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Biphasic recruitment of microchimeric fetal mesenchymal cells in fibrosis following acute kidney injury

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Fetal microchimeric cells (FMCs) enter the maternal circulation and persist in tissue for decades. They have capacity to home to injured maternal tissue and differentiate along that tissue's lineage. This raises the question of the origin(s) of cells transferred to the mother during pregnancy. FMCs with a mesenchymal phenotype have been documented in several studies, which makes mesenchymal stem cells an attractive explanation for their broad plasticity. Here we assessed the recruitment and mesenchymal lineage contribution of FMCs in response to acute kidney fibrosis induced by aristolochic acid injection. Serial *in vivo* bioluminescence imaging revealed a biphasic recruitment of active collagen-producing FMCs during the repair process of injured kidney in post-partum wild-type mothers that had delivered transgenic pups expressing luciferase under the collagen type I-promoter. The presence of FMCs long-term post injury (day 60) was associated with profibrotic molecules (TGF-β/CTGF), serum urea levels, and collagen deposition. Immunostaining confirmed FMCs at short term (day 15) using post-partum wild-type mothers that had delivered green fluorescent protein-positive pups and suggested a mainly hematopoietic phenotype. We conclude that there is biphasic recruitment to, and activity of, FMCs at the injury site. Moreover, we identified five types of FMC, implicating them all in the reparative process at different stages of induced renal interstitial fibrosis.

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Microchimerism, the presence of a rare population of cells within a genetically distinct host, occurs chiefly in pregnancy. Bidirectional trafficking of maternal and fetal cells is a well-described phenomenon occurring during most, if not all, pregnancies and results in long-term persistence of low levels of fetal or maternal cells in the mother and offspring, respectively.[1–3] Fetal microchimerism has been the subject of intensive research in the past decade, from which it has emerged that fetal microchimeric cells (FMCs) have capacity to home to injured maternal tissues and further differentiate along that tissue's lineage. The identification[4] of male FMC bearing epithelial, leukocyte, or hepatocyte markers in various tissue specimens from women with male offspring initiated the concept that fetal cells might have stem cell capabilities with multilineage potential. This was further supported by a range of studies demonstrating FMC plasticity along different lineages such as lymphoid[5,6] mesenchymal,[7–9] endothelial,[10–12] neuronal,[13] epithelial, and hepatic.[14,15]

This broad differentiation capacity raises the question of the origin of FMC transferred to the mother during pregnancy. Hematopoietic lineage cell types including mature T and B cells, dendritic cells, NK cells as well as more primitive lymphoid precursors[1,4,5,16–18] have been documented as transferring from fetus to mother. FMCs implying endothelial nature[12,19,20] have been detected in maternal blood and tissues during and after pregnancy as well as FMCs displaying characteristics of terminally differentiated hepatocytes[21] and epithelial cells.[4,22] However, other evidence implicates mesenchymal stem/stromal cells (MSC). FMCs in humans have been identified in trabecular bone, a known MSC niche.[23] In another study by our group, *ex vivo* expansion of the adherent cell population isolated from the blood of a mother undergoing first trimester termination, identified FMCs with tri-lineage differentiation capacity, displaying MSC characteristics *in vitro*.[24] Limited immunophenotyping of male adherent cells isolated from human female post-reproductive bone marrow was consistent with MSC.[24] Further studies on human fetal MSC populations suggested

increased differentiation potential beyond mesenchymal lineages, rendering them attractive candidates to explain the broad plasticity of FMC.[23,25] However, strictly mesenchymal phenotypes in the context of FMC have rarely been described.

In this study, we assessed the recruitment and mesenchymal lineage contribution of FMCs in response to acute kidney fibrosis. We used a transgenic approach to track FMCs in mice with aristolochic acid (AA) toxin-induced nephropathy. AA-nephropathy (AAN) was chosen as a model of renal fibrosis because of its simplicity and reproducibility in several mouse strains.[26,27] AA is a Chinese herbal extract, identified as a kidney toxin capable of inducing progressive interstitial fibrosis in humans and rodents. AAN is characterized by acute tubular injury and interstitial inflammation, and involves distinct long-term outcomes.[28] In this pathology, fibroblasts are involved in the production of excess extracellular matrix deposition resulting in interstitial fibrosis[29,30] involving type I collagen. Using two reporter models, COL1A2 transgenic mice and ROSA-GFP transgenic mice to track mesenchymal, and overall FMC, respectively, in the context of AAN, we describe biphasic recruitment of mesenchymal FMCs to renal injury sites. These two waves of recruitment coincide with the acute and chronic AAN process. Finally, we provide evidence for the active involvement of FMCs in the AAN repair process.

RESULTS

Generation of chimeric mice and induction of kidney toxicity

C57BL/6 females were crossed with males transgenic for the luciferase gene under the control of Col1a2 promoter (Col1-Luc). After serial pregnancies (at least two), mothers received intraperitoneal injections of AA toxin or saline solution over 5 days (Figure 1a). One group of mice ($n = 29$, 16 AA vs. 13 saline) was killed 60 days after first injection, and analyzed histologically using hematoxylin and eosin and Masson's trichrome staining (Figure 1bi and Supplementary Figure 1 online). Sections revealed patchy tubulointerstitial injury and fibrosis, characterized by tubular dilation or atrophy, interstitial volume expansion, interstitial fibrosis, and massive infiltration of inflammatory cells in AA-treated animals. These observations were quantified and each mouse assigned a histopathological score. Compared with saline-injected controls, AA-treated mice showed a significant increase in pathological manifestations (Figure 1bii) correlating with increased collagen deposition (Figure 1biii). The expression of transforming growth factor-beta (TGF-β), a profibrotic molecule, was increased, but not connective tissue growth factor (CTGF, CCN2; Figure 1c). AA-treated mice had increased plasma urea levels compared with control mice at day 60 ($P = 0.002$), albeit with blood creatinine level remaining normal (Figure 1d). These renal function results, suggesting half the AA-treated animals had measurable renal dysfunction, are consistent with moderate severity AAN at this time point.

Fetal cells recruited in maternal renal injury

Having established that the AA-toxin was capable of driving fibrosis in our model, we addressed the recruitment of collagen-producing FMC at the injury site. C57BL/6 females crossed with Col1-Luc transgenic males were examined for bioluminescence activity over 60 days. In chimeric mice carrying Col1-Luc transgenic fetal cells, bioluminescence was observed and localized at kidney sites (red circles, Figure 2ai). The bioluminescence was significantly increased over time in AA-treated animals ($P < 0.0001$) (Figure 2b). The bioluminescence kinetics in AA-treated mice revealed distinct phases between transgenic virgin mice, which showed a single peak that plateaued (Figure 2c, black triangle) while in wild-type mothers with Col1a2-luc fetal cells, two peaks of luminescence intensity were documented; an early (D16-18) and later time point (D45-52; Figure 2c, black circle). C57BL/6 virgin mice displayed minimal bioluminescence, considered background. Minimal bioluminescence was also observed at D0 in C57BL/6 virgin mice showing no difference between experimental and control groups before experiment (data not shown). The presence of few FMCs was confirmed by fluorescence *in situ* hybridization studies at day 60 (Supplementary data 4 online).

Early-recruited FMCs are mostly hematopoietic

After FMCs were detected at an early time point in the *in vivo* trafficking experiment, we used another model to investigate FMCs in kidney tissue at a shorter time point. The reason for the different model was the lack of a good luciferase antibody that could be used in wax-embedded samples. To assess FMC participation in tissue harvested during the acute injury stage, virgin C57BL/6 females were crossed with hemizygous GFP+ males, and after two serial pregnancies, females were injected with either AA-toxin or saline. Kidneys were harvested 15 days after first injection and their structure assessed histologically (Figure 3a). Altered renal morphology was confirmed in AA-treated animals ($P = 0.0002$; Figure 3bi and Supplementary Figure 1 online). Unsurprisingly, collagen deposition was also observed (Figure 3bii) but was not different between injured and healthy kidneys. TGF-β expression levels were increased in AA-injected mice ($P = 0.0008$), with the concomitant increase observed in both blood creatinine and urea levels (with $P = 0.016$ and $P < 0.0001$, respectively) reflecting the associated renal dysfunction (Figure 3c and d).

We next used anti-enhanced green fluorescent protein (EGFP) immunofluorescence to detect fetal cells in kidney sections from AA-treated versus saline-injected mice with scoring blinded to treatment allocation. Virgin mice injected with AA or saline buffer were used as negative controls. EGFP was detected most readily around vessels, forming clusters of two to three cells (Figure 4a). EGFP+ fetal cells were detected more frequently in kidney sections from AA-treated mice compared with controls (Figure 4b; 16 of 25 AA-treated vs. 3 of 21 saline-treated mice, $P = 0.0009$). Different shapes were observed suggesting the presence of various cell types