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Original Paper

Endothelial Repair in Childhood Arterial Ischaemic Stroke with Cerebral Arteriopathy

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Key Words
Children · Stroke · Endothelial progenitor cells · Repair

Abstract
Background: We have previously shown that recurrent arterial ischaemic stroke (AIS) in children with cerebral arteriopathy is associated with increased circulating endothelial cells and endothelial microparticles, consistent with ongoing endothelial injury. To date, however, little is known about endothelial repair responses in childhood AIS. We examined the relationship between the number and function of circulating endothelial progenitor cells (EPC), the levels of brain-derived neurotrophic factor (BDNF) and AIS recurrence. ***Methods:*** Flow cytometry was used to identify peripheral blood mononuclear cells positive for CD34/kinase insert domain-containing receptor (KDR). In a subgroup of patients (5 in each group selected at random), monocytic EPC function was assessed by colony-forming unit (EPC-CFU) capacity and incorporation into endothelial cell networks in Matrigel. BDNF was measured using ELISA. ***Results:*** Thirty-five children, aged 12 years (range: 5–16.5; 9 males), with AIS and cerebral arteriopathy were studied; 10 had recurrent AIS. CD34+/KDR+ cells were significantly higher in recurrent AIS compared to non-recurrent AIS patients ($p = 0.005$) and controls ($p = 0.0002$). EPC-CFU and EPC incorporation into endothelial cell networks were significantly reduced in recurrent compared to non-recurrent AIS patients ($p = 0.04$ and $p = 0.01$, respectively). Levels of BDNF were significantly higher in recurrent compared to non-recurrent AIS patients ($p = 0.0008$) and controls ($p = 0.0002$). ***Conclusions:*** Children with recurrent AIS and cerebral arteriopathy had increased circulating CD34+/KDR+ cells and BDNF consistent with an endothelial repair response. However, EPC function was impaired. Future studies are needed to examine whether suboptimal endothelial repair contributes to childhood AIS recurrence.

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Introduction

Abnormalities or arteriopathies of the cervical or intracranial circulation are implicated in the aetiology and recurrence of childhood arterial ischaemic stroke (AIS) [1]. We have previously shown that in children with AIS associated with arteriopathy, indices of endothelial injury (circulating endothelial cells and endothelial microparticles) are higher in those with a recurrent compared with a monophasic disease course suggesting that persistent endothelial injury could contribute to recurrent AIS [2]. Similar to other vascular disorders, it is likely that a series of endothelial repair responses are mobilized in response to this ongoing vascular injury to re-establish endothelial integrity [3]. For instance, circulating CD34+/kinase insert domain-containing receptor (KDR)+ cells are considered to play a key role as endothelial progenitor cells (EPC) in vascular maintenance and repair, and a reduced number has been proposed to result in endothelial dysfunction [4]. Monocyte-derived EPC are likely to have an additional role since a reduced function of these cells negatively influences their restorative capacities [3, 4]. Notably, previous studies have suggested that EPC release brain-derived neurotrophic factor (BDNF), and they have implicated this in neuronal protection and neurogenesis following ischaemic stroke [5].

We hypothesized that, in addition to persistent endothelial injury, recurrence of childhood AIS associated with cerebral arteriopathy is also characterized by impaired vascular repair responses that influence neurotrophic support to the ischaemic brain. The aim of this study was therefore to compare the number of circulating CD34+/KDR+ cells, the functional properties of monocyte-derived EPC, and the levels of BDNF in children with AIS associated with cerebral arteriopathy with and without recurrence.

Patients and Methods

A cross-sectional study was conducted of children aged >28 days with AIS (acute focal neurological deficit attributable to cerebral infarction in a corresponding arterial distribution) and imaging evidence of cerebral/cervical arteriopathy presenting to the Great Ormond Street Hospital NHS Foundation Trust from October 2007 to January 2012. The study had Institutional Ethics Committee approval. Informed consent/assent was obtained from the study participants/parents. Cerebral/cervical arteriopathy was defined as focal or segmental stenosis or occlusion, with regular or irregular abnormalities of the arterial wall and categorized according to published consensus definitions [6, 7]. Magnetic resonance imaging (MRI) and magnetic resonance angiography (MRA) were performed on a 1.5-tesla magnetic resonance scanner using a standard imaging protocol, including T2-weighted turbo spin-echo imaging in the axial plane, fluid-attenuated inversion recovery (FLAIR) sequence in the coronal plane, T1-weighted spin-echo imaging in the sagittal plane, and three-dimensional short-echo time-of-flight MRA of the circle of Willis. In the <2-year age group, the axial T2-weighted imaging was replaced by a double-echo short tau inversion recovery imaging sequence, and the coronal FLAIR sequence was not acquired. Catheter cerebral arteriography was performed at the clinician's discretion. Blood was taken prior to cerebral arteriography to avoid catheter-induced endothelial injury. All children underwent standardized investigations for AIS risk factors (online suppl. material; for all online material, see www.karger.com/doi/10.1159/000381963). The patients were categorized into 2 groups according to AIS recurrence (>1 week after the initial clinical event). We recruited 25 healthy controls aged 11.5 years (range: 2–16; 7 males) undergoing minor surgical procedures (preoperative blood samples obtained) with no identifiable chronic illnesses.

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Table 1. Study population characteristics

Demographics	AIS recurrence (n = 10)	AIS no recurrence (n = 25)	p value
Sex (M:F)	6:4	16:9	1
Median age, years	8.2 (0.9–15.4)	9.4 (2.7–17.4)	0.681
Median time from event to evaluation, months	7 (6–13)	11 (6–24)	0.1
Clinical features			
Focal neurological deficit	10 (100)	25 (100)	1.0000
Diffuse neurological deficits	7 (70)	2 (8)	0.0005
Sebire et al. [6] classification of cerebral arteriopathy	n = 3 arterial dissection (30) n = 1 PACNS (10) n = 1 PVA (10) n = 1 moyamoya (10) n = 4 unclassified (40)	n = 16 TCA/FCA (64) n = 4 arterial dissection (16) n = 3 PVA (12) n = 2 moyamoya (8)	n/a
Median ESR, mm/h (normal range: 0–10)	5.4 (1–12)	6.6 (1–28)	0.561
Median CRP, mg/l (normal range: <10)	5 (3–16)	5 (3–6)	0.873
MRI			
Multifocal and bilateral lesions	9 (90)	5 (20)	0.0002
MRA			
Multifocal and bilateral lesions	9 (90)	5 (20)	0.0002
Anterior and posterior circulation	7 (70)	2 (8)	0.0005
Collaterals	4 (40)	2 (8)	0.0400
Treatment			
Aspirin	10 (100)	25 (100)	1
Anticoagulation (heparin/warfarin)	3 (30)	5 (20)	0.6614
Corticosteroids	4 (40)	0 (0)	0.0040
Cyclophosphamide	2 (20)	0 (0)	0.0756
Surgical revascularization	2 (20)	4 (16)	1

Figures in parentheses are percentages or ranges. Focal neurological deficits included hemiparesis, facial weakness and hemisensory loss. Diffuse neurological deficits included neurocognitive dysfunction, personality changes and concentration difficulties. M = Male; F = female; TCA = transient cerebral arteriopathy; FCA = focal cerebral arteriopathy; PACNS = primary angiitis of the central nervous system; ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; MTHFR = methylene-tetrahydrofolate reductase; PVA = post-varicella arteriopathy; n/a = not applicable. p values were calculated using Fisher's exact test for categorical data and the Mann-Whitney U test for continuous variables. p values <0.05 (two-sided) were regarded as significant.

Flow Cytometric Identification of CD34+/KDR+ Peripheral Blood Mononuclear Cells

CD34+/KDR+ peripheral blood mononuclear cells (PBMC) were identified using flow cytometry as previously described [2]. Briefly, PBMC were isolated by density centrifugation (Lymphoprep™, Axis Shield, Dundee, UK). After purification with 3 washing steps, the cells were resuspended at a concentration of 2×10^6 cells/ml and 50 µl of Fc receptor blocking agent added per millilitre before incubating with KDR (clone KDR2, conjugated with biotin followed by streptavidin FITC; Sigma-Aldrich) and CD34 (clone 8G12, PerCP-conjugated; Becton Dickinson) antibodies. While assessing EPC numbers, we adjusted for their low event rate in flow cytometry by using IgG isotype controls for all fluorochromes (BD Biosciences). After washing, we counted the cells with FACSCalibur (BD Biosciences) and gated the mononuclear cells identifying CD34+/KDR+ cells.

Colony-Forming Unit Counts

EPC colony-forming units (CFUs) were assessed as previously described [3, 8]. Briefly, PBMC were isolated by density centrifugation (Lymphoprep). After purification with 3 washing steps, 2×10^6 PBMC were plated on fibronectin-coated 24-well plates. The cells were

cultured and maintained in EGM-2 culture medium supplemented with growth factors as per the manufacturer's recommendations (PromoCell), plus 20% foetal calf serum and 40 ng/ml of vascular endothelial growth factor. After 4 days of culture, the non-adherent cells were removed by washing with phosphate-buffered saline. The culture medium was changed to maintain the cells in culture until day 7. CFUs were defined as a central core of rounded cells surrounded by elongating and spindle-shaped cells and were counted after 7 days in culture. Colonies were counted manually in a minimum of 2 wells of each 24-well plate by 2 independent observers who were unaware of clinical profiles, and the results were expressed as average numbers of CFUs per well (online suppl. fig. 1). In selected samples, the endothelial phenotype was confirmed using endothelial-specific indicators, i.e., uptake of DiI-LDL, staining for UEA-1 lectin as previously reported [3, 8].

Matrigel Plate Assay

In 5 subjects per group, Matrigel assays were employed to examine the ability of putative EPC to incorporate into endothelial capillary networks. Growth factor-reduced Matrigel Matrix (Becton Dickinson Labware) was thawed and placed in 96-well plates at 37°C for 30 min to allow solidification. Putative EPC were labelled with 2 μg/ml of DiI-LDL to distinguish them from human umbilical vein endothelial cells (HUVEC) and were then coplated (3,000 cells/well) with HUVEC (10,000 cells/well) on top of a solidified Matrigel layer and cultured at 37°C for 18–20 h with EGM-2 medium. Incorporation of EPC into capillary networks of HUVEC on Matrigel was examined with fluorescence microscopy. Five independent fields were assessed for each well and the mean numbers of EPC incorporated into the HUVEC capillary networks was determined (online suppl. fig. 2).

Brain-Derived Neurotrophic Factor

Blood was collected into 3.2% trisodium citrate and centrifuged twice at 5,000 *g* for 5 min each to obtain platelet-poor plasma. BDNF levels were measured by sandwich ELISA using a commercially available kit from RayBio Systems Europe.

Statistical Analysis

Numeric results were summarized as median and range. The Kruskal-Wallis test followed by the Mann-Whitney U test was applied to compare experimental laboratory markers and Fisher's exact test for categorical data. Binary logistic regression examined the relationship between numbers of CD34+/KDR+ cells and recurrence. p values <0.05 (two-sided) were regarded as significant. Statistical analysis was performed using SPSS version 17.

Results

Patient Characteristics

We studied 35 children aged 12 years (range: 5–16.5; 9 males). These were a subgroup of the 46 previously described patients from a linked study [1], where sufficient blood was available to undertake the aforementioned assays. The median body mass index was 15 (range: 14–18). None of the children had hyperlipidaemia, arterial hypertension or received statins. Clinical characteristics of the study populations are summarized in table 1. Ten children had AIS recurrence and 25 had a single event. Evaluation took place at a median of 7 months (range: 6–13) following recent AIS for those children with AIS recurrence compared to 11 months (range: 6–24) for those without recurrence (p = 0.1000). All those with recurrence had progression of previously identified arteriopathy; they more commonly had diffuse neurological deficits (p = 0.0005) and multifocal, bilateral lesions on MRI (p = 0.0002).

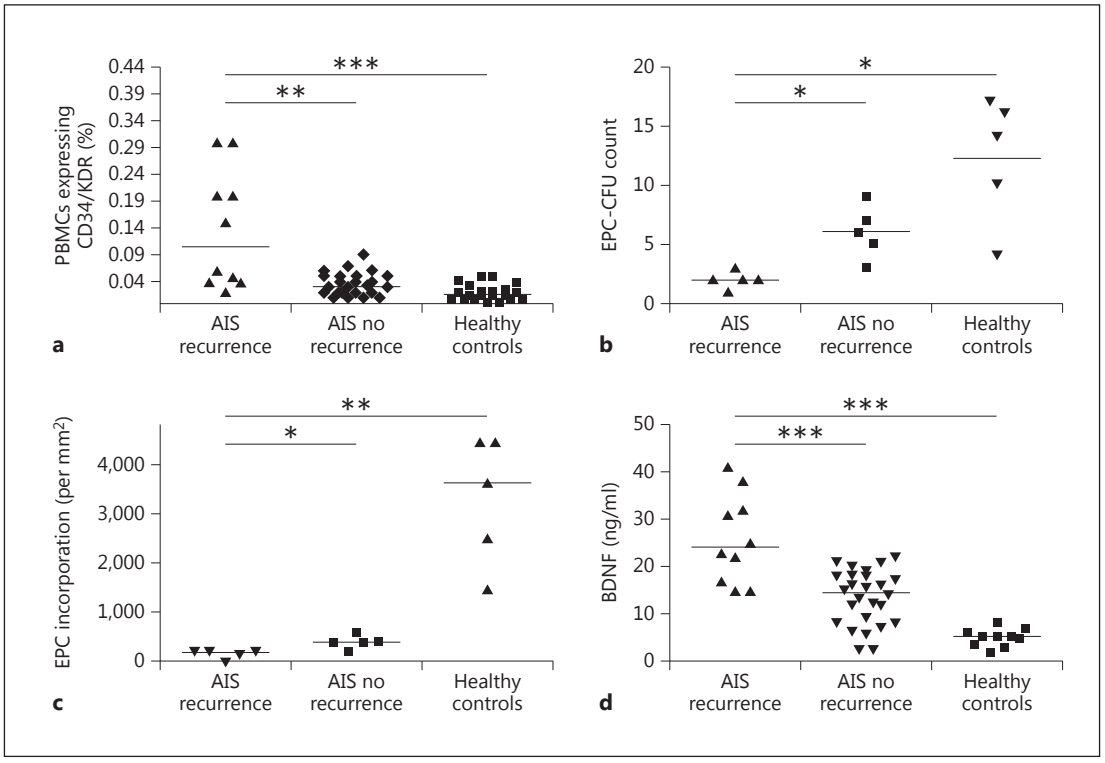

Fig. 1. Circulating EPC and BDNF in children with AIS. **a** CD34+/KDR+ cell counts were significantly higher in patients with AIS recurrence compared to those with no recurrence (p = 0.005) and controls (p = 0.0002). **b** EPC-CFUs were reduced in children with AIS recurrence compared to children with no recurrence (p = 0.04) and controls (p = 0.03). **c** Incorporation of EPC into HUVEC vascular networks in Matrigel was decreased in recurrent AIS compared to non-recurrent AIS (p = 0.01) and controls (p = 0.007). **d** Levels of BDNF were increased in children with recurrence compared to those with a single event (p = 0.0008) and controls (p = 0.0002). * p < 0.05; ** p < 0.005; *** p < 0.0005, with Mann-Whitney U test.

Circulating CD34+/KDR+ Cells Are Increased but EPC Function Is Impaired in Recurrent AIS

CD34+/KDR+ cell counts were significantly higher in patients with AIS recurrence (0.1% of gated PBMC, range: 0.02–0.3) compared to those with no recurrence (0.03% of gated PBMC, range: 0.01–0.09, p = 0.005; controls: 0.01% of gated PBMC, range: 0–0.05, p = 0.0002; fig. 1a). In binary logistic regression analysis, after correction for age/gender/time to sampling from recent AIS, higher numbers of circulating CD34+/KDR+ cells were significantly related to recurrence (odds ratio: 1.88; 95% confidence interval: 1.1–2.1, p = 0.03). In contrast, EPC-CFUs were reduced in children with AIS recurrence (2 CFUs/well, range: 1–2) compared to children with no recurrence (6 CFUs/well, range: 3–9, p = 0.04; controls: 14 CFUs/well, range: 4–17, p = 0.03; fig. 1b). Furthermore, incorporation of EPC into HUVEC vascular networks in Matrigel was decreased in children with recurrent AIS (193 cells/mm^2, range: 0–210) compared to those with non-recurrent AIS (396 cells/mm^2, range: 202–596, p = 0.01; controls: 3,636 cells/mm^2, range: 1,478–4,478, p = 0.007; fig. 1c).

Levels of BDNF Are Higher in Children with Recurrent AIS

Levels of BDNF were increased in children with recurrence (24 ng/ml, range: 15–41) compared to those with a single event (14.50 ng/ml, range: 2.4–22, p = 0.0008; controls: 5 ng/ml, range: 1.8–8.1, p = 0.0002; fig. 1d).

Discussion

We have demonstrated important differences in endothelial repair mechanisms between children with recurrence of AIS associated with cerebral arteriopathy. While CD34+/KDR+ cells were increased in the recurrence group, the angiogenic function of monocytic EPC was impaired. These novel exploratory observations could provide an important insight into the pathogenesis of childhood AIS.

Mobilization of CD34+/KDR+ cells in response to ongoing tissue ischaemia could be an attempt to mediate vascular repair, and hence higher levels of these cells may reflect the extent of ischaemic brain injury. In conditions of acute vascular ischaemia, an increase in the number of circulating CD34+/KDR+ cells has been observed in association with a concomitant increase in levels of EPC-mobilizing factors, such as vascular endothelial growth factor and stromal cell-derived factor-1, suggesting a direct causal relationship [4]. In addition, recent studies proposed that CD34+/KDR+ cells are generated from CD34+ cells at platelet-rich sites of vascular injury, thus reflecting the plasticity of CD34+ cells and their capacity to respond to and be taken up into ischaemic lesional tissue [9, 10]. These responses to ischaemic insult may still be relevant to patients with recurrent AIS sometime after the initial insult, as we have detected ongoing endothelial injury many months after an acute clinical event [1].

Our preliminary observations are in keeping with similar studies in systemic vasculitis that suggest that attempts at endothelial repair may be thwarted (for reasons as yet unidentified) in children with the most severe vasculitis [11]. The same may also be true of children with recurrent AIS. As BDNF mobilizes haematopoietic progenitor cells and promotes revascularization in ischaemic injury [5, 12], the increased levels we noted may represent a continuous effort to support endothelial repair and neuronal survival in children with recurrent AIS. It is uncertain which factors might result in the decreased circulatory EPC angiogenic function we observed. Possibilities include: increased uptake from the circulation of the most angiogenic active EPC into areas of cerebral ischaemia, inherently impaired angiogenic potential in children prone to AIS recurrence, or another as yet undefined neuro-immunological pathway secondary to brain injury adversely influencing angiogenic responses. Larger longitudinal studies of CD34+/KDR+ cells and EPC function over time and in response to therapy (aspirin, anticoagulation and/or immunosuppression) are now urgently needed.

We regard our findings as exploratory since the cross-sectional nature of this small study precludes any causal effect to be determined. In addition, although treatments were within recommended clinical guidelines, there remained some therapeutic heterogeneity. The timing of sampling from the index AIS was not standardized in our study. We did, however, perform binary logistic regression analysis after correction for this potentially important confounder and found that higher numbers of circulating CD34+/KDR+ cells were still significantly related to AIS recurrence. The effect of immunosuppression on endothelial repair responses was not formally assessed in our study; future longitudinal studies of EPC changes with time after the initial cerebrovascular event and in response to therapy would be of considerable interest in paediatric AIS and could enhance our understanding of the effect of immunosuppression on EPC biology. We were underpowered to perform formal subgroup analyses of the various arteriopathies studied herein; larger multicentre prospective studies to establish the specificity of these vascular repair responses across different vascular pathologies leading to AIS are now warranted.

In summary, children with recurrent AIS associated with cerebral arteriopathy had increased numbers of circulating CD34+/KDR+ cells but impaired EPC angiogenic function. Whether this contributes to the vascular dysfunction associated with AIS recurrence now requires further investigation.

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Disclosure Statement

The authors declare no conflict of interest.

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