Intra-Arterial Tumor Necrosis Factor-α Impairs Endothelium-Dependent Vasodilatation and Stimulates Local Tissue Plasminogen Activator Release in Humans

Stanley Chia, Motaz Qadan, Richard Newton, Christopher A. Ludlam, Keith A.A. Fox, David E. Newby

Objective—Inflammation contributes to the pathogenesis of cardiovascular disease, potentially through the actions of proinflammatory cytokines. We assessed the direct effects of local intra-arterial tumor necrosis factor-α (TNF-α), interleukin-6, and endotoxin on blood flow and endogenous tissue plasminogen activator (t-PA) release in vivo in humans.

Methods and Results—In a double-blind, randomized, placebo-controlled trial, blood flow, plasma cytokine, and fibrinolytic parameters were measured using venous occlusion plethysmography and blood sampling. Ten subjects received intrabrachial TNF-α, interleukin-6, and endotoxin infusions, and 8 additional subjects received intrabrachial infusions of bradykinin, acetylcholine, and sodium nitroprusside after pretreatment with TNF-α. TNF-α but not interleukin-6, endotoxin, or placebo caused a gradual and sustained 20-fold increase in plasma t-PA concentrations (P<0.001) that was associated with elevated plasma interleukin-6 concentrations (P<0.05) but without an effect on blood flow or plasminogen activator inhibitor type 1 antigen. Compared with placebo, TNF-α pretreatment impaired bradykinin- and acetylcholine-induced vasodilatation (P<0.03) and resulted in a doubling of bradykinin-induced t-PA release (P<0.05).

Conclusions—Intra-arterial TNF-α causes an acute local vascular inflammation that is associated with impaired endothelium-dependent vasomotion as well as a sustained and substantial increase in endothelial t-PA release. TNF-α has potentially both adverse vasomotor and beneficial profibrinolytic effects on endothelial function. (Arterioscler Thromb Vasc Biol. 2003;23:695-701.)

Key Words: cytokines ■ endothelium ■ fibrinolysis ■ inflammation ■ vasodilatation

There is emerging evidence that systemic inflammation plays a major role in the pathogenesis of cardiovascular disease. The proinflammatory cytokines, tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) in particular, have been implicated in the initiation and maintenance of systemic and vascular inflammation associated with atherosclerosis and coronary artery disease. Indeed, plasma concentrations of these cytokines are elevated in patients with ischemic heart disease and have been shown to predict the future risk of myocardial infarction in apparently healthy individuals.3

The vascular endothelium plays a vital role in the control of blood flow, hemostasis, fibrinolysis, and inflammation, and changes in endothelial function may underlie the association between inflammation and the risk of cardiovascular disease.

Tissue plasminogen activator (t-PA) is a fibrinolytic factor released from the endothelium and lyses intravascular fibrin.4 If endogenous fibrinolysis is to be effective, then the rapid mobilization of t-PA from the endothelium is essential, because thrombus dissolution is much more effective if t-PA is incorporated during, rather than after, thrombus formation.5 However, in the presence of proinflammatory states or an imbalance in endogenous fibrinolysis, intravascular thrombus may propagate, ultimately leading to arterial occlusion and tissue infarction.6

TNF-α and endotoxin have been reported to induce local vascular inflammation and impair endothelium-dependent vasodilatation in the venous circulation of humans.7 Although mild systemic inflammation has also been shown to alter endothelial function,8 the underlying mechanisms for these observations have not been elucidated, and the direct in vivo effects of cytokines and inflammatory stimuli on local arterial endothelial vasomotor and fibrinolytic function are unknown.

The aims of this study were to investigate the acute effects of local intra-arterial inflammatory cytokines (IL-6 and TNF-α) and bacterial endotoxin (lipopolysaccharide [LPS]) exposure on vasomotor function and endothelial t-PA release in vivo in humans.
Methods

Subjects
Eighteen healthy nonsmoking men 21 to 25 years of age participated in the study, which was undertaken with the approval of the local research ethics committee in accordance with the Declaration of Helsinki and the written informed consent of each subject. None of the subjects had infectious illnesses or received medication in the week before study. All subjects abstained from alcohol for 24 hours and from food and caffeine-containing drinks for at least 4 hours before each study. All studies were performed in a quiet, temperature-controlled room.

Cytokines and Drugs
TNF-α (Knoll Pharmaceuticals), IL-6 (Novartis Pharma AG), LPS (lot G-1, USPCI), bradykinin (Clinalfa), acetylcholine (CibaVision Ophthalmics), and sodium nitroprusside (David Bull Laboratories) were administered after dissolution in 0.9% saline. TNF-α, IL-6, and LPS were prepared as stock solutions and stored at −80°C in aliquots. All other drugs were freshly prepared on the study day.

Venous Sampling and Assays
Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges, as previously described. Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer.

Venous Sampling and Assays
Venous blood (10 mL) was withdrawn simultaneously from each arm and collected into tubes containing acidified buffered citrate (for t-PA assays), trisodium citrate (for plasminogen activator inhibitor type 1 [PAI-1] assays), and potassium ethylene diamine tetracetic acid (EDTA) (for cytokine assays). Citrate and acidified buffered citrate samples were centrifuged at 2000 g for 30 minutes at 4°C and EDTA samples at 1000g for 10 minutes at 20°C. Platelet-free plasma was decanted and stored at −80°C before assay. Plasma t-PA, PAI-1, TNF-α, IL-6, and von Willebrand factor (vWF) antigen concentrations were determined as previously described using enzyme-linked immunosorbent assays (Coatiza t-PA and PAI-1, Chromogene AB). Hematocrit and white cell count were determined using an automated Coulter counter (Beckman-Coulter AcT.II).

Hemodynamic Measurements
Blood flow was measured in both forearms by venous occlusion plethysmography using mercury-in-silastic strain gauges, as previously described. Blood pressure was monitored in the noninfused arm at intervals throughout each study with a semiautomated noninvasive oscillometric sphygmomanometer.

Data Analysis and Statistics
Plethysmographic data were extracted from the chart data files, and the last 5 linear recordings in each measurement period were averaged. Estimated net t-PA antigen and activity release was defined previously as the product of the infused forearm plasma flow and the concentration difference between the infused and noninfused forearms. Because basal t-PA concentrations were al-

Cytokine and Endotoxin Administration
In a randomized, double-blind study, 10 subjects attended on 3 occasions at least 1 week apart, and saline was infused for 30 minutes to allow time for equilibration. Subjects were then randomized to receive intra-arterial infusions of low-dose TNF-α (80 ng/min; n = 6), high-dose TNF-α (240 ng/min; n = 6), IL-6 (30 ng/min; n = 6), LPS (100 pg/min; n = 6), or saline placebo (1 mL/min; n = 6) over 60 minutes. This was followed by an additional 60-minute saline washout infusion. Venous samples were obtained at baseline, 10, 20, 40, and 60 minutes during drug infusion, and 30, 60, and 180 minutes after cessation of drug infusion. Cytokine and LPS doses were chosen to achieve local concentrations comparable with healthy volunteer studies and those seen in cardiovascular disease.

Effect of Tumor Necrosis Factor-α on Endothelial Function
Eight subjects attended on 2 occasions at least 1 week apart in a randomized, double-blind, placebo-controlled, crossover trial. They received an intra-arterial infusion of either TNF-α (80 ng/min) or saline placebo (1 mL/min) over 60 minutes. After an additional 60 minutes of saline infusion, intra-arterial bradykinin was administered at 100, 300, and 1000 pmol/min for 10 minutes at each dose, acetylcholine at 5, 10, and 20 pg/min, and sodium nitroprusside at 2, 4, and 8 µg/min for 5 minutes at each dose. Infusions of the vasoactive drugs were separated by 15-minute infusions of saline. Venous samples were obtained at baseline, after 60 minutes of TNF-α/placebo infusion, before and during each dose of bradykinin, and 15 minutes after cessation of bradykinin infusion. Venous samples were not obtained during acetylcholine or sodium nitroprusside infusion, because they do not affect plasma t-PA or PAI-1 concentrations in this forearm model.

Study Design
Subjects rested recumbent, strain gauges and cuffs were applied, and venous cannulae (17-G) were inserted into both antecubital fossae. The brachial artery of the nondominant arm was cannulated with a 27-SWG needle under local anesthesia. The total rate of intra-arterial infusion was maintained constant throughout at 1 mL/min. Forearm blood flow was measured every 6 to 10 minutes.

Data Analysis and Statistics
Plethysmographic data were extracted from the chart data files, and the last 5 linear recordings in each measurement period were averaged. Estimated net t-PA antigen and activity release was defined previously as the product of the infused forearm plasma flow and the concentration difference between the infused and noninfused forearms. Because basal t-PA concentrations were al-

Figure 1. Plasma concentrations of TNF-α (top) and IL-6 (bottom) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF-α at 80 ng/min (left) and 240 ng/min (right). P<0.01 (ANOVA) for all responses except IL-6 concentrations in the noninfused arm. P<0.05 (ANOVA, infused vs noninfused arm) for all responses. P<0.05 (paired t test; vs baseline).
TABLE 1. Forearm Blood Flow (mL/100 mL/min) During TNF-α, IL-6, LPS, and Saline Placebo Infusion

<table>
<thead>
<tr>
<th></th>
<th>TNF-α, 80 ng/min</th>
<th>TNF-α, 240 ng/min</th>
<th>IL-6, 30 pg/mL</th>
<th>LPS, 100 pg/min</th>
<th>Saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infused arm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.2±0.2</td>
<td>2.6±0.6</td>
<td>2.4±0.1</td>
<td>2.5±0.3</td>
<td>2.2±0.3</td>
</tr>
<tr>
<td>1 hour</td>
<td>1.9±0.3</td>
<td>2.4±0.9</td>
<td>1.9±0.2</td>
<td>2.3±0.3</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>2 hours</td>
<td>1.8±0.3</td>
<td>2.1±0.7</td>
<td>1.8±0.2</td>
<td>2.0±0.3</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Noninfused arm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>2.2±0.4</td>
<td>2.6±0.5</td>
<td>2.0±0.2</td>
<td>2.3±0.4</td>
<td>1.8±0.2</td>
</tr>
<tr>
<td>1 hour</td>
<td>2.6±0.5</td>
<td>2.6±0.8</td>
<td>2.0±0.3</td>
<td>2.0±0.3</td>
<td>1.7±0.2</td>
</tr>
<tr>
<td>2 hours</td>
<td>2.9±0.7</td>
<td>2.4±0.5</td>
<td>1.8±0.2</td>
<td>1.8±0.2</td>
<td>1.9±0.3</td>
</tr>
</tbody>
</table>

Values are mean±SEM.

tered by pretreatment with TNF-α, t-PA antigen and activity release during bradykinin infusion was calculated by subtracting the mean t-PA release before and 15 minutes after cessation of bradykinin infusion.

Data were examined, where appropriate, by ANOVA with repeated measures and 2-tailed paired Student’s t test using Statview (SAS Institute, Inc). All results are expressed as mean±SEM. Statistical significance was assigned at the 5% level.

Results

All subjects remained well throughout the study and reported no adverse effects. There were no effects on hematocrit, body temperature, or white cell count throughout all studies (data on file).

Cytokine and Endotoxin Administration

Cytokine Assays

Plasma TNF-α concentrations increased from 1±0 to 2±1 pg/mL to 539±71 and 1164±41 pg/mL in the infused arm (P<0.001 for both) and to 20±2 and 62±8 pg/mL in the noninfused arm (P<0.001 for both) during 80 and 240 ng/min of TNF-α, respectively (Figure 1). This was accompanied by a gradual increase in plasma IL-6 concentrations (Figure 1). In the infused arm, IL-6 infusion increased plasma IL-6 concentrations from 1±3 to 7±1 pg/mL (P=0.01) and LPS infusion increased plasma TNF-α and IL-6 concentrations from 1±3 to 7±1 pg/mL (P=0.01) and from 1±0 to 6±2 pg/mL (P=0.02), respectively.

Hemodynamic Effects

Intra-arterial saline placebo, TNF-α, IL-6, and LPS infusions had no effect on heart rate, blood pressure, or forearm blood flow throughout all studies (data on file; Table 1).

Fibrinolytic and Hemostatic Assays

There were no changes in plasma t-PA antigen concentrations during IL-6, LPS, or saline placebo infusions (data on file). Plasma t-PA antigen and activity concentrations increased in the infused arm by up to 20-fold after both low- and high-dose TNF-α infusions (P<0.001; Figure 2). Plasma t-PA concentrations increased slowly, being detectable at 20 minutes and peaking at 60 minutes of infusion. Thereafter, plasma t-PA concentrations fell but remained elevated 4 hours after initiation of the infusion, with an apparently stable elevation between 2 and 4 hours.

Plasma PAI-1 and vWF antigen concentrations were unchanged throughout all studies, although plasma PAI-1 activity was reduced in the infused arm only after high-dose TNF-α infusion (P=0.003; Table 2).

Effect of Tumor Necrosis Factor-α on Endothelial Function

Cytokine Assays

Intra-arterial TNF-α infusion (80 ng/min) increased plasma TNF-α concentrations in the infused and noninfused arm from 2±1 and 2±1 pg/mL to 561±108 and 15±1 pg/mL, respectively (P<0.001 for both).

Hemodynamic Effects

Intra-arterial TNF-α or placebo infusion had no significant effects on heart rate, blood pressure, or basal forearm blood flow up to 2 hours after commencement of the infusion (P=NS for both). Forearm blood flow increased in a dose-dependent manner during bradykinin, acetylcholine, and so-

Figure 2. Plasma concentrations of t-PA antigen (solid lines) and activity (dashed lines) in the infused (●) and noninfused (○) arms after 1 hour of intra-arterial infusion of TNF-α at 80 ng/min (left) and 240 ng/min (right). P<0.001 (ANOVA) for all responses except noninfused arm after TNF-α 80 ng/min infusion. P<0.001 (ANOVA, infused vs noninfused arm) for all responses. *P<0.05 (paired t test; vs baseline).
TABLE 2. Plasma PAI-1 Antigen and Activity Concentrations During TNF-α Infusion

<table>
<thead>
<tr>
<th></th>
<th>TNF-α, 80 ng/min</th>
<th>TNF-α, 240 ng/min</th>
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<tbody>
<tr>
<td></td>
<td>Infused Noninfused</td>
<td>Infused Noninfused</td>
</tr>
<tr>
<td>PAI-1 antigen, ng/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 minutes</td>
<td>17±4 19±5</td>
<td>23±5 24±5</td>
</tr>
<tr>
<td>60 minutes</td>
<td>18±4 20±4</td>
<td>25±6 26±8</td>
</tr>
<tr>
<td>120 minutes</td>
<td>14±3 16±3</td>
<td>18±5 24±5</td>
</tr>
<tr>
<td>240 minutes</td>
<td>15±4 14±3</td>
<td>21±5 36±17</td>
</tr>
<tr>
<td>PAI-1 activity, IU/mL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 minutes</td>
<td>12±2 11±1</td>
<td>10±2 9±2</td>
</tr>
<tr>
<td>60 minutes</td>
<td>9±2 10±2</td>
<td>5±2* 12±3</td>
</tr>
<tr>
<td>120 minutes</td>
<td>11±1 14±3</td>
<td>6±1* 10±2</td>
</tr>
<tr>
<td>240 minutes</td>
<td>8±1 11±2</td>
<td>4±1* 12±1</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *P=0.003, ANOVA, infused vs noninfused arm.

Discussion

We have demonstrated for the first time that local intra-arterial TNF-α administration can be used to generate an in vivo local vascular inflammatory model in humans that is associated with endothelial dysfunction and a unique profile of endogenous t-PA release. In contrast to IL-6 and LPS, TNF-α causes a slow-onset, sustained, and selective release of t-PA from the vascular endothelium in the absence of basal vasomotor effects. Moreover, pretreatment with TNF-α attenuates endothelium-dependent vasodilatation but augments acute bradykinin-induced t-PA release. These findings indicate that TNF-α and acute vascular inflammation have complex effects on endothelial function. Although the fibrinolytic actions may reflect a protective mechanism in acute inflammation, TNF-α also directly impairs endothelium-dependent vasomotor responses.

Model of Local Vascular Inflammation

We have here developed a model of local vascular inflammation in vivo in humans. Using unilateral intrabrachial infusions, we achieved high local plasma TNF-α concentrations that were comparable to the plasma concentrations seen...
in patients with severe heart failure. A direct local vascular and endothelial inflammatory response was confirmed by the local rise in plasma IL-6 and t-PA concentrations. However, the fibrinolytic effects of TNF-α were not mediated through IL-6 release, because isolated IL-6 infusion had no effect on t-PA release. Indeed, in pilot studies, we found that high-dose intrabrachial IL-6 infusion sufficient to increase plasma IL-6 concentrations to >100 pg/mL have also failed to produce significant effects on forearm blood flow or t-PA release.

Effects of TNF-α on t-PA Release
The profile of t-PA release during local intra-arterial TNF-α administration is unique and has not been previously described in vivo in humans. Previous studies in healthy volunteers have reported changes in plasma fibrinolytic and coagulation factors during systemic TNF-α administration. TNF-α has pleiotropic effects and may cause these effects through actions on specific tissues or via secondary mediators released from organs such as the liver. In the present study, we have assessed local peripheral vascular responses to direct intra-arterial TNF-α and have shown that it causes selective endothelial t-PA release in the forearm without demonstrable effects on plasma vWF or PAI-1 antigen concentrations. Although there was a modest rise in the IL-6 and t-PA concentrations in the noninfused arm, this may represent overspill from the infused arm, where the concentrations increased by up to 20-fold. In contrast to previous systemic studies, subjects here remained asymptomatic and there was no associated pyrexia, consistent with the absence of a major systemic response.

We and others have previously reported acute rapid t-PA release during intra-arterial substance P, bradykinin, and methacholine infusions. Using these agents, there is a near-instantaneous onset and offset of action with no sustained increase in t-PA release after cessation of administration. Moreover, there is always an associated change in vascular tone and regional blood flow, because these agents also cause vasodilatation. In contrast, TNF-α administration had no effect on basal blood flow and caused a slow onset and sustained release of t-PA that was not apparent until 20...
minutes after commencement of the infusion and continued for at least 3 hours after its cessation.

Endothelial cells synthesize and secrete t-PA both constitutively and facultatively. The facultative release of t-PA occurs in response to stimulation by several physiological agonists, including thrombin and bradykinin. This large and rapid release arises from the translocation of a dynamic intracellular storage pool of t-PA. Agonists such as bradykinin are likely to stimulate t-PA release via exocytosis of these granules because of the near-instantaneous release of t-PA and the ex vivo animal evidence that inhibition of protein synthesis by cycloheximide has no effect on bradykinin-induced acute t-PA release. The profile of t-PA release seen with TNF-α is distinct from this pathway. Although the mechanism has not been elucidated in our study, we speculate that it may arise from an increase in de novo t-PA synthesis and its constitutive release rather than through the previously described facultative pathways. However, the initial detectable rise in t-PA release seen at 20 minutes may be too early for protein synthesis to occur. Other potential mechanisms such as activation of adherent leukocytes may cause the generation of secondary mediators that enhance t-PA release.

Infusion of TNF-α seems to cause an isolated increase in local t-PA release without affecting other endothelium-derived fibrinolytic or hemostatic factors, namely PAI-1 and vWF, respectively. This suggests a selective and specific action of TNF-α on the endothelium and forearm vascular bed. The fall in plasma PAI-1 activity seen in the infused arm during high-dose TNF-α infusion is likely to reflect the magnitude of the local t-PA released and its immediate binding with PAI-1. Although other investigators have reported elevated plasma PAI-1 antigen concentrations within 3 hours of systemic administration of TNF-α and endotoxin, we did not detect any changes in PAI-1 antigen concentrations. This suggests that cytokine-induced increases in PAI-1 release are mediated through a systemic mechanism, such as hepatic or adipocyte synthesis, or produced in response to markedly elevated systemic plasma t-PA concentrations.

**Effects of TNF-α on Endothelium-Dependent Vasomotion**

Bhagat and Vallance have previously shown that TNF-α directly induced endothelial dysfunction in the venous circulation of healthy volunteers. We have extended these findings and demonstrated that TNF-α also impairs resistance vessel endothelium-dependent vasodilatation, possibly through the development of acute arterial endothelial injury. The effects of TNF-α were specific for the endothelium, because endothelium-independent vasodilatation to the nitric oxide donor, sodium nitroprusside, was unaltered.

TNF-α may alter endothelial vasomotor responses through various mechanisms including decreased constitutive nitric oxide synthase expression, increased inducible nitric oxide synthase expression, and enhanced production of reactive oxygen species. Additional studies are needed to clarify the precise mechanism, although it is tempting to speculate that TNF-α may, in part, be responsible for inducing endothelial dysfunction in cardiovascular conditions associated with inflammation.

**Effects of TNF-α on Acute Endogenous Fibrinolysis**

We have additionally demonstrated that TNF-α potentiates bradykinin-induced endothelial t-PA release despite reduced endothelium-dependent vasodilatation. This suggests that acute local vascular inflammation induces antithrombotic properties that may represent an adaptive response to inhibit intravascular thrombus deposition at sites of vascular injury. In particular, molecular and pharmacological evidence supports the role of bradykinin B2 receptors in the acute phase of inflammation, and upregulation of B2 receptors by TNF-α may account for the observed profibrinolytic effect. The augmented bradykinin response is also likely to reflect the proposed increase in de novo t-PA production induced by TNF-α pretreatment.

**Clinical Implications**

There is a consistent link between endothelial dysfunction and cardiovascular disease, with impaired endothelium-dependent vasodilatation having been described in atherosclerotic conditions and its associated risk factors, such as hypercholesterolemia. The major findings of our study will be particularly pertinent to cardiovascular conditions, such as acute coronary syndromes and congestive heart failure, in which inflammation and impaired endothelium-dependent vasodilatation occur.

We have found that although TNF-α adversely affects certain aspects of endothelial function, such as endothelium-dependent vasodilatation, it enhances other protective mechanisms, such as the endogenous fibrinolytic capacity. This reflects the complex and pleiotropic nature of TNF-α, which functions as part of the normal host surveillance mechanisms and responses to tissue injury. These diverse effects may explain some of the contradictory findings of recent clinical studies. For example, in patients with heart failure, TNF-α antagonism causes marked improvement in endothelium-dependent vasodilatation but has failed to demonstrate clinical benefit in the RECOVER and RENAISSANCE randomized controlled trials. Thus, the benefits of restoring endothelium-dependent vasoconstrictor function by TNF-α antagonism may be counterbalanced by inhibiting other potentially beneficial effects such as enhancing acute endogenous t-PA release.

**Conclusions**

This is the first study to delineate the direct effects of intra-arterial TNF-α administration on local vascular tone and endogenous fibrinolysis in vivo in humans. It supports the crucial role of TNF-α in cardiovascular disease and provides evidence for its direct and pleiotropic effects on the circulation and endogenous fibrinolysis. Our findings have particular implications for the future development of effective anticytokine and anti-inflammatory strategies in cardiovascular disease.

**Acknowledgments**

This study was supported by a grant from the British Heart Foundation (PG/2001068). Dr Chia is a recipient of the British Heart Foundation.
Foundation Junior Research Fellowship (FS/2001049). We would like to thank Pamela Dawson for her assistance with this study.

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Arterioscler Thromb Vasc Biol. 2003;23:695-701; originally published online March 6, 2003; doi: 10.1161/01.ATV.0000065195.22904.FA

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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