–I77.
17. Shreeniwas R, Ogawa S, Cozzolino F, et al. Macrovascular and microvascular endothelium during long-term hypoxia: alterations in cell growth, monolayer permeability, and cell surface coagulant properties. J Cell Physiol 1991; 146: 8–17.
18. Ogawa S, Gerlach H, Esposito C, et al. Hypoxia modulates the barrier and coagulant function of cultured bovine endothelium. J Clin Invest 1990; 85: 1090–1098.
19. Goerge T, Niemeyer A, Rogge P, et al. Secretion pores in human endothelial cells during acute hypoxia. J Membrane Biol 2002; 187: 203–211.
20. Pinsky DJ, Naka Y, Liao H, et al. Hypoxia-induced exocytosis of endothelial cell Weibel-Palade bodies. A mechanism for rapid neutrophil recruitment after cardiac preservation. J Clin Invest 1996; 97: 493–500.
21. Blann AD, Taberner DA. A reliable marker of endothelial cell dysfunction: does it exist? Br J Haematol 1995; 90: 244–248.
22. Olli R, Celermajer DS. Testing for endothelial dysfunction. Ann Med 2000; 32: 293–304.
23. Mavromatis BH, Kessler CM. D-dimer testing: the role of the clinical laboratory in the diagnosis of pulmonary embolism. J Clin Pathol 2001; 54: 664–668.
24. Morgan VL, Graham Jr TP, Roselli RJ, Lorenz CH. Alterations in pulmonary artery flow patterns and shear stress determined with three-dimensional phase-contrast magnetic resonance imaging in Fontan patients. J Thorac Cardiovasc Surg 1998; 116: 294–304.
25. Khambadkone S, Li J, de Leval MR, et al. Basal pulmonary vascular resistence and nitric oxide responsiveness late after Fontan-type operation. Circulation 2003; 107: 3204–3208.
26. Mahle WT, Todd K, Fyfe DA. Endothelial function following the Fontan operation. Am J Cardiol 2003; 91: 1286–1288.