Urokinase Activates the Jak/Stat Signal Transduction Pathway in Human Vascular Endothelial Cells

Inna Dumler, Angela Kopmann, Angelika Weis, Oleg A. Mayboroda, Kai Wagner, Dietrich C. Gulba, Hermann Haller

Abstract—Endothelial cells demonstrate high urokinase expression and upregulation of urokinase receptors in response to vascular injury. Urokinase receptor binding facilitates endothelial cell migration into an arterial wound; however, the signaling cascade induced by the urokinase receptor in this cell type is incompletely understood. Because the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway seems to be important for vessel function, we investigated the hypothesis that urokinase receptor binding activates Jak/Stat signaling in human vascular endothelial cells. Incubation of endothelial cells with urokinase-type plasminogen activator (uPA, 1 nmol/L) induced a rapid and pronounced increase in tyrosine phosphorylation of several proteins with a molecular weight between 80 and 90 and 130 to 140 kDa. The same pattern of tyrosine phosphorylation was found after treatment with 1 nmol/L ATF, the urokinase amino-terminal fragment, which is devoid of proteolytic activity but still binds to the urokinase receptor. Using coimmunoprecipitation techniques, we demonstrated that the activated urokinase receptor is associated with 2 cytoplasmic tyrosine kinases of the Jak family, viz, Jak1 and Tyk2. uPA and ATF induced a time-dependent activation of both kinases, as shown by immunoprecipitation and Western blot analysis. Using electrophoretic mobility shift and supershift assays, we then demonstrated that Stat1 is rapidly activated in endothelial cells in response to uPA and ATF. Furthermore, Stat1 specifically binds to the regulatory elements interferon-γ activation site/interferon-stimulated response element. The uPA-induced, time-dependent translocation of Stat1 to cell nuclei was confirmed by confocal microscopy study and immunoblotting of nuclear extracts with an anti-Stat1 antibody. This study provides evidence for a novel signaling pathway for uPA in human vascular endothelial cells. Direct activation of the Jak/Stat system via the uPA-receptor complex may be an important mechanism for endothelial cell migration and/or proliferation during angiogenesis and after vascular injury. (Arterioscler Thromb Vasc Biol. 1999;19:290-297.)

Key Words: endothelial cells • urokinase receptor • signal transduction • Jak/Stat pathway

Vascular endothelial cell proliferation and migration play an important role in a number of physiological and pathological conditions, such as embryogenesis, angiogenesis, wound healing, tumor invasion and metastasis, as well as neointima formation in atherosclerosis.1-3 Although not fully understood, cell migration is known to involve regulated adhesion and disadhesion events, contraction of nonmuscle myosin and actin, cytoskeletal plasticity, and protein synthesis.4 Cell adhesion to extracellular matrix proteins is controlled by integrins that link the cytoskeleton to the extracellular environment.5,6 One of the phenotypic hallmarks of migrating endothelial cells is a coordinated expression of the urokinase-type plasminogen activator (uPA), a key mediator of extracellular proteolysis, and of its specific receptor uPAR.7-9 Migrating cells express both uPA and uPAR selectively on their leading edge, thereby providing efficient and spatially restricted proteolysis.10-12 Blockage of uPAR with its antagonist amino-terminal fragment (ATF)-human serum albumin inhibits in vitro endothelial cell migration.13 Localized expression of uPA and uPAR in endothelial cells at the wound edge supports the hypothesis that uPA and uPAR are necessary for in vivo endothelial cell proliferation.14 In addition to promoting activation of cell-surface proteinases, uPAR induces intracellular activities that are independent of proteolysis and that imply transmembrane signaling of the receptor.15-19 Recently, it was shown that the activation of uPAR facilitates cell migration via a binding site for vitronectin on uPAR.20-22 The capacity of uPAR to act as an adhesion receptor depends on a functional and physical association with integrins.23 Therefore, uPAR is a multifunctional protein that controls cell migration and proliferation at multiple levels.

However, because of uPAR’s special topology in the cell membrane, the mechanisms of uPAR signaling and its effects on the activation of cell migration and proliferation remain unclear.24 A possible candidate for uPAR-related signaling is...
the Jak/Stat (Janus kinase/signal transducer and activator of transcription) system. This system was recently identified as a novel signaling mechanism correlating with pleiotropic functional responses induced by a variety of growth factors, cytokines, and interferons.\textsuperscript{25-28} Stat proteins bind to several consensus elements in the promoter regions of numerous interesting genes important for vascular cell functions.\textsuperscript{29,30} Moreover, the association of uPAR with some components of the Jak/Stat pathway was recently demonstrated for the human cancer cell line TCL-598\textsuperscript{31} and smooth muscle cells.\textsuperscript{32} We have explored whether or not uPA/uPAR can directly modify Jak/Stat signaling in human vascular endothelial cells. We found that uPAR is associated with 2 members of the Jak family, Jak1 and Tyk2, which are activated by uPA and ATF. This in turn leads to the activation of Stat1 and its rapid translocation to the endothelial cell nucleus, followed by selective Stat1 binding to DNA transcriptional elements interferon-γ activation site (GAS)/interferon-stimulated response element (ISRE).

**Methods**

**Materials**

Chemicals were of high-quality commercial grade and were purchased from Sigma Chemical Co, Pharmacia LKB Biotechnology, Merck, or Serva. Radiochemicals were obtained from NEN Life Science Products, and chemiluminescent signal enhancers were obtained from Tropix, Inc and NEN-DuPont. Aqua-Poly/Mount mounting medium was purchased from Polysciences, Inc. Oligonucleotides were from Santa Cruz Biotechnology, Inc; T4 polynucleotide kinase was purchased from Stratagene; and poly(dI-dC) and NAP-5 Sephadex G-25 DNA-grade columns were from Pharmacia LKB Biotechnology.

**Antibodies**

Anti-uPAR (CD87) monoclonal antibody (product No. 3937) was purchased from American Diagnostica, Inc. Monoclonal PY20 anti-phosphotyrosine antibody was from Transduction Laboratories, and monoclonal anti-Stat1α/β antibody (STAT 91/84, IgG2b and IgG1 as a gel supershift reagent) was from Dianova and Santa Cruz Biotechnology, Inc. Polyclonal and monoclonal antibodies for JakS were purchased from Santa Cruz Biotechnology, Inc and from Transduction Laboratories. Cy5-conjugated donkey anti-mouse IgG (heavy and light chains of IgG) was from Jackson Immuno Research Laboratories.

**Cell Culture**

Human umbilical vein endothelial cells were isolated from umbilical cords by chymotrypsin treatment. Primary cultured cells were grown for 3 to 4 days, then subcultured in essential growth medium (Clonetics) with 2% FCS and supplements, and used between passages 3 and 6. For uPA or ATF stimulation experiments, the cells were cultured for 24 hours in serum-free essential growth medium and then treated with uPA as described below.

**Tyrosine Phosphorylation, Western Blotting, and Stripping**

Subconfluent and serum-starved endothelial cells were washed twice with HEPES-NaCl buffer (10 mM HEPES, pH 7.5; 150 mM NaCl) and then treated with 1 mM uPA (Sigma) or 1 mM ATF (kindly provided by Dr J. Henkin, Abbott Laboratories, Abbott Park, Ill) at 37°C for 5 to 30 minutes. Cells were put on ice; washed with ice-cold, HEPES-buffered saline containing the protease inhibitors; and harvested by scraping. After centrifugation, the pellets were lysed in lysis buffer (20 mM HEPES, pH 8.0; 138 mM NaCl; 10% glycerol; 2 mM EDTA; 1% Triton X-100; and protease inhibitors as indicated above), left on ice for 5 minutes, and centrifuged. Supernatants were used for polyacrylamide gel electrophoresis (PAGE) and Western blotting. The blots were developed with the appropriate antibody; the immune complexes were visualized by an enhanced chemiluminescence detection system. Stripping of the membranes was performed with 200 mM/L β-mercaptoethanol, 62.5 mM/L Tris-HCl (pH 6.8), and 2% SDS for 30 minutes at 50°C.

**Immunoprecipitation**

For immunoprecipitation, cell lysates containing 800 to 1000 μg protein were preclariﬁed for 2 hours at room temperature with Gamma-Bind Sepharose (Pharmacia Biotech) and then immunoprecipitated overnight at 4°C by using 5 or 10 μg antibody coupled to protein A-agarose (Santa Cruz Biotechnology, Inc). Precipitates were washed in PBS-Tween buffer and were used for PAGE and Western blotting.

**Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)**

Nuclear extracts were prepared from endothelial cells that were either left untreated or treated with 1 mM uPA or ATF. Cells were put on ice; washed with ice-cold, HEPES-buffered saline containing the protease inhibitors; and harvested by scraping. The cell suspension was centrifuged at 800 rpm for 8 minutes at 4°C, and the cell pellet was resuspended in buffer A (10 mM HEPES, pH 7.9; 1.5 mM MgCl\(_2\); 10 mM KCl; and 0.5 mM DTT) containing the protease inhibitors and incubated on ice for 15 minutes. Large aggregates were dispersed by homogenization in a Wheaton 0.1-mL homogenizer, and the nuclei were collected by centrifugation. The pellet (nuclear fraction) was resuspended on ice in buffer B (20 mM HEPES, pH 7.9; 25% glycerol; 1.5 mM MgCl\(_2\); 420 mM NaCl; 0.2 mM EDTA; and 0.5 mM DTT) containing the protease inhibitors. After 30 minutes the nuclear fraction was centrifuged, and the supernatant was dialyzed against buffer C (20 mM HEPES, 20% glycerol, 100 mM NaCl, 0.2 mM EDTA, and 0.5 mM DTT) containing the protease inhibitors for 2 hours at 4°C followed by centrifugation. The supernatant proteins were used immediately or divided into aliquots and stored at −80°C.

EMSA was performed for 30 minutes at room temperature in a volume of 20 μL containing 0.5 μg of nuclear protein extracts, 40 ng of poly(dI-dC), 4 μL of 5× binding buffer (1× binding buffer is 20 mM HEPES, pH 7.9; 50 mM NaCl; 5 mM MgCl\(_2\); 1 mM EDTA; 1 mM DTT; and 10% glycerol) with or without a 50- or a 100-fold excess of a cold competitor or an unrelated competitor, and a radiolabeled probe (3×10\(^5\) counts per minute). In the supershift EMSA, nuclear extracts were incubated with 2 μg of experimental or isotopic control antibody before the addition of \(^32\)P-labeled probe. DNA-protein complexes were separated on a 5% polyacrylamide gel in Tris-glycine buffer (50 mM/L Tris, 0.4 mol/L glycine, and 2 mM/L EDTA).

The following double-stranded oligonucleotides were purchased from Santa Cruz Biotechnology, Inc and used in this study: GAS/ISRE, 27 bp (No. sc-2537); AP-1, 21 bp (No. sc-2501). Probes (5'-end-labeled) were prepared with 40 μCi of [γ-\(^32\)P]ATP by using T4 polynucleotide kinase and were gel-purified on NAP-5 Sephadex G-25 DNA-grade columns.

**Confocal Microscopy**

For staining with uPA- or ATF-induced nuclear translocation experiments, the cells were fixed on glass coverslips with 4% paraformaldehyde and permeabilized with 80% methanol at −20°C. After overnight incubation at 4°C with 1% BSA in PBS, the preparations were treated with the anti-Stat1 monoclonal antibody or control monoclonal antibodies, all diluted (5 μg/mL) in 0.2% BSA-PBS. The preparations were washed 3 times in PBS and incubated in a dark, humid chamber with Cy3-conjugated donkey anti-mouse IgG (diluted 1:400) in 0.2% BSA-PBS. The coverslips were washed 4 times in PBS and embedded in Aqua-Poly/Mount mounting medium. The images were acquired with an NORAN Instrument Odyssey XL laser scanning confocal microscope with an Ar-Kr laser, supported with Intevision 1.5 software.
Figure 1. uPA-induced tyrosine phosphorylation [(P)-Tyr] and identification of the Jak5 associated with and activated by uPA. A, The cells were treated with 1 nmol/L uPA (a) or ATF (b) and lysed as described in Methods. The phosphorylated proteins were fractionated by SDS-PAGE and visualized by chemiluminescence after immunoblotting with monoclonal anti-phosphotyrosine antibody (Ab). Arrows indicate the sets of phosphorylated proteins after uPA/ATF treatment that presumably were affiliated with the Jak5/Stat system. B, uPAR signaling complex was immunoprecipitated (IP) from the endothelial cell lysates with anti-uPAR or idiotypic control antibody and analyzed by immunoblotting with anti-Jak1, Jak2, Jak3, and Tyk2 antibodies, followed by chemiluminescence staining as indicated in Methods. C, Jak1 and Tyk2 were immunoprecipitated from control and uPA-treated, lysed endothelial cells with anti-Jak1 and anti-Tyk2 antibodies. Tyrosine-phosphorylated proteins in the immunoprecipitates were visualized by chemiluminescence after protein separation by SDS-PAGE and immunoblotting with anti-phosphotyrosine antibody. The blots were reprobed with anti-Jak1 and anti-Tyk2 antibodies, as indicated, to demonstrate equal protein loading.
Statistics
Each experiment was repeated at least 5 times, and representative figures of each are shown. For confocal microscopy studies, at least 20 to 40 cells from at least 7 separate experiments were examined under each experimental condition. The observers were unaware of the experimental design and antibodies used.

Results
uPA-induced Tyrosine Phosphorylation and Identification of Jaks Associated With uPAR in Endothelial Cells
We first examined tyrosine phosphorylation in endothelial cells after cell stimulation with uPA and ATF. Cells were preincubated with 1 nmol/L uPA or ATF for various periods of time (from 5 to 30 minutes); washed, scraped, and lysed as described in Methods; and subjected to PAGE followed by Western blotting with anti-phosphotyrosine monoclonal antibodies. After 5 minutes of uPA application, an increase in tyrosine phosphorylation of several proteins was observed (Figure 1A a). The increase in phosphorylation was rapid, peaking between 10 and 15 minutes, and decreased after 30 minutes. To exclude a potential contribution of proteolytic activity to the mechanism of tyrosine phosphorylation, the uPA ATF, which has no proteolytic activity, was used for cell stimulation. This peptide provided the same effect (Figure 1A b), confirming the involvement of proteolytically inactive uPA in signaling events. The 38-kDa phosphorylated band may contain members of the mitogen-activated protein kinase family, and the presence of 53- to 60-kDa phosphorylated proteins suggested identity with the previously described Src-phosphotyrosine kinase. The presence of phosphorylated proteins with molecular masses of ~130 to 140 and 80 to 90 kDa (indicated by arrows) supported their probable affiliation with the Jak/Stat system.

To identify the Jaks providing part of the tyrosine phosphorylation on uPA/ATF treatment, cell lysates were subjected to immunoprecipitation with anti-uPAR antibodies. The immunoprecipitated products were analyzed by SDS-PAGE and Western blotting using polyclonal antibodies against individual Jaks, as described in Methods. The presence of 2 Jaks, viz, Jak1 (p130) and Tyk2 (p140), was clearly demonstrated in the immunoprecipitates (Figure 1B). The antibodies against 2 other members of Jaks, Jak2 (p130) and Jak3 (p120), did not show any positive reaction.

uPA-Induced Jak1, Tyk2, and Stat1 Activation
To examine the rates of Jak1 and Tyk2 activation after endothelial cell treatment with uPA, the cells were treated with uPA or ATF for 5 to 30 minutes and washed as described above. Jak1 and Tyk2 were immunoprecipitated from the cell lysates and analyzed on blots by probing with anti-phosphotyrosine antibodies. A 15-minute treatment with uPA
or ATF stimulated the tyrosine phosphorylation of Jak1 and Tyk2, which decreased within 30 minutes (Figure 1C; shown for uPA stimulation). No tyrosine phosphorylation was observed at the 0-minute time-point stimulation and in the immunoprecipitates from the unstimulated cells. Reprobing of the blots with monoclonal anti-Jak1 and anti-Tyk2 antibodies confirmed equal protein loading on the gels (Figure 1C). We reasoned that the p91/84 phosphorylated double band (Figure 1A) might correspond to Stat1 protein. We then reprobed the blots with an anti-Stat1 monoclonal antibody. The data shown in Figure 2A strongly support this notion. To demonstrate the uPA-induced phosphorylation of Stat1 more directly, the Stat1 protein was immunoprecipitated from the activated cells and screened after Western blotting by anti-phosphotyrosine antibody. These experiments (Figure 2B) provide the evidence that Stat1 was indeed activated in response to uPA.

**uPA-Induced Nuclear Translocation of Stat1 in Endothelial Cells**

To further examine the uPA-dependent activation of Stat1, we performed nuclear translocation experiments. Endothelial cells were treated with 1 nmol/L uPA or ATF, then washed, fixed, and stained (before and after the stimulation) as described in Methods. In parallel, some of the treated cells were used for the nuclear extract preparation and immunoblotting analysis. Immunohistochemical staining with the anti-Stat1 monoclonal antibody revealed that Stat1 protein was localized diffusely within the cytoplasm and to a lesser extent in the endothelial cell nucleus (Figure 3A a). After a 5-minute treatment with 1 nmol/L uPA or ATF, anti-Stat1 staining was intensely increased in the perinuclear space. Furthermore, some Stat1 protein could also be found within the nucleus (Figure 3A b). Ten and 15 minutes‘ activation resulted in predominant nuclear staining (Figure 3A c and d). These observations were further confirmed by immunoblotting analysis of nuclear extracts isolated from uPA-treated endothelial cells (Figure 3B). Anti-Stat1 antibody revealed the enrichment of cell nuclei with Stat1 protein to be mostly pronounced after 15 minutes of uPA stimulation. These experiments demonstrate that uPA-dependent endothelial cell activation promotes Stat1 translocation in the nucleus.

**uPA Induces a Specific GAS/ISRE Binding Activity in Endothelial Cells**

To determine whether or not the activation of Jak1 and Tyk2 on uPA or ATF treatment could induce the activation of transcription factors, a DNA binding assay was performed. In our EMSA, 32P-labeled GAS/ISRE oligonucleotide containing a consensus binding site for Stats was used as a probe to analyze the nuclear extracts from activated endothelial cells. As shown in Figure 4A, stimulation of cells with uPA led within 15 minutes to the induction of the DNA-binding protein complex. The same results were observed after stimulation of the cells with 1 nmol/L ATF. The observed band was specific, because it could be inhibited by unlabeled oligonucleotide but not by an unrelated oligonucleotide (Figure 4B). The presence of Stat1 (p91/84) in the observed complexes was tested by using an anti-Stat1 antibody in gel retardation assays. Addition of a specific antibody diminished...
the corresponding band (Figure 4C). The kinetics of DNA binding activity (Figure 4A) was correlated with the rates of uPA-induced Jak1, Tyk2, and Stat1 tyrosine phosphorylation (Figures 1C and 2). In both instances, the effects were rather transient; the maximum uPA-induced activation was observed within 10 to 15 minutes, which declined to some extent after 20 to 30 minutes.

**Discussion**

We have demonstrated that uPAR can directly activate the Jak/Stat signaling pathway in human endothelial cells. Coimmunoprecipitation experiments showed the association of uPAR with 2 Jaks, viz, Jak1 and Tyk2. Both kinases were shown to be activated by uPA and its ATF, which is devoid of proteolytic activity. uPA induced rapid activation of Stat1, its translocation to the cell nucleus, and a selective binding to DNA transcriptional elements. The data obtained in the present study suggest that in human vascular endothelial cells, uPAR may provide transmembrane signal transduction through a novel mechanism involving the Jak/Stat pathway.

The recently identified Jak/Stat signal transduction pathway is utilized by a wide range of cytokines and growth factors to regulate gene expression in an accelerated fashion. The Jak/Stat signaling pathway has been presumed to be especially important in cardiovascular physiology. New findings about the association of uPAR with some components of the Jak/Stat signaling pathway in human tumor and smooth muscle cells have been published during the preparation of this article. With these facts in mind, we investigated the possibility of a relationship between uPA/uPAR and Jak/Stat signaling in human vascular endothelial cells. In our cell system, uPA and ATF treatment induced a specific, time-dependent increase in tyrosine phosphorylation of several proteins. No uPA/ATF-induced tyrosine phosphorylation was observed in phosphatidylinositol-specific phospholipase C–treated cells, as was shown in our previous experiments, which implies the necessity of a native uPAR for this signaling. The 80- to 90- and 130- to 140-kDa phosphorylated proteins are probably affiliated with the Jak/Stat system. These proteins have a molecular mass similar to the Jak family of protein tyrosine kinases and to the Stat family of transcription factors. The latter are known to be substrates for Jaks in a number of cytokine receptor systems. The immunoprecipitation experiments allowed us to identify 2 members of Jak, viz, Jak1 and Tyk2, which were associated with uPAR. In vivo, the activation of Jaks by cytokines or growth factors leads to the rapid phosphorylation of Stat proteins on tyrosine residues. The phosphorylated Stats translocate immediately to the nucleus and bind to specific DNA sequences such as GAS, ISRE, or the Sis-inducible element to activate specific gene expression. In our immunoprecipitation experiments the rapid and transient tyrosine phosphorylation of Stat1 in response to uPA and ATF was demonstrated. To follow uPAR-dependent modulations of Jak/Stat signaling, we examined uPA- and ATF-induced translocation of Stat1 in endothelial cell nuclei and its binding to the DNA sequences GAS/ISRE. We demonstrated that uPA/ATF activation leads to the rapid translocation of Stat1 to the nucleus. The gel shifts showed the specific formation of protein-DNA complexes in endothelial cell

**Figure 4.** EMSAs of uPA-induced protein-DNA complex formation. A, GAS/ISRE binding activity of nuclear extracts prepared from endothelial cells cultured with or without 1 nmol/L uPA for 0, 5, 15, 20, or 30 minutes as indicated. Open arrows indicate the position of the protein-32P-labeled GAS/ISRE complex; solid arrows indicate the position of the free probe. B, A 50-fold and 100-fold molar excess of unlabeled GAS/ISRE competitor or unrelated competitor (AP-1 element sequence) were included as indicated. C, Addition of anti-Stat1 antibody or the corresponding control antibody. EMSA and protein-DNA complex separation were performed as indicated in Methods.
nuclear fractions in response to uPA. The kinetics of uPA-induced DNA binding activity in cell nuclear extracts was rapid and had a rather transient character, correlating with the kinetics of uPA-dependent Stat1 translocation to the nucleus. In EMSA experiments, anti-Stat1 antibody inhibited protein-DNA complex formation. The EMISA is the method of choice to prove protein-DNA interactions. The addition of a specific antibody may result in a supershift of the band. If the antibody binds to a specific region involved in the formation of the complex, however, it may inhibit complex formation. The inhibition of formation of the protein-DNA complex band by the anti-Stat1 antibody in the EMISA as observed by us has been previously reported and is regarded as evidence for a Stat protein presence in these complexes. Taken together, these results provide evidence linking uPAR to a dynamic intracellular event, suggesting a novel mechanism for uPAR-dependent signal transduction and a novel function for uPA in the vascular endothelium.

Although our results suggest a direct activation of the Jak/Stat pathway via uPAR, some questions still remain open. It is not clear how uPAR, in the absence of a transmembrane or cytoplasmic domain, can be coupled to the cytoplasmic JakS. The presence of an unknown “adapter” molecule linking uPAR to the cytoplasmic signaling machinery has been postulated; however, the nature of this adapter remains unclear. We do not as yet have any evidence that the uPA-induced Stat1 translocation to the nucleus and that the activation of DNA binding activity of Stat1 observed in our experiments are realized via the activation of Jak1 and/or Tyk2, although the kinetics of their activation is very convincing. Some receptors, such as epidermal growth factor receptor, for example, do not require JakS in the activation of Stats, even though Jak1 has been shown to be activated in response to epidermal growth factor. Thus, in endothelial cells Jak activation may serve different functions for distinct classes of receptor. Further experiments will be necessary to test this hypothesis. Another question also to be answered is whether other Stats in addition to Stat1 might be modulated for signal transduction and a novel function for uPA in the vascular endothelium.

Acknowledgments

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to Hermann Haller. The authors thank Jana Krentler for excellent technical assistance and Dr Jack Henkin for providing us with the ATF sample.

References


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*Arterioscler Thromb Vasc Biol.* 1999;19:290-297
doi: 10.1161/01.ATV.19.2.290

*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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