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Original article

Genetic lineage tracing analysis of c-kit$^+$ stem/progenitor cells revealed a contribution to vascular injury-induced neointimal lesions

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ABSTRACT

Aims: Accumulating evidence indicates the presence of vascular stem/progenitor cells that may play a role in endothelial repair and lesion formation in the injured artery, in which c-kit$^+$ stem/progenitor cells have been reported to differentiate into endothelial and smooth muscle cells *in vitro* and in ischemic tissue. In this study, we investigated whether and how endogenous c-kit$^+$ stem/progenitor cells contribute to vascular injury and neointima formation *in vivo*.

Methods and results: We created Kit-CreERxRosa26-RFP mice and performed genetic lineage tracing analysis of c-kit$^+$ stem/progenitor cells in injury-induced neointima formation *in vivo*. We provide direct evidence that endogenous c-kit$^+$ stem/progenitor cells minimally differentiate into endothelial or smooth muscle cells facilitating vascular repair, but predominantly generate monocytes/macrophages and granulocytes contributing to vascular immuno-inflammatory response to endothelial injury. Although c-kit$^+$ cells reside in both bone marrow and vessel wall, bone marrow transplantation data indicate that bone marrow-derived c-kit$^+$ cells are the main source for enhancing neointima formation. Furthermore, treatment of ACK2, a c-kit receptor antagonizer, attenuates neointimal hyperplasia after injury at least in part by depleting c-kit$^+$ cells and their generated progeny.

Conclusions: c-kit$^+$ stem/progenitor cells are not a main source for endothelial regeneration and smooth muscle accumulation of the large artery injury, but a plausible interventional approach to reduce vascular immuno-inflammatory response and subsequently to ameliorate vascular lesions.

1. Introduction

Neointima formation occurring in response to numbers of stimuli such as endothelial injury and hemodynamic change is a unifying pathological event in vascular diseases, especially in atherosclerosis and restenosis after percutaneous coronary interventions [1]. Intensive research on neointima formation after vascular injury has advanced our understanding of the pathogenesis of vascular remodeling. It was widely accepted that endothelial denudation or dysfunction initiates the intimal hyperplasia via infiltration and proliferation of inflammatory and smooth muscle cells and extracellular matrix deposit [1, 2]. Recently, it has been found that multipotent stem/progenitor cells from vessel wall and circulation possess potential to differentiate into endothelial, smooth muscle, mesenchymal or hematopoitic cell progeny [3, 4], implicating a role of these cells in maintaining vessel homeostasis and mounting pathological responses in vascular diseases

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[3–5]. But, the quantitative data on how many cells forming neointimal lesions are derived from stem/progenitor cells is still lacking.

Reports from different laboratories demonstrated the presence of vascular stem/progenitor cells using a variety of cell markers, including CD34, Sca-1 and c-kit [6–9]. Although there is no specific marker for vascular progenitors, c-kit positive (c-kit$^+$) cells are one of the well-known stem/progenitor cells found in human and mouse. Studies reported that adult endogenous c-kit$^+$ stem cells were both necessary and sufficient for functional ischemic tissue repair by regenerating endothelial cells and smooth muscle cells [10–12]. A single c-kit$^+$ adult vascular stem cell could produce millions of endothelial daughter cells *in vitro* and give rise to functional blood vessels for angiogenesis [13]. Furthermore, lineage tracing studies revealed that c-kit$^+$ cells did generate abundant CD31$^+$ endothelial cells in ischemic tissues, which is important for angiogenesis and of potential medicinal value [14]. However, it is unknown whether endogenous c-kit$^+$ cells play a role in endothelial repair after injury of the large vessel, e.g. femoral artery, and whether these cells contribute to neointimal lesion formation via differentiating into smooth muscle cells or other cells. In the present study, we address these questions using the lineage-tracing models to investigate if endogenous c-kit$^+$ cells are truly used by the large vessel as part of the pathological endothelial repair, as well as to provide additional convincing evidence that these cells have the potential contribution to neointima formation. We used inducible CreER-loxP recombination system and kit locus to generate a genetically-modified mouse model for lineage tracing analysis. We performed vascular injury models on Kit-CreERxRosa26-RFP mice to examine if and how c-kit$^+$ cells contribute to vascular injury and neointima formation *in vivo*. We found that endogenous c-kit$^+$ cells minimally contributed to endothelial regeneration and smooth muscle cell accumulation after vascular injury, but markedly enhanced lesion formation via infiltration and differentiation within neointima.

2. Materials and methods

2.1. Animals

Kit-CreERxRosa26-RFP mice were obtained by crossing Kit-CreER with Rosa26-RFP mice as previously described [15, 16]. Mouse genotype was screened by PCR of genomic DNA prepared from tail. Tamoxifen (Sigma, T5648) was dissolved in corn oil and administrated by gavage (0.1–0.15 mg g^{-1} × 4 times) to induce Cre recombinase activity. Both male and female mice were used in experiments. Mouse husbandry and all experimental procedures were performed in accordance with the guidelines of Institutional Animal Care and Use Committee of School of Medicine, Zhejiang University.

2.2. Artery injury models

Mice were anesthetized by isoflurane and the surgical procedure was similar to the previously described [17]. Wire-induced femoral artery injury was induced by insertion of a 0.25 mm flexible wire (tips of cross-IT 200× guide wire, Abbott Laboratories, Illinois, USA). The wire was inserted into the femoral artery with 3 passages and left in place for 3 min to denude endothelium and dilate the artery. Ligation-induced carotid artery injury was performed by ligating the left common carotid artery proximal to the bifurcation with a 6–0 suture. The artery samples were collected at the time point indicated for further analysis.

2.3. Immunostaining

The mice were subjected to perfusion fixation with 4% paraformaldehyde and arteries were collected at the indicated time points. The arteries were further fixed in 4% PFA for 2–3 h at 4° followed by dehydration in 30% sucrose overnight. The dehydrated samples were immersed in optimum cutting temperature (OCT, Tissue-Tek) for half an hour at 4° and then embedded and frozen at −80°. Cryosections of 7 μm thickness were collected. Slides were blocked for 1 h at room temperature in blocking buffer (PBS with 5% normal serum, 1% bovine serum albumin, 0.1% Triton X-100), incubated with primary antibodies overnight at 4°, and then secondary antibodies for 1 h at room temperature. Primary antibodies applied in the study were anti-RFP (Rockland, 600-401-379, 1:1000), anti-SMαA (Sigma, A5228, 1:100), anti-CD31 (BD Phharmingen, 553370, 1:100), anti-CD45 (Abcam, ab23910, 1:100), anti-CD11b (Abcam, ab64347, 1:100) and anti-Gr-1 (Abcam, ab25377, 1:100). Alexa Fluor 488- or 555- conjugated secondary antibodies (Invitrogen, 1:1000) were used to detect primary antibodies. All slides were mounted with anti-fade mounting medium with DAPI (Vector Lab, H1200). Images were acquired on Zeiss confocal microscopy (LSM710) and analyzed by ImageJ software.

2.4. Cell isolation

We isolated vessel cells by enzyme digestion of the whole artery. In brief, the artery was deprived of perivascular connective tissue carefully and cut into cubes (1 mm × 1 mm). Tissue cubes were digested in 1 mg/ml collagenase I and 0.744 units/ml elastase at 37 °C for 30 min and agitated gently by pipetting. Then dissociated cells were passed through a 70 μm cell strainer (Corning, 352350) to obtain a single-cell suspension. Bone marrow cells were isolated by flushing femurs with hanks balanced salt solution (HBSS). A 25-gauge needle with a syringe was used to flush the bone marrow from femurs. Cells were pipetted over a 70 μm cell strainer (Corning, 352350) and kept on ice for flow cytometry analysis. Blood leukocytes were obtained from whole peripheral blood by adding red blood cell lysis buffer (eBioscience, 00–4333).

2.5. Flow cytometry analysis (FACS)

Harvested cells were washed and adjusted to a concentration of 1×10^6 cells/ml in flow cytometry staining buffer solution (eBioscience, 00–4222). These cells were subsequently stained with the antibodies including CD45-PERCP-CY5.5 (BD Pharmingen, 550994), CD11b-APC (BD Pharmingen, 553312) and Gr-1-FITC (BD Pharmingen, 553126) at a dilution of 1:100 for 30 min in the dark. Then the samples were washed and re-suspended in staining buffer for FACS analysis using a BD LSR™ II flow cytometer (BD Bioscience). The FACS raw data were processed by Flowjo (TreeStar).

2.6. Bone marrow reconstitution

Bone marrow transplantation were performed as previously described [18, 19]. Briefly, bone marrow cells were harvested by flushing femurs and tibias from donor mice. Ten-week-old male mice received a lethal dose (9.0 Gy) of X-ray irradiation from a RS2000 irradiator (Rad Source). Six hours later, the irradiated recipient mice received 1×10^7 unfractionated bone marrow cells by tail vein injection. Six weeks after bone marrow transplantation, recipient mice with reconstituted bone marrow were used for tamoxifen induction and artery injury models. The efficiency of bone marrow transplantation was > 95% as determined by Y-chromosome detection [27, 28].

2.7. Statistics

All data were presented as mean ± standard error of the mean (SEM). Graphpad Prism 5 was used to perform statistical analysis. Two-group comparisons were conducted by two-tailed unpaired Student's *t*-test, with $p < .05$ considered statistically significant.