Endothelial Abnormalities in Adolescents with Type 1 Diabetes: A Biomarker for Vascular Sequelae?

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Objective To evaluate whether counts of circulating colony forming unit-endothelial cells (CFU-ECs), cells co-expressing CD34, CD133, and CD31 (CD34+CD133+CD31+), and CD34+CD45- cells are altered in adolescents with type 1 diabetes and if the changes in counts correlate with endothelial dysfunction.

Study design Adolescents with diabetes (ages 18 to 22 years) and race- and sex-matched control subjects were studied. We assessed circulating CFU-ECs, using colony assays, and CD34+CD133+CD31+ and CD34+CD45- cells, using poly-chromatic flow cytometry. CFU-ECs and CD34+CD133+CD31+ are hematopoietic-derived progenitors that inversely correlate with cardiovascular risk in adults. CD34+CD45- cells are enriched for endothelial cells with robust vasculogenic potential. Vascular reactivity was tested by laser Doppler iontophoresis.

Results Subjects with diabetes had lower CD34+CD133+CD31+ cells, a trend toward reduced CFU-ECs, and increased CD34+CD45- cells compared with control subjects. Endothelium-dependent vasodilation was impaired in subjects with diabetes, which correlated with reductions in circulating CD34+CD133+CD31+ cells.

Conclusions Long-term sequelae of type 1 diabetes include vasculopathies. Endothelial progenitor cells promote vascular health by facilitating endothelial integrity and function. Lower CD34+CD133+CD31+ cells may be a harbinger of future macrovascular disease risk. Higher circulating CD34+CD45- cells may reflect ongoing endothelial damage. These cells are potential biomarkers to guide therapeutic interventions to enhance endothelial function and to prevent progression to overt vascular disease. *(J Pediatr 2010;157:540-6)*.

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The primary sources of morbidity and mortality in individuals with diabetes are microvascular and macrovascular atherosclerotic complications. Despite advances in diabetes care, early cardiovascular disease remains common in persons with type 1 diabetes.[1,2] Progression of vascular disease is related to the degree of glycemic control over time, and intensive management of glycemia in persons with type 1 diabetes can affect the rate of cardiovascular events.[3] Currently, hemoglobin A1C (A1C) is the primary variable used to predict risk of subsequent vasculopathy. Yet, the rate and timing of complication development varies from individual to individual and is not solely determined by glycemic measurements. No specific biomarkers are available to identify which children with type 1 diabetes are at highest risk of later vascular diseases.

Endothelial dysfunction is a predictor of cardiovascular disease.[4-6] Given that diabetes is characterized by inflammation[7] and oxidative excess,[8] the endothelium in individuals with type 1 diabetes is continually exposed to multiple stressors that promote the development of endothelial dysfunction.

Homeostatic regulation of the endothelium is complex, requiring dynamic interactions between endothelial cells resident in vessel walls and cells circulating in the peripheral blood to sustain endothelial integrity and function. Circulating endothelial progenitor cells (EPCs) are purported to participate in both of these processes by promoting neovascularization and reendothelialization. Vascular reactivity studies have documented endothelial dysfunction in adolescents and young adults with type 1 diabetes. These include studies demonstrating reductions in brachial flow-mediated dilation,[9-11] increases in carotid intima-media thickness,[10-12] and higher radial artery stiffness by tonometry.[13]

Studies in adults, including subjects with type 1 and type 2 diabetes, demonstrate an inverse correlation between vascular disease risk and circulating EPC numbers.[14-17] In these studies, a variety of methods were used to quantify circu-

A1C	Hemoglobin A1C
CFU-ECs	Colony forming unit-endothelial cells
CPCs	Circulating progenitor cells
ECFCs	Endothelial colony-forming cells
EPCs	Endothelial progenitor cells
hsCRP	High-sensitivity c-reactive protein
MNCs	Mononuclear cells
PU	Perfusion units

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lating EPCs including colony formation assays and poly-chromatic flow cytometry to detect cells that co-express CD34, CD133, CD31, and/or vascular endothelial growth factor receptor 2. However, because these important observations were made, the definition of an EPC has been questioned due to data demonstrating that these methods do not identify endothelial precursors, but rather subpopulations of hematopoietic progenitor cells that facilitate vascular repair and angiogenesis via secretion of paracrine growth factors (reviewed in Reference 18).[18] Specifically, circulating progenitor cells (CPCs) are primitive hematopoietic progenitor cells, and colony forming unit-endothelial cells (CFU-ECs) are not endothelial cells but angiogenic macrophages.[18-21] In addition, a distinct subpopulation of peripheral blood cells expresses CD34 but not CD45 (CD34+CD45-), which is enriched for endothelial colony forming cells (ECFCs).[21] ECFCs are true endothelial cells with robust proliferative, clonogenic, and vasculogenic potential. Interestingly, ECFCs are elevated in patients with severe coronary artery disease[22] and an increase in circulating CD34+CD45- cells positively correlates with vascular disease progression.[19]

Despite ongoing study of the identity of these cells, multiple studies in adults demonstrate that a reduction in the circulating numbers of CFU-ECs and CPCs correlate with increased vascular disease risk,[14,16,23,24] suggesting that rare populations of circulating cells have an important role in protecting from vascular morbidities. However, limited studies have been conducted in children or young adults to examine whether a reduction in CPCs is present early in disease and whether alterations in circulating progenitor subpopulations correlate with a measure of vascular dysfunction. Using colony formation assays and novel poly-chromatic flow cytometry methods, we questioned whether adolescents with type 1 diabetes for >5 years have reduced CFU-ECs and CPCs that correlate with evidence of endothelial dysfunction assessed by laser Doppler iontophoresis.

Methods

The study was approved by the Indiana University Institutional Review Board. Patients with type 1 diabetes for at least 5 years and healthy sex- and race-matched control subjects ages 18 to 22 years were recruited. Control subjects were recruited both through advertisements and by asking patients to ask a friend without diabetes to participate with them. Subjects were excluded if they had evidence of insulin resistance (acanthosis, extreme obesity), were smokers, or were on medications other than insulin or oral contraceptive pills. Consent was obtained from all subjects.

All assessments were done in the morning after subjects had been fasting for at least 3 hours. Subjects with diabetes took their morning insulin as usual (adjusting for the fasting conditions). Height, weight, and blood pressure were obtained. Subjects completed the International Physical Activity Questionnaire, Short Format, to assess physical activity over 7 days before the visit.[25] Peripheral blood was collected for A1C, fructosamine, lipid profiles, high-sensitivity c-reactive protein (hsCRP), colony formation assays, and flow cytometry. Vascular reactivity was assessed by laser Doppler iontophoresis.

Preparation of Mononuclear Cells

Peripheral blood was collected in citrate cell preparation tubes (BD Biosciences, Franklin Lakes, New Jersey) and processed at the Angiogenesis and Endothelial Progenitor Cell Core at Indiana University. Tubes were centrifuged at 1600*g* for 30 minutes, and low-density mononuclear cells (MNCs) were collected similar to previous studies[20] for colony formation assays and flow cytometry studies.

Colony Formation Assay

CFU-ECs were cultured from MNCs using the EndoCult Liquid Medium Kit (StemCell Technologies, Vancouver, British Columbia, Canada) per the manufacturer's protocol and as previously described.[20] Briefly, MNCs were resuspended in complete EndoCult medium (Cambrex, Walkersville, Maryland) and seeded on fibronectin-coated tissue culture plates (BD Biosciences). After 48 hours, nonadherent cells were collected and replated in fibronectin-coated tissue culture plates for 3 days. Colonies were identified as elongated sprouting cells radiating from a central core of round cells. Colony numbers were scored in a blinded fashion.

Poly-Chromatic Flow Cytometry

MNCs were incubated with Fc block for 10 minutes at 4°C (Miltenyi Biotec, Auburn, California) before staining with directly conjugated monoclonal antibodies against human antigens, CD31 fluoroscein isothyocyanate (FITC, BD Pharmingen, San Diego, California, cat. No. 555445), CD34 phycoerythrin (PE, BD Pharmingen, cat. No. 550761), CD133 allophycocyanin (APC, Miltenyi Biotec, Auburn, California, cat. No. 130-090-826), CD45 APC-AlexaFluor (AF) 750 (Invitrogen, Carlsbad, California, cat. No. MHCD4527), as well as the viability marker ViViD (Invitrogen) and CD41a (BD Pharmingen, cat. No. 555465), and CD235a (glyA, R&D Systems, Minneapolis, Minnesota, cat. No. MAB1228), both conjugated to Pacific Blue (PacB, Invitrogen) for the exclusion of platelets and red blood cells, respectively. Cells were incubated with antibodies for 30 minutes at 4°C, washed twice in PBS with 2% FBS, and fixed in 300μL 1% paraformaldehyde (Sigma Aldrich, St. Louis, Missouri). Fluorescence minus 1 controls were prepared as negative gating controls and anti-mouse Ig BD CompBeads (BD Biosciences, Bedford, Massachusetts) were stained with each of the individual test antibodies to serve as single-color compensation controls. The frequency of phenotypically defined cell populations was analyzed using a Becton Dickinson LSR II flow cytometer and FlowJo software, version 8.7.3 (Tree Star, Inc., Ashland, Oregon). At least 300 000 events were collected and analyzed for each sample.

Vascular Reactivity Studies

Participants' forearms were measured using a tape measure and a probe connected to a laser Doppler instrument