Uric Acid Heralds Ischemic Tissue Injury to Mobilize Endothelial Progenitor Cells

Daniel Patschan,* Susann Patschan,* Glenda G. Gobe,† Sreedhar Chintala,* and Michael S. Goligorsky*

*Department of Medicine, Renal Research Institute and Division of Nephrology, New York Medical College, Valhalla, New York; and †Centre for Kidney Disease Research, University of Queensland Department of Medicine, Princess Alexandra Hospital, Woolloongabba, Brisbane, Queensland, Australia

Understanding the nature of endogenous mechanisms for mobilization of stem/progenitor cells is predicated on the identification of injury-induced substances that are released from a damaged organ and capable of producing a distant effect. Although different substances that mobilize endothelial progenitor cells (EPCs) have been proposed, their potential to signal injury and afford posts ischemic renoprotection and repair remains obscure. Uric acid (UA) is consistently overproduced by ischemic tissues and has been shown to exert immunomodulatory functions. It was hypothesized that UA and/or its precursors might serve as injury signals that are capable of mobilizing EPCs in acute renal ischemia. Indeed, FVB/NJ mice that were subjected to acute renal ischemia showed a transient surge in UA level in the peripheral blood. Single-dose treatment with UA, as well as acute hyperuricemia induced by the inhibition of uricase, caused a robust mobilization of EPCs, whereas administration of adenosine or inosine seemed to lack this effect. Moreover, pretreatment of mice with a single dose of UA afforded significant renoprotection against ischemic injury. In animals with chronic hyperuricemia (induced by continuous 2-wk treatment with a uricase inhibitor oxonic acid), EPC mobilization was blunted and renoprotective effects were absent. In conclusion, acute elevation of UA acts as “physiologic,” fast-acting endogenous mediator of EPC mobilization and renoprotection, consistent with its novel function in pharmacologic preconditioning. Both of these actions are lacking in mice with chronic hyperuricemia. In summary, a transient surge in UA concentration may serve as a universal herald of tissue injury to accelerate the recruitment of EPCs.


There is growing, albeit a somewhat conflicting, evidence that transplantation of stem and progenitor cells may improve regeneration of various injured organs, such as the myocardium, arterial wall, and kidney, to name a few (1–3). Alternatively, various investigators used maneuvers to mobilize stem/(endothelial) progenitor cells—granulocyte colony-stimulating factor, stromal-derived factor 1 (SDF-1), vascular endothelial growth factor, placental growth factor, erythropoietin, and others—for improved organ regeneration (1,4–8), yet there is strong evidence demonstrating the presence of stem and progenitor cells at sites or attracted to the sites of injury even in the absence of any pharmacologic stimulation of their mobilization (7,9), suggesting that some intrinsic factors that are generated during organ damage may be responsible for their mobilization. In fact, there may even exist a dosageresponse relation between the severity of injury and stem cell mobilization: increasing duration of renal ischemia results in an increased mobilization of stem cells (10).

Understanding the nature of endogenous mechanisms for stress-triggered mobilization of stem/progenitor cells hinges on the identification of injury-induced substances that are capable of being released from a damaged organ or cell and eliciting a distant effect. Several such factors have been proposed. Discovery of hepatocyte growth factor was associated with the hypothetical existence of a factor termed injurin, which was deemed to be responsible for hepatocyte growth factor induction (11), yet the chemical identity of injurin has not been discovered so far. Alternatively, a series of studies from Burnstock’s laboratory have implicated ATP released from injured cells in signaling the damage (12). Although this may serve as a powerful mechanism for local regulation of the circulation, it is not entirely clear whether this type of signaling may remain sufficiently robust distantly as a result of the rapid degradation of ATP. Some purines and their degradation product, uric acid (UA), however, may have longer half-life in the circulation, especially in humans, and have been shown to exhibit a dramatic surge after injury. For instance, Miller et al. (13) described a rapid increase in the concentration of adenine, inosine, and hypoxanthine, all precursors of UA, in the peripheral blood after only 1 min of renal ischemia. UA, whose systemic concentration rises in a variety of pathologic conditions that are followed by severe tissue damage, has been implicated in alarm signaling, resulting in stimulation of dendritic cell maturation and the activity of CD8+ T cells (14).
Flow Cytometry Analysis

For quantification of peripheral circulating and splenic EPCs by FACS, mononuclear cells were isolated by density gradient centrifugation using Histopaque-1077 solution from either 500 µl of peripheral blood or splenic tissue homogenates. Cells were incubated for 30 min on ice with FITC-conjugated anti-mouse CD34 (RAM34) and PE-conjugated anti-mouse Flk-1 (Avas12a1; BD Biosciences, Rockville, MD). After incubation, cells were washed with PBS and fixed in 4% paraformaldehyde. Data were acquired using a FACScan cytometer equipped with a 488-nm argon laser and a 620-nm red diode laser and analyzed using CellQuest software (Becton Dickinson, San Jose, CA). The setup of FACScan was performed according to the manufacturer’s instructions using unstained and single antibody–stained cells. For quantification of EPCs, the number of CD34/Flk-1 double-positive cells within the monocyte cell population was counted.

Immunohistochemical Staining and Analysis

Tissue samples of the kidney were fixed in a 4% paraformaldehyde solution (Electron Microscopy Sciences, Hatfield, PA), followed by incubation in 30% sucrose overnight at 4°C. Embedding was performed in an OCT compound (Tissue-Tek, Torrance, CA), and embedded samples were stored at −80°C. Frozen samples were cut into 10-µm-thick sections (Cryomicrotome CM 1850; Leica Microsystems, Bannockburn, IL). Nonspecific protein binding was blocked by 1 h of incubation with PBS-BSA (1%). The following primary antibodies were used: FITC-conjugate anti-mouse CD117 (c-Kit, 1:1000 in PBS-BSA 1%; BD Biosciences) and anti-human Tie-2 (sc-9026, 1:100 in PBS-BSA 1%; Santa Cruz Biotechnology, Santa Cruz, CA). Secondary antibody for Tie-2 staining was Alexa Fluor 594 goat anti-rabbit IgG (A10102, 1:500 in PBS-BSA 1%; Molecular Probes, Eugene, OR). Incubations with primary antibodies were carried out overnight at 4°C, and incubations with the secondary antibody were performed for 1 h at room temperature. Control samples were stained with secondary antibodies only. For visualization of the nuclei, tissue sections were counterstained with DAPI (Molecular Probes). Sections were examined using a Nikon compound fluorescence microscope with the appropriate dichroic mirrors (Nikon, Melville, NY).

Morphologic Evaluation of Kidneys

Hematoxylin-eosin–stained kidney sections were scored in a blinded manner for the severity of renal damage, based on the previously established criteria (20,21).

Measurement of Serum Creatinine and UA

Serum creatinine concentration was measured using a commercially available kit (Raichem, San Diego, CA) according to the manufacturer’s protocol. Serum concentration of UA was measured using the Amplex Red UA/uricase assay kit (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions.

Statistical Analyses

The results were expressed as means ± SEM. The means of two populations were compared by t test. For multiple comparisons, ANOVA was used. Differences were considered significant at \( P < 0.05 \).

Results

UA Levels in the Blood after Renal Ischemia

To test the hypothesis that UA may serve as endogenous stimulus for EPC mobilization in acute ischemic renal injury and subsequently be involved in mediating renoprotection, we
performed bilateral renal pedicle clamping in FVB/NJ mice and examined systemic concentration of UA at various times after reperfusion. Already 5 min after release of the clamp after an ischemic period of 30 min, systemic UA concentration was significantly elevated, as compared with sham-operated controls (n = 4). No increase was detectable at 1 h after ischemia. The injection of 50 μg of UA significantly elevated systemic UA into the same range (measurement at 5 min after injection) ARF, acute renal failure (resulting from bilateral renal ischemia). Data are means ± SEM. *P < 0.05.

Injection of UA Is Sufficient to Mobilize EPC

To evaluate the potential EPC-mobilizing activity of UA alone, without inflicting ischemic injury, we examined dosage-response relations in FVB/NJ mice that were treated with increasing single doses of UA (6.25, 12.5, 25, 50, 75, and 100 μg per mouse). A single dose of 50 μg of UA induced a significant mobilization of EPC, as judged by the increased splenic sequestration of EPCs, at 3 h and was even more pronounced at 7 d after the injection (Figures 2 through 4). To analyze whether injection of 50 μg of UA would result in a systemic UA increase comparable to the increase after renal ischemia, we measured blood UA at 5 min after the injection. Systemic UA was elevated approximately 2.4-fold, which was within the same range as renal ischemia–induced surge in UA (Figure 1). None of the other tested dosages of UA led to a splenic EPC increase. Incidentally, the number of detectable circulating EPCs did not change at any checked time point after the administration, consistent with our previous observations (7).

Acute Stimulation of Endogenous UA Production Mobilizes EPCs

To investigate whether an acute elevation of UA as a result of the blockade of the degradation pathway is capable of mobilizing EPCs, we treated FVB/NJ mice with a single dose of the
uricase inhibitor OA. Because substantial increases in the UA levels have been documented after the injection of 280 mg/kg (17), this dosage was chosen as a starting dosage and then tapered (140, 70, 35, 17.5, and 8.75 mg/kg) to evaluate the dosage-response relations between OA and EPC mobilization. Mobilization of EPCs was detectable only with the use of 8.75 mg/kg of the injected dose, as judged by the splenic EPC sequestration at 3 h after the treatment. Importantly, UA was elevated already after 15 min but returned to the basal level at 3 h, the time when mobilization of EPCs was observed. These data further support the notion that a brief increase in UA, rather than its prolonged elevation, is sufficient to act as EPC-mobilizing stimulus (Figure 5).

Necessity of UA Signaling

The finding of EPC mobilization after a single systemically administered dose of UA led to the question of whether this stimulus was necessary and whether further uricase-induced metabolism of UA to allantoin can reverse its stimulatory effects on EPC mobilization. Therefore, uricase was intraperitoneally applied over a 3-d period before UA injection as described previously (22). Under these experimental conditions, UA-mediated EPC mobilization was completely curtailed at both 3 h and 1 wk after the treatment (Figures 2 and 3). Collectively, these data indicate that acute elevation of UA but not its degradation products is a necessary ischemia-induced signal for EPC mobilization.

Effects of Adenosine and Inosine on Peripheral EPC

Previous studies by Miller et al. (13) demonstrated an early reversible elevation in systemic levels of adenosine and inosine after an acute ischemic episode, even of a very short duration. To evaluate potential EPC stimulatory effects of these purine metabolites, we administered a single injection of either adenosine or inosine using the concentrations used previously (18). These treatments, however, resulted in neither increased numbers of circulating nor increased splenic EPCs at 3 h after injection (Figure 6).

UA Treatment Affords Pharmacologic Preconditioning

Because EPCs have been shown to exhibit renoprotective effect in acute ischemic renal injury (7,23) and because mobilization of EPCs was documented after a single injection of UA, the biologic consequences of short-term UA treatment in the setting of acute renal failure were tested. Mice were administered an injection of 50 μg of UA, and 3 h later (at the time when EPC mobilization commenced), bilateral renal artery clamping of 30 min was performed. A separate group of mice received uricase in addition to UA. Recipients of UA therapy were significantly protected from ischemic renal injury. This renoprotective effect was absent in mice that received combined UA/uricase treatment (Figure 7). Histologic examination of the kidneys, performed in a blind manner, demonstrated that the injury was significantly reduced in mice with UA pretreatment compared with the combined UA/uricase therapy (total renal
injury score in UA versus UA/uricase-treated animals 1.1 ± 0.3 versus 2.3 ± 1.6; \( P = 0.003 \). Finally, mice that received UA pretreatment showed sporadic c-Kit\(^+\)/Tie-2\(^+\) cells in the renal medulla (Figure 8). These observations are consistent with the novel function of UA in pharmacologic preconditioning.

Lack of EPC Mobilization in Mice with Chronic (OA-Induced) Hyperuricemia

OA is a potent inhibitor of the enzyme uricase and is responsible for degradation of UA to allantoin in rodents but is much less potent in humans (19). To evaluate the consequences of a chronic increase in the level of UA on mobilization of EPC (as opposed to its acute elevation after a single injection), we treated FVB/NJ mice with OA on a long-term regimen. This regimen has been shown to strongly increase systemic levels of UA (19). We confirmed a large increase of systemic UA at the end of the 2-wk feeding period. OA-induced chronic hyperuricemia, in contrast to the acute increase, failed to produce any elevation in circulating or splenic EPC (Figure 9). In the postischemic period (2 d after renal artery clamping for 30 min), renal function in chronically hyperuricemic mice was significantly impaired, as compared with both the untreated controls and the mice that received UA as a single-dose pretreatment (Figure 10). This observation demonstrated that only a short-term elevation, rather than a long-term increase in the level of UA, is capable of acting as the EPC-mobilizing stimulus.

Discussion

The data show for the first time agonistic effects of UA on the mobilization of EPCs. The stimulatory effects, reflected by splenic accumulation of EPCs (7), were detectable as early as 3 h after a single injection of UA as well as 3 h after indirect
elevation of UA by treatment with OA. The increase remained detectable until 1 wk after UA administration. The agonistic effect of UA on EPC mobilization was dosage dependent because it occurred (1) only with 50 μg of UA per mouse and (2) only with the administration of 8.25 mg/kg but not with higher dosages of OA. Neutralization of UA by uricase completely abolished its agonistic effect. As opposed to UA, its precursors adenosine and inosine did not promote mobilization of EPCs at concentrations that were used in this study. Systemic UA was elevated as early as 5 min after commencement of renal reperfusion; its increase was approximately 2.4-fold and thereby within the same range as 5 min after injection of 50 μg of UA. In accordance with our previous data demonstrating that EPC mobilization is renoprotective in the setting of renal ischemia-reperfusion injury (7), single-dose treatment with UA was found to be protective against acute renal ischemia. Chronic (OA-induced) hyperuricemia did not stimulate EPC mobilization or protect ischemic kidneys from ischemic injury. Therefore, UA seems to serve as a fast-acting, necessary, and self-sufficient endogenous mechanism to signal cell damage in renal ischemia and mediate mobilization of EPCs.

Stimulatory effects of UA have been documented in the immune system: Shi et al. (14) identified UA as the substance that promotes maturation of dendritic cells in vitro and enhances CD8+ T cell activity in vivo. Whether those functional changes in immune cells and the presently reported EPC mobilization and renoprotection are directly mediated by UA or require a second messenger(s) remains to be established. It has
been argued that TGF released by cells of the monocytic lineage after uptake of UA (24) could be involved in immunomodulation, but this would not explain the renoprotection that was observed in our study.

It is interesting that the tested precursors of UA did not elevate peripheral EPC at dosages used. Both adenosine and inosine have been reported to be elevated in the renal venous blood of various animals already a few minutes after a brief period of renal ischemia (13). Furthermore, the adenosine A$_2_A$ receptor agonist CGS-21680 has been shown to stimulate vasculogenesis in wounds of FVB/NJ mice with bone marrow transplantation (Tick-2 GFP mice served as donors) by mobilizing EPCs to the respective tissue areas (25). Comparable effects have been identified for dibutyryl cAMP, an adenosine metabolite (26). However, in both experimental settings, the drugs were administered topically on a long-term regimen, whereas in this study, adenosine and inosine were injected systemically and singularly. Because the effect of acute administration of UA could not be mimicked by its precursors and was not dependent of its catabolites (as judged from the results of uricase co-administration), we conclude that it is UA per se that acts as a “danger signal” in acute renal ischemia, leading to EPC mobilization and renoprotection. With regard to the dosage dependence of these effects, it has to be recognized that not only the total increase in systemic UA but also the duration of its elevation is of significant importance in eliciting this action: EPC mobilization was detectable only with “near-physiologic” its elevation is of significant importance in eliciting this action: EPC mobilization was detectable only with “near-physiologic” increases of UA (approximately 2.5-fold increase 5 min after injection of 50 $\mu$g), whereas a much more severe hyperuricemia suppresses this effect. However, the duration of the increase seems to be relevant as well. Three hours after the injection of either 140 or 75 mg/kg OA, UA concentration was persistently elevated two- to 2.5-fold, but EPC mobilization was absent. Thus, prolonged hyperuricemia is associated with the loss of EPC-mobilizing effect, even when the systemic level of UA is comparable to that observed after the transient increase after ischemia or injection of 50 $\mu$g of UA.

In contrast to the acute elevation of UA, heralding injury and mobilizing EPCs, chronic hyperuricemia lacks this function. This is a remarkable observation regarding the known deleterious consequences of persistent hyperuricemia not only in terms of kidney function. Chronic hyperuricemia has been associated with damage to various organs, including kidney, joints, and blood vessels (27). The responsible cellular/molecular mechanisms include the activation of vascular smooth muscle cells (28) and the stimulation of specific mitogen-activated protein kinases (19,29,30), nuclear transcription factors (29), COX-2 (19), PDGF A and C chains (29,31), PDGF-α receptor (29), and various inflammatory mediators, including C-re- active protein (30) and the monocyte chemoattractant protein-1 (15). Soluble UA has also been shown to activate primary human vascular endothelial cells and to block nitric oxide release, thereby inhibiting endothelial proliferation (30,32). Conversely, UA is a potent scavenger of peroxynitrite (33,34), and it has been shown to increase the activity of superoxide dismutases 1 and 3 in the aortic wall of ApoE$^{-/-}$ mice (35). Inosine, a precursor of UA whose administration results in hyperuricemia, has been proposed for treatment of experimental allergic encephalomyelitis (18). Potassium oxonate (uricase inhibitor used in this study) is one of three components of 5-1, an antineoplastic agent that additionally contains 5-fluorouracil and 5-chloro-2,4-dihydroxypyridine and reduces gastrointestinal and myelotoxicity (36). These disparate actions of UA may explain the disparity between the renoprotective effect of acute hyperuricemia and its lack in chronic hyperuricemia.

Conclusion

These findings identify UA as a physiologic, fast-acting endogenous mediator of EPC mobilization in response to tissue ischemia. This action of UA may be used for pharmacologic preconditioning. In addition, the data confirm our previous observations (7) on the renoprotective effect of EPCs in the course of acute ischemia. The lack of EPC-mobilizing action in chronic hyperuricemia should provide a cautionary note to the potential adverse effects of UA-elevating therapies. Finally, it is proposed that a transient surge in UA may serve as a universal herald of tissue ischemia and signal for recruitment of EPCs.

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Disclosures

None.

References


Figure 10. Effects of chronic (OA-induced) hyperuricemia on renal function in the setting of acute renal ischemia. The renal protective effect of a single injection of UA was completely abrogated in mice with chronic (OA-induced) hyperuricemia. Two days after bilateral renal artery clamping of 30 min, plasma creatinine concentration was comparably elevated in mice that had been treated with combined UA and uricase or with OA before the ischemic injury. Data are means ± SEM.


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