Intercellular Adhesion Molecule-1 Is Upregulated in Ischemic Muscle, Which Mediates Trafficking of Endothelial Progenitor Cells

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Background—Trafficking of transplanted endothelial progenitor cells (EPCs) to an ischemic organ is a critical step in neovascularization. This study was performed to elucidate the molecular mechanism of EPC trafficking in terms of adhesion molecules.

Methods and Results—Using murine hindlimb ischemia model, we examined expressions of E-selectin, intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and platelet-endothelial cell adhesion molecule-1 (PECAM-1) in ischemic muscle by immunofluorescence. ICAM-1 was overexpressed in ischemic muscle compared with nonischemic muscle, whereas expressions of E-selectin, VCAM-1, and PECAM-1 did not show that much difference. ICAM-1 was also upregulated by hypoxia in murine endothelial cells (ECs) as assessed by immunoblot and flow cytometry. EPCs were attached to ECs specifically through ICAM-1/β2 integrin interaction in vitro. When EPCs were labeled with fluorescent dye or radioisotope (Tc-99m-HMPAO) and systemically administrated in vivo, EPCs preferentially homed to ischemic muscle. By blocking ICAM-1, EPCs entrapment to ischemic limb in vivo was significantly reduced and neovascularization induced by EPC transplantation was attenuated.

Conclusions—ICAM-1 is upregulated by ischemia, and this is closely associated with EPCs entrapment to ischemic limb. Our findings suggest that ICAM-1 expression might be important in regulating the process of neovascularization through its ability to recruit EPCs. (Arterioscler Thromb Vasc Biol. 2006;26:1066-1072.)

Key Words: angiogenesis • adhesion molecules • endothelium • endothelial progenitor cells • ischemia

Neovascularization is a physiological response to ischemia that often produces insufficient collateral vessels to resolve its symptoms or signs.\(^1\) In ischemic heart disease, therapeutic angiogenesis leading to enhanced collateral vessels may be an ideal physiological therapeutic modality.\(^2\) At present, many trials with stem cells or endothelial progenitor cells have been conducted or are undergoing investigation to test this theory.\(^3-6\)

Because the number of circulating endothelial progenitor cells (EPCs) may limit the ultimate magnitude of therapeutic angiogenesis, strategies based on administering ex vivo expanded populations of EPCs, harvested from the patient’s circulating blood, appear promising.\(^3-5\) However, increased recruitment and specific lodging of EPCs into ischemic tissues may be another target. We may be able to increase the efficiency of therapeutic angiogenesis if we could enhance selective lodging, or “homing,” of EPCs to the recipient’s ischemic organ.

Recently, several molecules have been reported to be related with the homing process.\(^7-10\) However, these are not molecules that have been confirmed to be expressed in the ischemic tissue but rather investigational molecules, locally injected to the tissues, or those expressed in tumors.\(^10\) To maximize the homing of EPCs to the ischemic tissue, we need to elucidate the key molecules that take part in the physiological or pathologic process after ischemia. Therefore, we investigated the change in expression of adhesion molecules in endothelial cells after ischemia, and whether these molecules are involved in the homing of EPCs to ischemic tissue.

Materials and Methods

The basic design of the study was to: (1) identify a probable adhesion molecule for the homing of EPCs to ischemic tissues; (2) confirm the interaction of EPCs and endothelial cells through ICAM-1 in vitro adhesion assay; and (3) test whether the expression of intercellular adhesion molecule-1 (ICAM-1) is essential for EPC homing in a hindlimb ischemia model by using monoclonal neutralizing antibody against ICAM-1.

Original received August 18, 2005; final version accepted January 4, 2006.

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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000215001.92941.6c
Mouse Hindlimb Ischemia Model
All procedures were approved by the Institutional Review Board of Seoul National University and were performed in accordance with the Institutional Animal Care & Use Committee of Seoul National University Hospital. Six-week-old C57BL/6J mice (Biogenomics, Korea) were used for all animal experiments. Mice were anesthetized with 50 mg/kg intraperitoneal pentobarbital. To induce muscle ischemia, a unilateral femoral artery was removed as previously described.11,12

Tissue Preparation and Immunofluorescence
One day after unilateral femoral artery excision, mice were euthanized by administering an overdose of sodium pentobarbital. The calf muscle and tibial bone of either an ischemic hindlimb or a nonischemic one were rinsed in phosphate-buffered saline (PBS) to remove excess blood, snap-frozen in liquid nitrogen (LN2), and stored at −80°C. Ten-micrometer-thick histological sections were prepared from snap-frozen tissue samples. Immunofluorescent staining of adhesion molecules was performed using the following antibodies: for ICAM-1 or vascular cell adhesion molecule (VCAM-1), rat monoclonal antibodies against mouse ICAM-1 or VCAM-1 (R&D systems); fluorescein isothiocyanate (FITC)-conjugated rabbit anti-rat immunoglobulin as secondary antibody (DAKO); for E-selectin, goat polyclonal antibody against murine E-selectin (Santa Cruz technology); rat anti-goat immunoglobulin as secondary antibody (DAKO); and for platelet-endothelial cell adhesion molecule-1 (PECAM-1), FITC-labeled PECAM-1 (DAKO).

Western Blot of ICAM-1
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Fluorescence-Activated Cell Sorting
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Mouse Bone Marrow Harvesting and EPC Culture
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Adhesion Studies In Vitro
Cultured EPCs, fibroblasts (negative control) and Jurkat, and leukemic T-lymphocytes (positive control) were labeled with Dil, and then 10^5 cells per well were incubated on VEGF-stimulated (50 ng/mL) MS-1 (murine endothelial cells, ATCC) monolayers in HBSS supplemented with 2 mMol/L calcium and magnesium in the presence of ICAM-1 blocking monoclonal antibody (10 μg/mL; R&D Systems, Minneapolis, Minn) or isotype control antibody (10 μg/mL) for 1 hour. After removing nonadherent cells, the number of Dil-labeled cells bound to the endothelium was quantified under fluorescent microscopy.

EPC Injection
One day after unilateral femoral artery excision, 1 x 10^6 cultured EPCs in 100 μL EBMM-2 media without growth factors were administered systemically by cardiac puncture using an insulin syringe with 27-guage needle. EPCs were tagged with either fluorescent carbocyanine DiI dye (Molecular Probes) or Tc-99m hexamethylpropyleneamine oxime (HMPAO) for tracking and quantification. Ischemia Results in Overexpression of ICAM-1 on the Endothelial Cells In Vivo and In Vitro
In a model of unilateral hindlimb ischemia, immunofluorescence examinations of nonischemic and ischemic muscle showed different patterns of ICAM-1, VCAM-1, E-selectin, and PECAM-1 expression. In ischemic muscles, there was a significantly increased expression of ICAM-1 on ECs of small to medium sized vessels (Figure 1a) and capillaries (Figure 1b) compared with nonischemic muscles (Figure 1c, 1d). Bone marrow and spleen were stained as positive controls (Figure 1e, 1f). In contrast, VCAM-1 expression was observed on the vessel wall in the ischemic muscle (Figure 1g, 1h), which was not different from that in the nonischemic muscle (Figure 1i, 1j). VCAM-1 expression in the bone marrow and spleen was similar to ICAM-1 expression (Figure 1k, 1l). E-selectin was only scantily expressed on small to medium sized vessels in ischemic muscles (Figure 1m) and not found on the capillaries (Figure 1n), which were similar to the findings in nonischemic muscles (Figure 1o, 1p). Bone marrow and spleen, positive controls, showed high expression of E-selectin (Figure 1q, 1r). Although the expression of PECAM-1 was diffusely distributed on the endothelial cells per gram of tissue of each organ after transplantation of radiolabeled EPCs. Before cellular transplantation, EPCs in suspension were washed with PBS and incubated with Dil at a concentration of 2.5 μg/mL in serum-free basal medium for 10 minutes at 37°C or with Tc-99m HMPAO at a concentration of 1 mCi per 1 x 10^7 EPCs for 30 minutes. After PBS washes, the cells were resuspended in EBM-2 medium. Cell labeling and quantitation using Tc-99m HMPAO is a well-established method that showed stable labeling over 15 hours and ≤1% covariance when measured over 10 000 cpm.13 Furthermore, when we reattached and cultured the labeled cells, they showed as good survival and proliferation as unlabeled control EPCs (data not shown).

Harvested organs were weighed, and radioactivity was measured using a gamma counter after correcting for radioactive decay. To compensate for different muscle volumes, we compared radioactivity per milligram of tissue. For the direct counting of homed cells in the hindlimb, the middle belly of the thigh muscles were sectioned at 200-μm intervals. Dil positive cells were counted in each section by a fluorescent microscopy. Total cell number was divided by total muscle area, which was measured using Image-Pro Plus (MediaCybernetics).

Capillary Density and Laser Doppler Perfusion Analysis
For evaluation of the ICAM-1 blocking on therapeutic neovascularogenesis using EPCs, laser Doppler perfusion image analyzer (Moor Instrument, Wilmington, De) was used to record serial blood flow measurements of both blocking and nonblocking groups (n=12, respectively) over the course of 3 weeks after operation as was described in our previous study.13 Medium was also administered into another control group of mice to compare the effect of EPC transplantation. Capillary density in the ischemic muscle of the 3 groups was also measured by PECAM-1 immunofluorescent staining.

Statistical Analysis
Data are expressed as mean±SEM. The unpaired 2-tailed t test was used to compare continuous variables. Perfusion data were analyzed by repeated measure ANOVA with Tukey post hoc multiple analysis. All calculations were performed using SPSS 11.0, and P<0.05 was considered statistically significant.

Results

Ischemia Results in Overexpression of ICAM-1 on the Endothelial Cells In Vivo and In Vitro
In a model of unilateral hindlimb ischemia, immunofluorescence examinations of nonischemic and ischemic muscle showed different patterns of ICAM-1, VCAM-1, E-selectin, and PECAM-1 expression. In ischemic muscles, there was a significantly increased expression of ICAM-1 on ECs of small to medium sized vessels (Figure 1a) and capillaries (Figure 1b) compared with nonischemic muscles (Figure 1c, 1d). Bone marrow and spleen were stained as positive controls (Figure 1e, 1f). In contrast, VCAM-1 expression was observed on the vessel wall in the ischemic muscle (Figure 1g, 1h), which was not different from that in the nonischemic muscle (Figure 1i, 1j). VCAM-1 expression in the bone marrow and spleen was similar to ICAM-1 expression (Figure 1k, 1l). E-selectin was only scantily expressed on small to medium sized vessels in ischemic muscles (Figure 1m) and not found on the capillaries (Figure 1n), which were similar to the findings in nonischemic muscles (Figure 1o, 1p). Bone marrow and spleen, positive controls, showed high expression of E-selectin (Figure 1q, 1r). Although the expression of PECAM-1 was diffusely distributed on the endothelial cells
of vasculatures, it was not changed by ischemia (Figure 1s, 1t, 1u, 1v). Bone marrow and spleen were stained not only on the vessel wall but also on the cellular component of the tissue. (Figure 1w, 1x).

To confirm in vitro that ischemia upregulates expression of ICAM-1 on ECs, we exposed ECs to hypoxia and measured the changes in ICAM-1. On exposure to hypoxia, endothelial cells showed a significant upregulation of ICAM-1 protein expression compared with those in normoxic condition as shown by immunoblot assay (Figure 2a). The upregulation of ICAM-1 on the surface of ECs in response to hypoxia was also confirmed by FACS analysis (Figure 2b).

**Figure 2.** Hypoxia induced ICAM-1 expression on endothelial cells. Twenty-four-hour hypoxia significantly increased ICAM-1 expression of murine endothelial cells (a). Such increase of ICAM-1 was also demonstrated by FACS analysis (b). Black lines are isotype control and gray lines are anti-ICAM-1.

Bone Marrow-Derived EPCs Express β2-Integrin, the Ligand of ICAM-1

As we cultured the bone marrow mononuclear cells (BMMNC) in an EPC-culture condition for 7 days, they became spindle shaped and showed phenotypes such as BS-1 lectin binding and DiI-acLDL uptake (Figure 3a). They also expressed Flt-1, Flk-1, and von Willebrand factor (vWF), which was absent or very weak in negative control, fibroblast (NIH-3T3), or bone marrow mononuclear cells. FACS analysis (c) confirmed the expression of β2-integrin on the cell surface of both bone marrow mononuclear cells and cultured EPCs. Positive control, murine T lymphocytes (Jurkat), and negative control, fibroblast, were also demonstrated.

**Figure 3.** Bone marrow-derived cultured endothelial progenitor cells (EPC) expressed the counter-ligand of ICAM-1, β2-integrin. Bone marrow-derived murine EPCs (a) (LM: light microscopic figure) were cultured for 7 days and characterized with FITC-labeled BS-1 lectin (green) binding and by DiI-acLDL (red) uptake. On day 7, cultured EPCs (b) expressed Flt-1, Flk-1, and von Willebrand factor (vWF), which was absent or very weak in negative control, fibroblast (NIH-3T3), or bone marrow mononuclear cells. FACS analysis (c) confirmed the expression of β2-integrin on the cell surface of both bone marrow mononuclear cells and cultured EPCs. Positive control, murine T lymphocytes (Jurkat), and negative control, fibroblast, were also demonstrated.

## Blocking ICAM-1 Results in Significant Reduction of EPC Adhesion to Endothelial Cells In Vitro

To demonstrate that ICAM-1 plays a critical function in the adhesion of EPCs, we performed an in vitro adhesion assay with or without monoclonal blocking antibody against ICAM-1. The attachment between EPCs and MS-1 was significantly reduced by pretreatment with blocking antibody to ICAM-1 (number of attached EPCs on MS-1 monolayer: 19±2.4/mm² versus 9.6±2.9/mm² for nonblocked versus blocked, \(P<0.001\)) (Figure 4a to 4c). In comparison to EPCs, fibroblasts (negative control) did not attach to the endothelial
monolayer whereas Jurkat cells (positive control) showed a large amount of attachment to endothelial cells, which was also attenuated by ICAM-1 blocking (Figure 4c).

**ICAM-1 Plays an Important Role in EPC Homing and Neovasculogenesis in Ischemic Hindlimb Muscles In Vivo**

Next, we examined whether the blocking of ICAM-1 reduced EPC homing to ischemic limb in vivo. Scintigrams 2 hours and 15 hours after systemic administration of radiolabeled EPCs showed that most of the administered EPCs homed to spleen and bone marrow (Figure 5a, 5b). These scintigrams are not appropriate for quantification. Therefore, we measured the radioactivity of the removed tissues using a gamma counter after euthanizing the mice at 15 hours after administration. We could confirm the substantial homing of EPCs to bone marrow and spleen quantitatively with this method (Figure 5c). At 2 hours after administration, there was no difference between the nonblocking group and blocking groups (Figure 5a, 5b). However, at 15 hours after administration, the nonblocking group showed slightly higher radioactivity in the ischemic limb than the blocking group on the image. The difference, however, was so small that we could not compare them using the images. Therefore, quantification of the cells (c) that homed to the muscles was performed by gamma counter after removing the muscles. To compensate for different tissue volumes, we compared radioactivities per milligram of tissue. The distribution of administered EPCs was greatest in spleen and bone marrow, whereas it was little in muscles. But the distribution to ischemic muscle was greater than nonischemic muscles. The radioactivity of the nonischemic muscles (d) was not changed by blocking ICAM-1, whereas the radioactivity of the ischemic muscle was significantly reduced by blocking ICAM-1.

to the nonischemic limb (Figure 6a, 6b). The number of trafficked EPCs in the ischemic limb was significantly decreased by treatment of ICAM-1 neutralizing antibody (Figure 6c), suggesting that ICAM-1 expression plays an important role in the trafficking of EPCs to the ischemic hindlimb. The numbers of EPCs per unit area were demonstrated in Figure 6d.

The perfusion ratio of the ischemic limb to the nonischemic limb was significantly decreased by blocking ICAM-1 at postoperative days 14 and 21 (Figure 6e). Capillary density of the ischemic muscle at postoperative day 21 was also significantly reduced by blocking ICAM-1 (Figure 6f), which suggested that decreased EPC homing by blocking ICAM-1 resulted in decreased neovasculogenesis in the ischemic muscle.
Transplanted EPCs were found in the spleen or the bone ishemic limb in vivo. EPCs to endothelial cells in vitro and homing of EPCs to ICAM-1, which significantly decreased both adhesion of ICAM-1 was confirmed by using a blocking antibody for ing that ICAM-1/ICAM-1. Furthermore, we showed that bone marrow-derived EPCs expressed & non-blocking

![ischemic muscle & non-ischemic muscle](image)

**Figure 6.** Immunofluorescent staining showed that EPCs were preferentially entrapped in the ischemic muscle through ICAM-1. Dil-labeled human EPCs (a) (red) were trafficked in the BS-1 lectin-stained capillaries (green) of the ischemic hind-limb of nude mouse. In the nonischemic muscle (b), there was no EPC observed. After pretreatment of ICAM-1-blocking monoclonal antibody (c), the number of homed EPCs to the ischemic muscle significantly reduced. Compared with the nonblocking group (d), the blocking group showed less EPCs in the ischemic muscle. Serial measurement of the perfusion by laser Doppler image analysis (e) showed that blocking ICAM-1 significantly attenuated the enhanced recovery of perfusion in the ischemic limb by EPC transplantation. (n=12, \( \dagger P<0.05 \); \( \ddagger P<0.01 \)). Capillary density (f) in the ischemic muscle was also decreased by blocking ICAM-1.

**Discussion**

In this study, to investigate the molecular mechanism of EPC homing to ischemic limb, we found that ischemia in muscle tissue significantly and selectively increased expression of ICAM-1. Furthermore, we showed that bone marrow-derived EPCs expressed \( \beta2 \)-integrins, the ligand of ICAM-1, suggesting that ICAM-1/\( \beta2 \)-integrin binding plays a role in homing of EPCs to ischemic muscle tissue. The importance of ICAM-1 was confirmed by using a blocking antibody for ICAM-1, which significantly decreased both adhesion of EPCs to endothelial cells in vitro and homing of EPCs to ischemic limb in vivo.

When we evaluated the distribution of the EPCs that we introduced systemically in the animals, the majority of the transplanted EPCs were found in the spleen or the bone marrow, which was also demonstrated by another group. These results confirmed our preliminary thoughts that the mere mobilization or transplantation of EPCs cannot guarantee its incorporation in ischemic tissues. Several groups have previously reported similar observations and suggested that an adjunctive method is necessary to increase the efficiency of cell therapy. The elucidation of the molecular mechanism of homing may give the answer to such questions.

**Multi-Step Process of EPC Homing**

In the light of the results up to now, EPC homing may involve multistep process. Certain factors (eg, VEGF, SDF-1, and MCP-1) in the blood mobilize and enhance differentiation of stem cells toward the EPC lineage. At the same time, several mechanisms are activated at the tissue site requiring neovasculogenesis, which increase chemoattractant molecules and endothelial adhesion molecules so that EPCs can bind and transmigrate through the vascular wall into the interstitial tissue. EPCs, after homing to these tissues, form clusters contributing to neovasculogenesis. Among these steps, the adhesion and transmigration is the key step for local accumulation of EPCs at the ischemic site. Consequently, SDF-1, VEGF, and MCP-1 have been investigated and reported as local chemoattractant factors, which can possibly increase the selective lodgment of EPCs when they are locally injected. However, the role of adhesion molecules has not been clearly elucidated for homing of EPCs, especially in the ischemic site.

**Adhesion Molecules**

There are a lot of adhesion molecules that can be expressed on the endothelium and each of them plays its own role in the attachment of blood cells as follows: E- and P-selectins for initial rolling; Integrin, ICAM-1, and VCAM-1 for firm adhesion; and ICAM-1 and PECAM-1 for transmigration.

In this study, we found that in response to ischemia, ICAM-1 was selectively overexpressed in the ischemic muscle compared with the nonischemic muscle. We also observed that blocking ICAM-1 significantly reduced homing of EPCs to the ischemic muscle.

Recently, E- and P-selectin have been shown to play important roles in the trafficking of the embryonic EPCs during tumor angiogenesis. Yet it is important to underscore that this work was performed with embryonic EPCs and a tumor model, whereas we used adult EPCs and a murine hindlimb ischemia model. It is likely that different cell types and different organ types may use distinct mechanisms for homing of EPCs. In the present study, we also found that bone marrow and spleen entrapped most of the transplanted EPCs probably caused by the high expression of E-selectin as well as ICAM-1. It is well-established that interactions of selectins with selectin-ligands mediate the rolling of cells on the surface of endothelial cells as the initial step of homing.

Based on our observation, relatively low expression of E-selectin in the ischemic limb might result in the low rate of EPC incorporation into the hindlimb compared with the bone marrow and spleen and, at the same time, make ICAM-1 essential for homing of EPCs to the ischemic hindlimb. Further studies are needed to elucidate a potentially synergistic role of other adhesion molecules and their counterligands for the multi-step recruitment process of adult endothelial progenitor and stem cells to ischemic tissue.

PECAM-1 has a role in transmigration rather than attachment. More importantly, PECAM-1 expression did not change in response to ischemia in the present study. Therefore, although PECAM-1 may participate in the multi-step process, it does not seem to be an essential molecule for the preferential homing of EPCs to ischemic tissue.
ICAM-1 has been shown to induce the firm attachment of the rolling cells and facilitate angiogenesis. Its expression was under control of VEGF, Akt, and nitric oxide, which are closely related with angiogenesis. Recently, Dimmeler et al showed that β2-integrin, counter-ligand of ICAM-1, on EPCs was related to homing of EPCs. Their data support that ICAM-1 overexpression in the ischemic muscle, may be the key molecular change to induce preferential recruitment of EPCs into the site.

ICAM-1: Inflammation Versus Angiogenesis and Physiological Versus Pathologic Role of ICAM-1 in Ischemia

ICAM-1 has been known to be an adhesion molecule for recruiting leukocytes, lymphocytes, neutrophils, monocytes, etc, to increase inflammatory response to the sites. Therefore, overexpression of ICAM-1 may recruit leukocytes to the ischemic muscle and then enhance pathologic or hazardous effects as described in several previous reports. However, inflammation always accompanies angiogenesis. Recruited monocytes/macrophages or lymphocytes were reported to enhance angiogenesis and collateral growth in the ischemic organ. In this regard, ICAM-1 overexpression may be the key coordinator to organize inflammation and angiogenesis in the ischemic organ through recruiting inflammatory cells and EPCs. It is likely that the net result in the ischemic organ may lie anywhere on a spectrum from physiological responses to pathologic changes depending on which kinds of cells are dominant in that process. If EPCs are sufficient and its function is good, more angiogenesis would be expected with aid of inflammatory cells. Further studies are warranted to better understand this issue.

Radioisotope in Small Animal Study

The quantification of cell lodging is a prerequisite for any analysis of the homing mechanism, especially in vivo. However, counting cells in tissue is too time-consuming and laborious to be used for assessing distribution of EPCs to various organs after systemic administration. Therefore, we used an easier, relatively straightforward method, Tc-99m HMPAO labeling and quantification of EPC homing by gamma counter. A recent report supported the use of this method for demonstrating the distribution of transplanted human EPCs in a nude rat acute myocardial infarction model and showed the selective accumulation of cells in the ischemic heart. In the present study, we found a good correlation between counting cells and measuring radioactivity and used these methods to investigate the homing mechanism. Radioisotope imaging was qualitatively used to see the distribution of transplanted EPCs in the mice. However, it was not appropriated for quantitative analysis because low signal and low image resolution because of the small size of a mouse.

We observed small number of EPCs in the nonischemic muscle as shown in Figures 5c and 6d. It is possible that some EPCs were physically entrapped in the vascular lumen because of the relatively large size of the transplanted EPCs. Vajkoczy et al showed similar results in their recent article using intravital fluorescent videomicroscopy.


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Arterioscler Thromb Vasc Biol. 2006;26:1066-1072; originally published online February 23, 2006;
doi: 10.1161/01.ATV.0000215001.92941.6c
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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