Original Article

Effect of Estrogen on Differentiation and Senescence in Endothelial Progenitor Cells Derived from Bone Marrow in Spontaneously Hypertensive Rats

Toshio IMANISHI, Katsunobu KOBAYASHI, Takuzo HANO, and Ichiro NISHIO

The functional impairment associated with atherogenic factors, including hypertension, constitutes a limitation to the ability of endothelial progenitor cells (EPCs) to repair. In addition, estrogens have been shown to play a role in reendothelialization after vascular injury. We investigated the effects of estrogens on differentiation and senescence of EPCs derived from bone marrow (BM-EPCs) in spontaneously hypertensive rats (SHR/lzm). Bone marrow (BM) cells were obtained from the tibias and femurs of age-matched, male SHR/lzm and Wistar-Kyoto rats (WKY/lzm). The number of differentiated, adherent BM-EPCs derived from SHR/lzm was significantly smaller than the number derived from WKY/lzm. 17 β-Estradiol (E_2) significantly increased the number of adherent BM-EPCs from SHR/lzm, and this effect was significantly attenuated by pharmacological phosphatidylinositol 3-kinase (PI3-K) blockers. Immunoblotting analysis revealed that E_2 treatment led to phosphorylation of Akt. Senescence, as assessed by acidic β-galactosidase staining, occurred at a significantly greater rate in the BM-EPCs from SHR/lzm than in those from WKY/lzm, but E_2 treatment dramatically delayed the senescence of BM-EPCs from SHR/lzm. A polymerase chain reaction (PCR)-ELISA based assay revealed that telomerase activity in BM-EPCs from SHR/lzm was significantly lower than in those from WKY/lzm, but that E_2 treatment significantly augmented it. Both MTS and colony forming unit assay revealed that E_2 treatment significantly augmented the functional activity in BM-endothelial cell (EC)-like cells from SHR/lzm compared to that in control BM-EC-like cells (no treatment). In conclusion, the differentiation of BM-EPCs derived from SHR/lzm was significantly decreased compared with that of BM-EPCs from WKY/lzm. In addition, the rate of senescence was significantly greater in the BM-EPCs from SHR/lzm than in those from WKY/lzm. Estrogen was shown to augment differentiation and delay the onset of senescence in BM-EPCs from SHR/lzm. (*Hypertens Res* 2005; 28: 763–772)

***Key Words*: endothelial progenitor cells, estrogen, senescence, hypertension**

Introduction

The integrity and functional activity of the endothelial monolayer play a crucial role in the prevention of hypertension and atherosclerosis. Recent insights suggest that the injured endothelial monolayer is regenerated by circulating bone marrow-derived endothelial progenitor cells (BM-EPCs), which accelerates reendothelialization and limits atherosclerotic lesion formation (*1–4*). This beneficial property of BM-EPCs

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makes them attractive for cell therapy targeting the regeneration of ischemic tissue. Since BM-EPCs may contribute to postnatal neovascularization, further understanding of the regulation of endothelial progenitor cells (EPCs) kinetics may provide new insights into the pathogenesis of vasculogenesis. On the other hand, various risk factors for coronary artery disease (CAD), such as diabetes, hyperlipidemia, hypertension, and smoking, affect the number and functional activity of EPCs in healthy volunteers (*5*) and in patients with CAD (*6*). By multivariate analysis of various risk factors, hypertension has been identified as a major independent predictor for impaired EPCs migration (*6*).

BM-EPCs are not pluripotent, self-renewing stem cells, but rather lineage-committed progenitors, and are thus subject to a Hayflick life span *via* replicative senescence (*7*). Therefore, an important question with respect to potential cell therapeutic approaches is whether the endogenous BM-EPCs pool can be revitalized *in vivo* with pharmacological agents, cytokines, or even gene therapy. Interestingly, Murasawa *et al*. (*8*) have demonstrated that constitutive human telomerase reverse transcriptase (hTERT) expression in cultured EPCs enhances their mitogenic and migratory activity, delays senescence, and augments neovascularization in a murine model of hindlimb ischemia. Moreover, recent studies have shown that estrogen accelerates reendothelialization and attenuates medial thickening after carotid artery injury in part by augmenting mobilization and proliferation of BM-EPCs and their incorporation into the recovered endothelium at the site of injury (*9*, *10*). Therefore, in the present study, first, we investigated the influences of hypertension on differentiation and senescence of BM-EPCs in spontaneously hypertensive rats (SHR/Izm). Secondly, keeping estrogen-induced reendothelialization after balloon injury in mind, we examined the effects of estrogen on hypertension-induced differentiation and senescence of BM-EPCs in SHR/Izm.

Methods

The Ethics Committee for Animal Experiments at the Wakayama Medical University approved the experimental protocol used in this study. The experiment was performed according to the Guidelines for Animal Experimentation at the Wakayama Medical University.

SHR/Izm

Male SHR/Izm aged 5 weeks ($n=6$) or 12 weeks ($n=6$) and equal numbers of age-matched male Wistar Kyoto rats (WKY/Izm) were obtained from the Disease Model Cooperative Research Association (Kyoto, Japan) and housed under a 12-h light/dark cycle for 1 week. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography according to the procedure described by Bunag (*11*).

Isolation of Mononuclear Cells and Cell Culture

Bone marrow (BM) cells were obtained from the tibias and femurs of 5- or 12-week-old male SHR/Izm and age-matched male WKY/Izm. BM-derived mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation with Histopaque 1083 (Sigma Chemical Co., St. Louis, USA). After purification with 3 washing steps, 10×10^6 BM-MNCs were plated on vitronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2; Clonetics, Walkersville, USA) with supplements (3 μg/ml bovine brain extract, 30 μg/ml gentamycin, 50 μg/ml amphotericin B, 10 μg/ml human epidermal growth factor, and 5% fetal bovine serum [FBS]). The 6-well plates were replated at day 4.

Western Blotting

The general protocol for Western blotting was performed as previously described (*12*). Briefly, the proteins from BM-MNCs (day 3) were size-separated using 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and electrotransferred to a PVDF membrane (Millipore, Bedford, USA). Membranes were blocked by incubation in Tris-buffered saline (10 mmol/l Tris, pH 7.5, 100 mmol/l NaCl) containing 0.1% (v/v) Tween 20 and 5% (v/v) non-fat dry milk for 2 h, followed by a 2 h incubation at room temperature with rabbit polyclonal anti-phospho-Akt-Ser^{473} or anti-Akt antibodies (Cell Signaling Technology, Inc., Beverly, USA). The filters were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween 20, before incubation for 1 h with a secondary anti-rabbit antibody conjugated to horseradish peroxidase. Membranes were then washed and developed using enhanced chemiluminescence substrate (ECL; Amersham Pharmacia Biotech, CA, USA). The profile of each band was plotted using NIH Image software, and the densitometric intensity corresponding to each band was measured as an intensity value.

Senescence-Associated β-Galactosidase (SA-β-Gal) Activity Assay

SA-β-Gal activity was measured as previously described (*13*). Briefly, BM-EPCs were washed in PBS, fixed for 3 min (room temperature) in 2% paraformaldehyde, washed, and incubated for 24 h at 37°C (no CO_2) with fresh SA-β-Gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indyl β-D-galactopylanoside (X-Gal), 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium fericyanide, 150 mmol/l NaCl, 2 mmol/l $MgCl_2$, 0.01% sodium deoxycholate, and 0.02% Nonidet-40). BM-EPCs were counterstained with 4′,6-diamino-phenylindole (DAPI) (0.2 μg/ml in 10 mmol/l Tris-HCl, pH 7.0, 10 mmol/l EDTA, 100 mmol/l NaCl) for 10 min to count the total cell number.

Table 1. Body Weight, Systolic Blood Pressure, and Heart Rate in WKY and SHR

	5 week			12 week		
	WKY(n=6)	SHR(n=6)	p value	WKY(n=6)	SHR(n=6)	p value
BW	105±2	110±4	n.s.	313±4	301±4	n.s.
SBP (mmHg)	109±6	110±10	n.s.	108±4	182±10	*
HR (/min)	486±24	476±41	n.s.	438±9	456±5	n.s.

Data shown are mean±SEM. *Significantly different compared to WKY/Izm, $p<0.01$. WKY, Wistar Kyoto rats; SHR, spontaneously hypertensive rats; BW, body weight; SBP, systolic blood pressure; HR, heart rate.

Telomeric Repeat Amplification Protocol (TRAP) Assay

For quantitative analyses of telomerase activity, TRAP assay, in which the telomerase reaction product is amplified by polymerase chain reaction (PCR), was performed using a *Telo TAGGG* PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol as previously described (*14*, *15*).

Proliferative Activity Assay

Mitogenic activity was assayed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQ; Promega, Madison, USA). BM-endothelial cell (EC)-like cells were harvested 14 days after culture and re-seeded with a 96-well plate (1×10^4 cells) in 0.1 ml of EBM-2 medium supplemented with 0.5% bovine serum albumin (BSA) in the presence of human recombinant VEGF (100 ng/ml; R & D Systems, Minneapolis, USA) overnight. After 24 h in culture, MTS/phenazine methosulfate (PMS) solution was added to each well for 3 h, at which time the light absorbance at 490 nm was detected using an ELISA plate reader (Bionetics Laboratory, Kensington, USA). In addition, a colony assay was performed to evaluate the proliferative activity. After 4 days of culture, adherent cells were gently detached by EDTA. Cells (1×10^5) were seeded in methylcellulose plates (Methocult GF H4434; Cell Systems, Kirkland, USA) treated with or without 17β-estradiol (E_2; 100 nmol/l) in the presence of 100 ng/ml human recombinant VEGF. Plates were studied under phase contrast microscopy and colonies were counted after 10 days of incubation.

Statistical Analysis

Data were expressed as the mean±SEM based on at least five independent experiments. Statistical analysis was performed by 1-way ANOVA (LSD test) for multiple testing. Probability values were considered significant at $p<0.05$.

Results

Effects of Hypertension on Differentiation and Senescence of EPCs in SHR/Izm

In terms of body weight, blood pressure, and heart rate, there were no significant differences between 5-week-old SHR/Izm and WKY/Izm (Table 1). In the 12-week-old animals, however, there was a significant difference in blood pressure between SHR/Izm and WKY/Izm (Table 1). BM-EPCs were characterized as adherent cells that were double-positive for both lectin and DiLDL uptake. There were no significant differences in the number of differentiated, adherent BM-EPCs between 5-week-old SHR/Izm and WKY/Izm (Fig. 1A). However, the number of differentiated, adherent BM-EPCs derived from 12-week-old SHR/Izm was significantly lower than that from 12-week-old WKY/Izm (Fig. 1B). The rate of senescence, as assessed by acidic β-Gal staining, was significantly higher in the BM-EPCs of 12-week-old SHR/Izm than in those of age-matched WKY/Izm (Fig. 2A). Finally, a PCR-ELISA based assay revealed that telomerase activity in BM-EPCs was significantly lower in 12-week-old SHR/Izm than age-matched WKY/Izm (Fig. 2B).

Estrogen Accelerates EPC Differentiation in SHR/Izm

When isolated BM-MNCs derived from SHR/Izm as well as WKY/Izm were incubated with E_2 (100 nmol/l) in the presence of VEGF (100 ng/ml), the number of differentiated, adherent EPCs were increased compared with that in controls (no treatment) (Fig. 3A, B). Dimmeler *et al*. (*16*) have demonstrated that statins require Akt to augment the number of EPCs, suggesting that Akt plays an essential role in regulating haematopoietic progenitor cell differentiation. Therefore, we examined whether the augmentation of the differentiation of BM-EPCs induced by E_2 depends on the phosphatidylinositol 3-kinase (PI3-K)/Akt pathway. E_2-induced EPC differentiation was significantly inhibited by the PI3-K blockers (either wortmannin [10 nmol/l] or LY294002 [10 μmol/l]) (Fig. 3B). In addition, we evaluated the differentiation of BM-MNCs in the presence of lower doses of VEGF (20 ng/ml), because

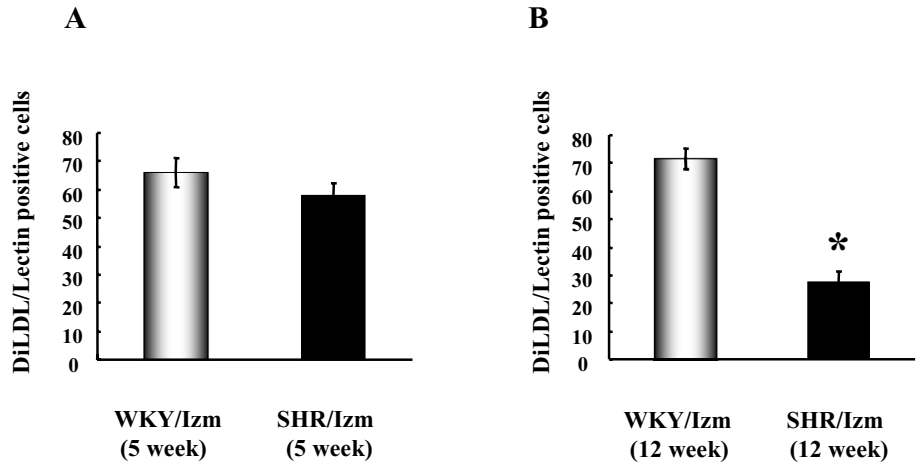

Fig. 1. *Effects of hypertension on BM-EPC differentiation. The differentiation of BM-EPCs was assessed by double-positive cells for DiLDL uptake and lectin binding. Quantification of BM-EPC differentiation was evaluated in 5- (A) and 12-week-old (B) WKY/Izm and SHR/Izm. Data are expressed as the means±SEM (*n = *6 in each group).* *p < *0.01 compared with WKY/Izm.*

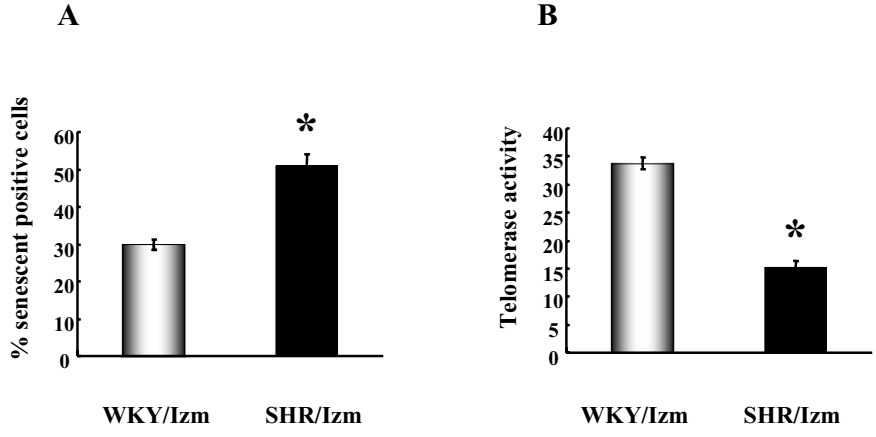

Fig. 2. *Effects of hypertension on BM-EPC senescence and telomerase activity. A: The senescence of BM-EPCs was assessed by senescence-associated β-galactosidase (SA-β-Gal) activity. Quantification of SA-β-Gal-positive cells was evaluated in 12-week-old WKY/Izm and SHR/Izm. Data are expressed as the means±SEM (*n = *6).* *p < *0.01 compared with WKY/Izm. B: Freshly isolated mononuclear cells were cultivated in endothelial basal medium-2 (EBM-2) supplemented with EBM-2 simple aliquots. The six-well plates were replated at day 4. After an additional 3 days of cultivation, telomerase activity was measured by PCR-ELISA based assay. Data are expressed as the means±SEM (*n = *6 in each group).* *p < *0.01 compared with WKY/Izm.*

BM-MNCs may easily differentiate into endothelial lineage cells in the presence of high doses of VEGF without estrogen. The results were similar to those obtained using high doses of VEGF (100 ng/ml). That is, the number of differentiated, adherent BM-EPCs derived from SHR/Izm was significantly lower than that from WKY/Izm (19.9±3.6 *vs.* 53.5± 4.2, respectively). Next, we investigated the effects of E_2 on Akt activation. Isolated BM-MNCs were stimulated with E_2 (100 nmol/l) for 1 h and immunoblots were performed with a phosphospecific Akt antibody directed at the Ser^{473} phosphorylation site. As shown in Fig. 4, E_2 augmented the phosphorylation of Akt in BM-MNCs derived from WKY/Izm as well as in those derived from SHR/Izm.

Estrogen Prevents EPC Senescence in SHR/Izm

Coincubation with E_2 significantly inhibited the increase in SA-β-Gal-positive cells in both SHR/Izm and WKY/Izm (Fig. 5A, B). Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the telomere length reduction induced by cell division. Therefore, we measured telomerase activity by a *TeloTAGGG*

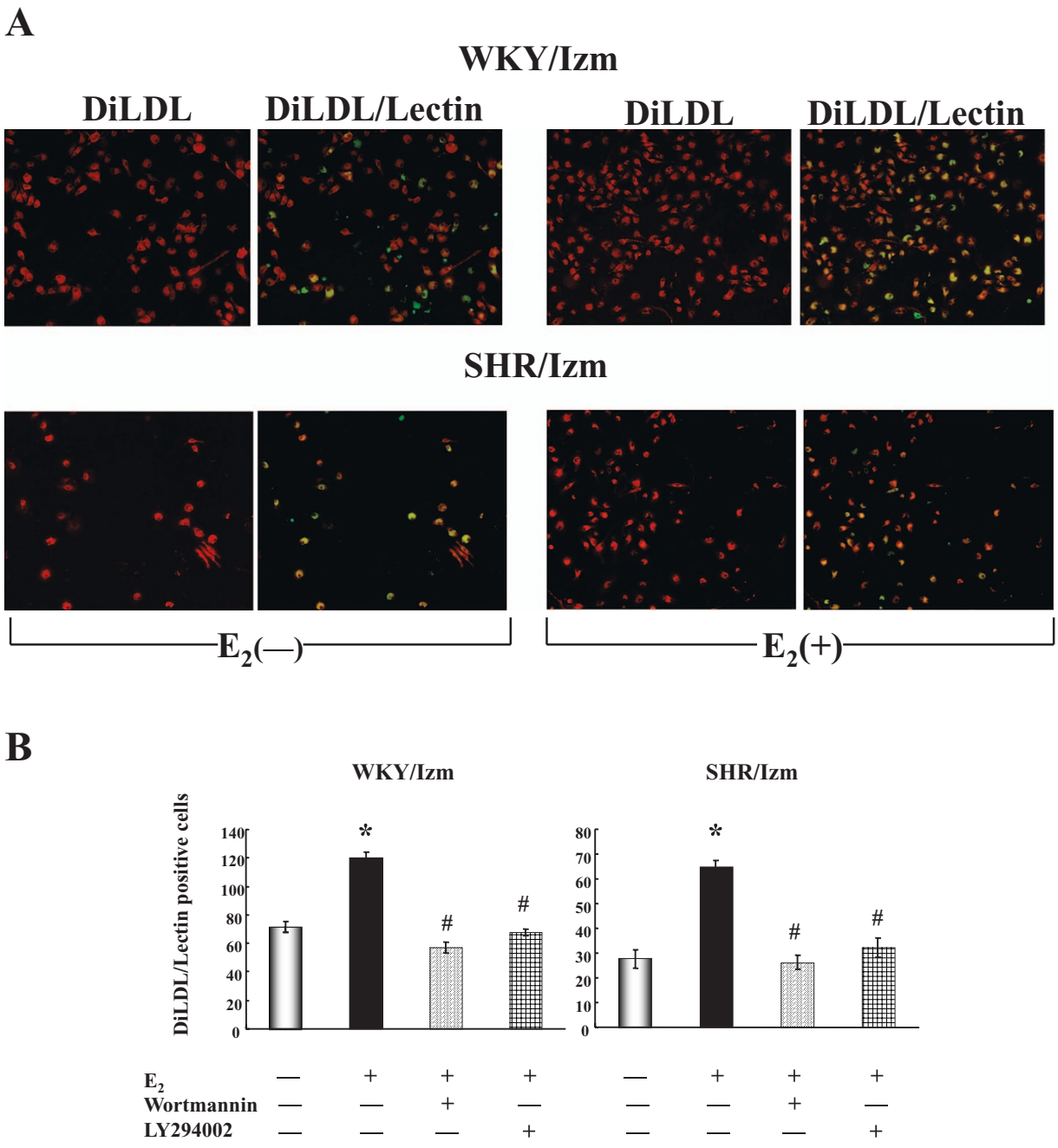

Fig. 3. *E_2 augments BM-EPC differentiation. A: The BM-MNCs at day 3 were treated with or without E_2 (100 nmol/l) in the presence of VEGF (100 ng/ml) for 24 h. Representative photomicrographs show double-positive cells for DiLDL uptake and lectin binding in BM-EPCs treated without (left) or with (right) E_2. B: Treatment of BM-MNCs with E_2 significantly augmented the differentiation of BM-EPCs. Data are expressed as the mean±SEM (*n = 6). *$p < 0.01$ compared with control (no treatment); #$p < 0.01$ compared with E_2 (100 nmol/l).*

Telomerase PCR ELISA. As demonstrated in Fig. 6, E_2 significantly increased telomerase activity in SHR/Izm as well as WKY/Izm.

Estrogen Increases the Proliferative Activity of EPCs in SHR/Izm

Having demonstrated that E_2 delayed the onset of senescence, we next examined whether this effect translates into an

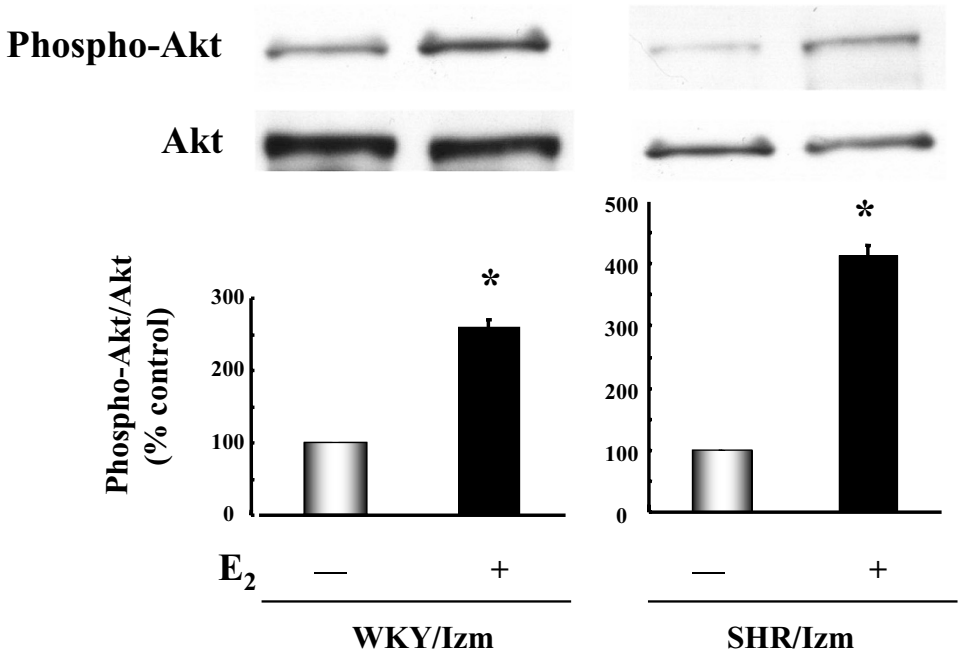

Fig. 4. *Effect of E_2 on Akt phosphorylation on Ser^{473}. Mononuclear cells (MNCs) at day 3 were stimulated for 1 h with E_2 (100 nmol/l), and phosphorylation of Akt was determined with a phosphospecific Akt antibody. A representative blot from six independent experiments is shown (upper). Bar graphs show phospho-Akt/normalized Akt (lower). Data are expressed as a ratio of the test value to the value for unstimulated cells (set at 100 %).* *$p < 0.01$ *compared with control (no treatment).*

increase in proliferation and clonal expansion. A MTS assay demonstrated that the mitogenic potential in BM-EC-like cells treated with E_2 exceeded that in untreated (control) BM-EC-like cells in SHR/Izm as well as WKY/Izm (Fig. 7A). To investigate the clonal expansion potential of the cultivated BM-EC-like cells, we further performed an outgrowth assay. For this purpose, BM-EPCs were cultivated for 4 days in the presence or absence of E_2. Then, cells were detached, and 1×10^5 BM-EC-like cells were seeded in methylcellulose plates. As shown in Fig. 7B, the number of colonies was significantly higher in BM-EC-like cells that had been pretreated with E_2 in SHR/Izm as well as WKY/Izm.

Discussion

The functional regeneration of ischemic tissue by improved neovascularization and possibly tissue repair is critically dependent on the mobilization and integration of EPCs into the ischemic tissue. Moreover, infusions of EPCs expanded *ex vivo* can limit scar extension in the ischemic myocardium (*17*) and improve the recovery of contractility, and thus may be useful as a novel therapeutic approach (*18*). However, it is conceivable that unfavorable clinical situations might be associated with dysfunctional BM-EPCs, defective vasculogenesis, and thus, impaired neovascularization. Indeed, Tepper *et al*. (*19*) reported that the proliferation and tube formation of EPCs were impaired in patients with type 2 diabetes compared with normal subjects. Therefore, an important question with respect to potential cell therapeutic approaches is whether BM-EPCs can be revitalized *in vitro*. Ikeda *et al*. (*20*) have demonstrated that human VEGF gene transfer is effective at enhancing the endothelialization of EPCs. Furthermore, the transplantation of cord blood MNCs expressing VEGF may be feasible as an angiogenic cell and gene therapy for the treatment of ischemic tissue. In the present study, we have shown that the differentiation of BM-EPCs derived from SHR/Izm at 12 weeks of age was significantly lower than that of BM-EPCs from age-matched WKY/Izm. Interestingly, in 5-week-old SHR/Izm and WKY/Izm, there were no significant differences in either differentiation or blood pressure. These results indicate that hypertension or hypertension-related factors might affect the differentiation of BM-MNCs into BM-EPCs. In addition, the senescence rate of BM-EPCs from SHR/Izm was significantly increased compared to that of BM-EPCs from WKY/Izm. More importantly, we have shown for the first time that estrogens can augment the differentiation of BM-EPCs and delay the onset of BM-EPC senescence in both SHR/Izm and WKY/Izm. The results from this study thus suggest that estrogens might have utility for therapeutic postnatal vasculogenesis of ischemic tissue, potentially including populations with hypertension.

We have shown previously that oxidized low-density lipoprotein (LDL) reduces the number of adherent EPCs in the presence of VEGF through the dephosphorylation of Akt

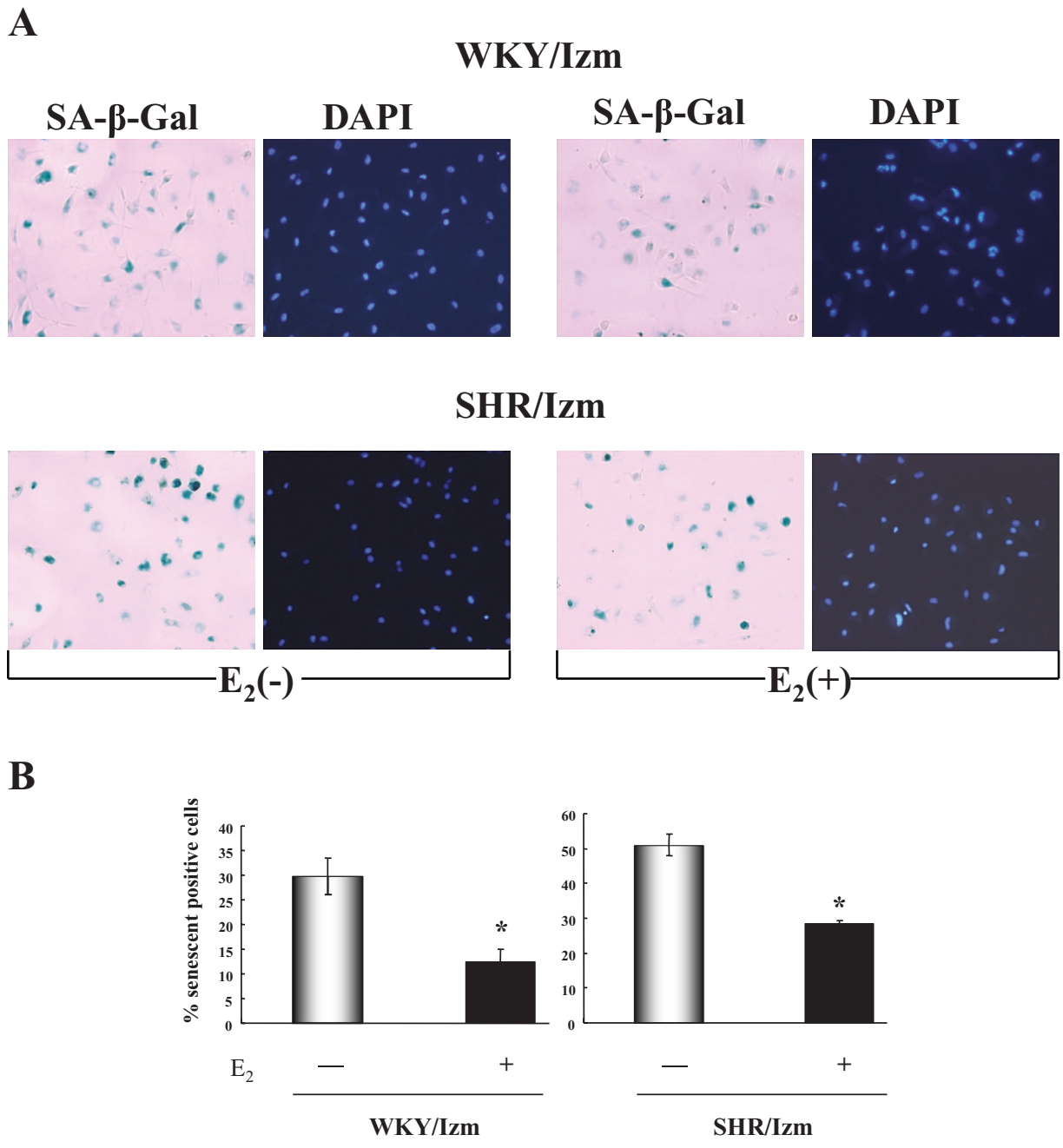

Fig. 5. *E_2 inhibits BM-EPC senescence. A: Representative photomicrographs show senescence-associated β-galactosidase (SA-β-Gal)-positive cells (blue) in BM-EPCs treated without (left) or with (right) E_2 in SHR/Izm and WKY/Izm. B: Quantification of SA-β-Gal-positive cells was performed in SHR/Izm and WKY/Izm. The number of blue cells was counted manually from a total of 200 cells. Data are expressed as the mean±SEM (*n = 6). *$p < 0.01$ compared with the control (no treatment).*

(*21*). Akt is a serine threonine protein kinase that is activated by a number of growth factors and cytokines in a PI3-K-dependent manner. In the present study, we have demonstrated that E_2 augments the differentiation of BM-EPCs derived from WKY/Izm as well as SHR/Izm. Interestingly, E_2 treatment led to the augmentation of the differentiation of BM-EPCs derived from SHR/Izm as well as WKY/Izm to almost the same degree. These results may be explained by the effects of E_2 on Akt, because we have demonstrated that E_2 treatment led to the phosphorylation of Akt in BM-EPCs derived from SHR/Izm as well as WKY/Izm to almost the same degree. In the present study, the cellular and molecular

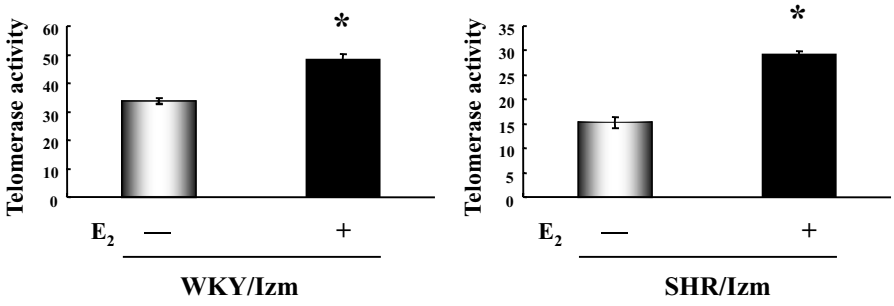

Fig. 6. *Effect of E_2 on telomerase activity. Freshly isolated mononuclear cells were cultivated in endothelial basal medium-2 (EBM-2) supplemented with single aliquots of EGM-2MV. The six-well plates were replated at day 4. After an additional 3 days of cultivation, E_2 was added for 24 h and telomerase activity was measured. Data are expressed as the mean±SEM (*n = 6). *$p < 0.01$ compared with the control (no treatment).*

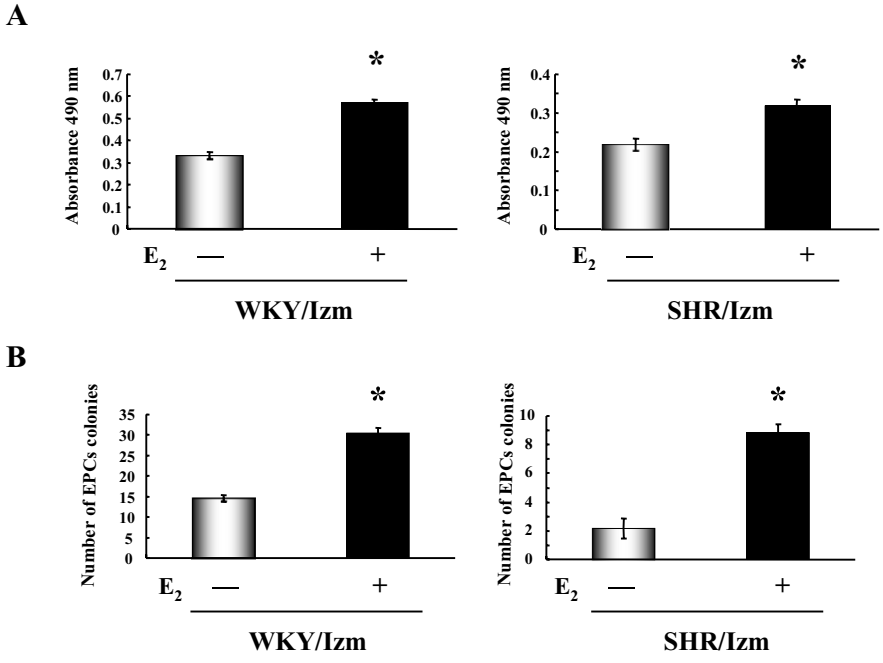

Fig. 7. *Effects of E_2 on the proliferative activity of BM-EC-like cells. A: BM-EC-like cells treated with or without E_2 (100 nmol/l) were harvested 14 days after culture. The mitogenic activity was detected as described in the Methods. Data are expressed as the mean±SEM (*n = 6). *$p < 0.01$ compared with E_2 (100 nmol/l) treatment. B: At day 4, BM-EC-like cells were seeded in methylcellulose plates, and colonies were counted after an additional 10 days of cultivation. Data are expressed as the mean±SEM (*n = 6). *$p < 0.01$ compared with E_2 (100 nmol/l) treatment.*

events involved in E_2-induced Akt activation remain to be determined. In this context, however, it is interesting to note that E_2 has been shown to activate Akt signaling in mature ECs *via* nonnuclear estrogen receptor α (*22*), suggesting one possible mechanism for the modulation of BM-EPCs.

The mechanisms by which E_2 delays the onset of BM-EPCs senescence remain to be determined. Telomerase is a cellular reverse transcriptase which catalyzes the synthesis and extension of telomeric DNA (*23*, *24*). Telomerase activity is constitutively expressed in germline cells and in the majority of malignant tissue cells and is repressed in most human normal somatic cells (*25*, *26*). Strikingly, however, telomerase activity is expressed in a highly regulated manner in certain somatic cell populations, such as lymphocytes and hematopoietic stem cells (*27–29*). Studies on telomerase regulation in normal somatic cells have focused on expression of the two essential components of telomerase, telomerase RNA template (hTER) (*30*) and hTERT (*31*). There is a good correla-

tion between the expression of hTERT mRNA and the presence of telomerase activity in extracts from tissue culture cells and normal and cancer tissues (*32*), whereas hTER is expressed constitutively in both cancer and normal cells, irrespective of the status of telomerase expression (*30*). Interestingly, Murasawa *et al*. (*8*) have revealed that overexpression of hTERT by adenovirus-mediated gene delivery could result in the delay of senescence and the recovery/enhancement of the regenerative properties of EPCs. In this context, we have recently shown that E_2 dose-dependently increased hTERT mRNA in a PI3-K/Akt dependent manner (*33*). Keeping these findings in mind, we speculated that estrogen delays the onset of senescence through telomerase activation. However, further studies will be needed to confirm our hypothesis.

In conclusion, the results of the present study demonstrated that E_2 delays the onset of the senescence of BM-EPCs in both SHR/Izm and WKY/Izm, which may be related to telomerase activation. Importantly, the inhibition of BM-EPC senescence by estrogen *in vitro* may improve the functional activity of BM-EPCs for potential cell therapy.

References

1. Asahara T, Murohara T, Sullivan M, *et al*: Isolation of putative progenitor endothelial cells to angiogenesis. *Science* 1997; **275**: 964–967.
2. Shi Q, Rafii S, Wu MH, Wijelath ES, *et al*: Evidence for circulating bone marrow-derived endothelial cells. *Blood* 1998; **92**: 362–367.
3. Kong D, Melo LG, Mangi AA, *et al*: Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. *Circulation* 2004; **109**: 1769–1775.
4. Iwakura A, Luedemann C, Shastry S, *et al*: Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendotheliazation after arterial injury. *Circulation* 2004; **108**: 3115–3121.
5. Hill JM, Zalos G, Halcox JP, *et al*: Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N Engl J Med* 2003; **348**: 593–600.
6. Vasa M, Fichtlscherer S, Aicher A, *et al*: Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ Res* 2001; **89**: e1–e7.
7. Hayflick L, Moorhead P: The serial cultivation of human diploid strains. *Exp Cell Res* 1961; **25**: 585–621.
8. Murasawa S, Llevadot J, Silver M, Isner JM, Losordo DW, Asahara T: Constitutive human telomerase reverse transcriptase expression enhances regenerative properties of endothelial progenitor cells. *Circulation* 2002; **106**: 1133–1139.
9. Iwakura A, Luedemann C, Shastry S, *et al*: Estrogen-mediated, endothelial nitric oxide synthase-dependent mobilization of bone marrow-derived endothelial progenitor cells contributes to reendotheliazation after arterial injury. *Circulation* 2003; **108**: 3115–3121.
10. Strehlow K, Werner N, Berweiler J, *et al*: Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation* 2003; **107**: 3059–3065.
11. Bunag RD: Validation in awake rats of a tail-cuff method for measuring systolic pressure. *J Appl Physiol* 1973; **34**: 279–282.
12. Imanishi T, McBride J, Ho Q, O'Brien KD, Schwartz SM, Han DK: Expression of cellular FLICE-inhibitory protein in human coronary arteries and in a rat vascular injury model. *Am J Pathol* 2000; **156**: 125–137.
13. Dimri GP, Lee X, Basile G, *et al*: A biomarker that identifies senescent human cells in culture and in aging skin *in vitro*. *Proc Natl Acad Sci USA* 1995; **92**: 9363–9367.
14. Imanishi T, Hano T, Sawamura T, Nishio I: Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clin Exp Pharmacol Physiol* 2004; **31**: 407–413.
15. Imanishi T, Hano T, and Nishio I: Angiotensin II accelerates endothelial progenitor cell senescence through induction of oxidative stress. *J Hypertens* 2005; **23**: 97–104.
16. Dimmeler S, Aisher A, Vasa M, *et al*: HMG-CoA reductase inhibitors (statins) increase endothelial progenitor cells *via* the PI 3-kinase/Akt pathway. *J Clin Invest* 2001; **108**: 391–397.
17. Kawamoto A, Gwon HC, Iwaguro H, *et al*: Therapeutic potential of *ex vivo* expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 2001; **103**: 634–637.
18. Assmus B, Schachinger V, Teupe C, *et al*: Transplatation of Progenitor Cell and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 2002; **106**: 3009–3017.
19. Tepper OM, Galiano RD, Capla JM, *et al*: Human endothelial progenitor cells from type II diabetes exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation* 2002; **106**: 2781–2786.
20. Ikeda Y, Fukuda N, Wada M, *et al*: Development of angiogenic cell and gene therapy by transplantation of umbilical cord blood with vascular endothelial growth factor gene. *Hypertens Res* 2004; **27**: 119–128.
21. Imanishi T, Hano T, Matsuo Y, Nishio I: Oxidized LDL inhibits vascular endothelial growth factor-induced endothelial progenitor cell differentiation. *Clin Exp Pharmacol Physiol* 2003; **30**: 665–670.
22. Simoncini T, Rabkin E, Liao JK: Molecular basis of cell membrane estrogen receptor interaction with phosphatidylinositol 3-kinase in endothelial cells. *Arterioscler Thromb Vasc Biol* 2003; **23**: 198–203.
23. Greider CW, Blackburn EH: Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell* 1985; **43**: 405–413.
24. Greider CW, Blackburn EH: A telomeric sequence in the RNA of Tetrahymena telomerase required for telomere repeat synthesis. *Nature* 1989; **337**: 331–337.
25. Crow JP, Beckman JS: The importance of superoxide in nitric oxide-dependent toxicity: evidence for peroxynitrite-mediated injury. *Adv Exp Med Biol* 1996; **387**: 147–161.
26. Bodnar AG, Ouellette M, Frolkis M, *et al*: Extension of lifespan by introduction of telomerase into normal human cells. *Science* 1998; **279**: 349–352.
27. Broccoli D, Young JW, de Lange T: Telomerase activity in normal and malignant hematopoietic cells. *Proc Natl Acad*

Sci USA 1995; **92**: 9082–9086.
28. Hiyama K, Hirai Y, Kyoizumi S, *et al*: Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J Immunol* 1995; **155**: 3711–3715.
29. Morrison SJ, Prowse KR, Ho P, Weissman IL: Telomerase activity in hematopoietic cells is associated with self-renewal potential. *Immunity* 1996; **5**: 207–216.
30. Feng J, Funk WD, Wang SS, *et al*: The RNA component of human telomerase. *Science* 1995; **269**: 1236–1241.
31. Nakamura TM, Morin GB, Chapman KB, *et al*: Telomerase catalytic subunit homologs from fission yeast and human. *Science* 1997; **277**: 955–959.
32. Greider CW: Telomerase activity, cell proliferation, and cancer. *Proc Natl Acad Sci USA* 1998; **95**: 90–92.
33. Imanishi T, Hano T, Nishio I: Estrogen reduces endothelial progenitor cell senescenes through augmentation of telomerase activity. *J Hypertens* 2005; **23**: 1699–1706.