FEBS Letters 582 (2008) 3509–3514

Dexamethasone increases angiopoietin-1 and quiescent hematopoietic stem cells: A novel mechanism of dexamethasone-induced hematoprotection

Hyongbum Kim[a,b,1], Ji-Young Choi[c,1], Jung Min Lee[a,d], Yoon Shin Park[a], Hwal Suh[b], Hae-Ryong Song[e], Sangmee Ahn Jo[a], Inho Jo[c,*]

[a] *Department of Biomedical Sciences, National Institute of Health, Seoul, South Korea*
[b] *Department of Medical Engineering, Yonsei University College of Medicine, Seoul, South Korea*
[c] *Department of Molecular Medicine and Ewha Medical Research Institute, Ewha Womans University School of Medicine, Mok-6-dong, Yangchun-gu, Seoul 158-710, South Korea*
[d] *Department of Biotechnology, Korea University School of Life Sciences and Biotechnology, Seoul, South Korea*
[e] *Department of Orthopaedic Surgery, Korea University College of Medicine, Institute of Scoliosis Research, Guro Hospital, Seoul, South Korea*

Received 28 April 2008; revised 2 September 2008; accepted 9 September 2008

Available online 18 September 2008

Edited by Masayuki Miyasaka

Abstract **Angiopoietin-1 (Ang-1) is known to have hematoprotective effects by increasing the quiescence of hematopoietic stem cells. However, it remains to be determined if the upregulation of Ang-1 and the subsequent increase in the quiescence of hematopoietic stem cells are also involved in the dexamethasone (Dex)-mediated bone marrow protection. Here Western blotting and flow cytometric analyses demonstrate that Dex increases the levels of Ang-1 in mouse bone marrow and the quiescence of hematopoietic stem cells. Our data for the first time suggest that the increased quiescence of hematopoietic stem cells provides a novel mechanism of Dex-induced hematoprotection.**

Keywords: Dexamethasone; Hematopoietic stem cells; Angiopoietin-1; Hematoprotection; Bone marrow

1. Introduction

Hematopoietic suppression is the major dose-limiting toxicity of many anticancer chemotherapy agents [1]. Glucocorticoid pretreatment has been shown to protect hematopoietic stem cells (HSCs) from bone marrow-suppressing chemotherapy in mice [2] and humans [3]. It remains unknown if glucocorticoids increase quiescent HSCs that are more invulnerable to chemotherapeutic agent.

Tie-2 is a receptor tyrosine kinase expressed on endothelial cells and HSCs [4,5]. Angiopoietins (Angs) are ligands for Tie-2 and relatively little is known about Ang-3 and -4 [6]. Angiopoietin-1 (Ang-1) is a well known agonist for Tie-2, whereas Angiopoietin-2 (Ang-2) acts as an antagonist in the context of the endothelial cells [7]. In hematopoietic cells, Ang-1 effectively induces phosphorylation of Tie-2 as an agonist, whereas Ang-2 weakens the phosphorylation induced by Ang-1 although Ang-2 also induces a low level of phosphorylation of Tie-2 in the absence of Ang-1 [8]. Arai et al. reported that exogenous Ang-1 treatment protected bone marrow from 5-fluorouracil, a chemotherapeutic agent, by increasing the quiescent HSCs that are more resistant to cytotoxic chemotherapy [9]. However, it is yet unknown if the Ang-1/Tie2 signaling pathway and the subsequent increase in quiescent HSCs are also associated with glucocorticoid-mediated hematoprotection during cytotoxic chemotherapy.

Here, we show that intraperitoneal injection of dexamethasone (Dex, a synthetic glucocorticoid) upregulated Ang-1 expression in mouse bone marrow and increased a quiescent subset of HSCs. Thus, to our knowledge, Dex is the first small molecule reported to increase the quiescence of HSCs in bone marrow.

2. Materials and methods

2.1. Dex treatment in mice and harvest of the bone marrow

All animal experimental procedures were carried out in accordance with the Guidelines and Regulations for the Use and Care of Animals of the National Institute of Health in Korea. C57/BL6 mice (7–8 weeks old) were intraperitoneally injected with dexamethasone-21-phosphate (Dex-P, 0.1 mg/kg/day), which is converted to active Dex in vivo [10], every 12 h for 30 h (total three injections). The bone marrow was harvested by flushing the femurs and tibia using PBS (phosphate buffered saline without calcium and magnesium).

From the isolated bone marrow, total bone marrow nucleated cells were obtained by lysing red blood cells using red blood cell lysis buffer (Roche Applied Science, Indianapolis, IN) and mononuclear cell (MNC) fraction was isolated using Ficoll-paque™ Plus (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer's instruction.

2.2. Flow cytometric analysis

All antibodies used for flow cytometric analysis were from BD bioscience (San Jose, CA), and all flow cytometric analyses and sorting were performed using a FACSVantage (BD Biosciences). To isolate $CD45^+$ and $CD45^-$ cells, total nucleated cells were stained with

*Corresponding author. Fax: +82 2 2650 5786.
E-mail address: inhojo@ewha.ac.kr (I. Jo).

[1]Contributed equally to this work.

Abbreviations: Dex, dexamethasone; Dex-P, dexamethasone-21-phosphate; Ang-1, angiopoietin-1; Ang-2, angiopoietin-2; GR, glucocorticoid receptor; GRE, glucocorticoid responsive element; HSCs, hematopoietic stem cells; LSK, $Lin^-Sca\text{-}1^+c\text{-}kit^+$; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; SP, side population; VEGF, vascular endothelial growth factor

0014-5793/$34.00
doi:10.1016/j.febslet.2008.09.021

APC-conjugated anti-CD45 antibody, washed, and sorted. To determine $Lin^-Sca\text{-}1^+c\text{-}kit^+$ (LSK) cells, MNCs were stained using APC-conjugated anti-c-kit, PE-conjugated anti-Sca-1, and biotinylated anti-lineage (followed by staining with streptavidine-PerCP-Cy5.5) antibodies at 4 °C for 30 min, washed and analyzed. The cell-cycle status of LSK cells was determined as previously described [11]. Briefly, LSK cells were sorted, fixed with BD Cytofix/Cytoperm buffer (BD Biosciences), and washed with BD Perm/Wash buffer (BD Biosciences). Cells were stained with FITC-conjugated anti-Ki-67 antibody in EFPBS (PBS supplemented with 5 mM EDTA and 2% fetal bovine serum, pH 7.4) at room temperature for 30 min, washed, resuspended in EFPBS containing 5 μg/ml of 7-aminoactinomycin-D (7-AAD), and analyzed. To identify $CD34^-$ LSK cells, MNCs were stained with APC-conjugated anti-c-kit, PE-conjugated anti-Sca-1, biotinylated anti-lineage (followed by streptavidine-PerCP-Cy5.5), and FITC-conjugated anti-CD34 antibodies at 4 °C for 30 min, washed and analyzed. The side population (SP) cells among LSK cells (LSK-SP) were determined as previously described [12]. Briefly, MNCs were stained with 1 μg/ml Hoechst (Sigma) at 10^6 cells/ml for 90 min at 37 °C. Cells were resuspended at 10^7 cells/ml, stained with anti-c-kit, anti-Sca-1, and anti-linage antibodies at 4 °C, and analyzed. Vital staining was carried out using 7-AAD (BD Pharmingen). Cells treated with 50 μM verapamil (Sigma–Aldrich) during the entire Hoechst staining procedure served as control to determine SP cells.

2.3. Culture and Dex treatment of human mesenchymal stem cells (MSCs)

Human MSCs were purchased from Cambrex BioScience (Walkersville, MD), cultured in complete media which consisted of Dulbecco's Modified Eagle's Media (low glucose) containing 20% fetal bovine serum (Invitrogen corporation, Grand Island, NY) as previously described [13]. Human MSCs were stimulated with various concentrations of Dex or solvent control (0.05% DMSO) for 24 h or indicated times.

2.4. Determination of Ang-1 levels in mouse bone marrow and human MSCs

The levels of Ang-1 protein in the bone marrow, $CD45^+$ and $CD45^-$ bone marrow cells were measured by Western blotting analysis using anti-Ang-1 antibody (Abcam, Cambridge, UK) as previously described [14]. The concentrations of Ang-1 and Ang-2 in culture supernatants from human MSCs were determined using ELISA kits (R&D Systems Inc., Minneapolis, MN). To determine Ang-1 mRNA, total RNA was isolated with Trizol and subjected to quantitative real-time RT-PCR analysis as previously described [13] using the following primers: for human Ang-1, forward 5′-GGAAGGGAACCGAGCCTATT-3′, reverse 5′-TTCCTGCTGTCCCAGTGTGA-3′; for human β-actin, forward 5′-GCCCAGTCCTCTCCCAAGTC-3′, reverse 5′-GGCACGAAGGCTCATCATTC-3′.

2.5. mRNA stability assay

To determine the stability of the Ang-1 mRNA, human MSCs were exposed to 100 nM Dex for 24 h, after which actinomycin D (10 μg/ml) was added to stop the RNA synthesis [15]. Total RNA was isolated at the indicated times and subjected to real-time RT-PCR.

2.6. Promoter activity assay

The Ang-1 promoter (−3040/+199) construct was kindly provided by Dr. Peter Oettgen (Harvard Institutes of Medicine, Boston, MA) and the promoter activities were determined as previously described [14].

2.7. Actinomycin D inhibition test

One hour after pretreatment with 10 μg/ml actinomycin D, cells were treated with 100 nM Dex or solvent control (0.05% DMSO) for 24 h in the presence of actinomycin D. Total RNA was isolated and subjected to real-time RT-PCR as described above.

2.8. Statistical analysis

All values are presented as the mean ± standard error (S.E.). Significance was determined by a Student's *t*-test or ANOVA followed by multiple comparisons by Tukey's test using SPSS for Windows (SPSS Inc., Chicago, IL). A P value of <0.05 was considered to be statistically significant.

3. Results and discussion

3.1. Expression and regulation of Ang-1 by Dex in mouse bone marrow

As Ang-1 has been reported to play a pivotal role in hematoprotection [9], we investigated if Dex increases the level of Ang-1 in mouse bone marrow. Intraperitoneal Dex-P increased the level of Ang-1 protein in mouse bone marrow in vivo (Fig. 1A). To further determine which subset of cells is responsible for Dex-induced Ang-1 upregulation, we sorted total bone marrow nucleated cells using a pan-hematopoietic cell marker, CD45, which detects all hematopoietic cells. As shown in Fig. 1B, Ang-1 was detected only in $CD45^-$ cells, indicating that non-hematopoietic cells are the main producer of Ang-1 in bone marrow. This is compatible with previous reports that Ang-1 is mainly produced by non-hematopoietic cells such as osteoblasts [9] or self-renewing osteoprogenitor cells [16]. In contrast, Ang-1 was barely detected in $CD45^+$ cells. Moreover, when mice were treated with Dex-P, the Ang-1 levels increased only in $CD45^-$ cells, suggesting that Dex increases Ang-1 in non-hematopoietic cells.

3.2. Dex increases quiescent HSCs in mouse bone marrow

As Ang-1 increases the quiescence of HSCs [9], leading to subsequent bone marrow protection against cytotoxic chemotherapy, this study was aimed to examine if Dex-P also increases the quiescence of HSCs in mouse bone marrow. The number of neither total bone marrow nucleated cells nor LSK cells was changed by Dex-P treatment (Fig. 2A–C). To identify the quiescent HSCs among LSK cells, cells were then stained with 7-AAD and Ki-67 antibody and the quiescent HSCs in G0 phase were determined by flow cytometric analysis [11,17]. The percentage of cells in G0 phase among LSK cells significantly increased from 25.5 ± 2.0% (mean ± S.E.) to 34.2 ± 2.2% ($P < 0.05$, Fig. 2D and E), suggesting that Dex-P increases the quiescence of HSCs. As quiescent HSCs can be also identified as $CD34^-$ LSK cells [18,19], we further investigated if Dex-P treatment increases $CD34^-$ cells among LSK cells. Flow cytometric analysis revealed that $CD34^-$ cells among LSK cells dramatically increased from 7.5 ± 1.1% to 23.7 ± 2.3% ($P < 0.001$, Fig. 2F and G), indicating that Dex in-

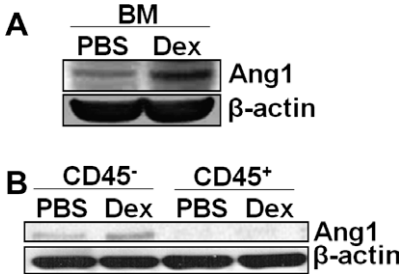

Fig. 1. Dex increases the level of Ang-1 in mouse bone marrow. C57/BL6 mice were intraperitoneally injected with Dex-P (0.1 mg/kg/day) every 12 h for 30 h (total three injections). (A) The whole bone marrow was then isolated and subjected to Western blotting analysis to determine the levels of Ang-1. Dex-P increased Ang-1 protein. (B) The bone marrow nucleated cells were sorted using CD45, panhematopoietic cell marker, into two groups ($CD45^+$ and $CD45^-$ cells) and subjected to Western blotting analysis to examine the levels of Ang-1. Dex increased Ang-1 levels only in $CD45^-$ cells, whereas the expression of Ang-1 was barely detected in $CD45^+$ cells.

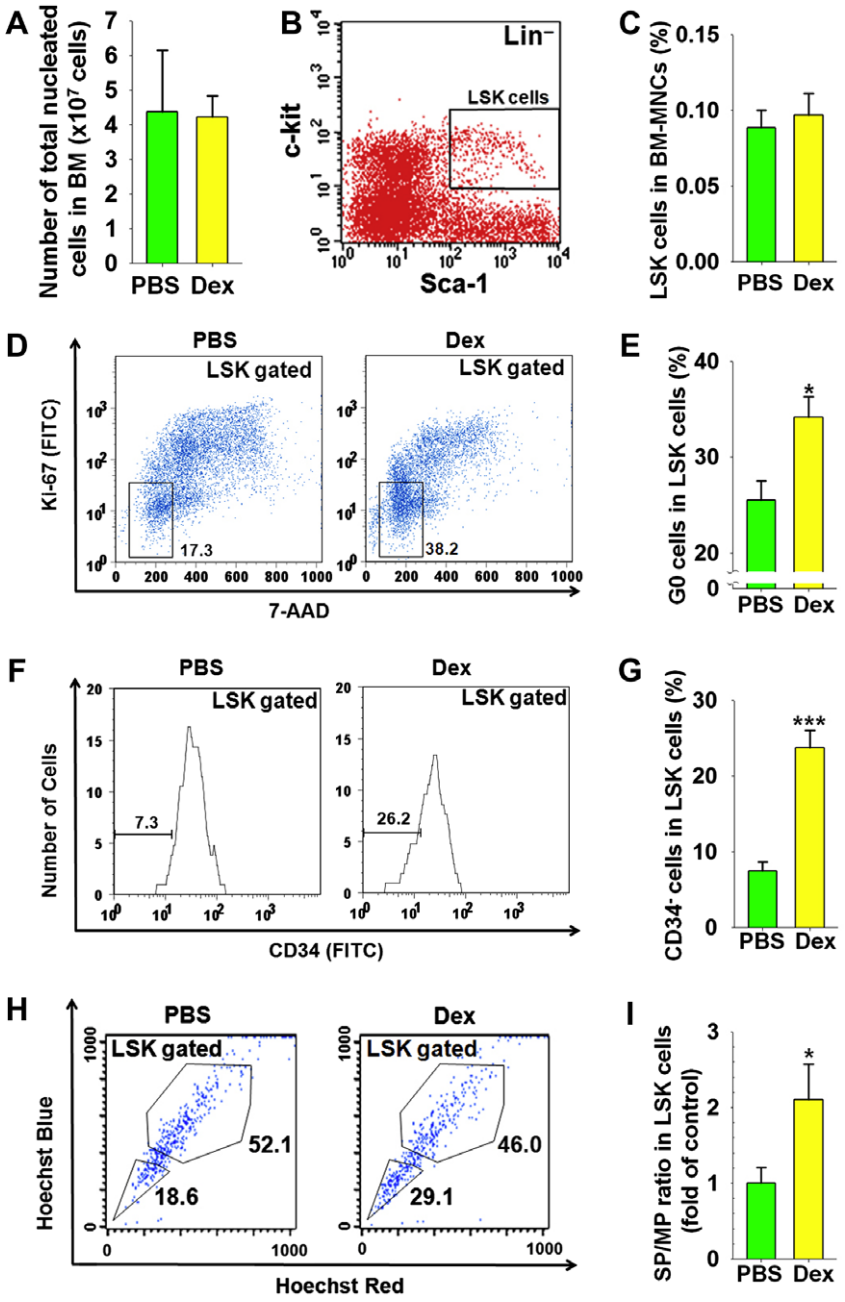

Fig. 2. Dex increases quiescent of HSCs in mouse bone marrow. (A) The number of bone marrow (BM) cells was measured by cell counting after Dex-P treatment. The number of total nucleated cells was not changed by Dex-P treatment. (B–H) The bone marrow MNCs were isolated and subjected to flow cytometric analysis. (B) Determination of LSK cells. (C) The number of LSK cells was not changed by Dex-P treatment ($n = 4$). (D) Determination of quiescent HSCs in G0 phase by Ki-67 and 7-AAD staining in LSK cells. (E) Dex-P treatment significantly increased the percentage of G0 cells among LSK cells ($^{*}P < 0.05$, $n = 6$). (F) Determination of $CD34^{-}$ cells in LSK cells. (G) Dex-P treatment significantly increased the percentage of $CD34^{-}$ cells among LSK cells ($^{***}P < 0.001$, $n = 4$). (H) Determination of side population (SP) and main population (MP) by Hoechst staining. (I) Dex-P treatment significantly increased the SP/MP ratio ($^{*}P < 0.05$, $n = 17$–19).

creases a subset of quiescent HSCs. Moreover, as it has been reported that SP is another marker of quiescent HSCs and that LSK-SP is a subset of HSCs that are resistant to 5-fluorouracil which induces apoptosis in actively cycling cells [9,20], we examined SP among LSK cells. As shown in Fig. 2H and I, Dex-P significantly increased the ratio of SP/MP in LSK cells by 2.1 ± 0.5-fold compared to the PBS-treated control. Taken all together, Dex-P treatment increased the number of quiescent HSCs that is more invulnerable to cytotoxic chemotherapy, providing a novel mechanism of Dex-induced bone marrow protection. To our knowledge, Dex is the first small molecule that has been identified to increase the quiescence of HSCs.

3.3. *Dex increases the level of Ang-1 protein and mRNA in a glucocorticoid receptor (GR)-dependent manner in human MSCs*

To investigate the molecular mechanism underlying Dex-induced Ang-1 upregulation, we used bone marrow-derived human MSCs, the major producers of Ang-1 in bone marrow

[16], which are known to reconstitute the hematopoietic microenvironment [16,21,22]. ELISA revealed that Dex increased Ang-1 protein secretion from human MSCs in a dose-dependent manner, with a maximum of 48.4 ± 7.0 pg/μg cellular protein at 100 nM Dex treatment (Fig. 3A). Although Ang-2, a natural antagonist for Tie-2, was detected only in Dex-treated cells, its level was ~500 times less than that of Ang-1 (data not shown). A GR antagonist RU486 completely attenuated this effect of Dex ($P < 0.001$) in a dose-dependent manner (Fig. 3B), suggesting a GR-mediation. We next determined if the increase in Ang-1 protein is attributable to an increase in the mRNA level. Real-time RT-PCR analysis showed that Dex increased Ang-1 mRNA levels in a dose-dependent manner, with a maximal increase of 7.8 ± 1.1-fold at 100 nM (Fig. 3C). This increase was also time-dependent, with a maximum increase of 6.3 ± 0.8-fold at 24 h (Fig. 3D).

Previously, it was reported that adipocytic conversion from fibroblasts is accompanied by the increased expression of Ang-1 [23]. This previous report, together with a possibility that Dex may induce adipogenic differentiation from hMSCs, prompted us to investigate if the observed effects depend on Dex-derived cell differentiation. In this regard, we have recently reported that Dex directly increased the levels of Ang-1 protein and mRNA in fully differentiated cells such as human brain pericytes and astrocytes [24]. Furthermore, we also found a significant increase in Ang-1 mRNA by Dex treatment in several other tissues, retina, brain cortex, and bone marrow (data not shown). These data support our conclusion that Dex directly increases Ang-1 independent of cell differentiation. Furthermore, under our experimental conditions, using Oil Red O staining we failed to find Dex-derived adipogenic differentiation from hMSCs (data not shown).

3.4. Dex increases the level of Ang-1mRNA without changing mRNA stability in human MSCs

As the increase in mRNA may have occurred as a result of the increase in mRNA stability and/or the activation of transcription, we examined which of these factors contributes to the upregulation of Ang-1 mRNA. As shown in Fig. 4A, Ang-1 mRNA was very stable, and the stability was not changed by Dex treatment. The high stability of Ang-1 mRNA was previously reported in retinal pigment epithelial cells [25]. Under the same experimental conditions, vascular endothelial growth factor (VEGF) mRNA measured as a reference decreased rapidly (Fig. 4A, inset). We also found that Dex significantly increased the Ang-1 promoter activity by 1.9 ± 0.2-fold ($P < 0.05$) (Fig. 4B), and that RU486 completely inhibited this Dex-stimulated Ang-1 promoter activity in a dose-dependent manner, further indicating a GR-mediation. To further confirm that transcriptional activation, but not alteration of

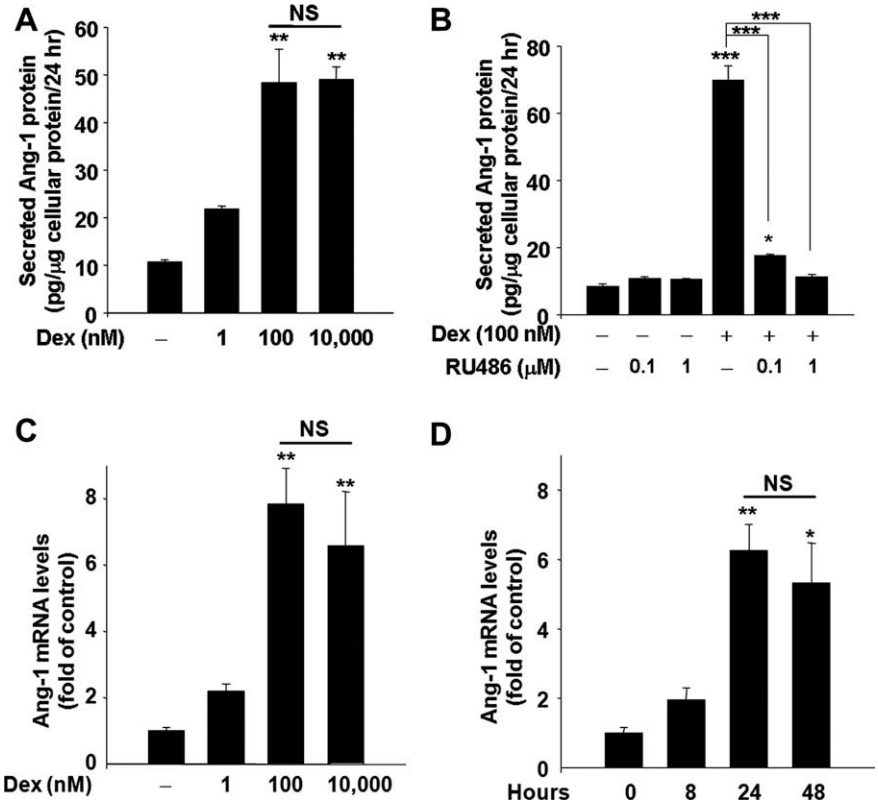

Fig. 3. Dex increases the levels of Ang-1 protein and mRNA in a GR-dependent manner. Human MSCs were stimulated with 0.05% DMSO (solvent control) or Dex in the absence or presence of RU486. (A and B) The concentrations of Ang-1 in the supernatants of human MSC culture were determined using ELISA. Each bar represents the amount of secreted Ang-1 normalized to that of total cellular protein. Dex increased Ang-1 protein in a dose-dependent manner (A). This increase was abrogated by RU486, a GR antagonist, suggesting that GR mediates the Dex-induced increase in Ang-1 (B). (C and D) Total RNA was isolated using TRIzol and subjected to real-time RT-PCR. Cells were treated for 24 h with various doses of Dex (C) or with 100 nM Dex for the indicated times (D) and the Ang-1 mRNA levels were then determined. Each bar represents the level of Ang-1 mRNA normalized to that of control. Dex increased Ang-1 mRNA in a dose (C)- and time (D)-dependent manner. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ (A, B, and D, $n = 3$; C, $n = 5$).

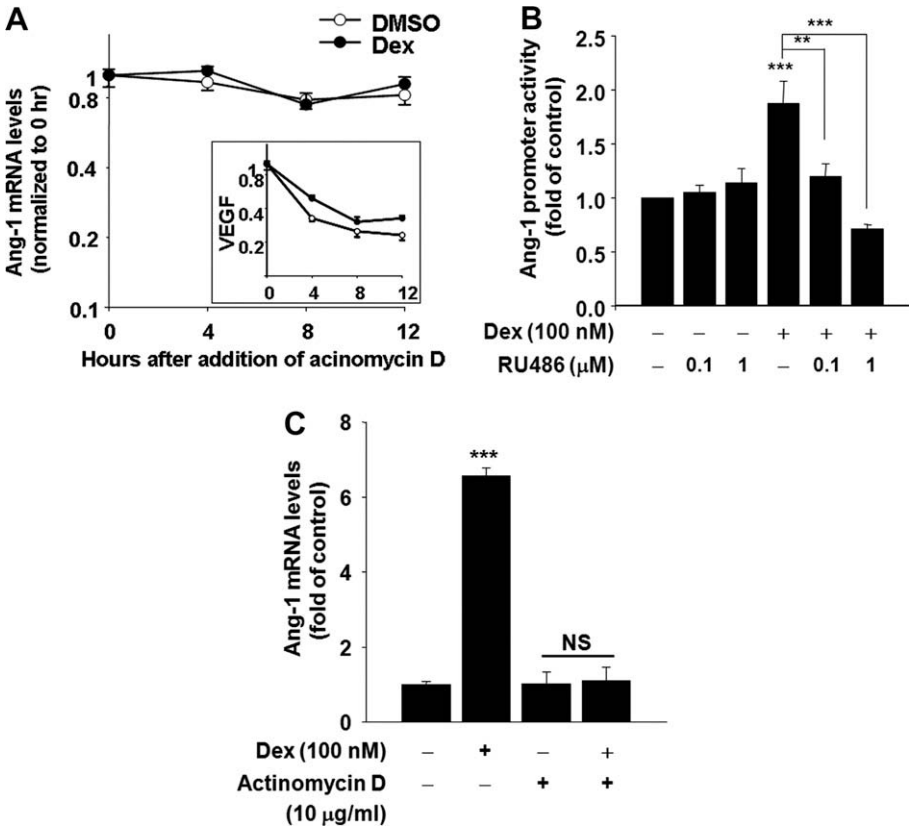

Fig. 4. Dex increases the levels of Ang-1 mRNA through a transcriptional activation. (A) Ang-1 mRNA stability assay. Human MSC were treated with DMSO (solvent control) or 100 nM Dex for 24 h and RNA synthesis was blocked by adding actinomycin D. Total RNA was isolated at the indicated times after actinomycin D treatment and the levels of Ang-1 and VEGF mRNA (inset) were determined by real-time RT-PCR. Each point represents the Ang-1 or VEGF mRNA levels normalized to the levels that were present just before the addition of actinomycin D. Ang-1 mRNA was very stable, and this stability was not altered by Dex treatment. (B) Promoter activity assay. Human MSCs were transfected with a luciferase vector containing Ang-1 promoter followed by treatment with Dex for 24 h and the luciferase activities of the cell lysates were then determined. Each bar represents the promoter activity relative to the solvent control-treated group. Dex treatment increased Ang-1 promoter activity, which was attenuated to the basal level by the addition of RU486. (C) Human MSCs were pretreated with 10 μg/ml actinomycin D or solvent control (0.05% DMSO) for 1 h and stimulated with 100 nM Dex or solvent control in the presence or absence of actinomycin D for 24 h. Total RNA was isolated and subjected to real-time RT-PCR to determine the Ang-1 mRNA levels. Each bar represents the Ang-1 mRNA level. Actinomycin D completely inhibited Dex-induced Ang-1 increase. $^{**}P < 0.01$, $^{***}P < 0.001$ (A, $n = 9$; B, $n = 6$–8; C, $n = 7$).

mRNA stability, is responsible for the observed effects, we treated human MSCs with Dex in the presence of actinomycin D and found that the Dex-induced increase in Ang-1 mRNA was completely inhibited (Fig. 4C).

3.5. Ang-1 promoter has putative glucocorticoid response elements (GREs)

It has been shown that several proteins are induced by glucocorticoids through transcriptional activation as a result of the binding of activated GR to the GREs in the regulatory regions of the genes [26–29]. Therefore, we identified putative GREs in the Ang-1 promoter in silico using two web-based transcription factor binding site searching programs, Match (http://www.gene-regulation.com/pub/programs.html#match) and MatInspector (http://www.genomatix.de/products/MatInspector). MatInspector proposed −2696, −1932, and +294 regions as putative GREs, all of which are also identified by Match. Taken together, our data suggest that Dex increases the level of Ang-1 protein in human MSCs via a transcriptional activation, possibly resulting from the binding of activated GR to the putative GREs in the Ang-1 promoter. However, the elucidation of the mechanisms by which Dex-stimulated Ang-1 expression occurs requires further experiments, including nuclear run-on assay, promoter activity assay using serially deleted promoter constructs, mutational analysis of GREs in the Ang-1 promoter, and DNA–protein binding assay.

4. Conclusion

In summary, Dex increased Ang-1 in bone marrow and increased quiescent HSCs which are invulnerable to cytotoxic chemotherapy. Investigation of the molecular mechanism by which Ang-1 upregulation occurred suggests that Dex increases Ang-1 mRNA at the transcriptional level. Our findings propose the increase in quiescent HSCs as a novel mechanism of Dex-induced protection of HSCs in the setting of chemotherapy.

Acknowledgments: We thank Dr. Peter Oettgen (Beth Israel Deaconess Medical Center, Boston, MA) for providing Ang-1 promoter and Dr. Fumio Arai (Keio University, Tokyo, Japan) for technical support regarding the flow cytometry. This work was in part supported by

Korean NIH Intramural Grant and the BK project from the Korean Research Foundation Grant funded by the Korean Government (MOEST).

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