Increased Levels of Circulating Endothelial Progenitor Cells in Patients With Moyamoya Disease

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Background and Purpose—Chronic cerebral ischemia leads to higher risk for strokes attributable to insufficient collateralization, resulting from inadequate capacity for arteriogenesis and angiogenesis. Patients with Moyamoya disease (MMD) have similar transient ischemic attack frequencies compared to patients with chronic cerebral ischemia with other etiologies, but a strong capacity for arteriogenesis and angiogenesis. The mechanisms involved in the upregulation of the arteriogenesis and angiogenesis in MMD still remain unknown. In the present study we investigated if circulating endothelial progenitor cells are increasingly mobilized during MMD.

Methods—Twenty MMD patients, 8 patients with atherosclerotic cerebrovascular disease, and 15 healthy individuals were included in this study. Peripheral blood mononuclear cells were isolated by Ficoll density gradient centrifugation and circulating endothelial progenitor cells were characterized by triple staining using antibodies against CD133, CD34, and vascular endothelial growth factor receptor-2. Serum concentrations of vascular endothelial growth factor and granulocyte-macrophage colony-stimulating factor were determined by enzyme-linked immunosorbent assay.

Results—In MMD patients the number of circulating endothelial progenitor cells was significantly higher than in atherosclerotic cerebrovascular disease patients \( (P<0.002) \) and healthy controls \( (P<0.0001) \). Serum vascular endothelial growth factor concentrations in MMD patients and in atherosclerotic cerebrovascular disease patients were significantly higher compared to those in healthy controls \( (P<0.0001) \). Similar findings were observed for granulocyte-macrophage colony-stimulating factor. An inverse correlation between circulating endothelial progenitor cell numbers and serum levels of vascular endothelial growth factor \( (r=-0.53; \ P<0.02) \) was found in the MMD group.

Conclusion—Our results show increased circulating endothelial progenitor cell numbers in MMD, which may play a role in the increased arteriogenesis and angiogenesis in MMD. Moreover, our results suggest that increased circulating endothelial progenitor cell mobilization in MMD may not be entirely mediated by vascular endothelial growth factor or granulocyte-macrophage colony-stimulating factor. \( \text{(Stroke. 2009;40:432-438.)} \)

Key Words: cerebral revascularization ■ circulating endothelial progenitor cells ■ Moyamoya disease ■ vascular endothelial growth factor

Moyamoya disease (MMD) is a rare cerebrovascular disease that is caused by a spontaneous and progressive occlusion of the intradural internal cerebral artery and the proximal anterior cerebral artery and middle cerebral artery, but the etiology remains unknown.\(^1\) On histology, the supraclinoid internal cerebral artery and proximal anterior cerebral artery and middle cerebral artery have intimal hyperplasia, thereby causing progressive occlusion.\(^2\) These unremitting changes of the basal cranial vessels lead to repeated hemodynamic stroke. MMD is a unique occlusive disease because it occurs at a specific location and has characteristic transdural anastomoses as well as proliferation of the lenticulostriate perforating arteries, which are referred to as “Moyamoya vessels.” The resulting vascular blush observed on angiograms of patients with MMD gave this disease its name, which means in Japanese “puff of smoke.” The increased arteriogenesis and angiogenesis are assumed to be a reaction to the progressive ischemia.\(^3\)

In general, chronic cerebral ischemia leads to a higher risk for strokes attributable to insufficient collateralization. This is attributable to an inadequate capacity for arteriogenesis and angiogenesis. Chronic cerebral ischemia can be the result of various diseases (eg, arteriosclerosis) or the result of arterial obstruction. MMD patients, in contrast to patients with chronic cerebral ischemia with other etiologies, have a strong capacity for arteriogenesis and angiogenesis. Just recently,
we could show that cortical microvascularization in MMD is characterized by significantly increased microvascular density and microvascular diameter, leading to increased microvascular surface. But because both patient groups have similar TIA frequencies and are thereby likewise limited, the increased ischemia cannot be the source of the augmented arteriogenesis and angiogenesis in MMD. Therefore, other mechanisms must be involved in the upregulation of the arteriogenesis and angiogenesis.

Until recently, the adult neovascularization was thought to arise only through angiogenesis, the mechanism by which new blood vessels form from preexisting vessels through endothelial cell migration and proliferation. However, recent studies have provided evidence that postnatal neovascularization can also arise though vasculogenesis, a process by which endothelial progenitor cells (EPC) are recruited and differentiate into mature endothelial cells to form new blood vessels.

EPC are bone marrow-derived cells and have the propensity to differentiate into mature endothelial cells. Their phenotype is characterized by the expression of the specific hematopoietic marker CD34, the stem cell marker CD133, and the endothelial marker vascular endothelial growth factor receptor-2 (VEGFR-2). Mobilization of EPC from the bone marrow critically depends on the activation of metalloproteinases and upregulation of adhesion molecules. This is most likely mediated by soluble factors such as vascular endothelial growth factor (VEGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF). Serum concentrations of these factors dramatically increase under pathological conditions, concomitantly with an increase in the number of EPC.

To understand the mechanisms of the augmented arteriogenesis and angiogenesis in MMD patients, we investigated in the present study if EPC are increasingly mobilized during MMD and if there is a correlation between EPC and the growth factors VEGF and the proinflammatory cytokines GM-CSF and IL-8.

Materials and Methods

Subjects
Patients with MMD (n=20) were enrolled consecutively over a 2-year time period (2004–2006) before their revascularization operation. Clinical data of each patient were recorded.

For controls we recruited 8 patients with atherosclerotic cerebrovascular disease (ACVD) and 15 healthy volunteers from our laboratory staff, hereafter referred to as healthy controls. The ACVD patients had either an internal cerebral artery occlusion or stenosis, or a middle cerebral artery occlusion from atherosclerotic origin and had similar TIA frequencies as the MMD patients.

MMD and ACVD patients underwent conventional catheter digital subtraction angiography and morphological imaging by MRI. Functional regional cerebral blood flow measurements were made at rest and after the administration of acetazolamide (15 mg/kg body weight) by stable xenon CT (DDP Inc). Cerebrovascular reserve capacity was calculated as described elsewhere in detail. To obtain cerebral blood flow values for the region analyzed by indocyanine green video-angiography, regions of interest on the xenon CT images were located in the vascular territory undergoing investigation (eg, vascular territory of the middle cerebral artery).

Blood Sampling
In MMD patients, ACVD patients, and healthy controls, 25 mL of blood was obtained by insertion of a 20-gauge cannula intravenously and collected in tubes containing sodium citrate (0.105 mol/L) as anticoagulant. The initial 5-mL blood was discarded to minimize endothelial cell contamination from the puncture wound of the vascular wall.

Flow Cytometry
All blood samples were processed within 1 hour after collection. Peripheral blood mononuclear cells were prepared by gradient centrifugation using Ficoll-Hypaque (Amersham Biosciences). The expression of cell-surface antigens was determined by 4-color immunofluorescence-staining as described previously. Briefly, 100 μL of peripheral blood mononuclear cell (containing 1×10⁶ cells) were incubated with 10 μL ofFc receptor–blocking reagent (Miltenyi Biotec) for 10 minutes to inhibit nonspecific bindings. Hereafter, the cells were incubated at 4°C for 30 minutes with 10 μL phycoerythrin–conjugated antihuman CD133 mAb (Miltenyi Biotec), 10 μL peridinin chlorophyll protein–conjugated antihuman CD34 mAb (BD Biosciences), 10 μL allophycocyanin-conjugated VEGF R² mAb (R&D Systems), and 10 μL fluorescein isothiocyanate–conjugated Annexin V mAb (BD Biosciences). Phycoerythrin-, peridinin chlorophyll protein-, allophycocyanin-, and fluorescein isothiocyanate–conjugated isotype-matched immunoglobulin G1 and immunoglobulin G2a antibodies (DakoCytomation) were used for each patient and measurements and served as negative controls. The cells were washed 3 times to remove unbound antibodies and finally resuspended in 400 μL of fluorescence-activated cell sorting (FACS) solution (BD Biosciences). FACS analysis was performed on a FACS Calibur flow cytometer (BD Biosciences) and the data were analyzed using WinMDI 2.8 software developed by Joseph Trotter at The Scripps Research Institute, La Jolla, California (copyright 1993–1998). A minimum of 500,000 events were collected. FACS analysis of each probe was performed in triplicate. The frequency of EPC in peripheral blood was determined by a 2-dimensional side-scatter/fluorescence dot-plot analysis of the samples after exclusion of Annexin V-positive cells and appropriate gating. The exclusion of Annexin V-positive cells was performed to rule out contamination with apoptotic cells in our positive population. EPC counts are expressed as percentage of total peripheral blood mononuclear cells in each patient or control.

Enzyme-Linked Immunosorbent Assay
The serum concentrations of VEGF, GM-CSF, and IL-8 were assessed using highly sensitive enzyme-linked immunosorbent assay kits (R&D Systems) in triplicate samples obtained from 5 mL of serum. Enzyme-linked immunosorbent assay was performed according to the manufacturer’s instructions.

Statistical Analysis
All data are presented as mean±SEM. Both parametric and nonparametric methods have been used. Analysis of variance and Kruskal-Wallis test have been performed when comparing the 3 groups. When the result was significant, we have performed t test analysis. Nonparametric statistical analysis (Kruskal-Wallis) was especially used to compare the 3 groups regarding GM-CSF, because the concentration in healthy controls was below the detection limit. Correlation analyses (Pearson/Spearman) have been considered for all target variables. P<0.05 was considered to be statistically significant. All analyses have been performed using the SAS system (Version 8.2).

Results
Patient Population
There was no statistical difference in mean age between the MMD group (35.7±15.9 years) and the healthy controls.

Informed consent was obtained from all study subjects. This study was approved by the Ethics Committee of the University of Heidelberg.
(34.8±7.3 years; P<0.82). There was a significant statistical difference in mean age between the ACVD patients (51.4±10.8 years) compared to the MMD group (P<0.02) and the healthy controls (P<0.0002). Relevant characteristics of all MMD and ACVD patients included in this study are summarized in the Table. The functional regional cerebral blood flow measurements in our patients demonstrated that both of our patient groups had similar extents of impaired reserve capacity and therefore strongly suggest that the strokes were hemodynamic. There was no evidence of hemorrhages in our patients. Furthermore, both patient groups had similar disease distribution and symptomatology (as seen in the Table) and, therefore, the same ischemic stimulus.

Hematopoietic Stem Cells
The percentage of the hematopoietic stem cells, defined as positive staining for CD34 and CD133, was low in healthy controls (0.09±0.03%). Whereas it was already significantly increased in ACVD patients (0.18±0.08; P<0.02), there was still a significant increase of CD34+/CD133+ cells in MMD patients (0.29±0.14%; P<0.0001; Figure 1).

Endothelial Progenitor Cells
In addition, the percentage of VEGFR-2+ cells within the population of CD34+/CD133+ cells was measured. In healthy controls only 17±5% of CD34+/CD133+ cells stained positive for VEGFR-2. Both in ACVD (20±0.7%) and in MMD patients (36±17%) this was significantly higher. This corresponds to a percentage of cEPC (CD34+/CD133+/VEGFR-2+ cells) in the total peripheral blood mononuclear cell fraction: 0.02±0.01% in healthy controls, 0.03±0.02% in ACVD patients, and 0.11±0.09% in MMD patients (Figure 2).

Circulating Growth Factors and Proinflammatory Cytokines
The measurement of the circulating growth factors and proinflammatory cytokines showed the following findings. In healthy controls, the mean serum VEGF concentration was low (35.9±20.4 pg/mL). In both patient groups, ie, ACVD (352.1±211.6 pg/mL; P<0.004) and MMD patients (299.4±131.1 pg/mL; P<0.0001), there was a significant increase in serum VEGF concentrations compared to that in healthy controls (Figure 3, A). Interestingly, there was no significant difference in serum VEGF concentrations between ACVD and MMD patients (P<0.43). Similar to VEGF, GM-CSF-concentrations were significantly increased in ACVD (0.39±0.17 pg/mL; P<0.0005) and MMD patients (0.27±0.4 pg/mL; P<0.04) compared to healthy controls (0.07±0.09 pg/mL; Figure 3B). There was no significant difference in serum GM-CSF concentrations between ACVD and MMD patients (P<0.26). The mean serum IL-8 concentration did not show a significant difference between the 3 groups (healthy controls, 17.05±10.76 pg/mL; ACVD, 17.21±11.77 pg/mL; MMD, 21.73±15.16).

Correlations
No significant correlation between cEPC numbers and serum levels of VEGF (r=0.09; P<0.56) and GM-CSF (r=−0.02; P<0.89) could be detected for all the groups. Unexpectedly, we found an inverse correlation in the MMD group between cEPC numbers and serum levels of VEGF (r=−0.53; P<0.02). Consequently, high numbers of cEPC were correlated to low levels of VEGF. We also found an inverse correlation between cEPC numbers and age for all the groups (r=−0.32; P<0.03). Consequently, high numbers of cEPC were correlated to a young age. However, we could not demonstrate a significant correlation between cEPC numbers and age in the respective groups.

Statins
We next investigated if changes in the number of cEPC were associated with the use of statins, because statins are reported to increase the mobilization of EPC.17 In the MMD group we did not observe a significant difference in EPC numbers between patients treated with statins and patients without statin treatment (P<0.27). Similar to EPC, there was also no significant difference in serum VEGF (P<0.44) and serum GM-CSF concentrations (P<0.73) between patients treated with statins and patients without statin treatment.

Discussion
The present study demonstrated an increased mobilization of cEPC in MMD patients compared with ACVD patients and healthy individuals. Serum concentrations of VEGF and GM-CSF were significantly increased in MMD and ACVD patients compared to healthy controls, but there was no significant difference in serum concentrations of these factors between MMD and ACVD patients. Within the MMD group we found an inverse correlation between cEPC numbers and serum levels of VEGF. We did not observe a significant difference in EPC numbers or serum concentrations of VEGF and GM-CSF between patients treated with statins and patients without statin treatment.

To our knowledge, this is the first study to detect cEPC, defined as CD133+/CD34+/VEGFR-2+ cells, in peripheral blood of MMD patients. Recently, Yoshihara et al18 have observed an increase in circulating CD34+ cells in patients with angiographic evidence of Moyamoya-like vessels compared with control subjects and with patients with major cerebral artery occlusion (or severe stenosis) but without angiographic evidence of Moyamoya-like vessels, suggesting a correlation of CD34+ cells with neovascularization at human ischemic brain. But, in their study, they have not used the important cell surface markers VEGFR-2 and CD133, which together with CD34 characterize EPC. CD34 is also found on mature endothelial cells or monocytes and therefore cannot alone characterize bone marrow-derived immature cells as postulated by them. Furthermore, they have only included 4 patients with angiographic evidence of Moyamoya-like vessels.

We have used standard flow cytometry to detect cEPC,19 although different approaches have been used to quantify EPC in a variety of patients and controls.20–23 Flow cytometry and colony-forming assays are the 2 main methods used for counting and functional assessment of endothelial progenitor cells. Both of these techniques, however, have serious limitations. Although endothelial cell colony-forming units are widely accepted as a surrogate as an estimate of EPC number...
Table. Summary of Patients Included in Our Study

<table>
<thead>
<tr>
<th>Case</th>
<th>Age, Gender</th>
<th>Diagnosis</th>
<th>Presentation</th>
<th>Angiographic Findings</th>
<th>Cardiovascular History</th>
<th>Smoker</th>
<th>cEPCs, % of PBMCs</th>
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<tbody>
<tr>
<td>1</td>
<td>45, F</td>
<td>MMD</td>
<td>L TIA</td>
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<td>2</td>
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<tr>
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</tr>
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</tr>
<tr>
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<td>Bilateral ICA intracranial stenosis</td>
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<td>No</td>
<td>0.13</td>
</tr>
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<td>Hypertension</td>
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<td>Hypertension</td>
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<td>DM</td>
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<td>R MCA stenosis, L ICA bifurcation stenosis</td>
<td>Hypertension, DM, hyperlipidemia</td>
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<tr>
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<td>L MCA stroke, L hemiparesis</td>
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<td>L ICA stenosis, L MCA stenosis</td>
<td>Hypertension, hyperlipidemia</td>
<td>No</td>
<td>0.04</td>
</tr>
</tbody>
</table>

ACA indicates anterior cerebral artery; DM, diabetes mellitus; F, female; ICA, internal carotid artery; L, left; M indicates male; MCA, middle cerebral artery; MMD, Moyamoya disease; PBMC, peripheral blood mononuclear cells R, right.
and function in cell culture, some important limitations may restrict the assumption that endothelial cell colony-forming units reflect EPC numbers accurately. Shantsila et al., comparing in their study colony-forming units to flow cytometry, described that endothelial colony-forming unit counts represent the cumulative characteristics of EPC quantity and their functional characteristics, and cannot be reliably used for the estimation of EPC numbers in peripheral blood or the bone marrow. They conclude by suggesting that flow cytometry may be the more optimal technique for EPC quantification. Although the exact phenotype of cEPC is still controversially discussed, the presence of CD34, CD133, and VEGFR-2 seems to be well-supported and therefore used in this study. These markers are easily detected using flow cytometry, which essentially estimates the number of cells from peripheral blood, bone marrow, or other tissues that possess a specific cell-type profile of surface markers.

In our study we could observe an increased mobilization of cEPC in MMD patients compared with ACVD patients, suggesting that the recruitment of EPC may play an important role in the augmented arteriogenesis and angiogenesis in MMD. Ischemia, resulting in endogenous production of VEGF, is especially known to be an important stimulus in EPC mobilization. Although MMD and ACVD patients in our study had similar TIA frequencies, ACVD patients had only slightly increased EPC numbers. Therefore, the increased ischemia, occurring in both conditions, cannot be the only source of the increased EPC numbers in MMD.

Many factors are described to have important roles for mobilization of EPC. Among them are growth factors, such as VEGF, placental growth factor, erythropoietin, and angiopoietin-1, proinflammatory cytokines, such as GM-CSF and granulocyte-colony stimulating factor, chemokines, such as stromal cell-derived factor-1, hormones, such as estrogens and lipid-lowering and anti-diabetic drugs, and physical activity.

### Figures

**Figure 1.** Percentage of circulating hematopoietic progenitor cells. FACS analysis from CD34/CD133-positive cells in the peripheral blood mononuclear cell fraction of healthy volunteers (n=15), ACVD patients (n=8), and MMD patients (n=20). Significant differences were found between all 3 groups. The results are expressed as mean simple linear regression±SEM. *Significant differences vs healthy controls. **Significant differences vs ACVD patients and healthy controls. P<0.05 was considered to be statistically significant.

**Figure 2.** Upregulation of cEPC. FACS analysis from CD34/CD133/VEGFR-2-positive cells in the peripheral blood mononuclear cell fraction of healthy volunteers (n=15), ACVD patients (n=8), and MMD patients (n=20). Significant differences were found between all 3 groups. The results are expressed as mean simple linear regression±SEM. *Significant differences vs healthy controls. **Significant differences vs ACVD patients. P<0.05 was considered to be statistically significant.

**Figure 3.** Upregulation of growth factors in serum. VEGF (A), GM-CSF (B), and IL-8 (C) concentrations in serum of healthy volunteers (n=15), ACVD patients (n=8), and MMD patients (n=20). The results are expressed as mean simple linear regression±SEM. *Significant differences only vs healthy controls. P<0.05 was considered to be statistically significant.
In our study we could show increased serum concentrations of VEGF and GM-CSF in MMD and ACVD patients compared to healthy controls. But we could not observe a significant difference in serum concentrations of these growth factors between the 2 patient groups, suggesting that VEGF and GM-CSF might not be the key factors stimulating EPC mobilization in MMD. This was further supported by our finding of an inverse correlation between cEPC numbers and serum levels of VEGF. VEGF has been shown to be involved in vasculogenesis and vascular permeability in different intracranial lesions. In ischemic disease without corresponding MMD, ischemia leads to cerebral angiogenesis by the release of VEGF.32–37 Recently, Sakamoto et al31 could demonstrate that meningevalcellularity and VEGF expression are significantly increased in dura mater of patients with MMD compared to control patients. Molecular studies have shown that fibroblast growth factor-2 is thought to play a major role in observed angiogenesis by demonstrating elevated levels in the cerebrospinal fluid and through immunohistochemistry postmortem of the involved vessels in MMD patients. However, unlike tumor angiogenesis, the role of VEGF may be minimal, as evidenced by variable levels of VEGF in the cerebrospinal fluid.32–37 In addition IL-8, platelet-derived growth factor, endothelial growth factor, and transforming growth factor-β were not elevated in the cerebrospinal fluid, suggesting a different signaling pathway and mechanism of angiogenesis.3 Therefore, our findings were not surprising to us.

Statin treatment has also been shown to increase the number of cEPC.17 However, recently, a reduced number of cEPC was observed in patients with coronary artery disease associated with long-term statin treatment.38 We did not observe a significant difference in EPC numbers or serum concentrations of VEGF and GM-CSF between patients treated with statins and patients without statin treatment. But in the MMD group only 9 out of 20 MMD patients were treated with statins, and the length of usage (from 3 week until 12 months) and the dosage varied among them. Therefore, we cannot derive any conclusion about the influence from statin treatment on EPC mobilization in MMD from our study.

Increasing age was shown to be associated with reduced levels of circulating EPC in patients with coronary artery disease.39 Because we observed a significant statistical difference in mean age between MMD and ACVD patients, it could be suggested that age-dependent impairment of EPC mobilization could be responsible for the lower EPC numbers in ACVD patients. This would be supported by the fact that we found an inverse correlation between cEPC numbers and age for all the groups. There has been speculation about the hypothesis that aging may result from primary progenitor cell dysfunction or exhaustion.40–41 However, it has been demonstrated that senescent bone marrow cells retain the capacity to repopulate depleted bone marrow over multiple successive generations.42 This suggests that progenitor cells remain fully functional even with aging, although there is plenty of evidence to the contrary.43 Furthermore, it has been recently demonstrated that impaired neovascularization resulted from defects in the response of aged tissue to hypoxia and not from intrinsic defects in EPC function or decreased total numbers.44 Because of these contradicting observations, we cannot exclude a possible influence of age on the reduced levels of circulating EPC in ACVD patients.

Although our study suggest that the recruitment of EPC may play an important role in the augmented arteriogenesis and angiogenesis in MMD, the functional role of EPC and its contribution to neovascularization is not yet understood. EPC are described to contribute to neovascularization either by differentiating into mature endothelial cells or by paracrine effects, which stimulate angiogenic activity of resting mature endothelial cells, leading to their proliferation and sprouting.25,45 A recent study46 gives some indication that there might be a specific molecular pattern for the mobilization and homing of EPC in MMD, endowing it with its own molecular signature. Therefore, the exact mechanisms underlying the augmented arteriogenesis and angiogenesis in MMD still have to be studied.

Conclusion

From the observations in our study we conclude that increased amount of EPC are recruited in the course of MMD, most likely for vasculogenesis and paracrine effects, which stimulate angiogenic activity of resting mature endothelial cells. Our data also indicate that VEGF and GM-CSF may not be the main mediators for EPC mobilization in MMD. Future studies are required to examine the key factors responsible for EPC mobilization in MMD and whether EPC numbers will decrease after bypass surgery, because chronic ischemia and TIA will improve.

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Disclosures

None.

References

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