

# NO Bioavailability, Endothelial Dysfunction, and Acute Renal Failure: New Insights Into Pathophysiology

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**This brief overview sketches current evidence of imbalance between inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS), role of oxidant stress, and generation of peroxynitrite in the pathophysiology of acute ischemic renal injury. The development of endothelial cell dysfunction at early stages of experimental acute renal ischemia is the focus of the review, with the results of recent studies on amelioration of renal injury by the infused endothelial cells engrafting in the renal microcirculation. Finally, this article provides some future perspectives on the potential usefulness of endothelial progenitor cells in the prevention and treatment of acute renal failure.**

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**A**DVANCES IN vascular biology over the past 2 decades led to significant cross-fertilization of other fields of medicine with the ideas and approaches initially developed in relation to microvascular homeostatic mechanisms. Principles and strategies of therapeutic correction of endothelial dysfunction or therapeutic angiogenesis and anti-angiogenesis as applied to treatment of cardiovascular diseases and tumors, respectively, exemplify such a penetration of vascular biology to the areas traditionally focused on studies of the myocardium or neoplastic cells. In the field of renal diseases, there is emerging interest in disclosing the contribution of vascular pathobiology to the pathophysiology of several major syndromes, from the progression of chronic renal disease to the dysfunction of the aging kidney.<sup>1,2</sup> In parallel, it is becoming increasingly recognized that endothelial dysfunction may accompany acute renal diseases, such as hemolytic uremic syndrome or acute renal failure.<sup>3-6</sup> Because altered generation of nitric oxide (NO) by the endothelial cells or reduced NO bioavailability represent an important feature of endothelial dysfunction, this brief review is focused on these subjects and their contribution to the pathophysiology of ischemic acute renal injury.

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The idea of imbalance between the expression and activity of the inducible and constitutive endothelial isoforms of nitric oxide synthase (NOS) as an important contributor to the pathophysiology of acute renal failure is based on the heterogeneity of NO effects depending on the site of its production, duration of its effect, and the level of concomitantly present reactive oxygen intermediates. Transient spike-like generation of NO, characteristic of endothelial NOS (eNOS) activation,<sup>7</sup> is critical for turning on heme-containing enzymes such as the soluble guanylate cyclase, mediation of vasorelaxation, and anti-apoptotic and antioxidant phenotype.<sup>8</sup> Sustained, high-output generation of NO by iNOS, depending on the cellular context, may turn on sequelae as broad as lipid peroxidation, DNA damage, and anti- and pro-apoptotic effects.<sup>9</sup>

## EFFECTS OF NOS INHIBITORS AND iNOS DEFICIENCY

Yu et al showed that a general NOS inhibitor prevented hypoxic cellular damage in freshly prepared proximal tubules.<sup>10</sup> Peresleni et al<sup>11</sup> showed that oxidant stress to African green monkey epithelial cells (BSC-1) resulted in increased immunodetectable iNOS, increased NO release and nitrite production, and decreased cell viability. When BSC-1 cells were transfected with an antisense oligodeoxynucleotide (AS-ODN) to iNOS, this treatment abrogated an increase in nitrite production, reduced the intensity of immunocytochemical and the reduced form of nicotinamide-adenine dinucleotide phosphate diaphorase staining, as well as prevented lethal cell damage. In parallel, staining with antibodies to nitrotyrosine, conspicuous in stressed cells, was undetectable in AS-ODN-treated cells. These data support the possibility of NO conversion to peroxynitrite during oxidative

stress and implicate it in cytotoxicity to BSC-1 cells.<sup>12-14</sup> Several lines of evidence suggest the possible link between oxidant stress and NO production. It has been shown that both the myocardial reoxygenation<sup>15</sup> and cerebral ischemia and reperfusion<sup>16</sup> result in the increased generation of NO. It has been hypothesized recently that cytotoxic sequelae of NO production depend on the redox state of the cell and its ability to generate peroxynitrite (ONOO<sup>-</sup>) anion.<sup>13</sup>

Studies by Ling et al<sup>17</sup> have shown that proximal tubules isolated from mice with the targeted deletion of iNOS were resistant to hypoxia, whereas tubules obtained from mice lacking eNOS or neuronal NOS (nNOS) were damaged lethally by the same degree of hypoxia. These studies strongly support the idea that, despite the shared substrate and end-product by all 3 enzymes, the sequelae of their activation may be vastly different. This most probably is owing to the differences in the temporary profile of NO output and the topography of NO release by each NOS isoform.

Results of *in vivo* application of AS-ODN targeting iNOS further support the role of NO generated by this high-output enzyme in renal injury.<sup>18</sup> Administration of AS-ODN resulted in a dramatic functional protection of kidneys from acute ischemia. The concentration of plasma blood urea nitrogen and creatinine in this group was not different from sham-operated animals and was significantly less than that detected in experimental animals receiving sense and scrambled constructs. The major registered effects of antisense ODN were in the prevention of tubular necrosis, diminution of the loss of the brush border membrane, and reduction in cast formation. More recently, with the development of a selective iNOS inhibitor, L-N<sup>6</sup>-(1-iminoethyl)lysine (L-Nil), we have revisited the problem and were able to confirm previous findings.<sup>19</sup> Administration of L-Nil to rats subjected to renal artery cross-clamping ameliorated renal dysfunction.

Hence, selective inhibition, depletion, or deletion of iNOS, not affecting constitutive isoforms, clearly showed its renoprotective effect against ischemia. This effect was owing to, at least in part, the rescue of tubular epithelial cells from injury induced by a product of iNOS concomitant with increased production of reactive oxygen intermediates.

## POSSIBLE ROLE OF PEROXYNITRITE IN CELL INJURY

Generation of superoxide and NO in ischemia/reperfusion injury results in formation of a cytotoxic metabolite, peroxynitrite, that is capable of causing lipid peroxidation and DNA damage. We studied the contribution of oxidative and nitrosative stress to the renal damage in rats after 45 minutes of renal artery clamping. Renal ischemia resulted in lipid peroxidation, DNA damage, and nitrotyrosine modification, as detected by using Western blot and immunohistochemical analyses. Animals were treated randomly with an inhibitor of iNOS (L-Nil), cell-permeable lecithinized superoxide dismutase (SOD), or both. The results showed that L-Nil or lecithinized SOD treatments improve renal function after renal ischemia by suppressing iNOS and oxidative stress, with the combination of agents eliciting a near-correction of renal dysfunction.<sup>19</sup>

Having shown the role of anti-oxidative and antinitrosative compounds (lecithinized SOD and L-Nil, respectively) and their additive effect in ameliorating ischemia-induced renal dysfunction, we next attempted to directly test the identity of a noxious product, either superoxide, NO, or peroxynitrite, by using a bona fide peroxynitrite scavenger, ebselen. Peroxynitrite scavenging by ebselen shows the second-order rate constant of  $2 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$  at pH 7.4 and 37°C, exceeding the rate of peroxynitrite reaction with the natural antioxidants such as ascorbate or glutathione by approximately 3 orders of magnitude. On the other hand, the rate of peroxynitrite decomposition, after protonation to form peroxynitric acid (ONOOH), which potentially can yield approximately 30% of hydroxyl radicals, proceeds with the rate constant about 3 orders of magnitude faster than its scavenging.<sup>20</sup> Therefore, efficient scavenging of peroxynitrite can be achieved by creating an excess of ebselen. Our studies showed that ebselen resulted in amelioration of renal dysfunction, associated with a decrease in nitrotyrosine formation.<sup>19</sup>

It has been shown that ischemic and endotoxin-induced renal injury are accompanied by nitrotyrosine formation.<sup>18,21</sup> Apart from inhibiting the activity of several highly susceptible enzymes (ie, prostacyclin synthase,<sup>22</sup> prostaglandin endoperoxide synthase,<sup>23</sup> and Mn-SOD<sup>24</sup>), detection of nitrotyrosine-modified proteins is indicative of the con-

comitant oxidative and nitrosative stress resulting in peroxynitrite formation.<sup>25</sup> Recently, the uniqueness of peroxynitrite in generating nitrotyrosine residues has been questioned.<sup>26</sup> Therefore, the finding that peroxynitrite scavenger ebselen reduces (1) nitrotyrosine formation, (2) DNA damage, (3) lipid peroxidation, and (4) improves the functional outcome of renal ischemia lends additional support to the notion that oxidative and nitrosative stress occur in this condition *in vivo* and are involved mechanistically in the ensuing loss of kidney function. Interestingly, iNOS *per se* can be responsible for generation of reactive oxygen intermediates, especially when L-arginine becomes depleted in macrophages.<sup>27</sup> Furthermore, the presence of SOD even may catalyze the peroxynitrite-induced nitrotyrosine modification of target proteins, thus making this particular therapeutic choice somewhat less desirable.<sup>24</sup> Hence, theoretically a preferred pathway for limiting oxidative and nitrosative stress could be found in a highly selective inhibition of iNOS or scavenging of peroxynitrite.

#### POSSIBLE PATHOPHYSIOLOGIC ROLE OF DEFECTIVE eNOS/NO SYSTEM

Inhibition of eNOS is one of the hallmarks of the developing endothelial cell dysfunction, which may accompany some forms of acute renal injury. It has been known for a long time that ischemic kidneys do not respond to acetylcholine with a decrease in the vascular resistance, but rather show an increase in resistance.<sup>28</sup> The observation of Conger et al<sup>28</sup> of the lack of responsiveness to acetylcholine in rats with acute renal failure is one of many examples of poor responses to endothelium-dependent vasodilators in this condition. The question is whether this phenomenon is a reflection of inhibition of the enzyme or rather its maximal stimulation and possible reduction in NO availability.

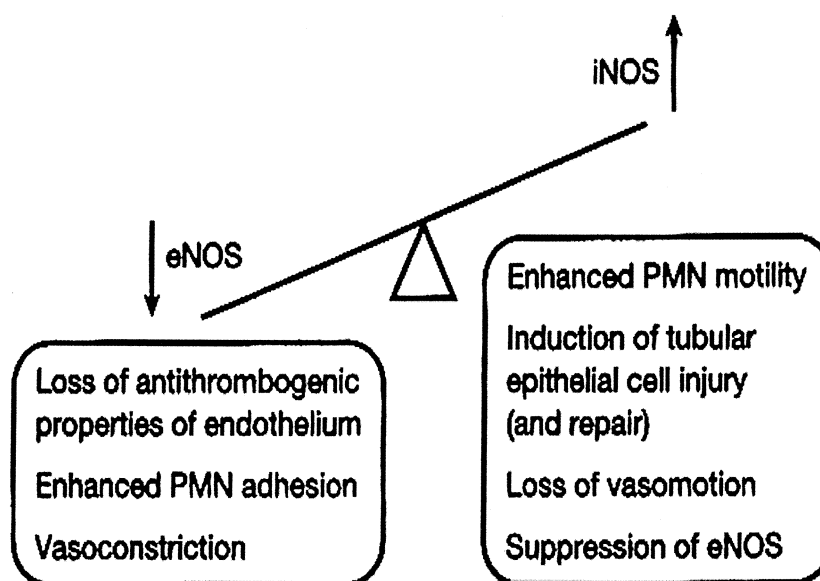
To gain insights into the possibility of suppression of eNOS activity by the high-output NO production via iNOS, we performed *in vivo* monitoring of NO release from control and ischemic kidneys by using an NO-selective electrochemical technique.<sup>18</sup> Intravenous administration of bradykinin resulted in an increase of NO release, as judged by the amplitude of the current, in control rats. In contrast, bradykinin had only a marginal

effect in kidneys of ischemic rats. These observations on the blunted NO release in renal ischemia corroborate the previously reported findings of the absence of vasorelaxation in response to endothelium-dependent vasodilators.<sup>29</sup> The baseline current recorded from ischemic kidneys was higher than that detected in control and AS-ODN-treated ischemic kidneys. Furthermore, although profoundly suppressed in nontreated ischemic kidneys, the bradykinin-induced increase in NO release was spared in AS-ODN-treated animals. These data suggest that the high-output NO production by iNOS may suppress the activity of eNOS (without changing its abundance). Collectively, these observations are consistent with the imbalance of NOS function in acute renal injury: the activity of the transient-output system, eNOS, is suppressed, whereas the activity of the high-output system, iNOS, is enhanced. The functional consequences of such an imbalance could explain some peculiar vascular phenomenology of this syndrome, as detailed in Figure 1.

#### ENDOTHELIAL DYSFUNCTION IN ACUTE RENAL ISCHEMIA

Flores et al<sup>30</sup> have shown that endothelial cells in the renal vasculature undergo an early swelling, leading to the narrowing of the lumen during renal ischemia. This observation was conceptualized in a no-reflow hypothesis.<sup>31,32</sup> It has been shown that ischemic renal vasculature is characterized by the profound loss of a vasorelaxing effect of acetylcholine.<sup>33</sup> Conger et al<sup>28,29</sup> have shown that vasorelaxation in response to stimuli generating endothelium-derived relaxing factor was inhibited in acute renal failure. In addition, NO production in response to bradykinin was found to be suppressed in ischemic kidneys.<sup>18</sup> Overexpression of intercellular adhesion molecule-1 by the vascular endothelium of the ischemic kidney has been shown and neutralizing anti-intercellular adhesion molecule-1 antibodies significantly improved the outcome of renal ischemia.<sup>34</sup> Furthermore, we have shown that the endothelium of renal microvessels in ischemic kidneys showed an enhanced expression of the Arg-Gly-Asp (RGD) peptide binding integrins.<sup>35</sup> Collectively, these observations are suggestive of endothelial cell dysfunction occurring in acute renal failure.

Hence, 3-decade-old ideas<sup>30</sup> incriminating dam-



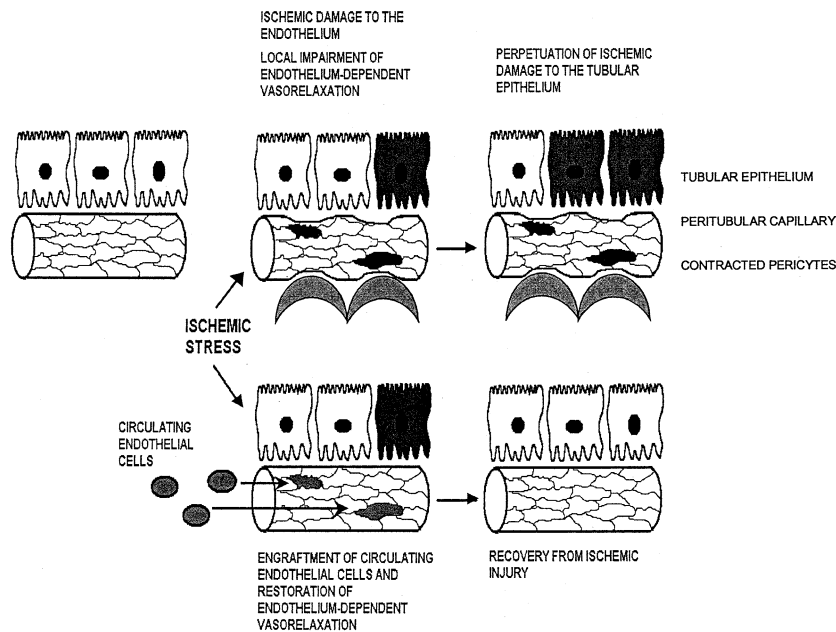
**Fig 1.** Hypothetical schema of the relations between iNOS and eNOS in ischemic acute renal injury.

aged endothelium in the development of a no-reflow phenomenon at early stages of acute renal ischemia seem to be resurrected and become enriched with a deeper understanding of endothelial cell pathobiology. By using minimally invasive intravital microscopy of glomerular and peritubular capillary blood flow immediately after ischemic insult to the rat kidney, we documented no-reflow phenomenon manifested by the cessation, deceleration, or reversal of blood flow, all occurring sporadically in pre- and postglomerular microvasculature. Morphologic analysis revealed the loss of endothelial integrity in the renal microvasculature. Transplantation of functionally competent mature endothelial cells into the circulation of postischemic rats resulted in a remarkable protection of the kidney against ischemic injury. This functional protection was linked to the engraftment of transplanted cells into renal microcirculation. A similar, albeit less profound, effect was achieved by transplantation of surrogate cells expressing a single endothelium-specific enzyme, eNOS. Based on these findings we hypothesized that (1) endothelial dysfunction develops early in the course of ischemic acute renal failure and manifests both structurally in the loss of endothelial integrity and functionally in the defective endothelium-dependent vasorelaxation, reminiscent of the phenomenon described by Furchgott and Zawadzki<sup>36</sup> in denuded arteries (Fig 2). Furthermore, the observed renoprotective effect of transplanted endothelial cells

lead to the second hypothesis that (2) the preexisting circulating endothelial cells or endothelial progenitor cells could be boosted to improve natural defenses against renal ischemia, thus supplanting the need to transplant exogenous and heterologous cells.

#### INTEGRITY OF ENDOTHELIAL BARRIER IS IMPAIRED IN PATHOLOGIC PROCESSES

By using scanning electron microscopy, we previously have shown endothelial cell desquamation and formation of gaps between confluent endothelial cells treated with thrombin.<sup>37</sup> In the *in vivo* studies of venules, McDonald<sup>38</sup> provided direct evidence for increased gaps between endothelial cells during inflammatory processes. Most recently, a permanent damage to peritubular capillaries was discovered in rats subjected to renal ischemia.<sup>4</sup> Our own *in vitro* and *in vivo* studies have provided direct morphologic evidence for the loss of endothelial integrity in stressed endothelial monolayers or microvasculature of ischemic kidneys. Moreover, we were able to attenuate postischemic renal dysfunction by transplanting athymic rats with circulating heterologous endothelial cells. Collectively, these data provide a foundation for the search of endogenous and/or autologous sources of endothelial cells, which could be used to correct renal dysfunction—the main theme of the current research in our laboratory.

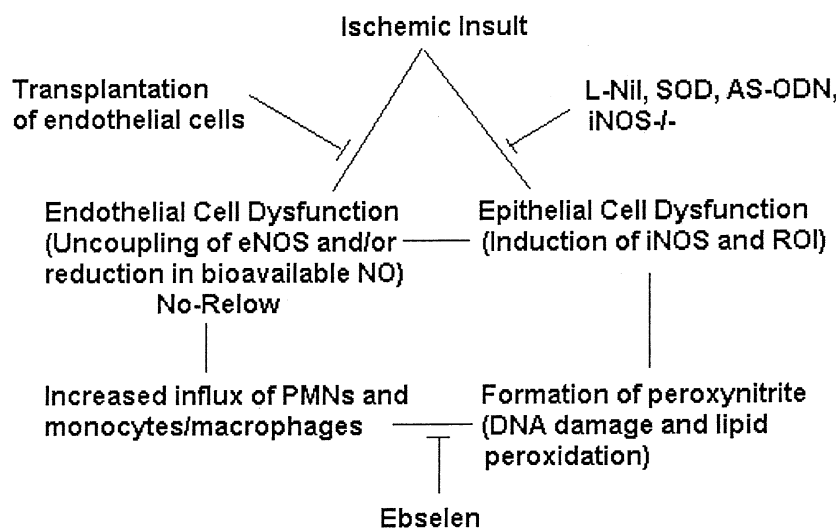


**Fig 2. A schematic view of the hypothesis. Black endothelial cells depict injured endothelium, gray endothelial cells depict circulating exogenous engrafted cells at sites of endothelial injury, dark epithelial cells depict injured epithelium.**

#### CIRCULATING ENDOTHELIAL CELLS AND THEIR PROGENITORS

More than 30 years ago, Boubier et al<sup>39</sup> were the first to detect endothelial cells in circulation. This observation was confirmed by Hladovec and Rossmann,<sup>40</sup> who have found approximately 3 endothelial cells/1  $\mu$ L of plasma in rats, a number that increased after treatment with citrate. The recent rediscovery of endothelial cells in the circulation,<sup>41,42</sup> which occurred on the background of a deeper appreciation of a multitude of endothelial functions, has a profound pathophysiologic and therapeutic significance. A pool of circulating endothelial cells appears to be heterogeneous. On the one hand, it contains mature endothelial cells, which have desquamated from the tunica intima of blood vessels, a process that is stimulated by vascular injury.<sup>41</sup> This phenomenon may explain the increased numbers of circulating endothelial cells in sickle cell anemia, myocardial infarction, unstable angina, thrombotic thrombocytopenic purpura, or primary pulmonary hypertension. On the other hand, this circulating cellular pool contains endothelial progenitor cells derived from bone marrow.<sup>42,43</sup> These cells appear to be recruited in response to injury or hypoxia.<sup>44</sup> The latter cohort contains endothelial progenitor cells at different stages of maturation, a process that remains poorly understood and insufficiently documented. By us-

ing bone marrow harvested from transgenic Tie2-b-lacZ mice and transplanting it to wild-type mice, Asahara et al<sup>45</sup> detected cells expressing lacZ reporter in newly formed vessels in ischemic tissues, thus establishing the functional activity of bone marrow-derived circulating endothelial progenitor cells. Notably, the functional profile of circulating mature and immature endothelial cells has been characterized too sparsely; it is known that endothelial progenitor cells could be recruited from the bone marrow and destined for angiogenesis in ischemic tissues, but no comparative information on the functional potential of mature endothelial cells exists so far. It has been hypothesized that circulating endothelial progenitor cells are involved in wound healing and tumor growth.<sup>42,45,46</sup> In this vein, our recent findings that transplanted mature endothelial cells can engraft in the renal microvasculature after ischemic injury resulting in improved renal function, represent the only indication that these cells possess the ability to afford a rapid protective effect on the damaged vasculature. In fact, one of the main topics of the laboratory is that circulating endothelial cells or their progenitors may rescue organs not only through stimulation of neovascularization (the process requiring several weeks), but also by restoring endothelial integrity of injured microvasculature and preventing the development of vasoconstriction



**Fig 3.** Flow chart of the pathophysiologic mechanisms operating in acute ischemic renal injury and the potential therapeutic interventions. Hypothetical sequence of events triggering renal dysfunction after renal artery cross-clamping.

and no-reflow in an ischemic organ (the process requiring several hours). But what is the evidence to support the notion that the integrity of endothelial barrier is impaired in pathologic processes? There is growing realization that diverse pathologic processes are accompanied by the injury to vascular endothelium. Progressive, but not passive Heyman nephritis, remnant kidney model, and acute renal injury are a few examples.

#### THERAPEUTIC EFFECTS OF ENDOTHELIAL PROGENITOR CELL TRANSPLANTATION

Therapeutic strategies based on the use of endothelial progenitor cells have been described. In rats with acute myocardial ischemia induced by ligation of the left anterior descending coronary artery, transplantation of endothelial progenitor cells partially rescued left ventricular function.<sup>47</sup> This effect was attributed to improved angiogenesis in the ischemic myocardium, although the possible role of improved vascular function was not addressed in the study. In diabetic mice, but not in control animals, transplantation of blood-derived angioblasts accelerated the restoration of blood flow to ischemic hind limbs.<sup>48</sup> This differential response in diabetic and control animals may be related to the preexisting endothelial dysfunction in animals that benefited from angioblast transplantation. Later we describe the beneficial effect of endothelial cell transplantation into rats subjected to acute renal ischemia. We reasoned that initially the use of endothelial progenitor cells did not appear to represent the strategy of choice owing to the pro-

tracted period of differentiation of transplanted cells<sup>49,50</sup> and gave preference to the use of fully differentiated human umbilical vein endothelial cells (HUVEC) for transplantation. The earlier-mentioned strategies to ameliorate acute renal failure are schematically depicted in Figure 3.

Having proved the main point—the existence of endothelial dysfunction in acute renal ischemia and the potential for functional salvage by transplanted endothelial cells—it has become much more rational to attempt boosting the mobilization and accelerating the maturation of circulating endothelial progenitor cells. The existing obstacles to this strategy are summarized next.

#### CURRENTLY EXISTING OBSTACLES TO ENDOTHELIAL PROGENITOR CELLS TRANSPLANTATION

As mentioned earlier, endothelial progenitor cells are rather scarce under normal conditions (2 cells/uL) and their detection, as well as harvesting, represent a major obstacle for transplantation experiments. Vasa et al,<sup>51</sup> by using fluorescence-activated cell sorter analysis of CD34+ and KDR+ cells, showed that they represent about 0.028% of circulating leukocytes in healthy volunteers and about one half of that value in patients with coronary artery disease. Ex vivo expansion of endothelial progenitor cells is defined poorly because about one third of plated individual samples show no attachment to fibronectin-coated dishes and fail to differentiate.<sup>48</sup> Furthermore, circulating progenitors could be present at different stages of

maturation—some expressing only undifferentiated markers (eg, AC133), and others expressing eNOS among other differentiated markers of endothelial cells. Maturation of nondifferentiated cells may require a protracted period of time for these transplanted cells.<sup>45,49</sup> Currently, used markers for harvesting endothelial precursor cells of hematopoietic origin include CD34 positivity, expression of vascular endothelial growth factor receptor-2, Tie-2, and AC133 (+). The process of maturation appears to be associated with an increasing expression of eNOS and a decreasing expression of AC133.<sup>52</sup> In bone marrow-derived hematopoietic stem cells, the loss of Thy-1.1 and gain of vascular endothelial growth factor R2 heralds the loss of self-renewal.<sup>53</sup> It is, however, imperative to arrive at a deterministic genomic profile of different subsets of circulating endothelial progenitor cells of hematopoietic lineage—one of the goals of this proposal. The potential ways of overcoming the earlier-mentioned obstacles and shortcomings of strategies dealing with transplantation of endothelial progenitor cells may include: (1) an increased harvest of cells through cell culture and (2) an accelerated differentiation by using a defined culture medium.

#### FUTURE DIRECTIONS

Several corollaries for future directions of research can be drawn from the earlier discussion. These directions include (1) establishing a more detailed mechanistic view on the renoprotective effect of transplanted differentiated endothelial cells in renal ischemia; (2) obtaining information on the population of circulating endothelial cells and their progenitors in acute renal ischemia, (3) investigating possible strategies for preconditioning leading to the increased mobilization and accelerated maturation of these cells derived from endogenous sources, and (4) identifying the optimal conditions for harvesting and *in vitro* expansion of endothelial progenitors as a possible exogenous source of autologous endothelial cells.

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