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Genetic overexpression of COMP-Ang1 impairs BM microenvironment and induces senescence of BM HSCs

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ABSTRACT

Supplemental Angiopoietin 1 (Ang1) exerts its therapeutic potential on microvascular regression-associated diseases, and this potential is linked with the function of hematopoietic stem cells (HSCs). However, the underlying mechanisms of the effect of enhanced angiogenesis on the modulation of HSCs are not yet defined. Here, we generated transgenic mice expressing **C**artilage **O**ligomeric **M**atrix **P**rotein (COMP)-Ang1 in keratin 14-expressing cells. The mutant animals expressed excessive angiogenic characteristics in the skin and bone marrow (BM) along with redder skin with more numerous and branched vessels compared with their wild-type (WT) littermates. The mutants displayed reduced long bone formation and osteoclast activity than did WT littermates and had fewer $CD150^{+}CD48^{-}$ **L**ineage^{-}**S**ca-1^{+}c-**K**it^{+} (LSK) cells in the BM. The mutants also exhibited greater senescence-associated (SA) β-gal activity, $p16^{INK4a}$ protein expression, and superoxide anion levels in $CD150^{+}CD48^{-}$LSK cells in the BM. Furthermore, transplantation assay revealed that the mutant-derived LSK cells were inferior to the cells derived from WT littermate in inducing competitive repopulating capacity in the recipients. Collectively, our results demonstrate that persistent and prolonged administration of COMP-Ang1 by inducible transgenic expression mediates excessive angiogenesis in the body and impairs BM microenvironment, eventually leading to senescence of BM HSCs.

1. Introduction

Angiogenesis, the process by which new blood vessels are formed from pre-existing vessels, is essential for organ growth and development, as well as for tissue repair [1,2]. Abnormal angiogenic processes contribute to numerous disorders in many organ systems including blood vessels, skin, lungs, bone, and the reproductive system [3,4]. Specifically, hematopoiesis is closely linked with angiogenesis during embryogenesis, and angiopoietin-1 (Ang1) plays an important role in maintaining quiescence and survival of hematopoietic stem cells (HSCs) via the Ang1/Tie2 signaling axis [5,6]. Ang1/Tie2 signaling is required for the maintenance of HSCs in adult bone marrow (BM) [7]. In addition, Ang1 promotes endothelial cell differentiation from embryonic stem cells and induced pluripotent stem cells, mainly through its cognate receptor, Tie2 [8], suggesting that the Ang1/Tie2 signaling axis closely regulates the processes required for angiogenesis and hematopoiesis and regulates vascular and hematopoietic development at both the embryonic and adult stages.

Based on its essential roles in vessel assembly, maturation, and stabilization [9,10], Ang1 has been used to treat microvascular regression-associated diseases to improve endothelial cell (EC) survival and to suppress vascular inflammation and leakage [11]. Indeed, treatment with Ang1 enhances blood vessel remodeling and formation in several organs and reduces ischemic side effects [12–14]. Supplemental Ang1 also attenuates inflammation-induced vascular leakage and inflammatory cell infiltration in the targeted ECs [15,16]. Ang1-deficiency exacerbates laser-induced choroidal neovascularization and vascular leakage, while genetic

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overexpression of Ang1 suppresses it by inhibiting the recruitment and infiltration of macrophages from the BM [17]. In addition, genetic overexpression of Ang1 using the skin-specific keratin-14 (K14) promoter produces leakage-resistant and enlarged vessels with an increased EC number in the skin [18,19]. Therefore, it is considered that that supplemental Ang1 positively regulates the development and growth of various organs by activating angiogenesis through the Ang1/Tie2 signaling axis, and that this regulation is closely linked with hematopoietic development. However, the underlying mechanisms of how excessive angiogenesis affects BM microenvironment and hematopoietic system are not completely understood.

In this study, we generated transgenic mice expressing **C**artilage **O**ligomeric **M**atrix **P**rotein (COMP)-Ang1; the transgene was created by replacing the N-terminal portion of Ang1 with the short coiled-coil domain of COMP utilizing the human K14 promoter. This was because COMP-Ang1 showed higher solubility and stability with greater angiogenic potential than did native Ang1 [20], and the K14 promoter is highly active in the basal layer of stratified squamous epithelia and in the outer root sheath of the hair follicles in mice, matching that of endogenous K14 [21]. We firstly examined the effects of genetic overexpression of COMP-Ang1 on the formation of vessels and lone bones. We also investigated how the enhanced angiogenesis influences the survival and senescence of HSCs in the BM. Our current findings suggest that transgenic overexpression of COMP-Ang1 induces excessive angiogenesis in the body, whereas it impairs the BM microenvironment and leads to senescence of HSCs.

2. Materials and methods

2.1. Generation of transgenic mice

All the animal procedures were performed in accordance with the guidelines of the Animal Care Committee of Chonbuk National University. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Chonbuk National University (Permit Number: CNU 2014-00055). We generated *K14-Cre;inducible (IND)-COMP-Ang1-transgenic (Tg)* mice [22,23] by crossing *K14-Cre* mice with *IND-COMP-Ang1-Tg* mice. Mouse offspring (3–4-week-old) were genotyped using polymerase chain reaction analysis according to the methods described previously [22,23].

2.2. Flow cytometric analysis of BM cells

BM cells were obtained and treated with red blood cell lysis buffer (Sigma-Aldrich Co. LLC) for 15 min in ice followed by washing with phosphate buffered saline (PBS). The levels of **L**ineage$^-$**S**ca-1$^+$c-**K**it$^+$ (LSK) and CD150$^+$CD48$^-$LSK cells in the BM were analyzed using a flow cytometer (BD Calibur or BD Aria, BD Biosciences). The populations of these cells were sequentially gated using a FlowJo software program. LSK and CD150$^+$CD48$^-$LSK cells were phenotypically identified using the following antibodies: lineage markers PE-Cy7-conjuaged anti-CD3 (cat.#552774), anti-B220 (CD45R; cat.#552772), anti-CD11b (cat.#552850), anti-Gr-1 (cat.#552958), or anti-TER-119 (cat.#557853) (All of these markers were from BD Biosciences); FITC-conjugated anti-Sca-1 (cat.#557405; BD Biosciences) or PE-conjugated anti-Sca-1 (cat.#553108; BD Biosciences); APC-conjugated anti-c-Kit (cat.#553356; BD Biosciences); perCP/Cy5.5-conjuated anti-CD150 (cat.#46-1502; eBioscience); and APC-Cy7conjugated anti-CD48 (cat.#561826; BD Biosciences). Senescence-associated β-gal (SA-β-gal) activity and mitochondrial superoxide anion levels in LSK and CD150$^+$CD48$^-$LSK cells that had already been incubated with the cell surface markers mentioned above were analyzed with C_{12}FDG (cat.#I2904; Molecular Probes) and MitoSox™ Red (cat.#M36008; Invitrogen), respectively. The level of p16^{INK4a} protein in these cells was determined using an Alexa Flour 488-conjugated antibody (cat.#sc-1661; Santa Cruz Biotechnology) after fixation and permeabilization (BD Biosciences).

2.3. Transplantation

To investigate the competitive repopulating capacity of donor cells derived from the mutants or their wild type (WT) littermates, recipient mice were lethally irradiated 12–24 h prior to transplantation. LSK cells (3×10^3 cells) from the mutants or their WT littermates (CD45.2) with BM cells (4×10^5 cells) from competitor mice (CD45. 1) were transplanted into conditioned recipient mice (CD45.1/2) by tail vein injection. Donor-mediated blood repopulating capacity was assessed using peripheral blood of the recipient mice after 5 months of serial transplantation.

2.4. Immunohistochemistry and TRAP staining

Immunohistochemistry was performed using the Histostain Plus Rabbit Primary (DAB) kit (Zymed Laboratories) and the Histostain Plus goat Primary (DAB) kit (Zymed Laboratories) according to the manufacturer's instruction. Hind limbs dissected from the mutants or WT littermates were fixed in 4% PFA at 4 °C for 12 h. After rinsing with PBS, the specimens were decalcified in 10% EDTA for 4 weeks, dehydrated, embedded in paraffin solution, and then sectioned at a thickness of 5 μm. The dried slides were incubated at 60 °C for 15 min before treatment with xylene I and II for 10 min each. Thereafter, the slides were hydrated through a descending series of ethanol concentrations (70–100%), followed by treatment with 3% hydrogen peroxide. The slides were incubated with anti-goat receptor activator of nuclear factor kappa-B ligand **(**RANKL) (cat.#sc-7628, 1:50; Santa Cruz Biotechnology). To identify osteoclast activity, the hind limb sections were also visualized using a tartrate-resistant acid phosphatase (TRAP) staining kit (cat.#sc-386A; Santa Cruz Biotechnology) according to the manufacturer's instructions.

2.5. Western blotting and ELISA

Equal amounts (20 μg/sample) of protein extract collected from BM cells flushed from the tibia and femur were run on 12% SDS–PAGE and blotted onto polyvinyl difluoride membranes. The blots were probed with primary antibodies specific for anti-rabbit Ang1 (cat.#ab95230, 1:100; Abcam) and anti-mouse monoclonal β-actin (cat.#sc-81178; 1:200; Santa Cruz Biotechnology) at 4 °C. An ELISA assay was also performed with RANKL and osteoprotegerin (OPG) mouse ELISA kits (Abcam) following the manufacturers' instructions.

2.6. Micro-CT analysis

The hind limbs dissected from the mutants or WT littermates were scanned using a desktop scanner (1076 Skyscan Micro-CT, Skyscan, Kontich, Belgium), followed by analysis with CTscan software (Skyscan).

2.7. Statistical analyses

All data were analyzed using SPSS program (ver. 12.0) and are expressed as mean ± SD. Differences between two groups were analyzed by Student's *t*-test. A value of $p < 0.05$ was considered statistically significant.

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