Loss of Bcl-2 During the Senescence Exacerbates the Impaired Angiogenic Functions in Endothelial Cells by Deteriorating the Mitochondrial Redox State

Maki Uraoka, Koji Ikeda, Ritsuko Kurimoto-Nakano, Yusuke Nakagawa, Masahiro Koide, Yoshiki Akakabe, Youhei Kitamura, Tomomi Ueyama, Satoaki Matoba, Hiroyuki Yamada, Mitsuhiko Okigaki, Hiroaki Matsubara

Abstract—Ageing is an important risk factor for ischemic cardiovascular diseases, although its underlying molecular mechanisms remain to be elucidated. Here, we report a crucial role of Bcl-2 in the impaired angiogenic functions in senescent endothelial cells (ECs) by modulating the mitochondrial redox state. Cellular senescence impaired angiogenic functions in ECs without attenuating the mitogen-activated protein kinase or Akt signaling, and vascular endothelial growth factor receptor 2 or Tie-2 expressions. We identified that Bcl-2 expression was markedly reduced in 3 independent models for senescent ECs, and pharmacological inhibition, as well as small interfering RNA-mediated gene silencing of Bcl-2, significantly impaired the angiogenic functions in young ECs. Bcl-2 has an antioxidative role by locating the glutathione at mitochondria, and we found that mitochondrial oxidative stress was significantly augmented in senescent ECs, in association with reduced mitochondria-associated glutathione. Transfection of Bcl-2 in senescent ECs significantly reduced the mitochondrial oxidative stress, restored the mitochondrial membrane potential, and improved the angiogenic capacity. Furthermore, gene transfer of Bcl-2 using adenovirus significantly improved the in vivo angiogenesis in the Matrigel plugs implanted into aged mice, whereas the Bcl-2 inhibitor reduced the angiogenesis in the Matrigel plugs implanted into young mice. Together, Bcl-2 plays a crucial role in the regulation of the mitochondrial redox state in ECs, and, thus, loss of Bcl-2 during the senescence exacerbates the impaired angiogenesis by augmenting the mitochondrial oxidative stress. (Hypertension. 2011;58:254-263.)

Key Words: cellular senescence ■ endothelial cells ■ mitochondrial oxidative stress ■ ageing ■ angiogenesis

Ischemic cardiovascular and cerebrovascular diseases are the leading causes of death in elderly population, and aged patients have greater morbidity after myocardial infarction. Furthermore, analyses of coronary angiographs revealed that older patients demonstrated decreased collateral circulation to an infarct-related artery as compared with younger patients, which contributes to the poor prognosis of elderly patients with acute myocardial infarction. Therefore, impaired angiogenesis plays an important role in the higher incidence and morbidity of ischemic diseases in an elderly population.

It has been revealed that angiogenesis in the ischemic limb and the tumor xenograft is impaired in aged animals. Tissues from aged individuals display a substantially lower capillary density than do tissues from nonaged counterparts. Decreased expression of vascular endothelial growth factor and/or basic fibroblast growth factor in the ischemic tissues, as well as impaired vasodilation, have been identified in aged animals as possible causative factors leading to the defective angiogenesis associated with ageing. In addition, senescent endothelial cells (ECs) demonstrated higher sensitivity toward apoptotic stimuli because of the reduction of endothelial NO synthase (eNOS) expression, and a role of microRNA-217 was reported in the altered EC functions in association with ageing. However, detailed molecular mechanism(s) underlying the impaired angiogenesis observed in elderly population and a role of cellular senescence on endothelial angiogenic functions still remain to be elucidated.

In the present study, we found that cellular senescence significantly attenuated the angiogenic capacity of ECs, in association with the Bcl-2 depletion. Furthermore, we revealed that the loss of Bcl-2 during the senescence exacerbated the impaired endothelial functions, such as migration and tube formation, by augmenting the mitochondrial oxidative stress. In vivo association between Bcl-2 and impaired angiogenesis in aged mice was also examined.

Methods

Cell Culture

Human umbilical vein ECs (HUVECs) were cultured in HuMedia EG2 (Takara, Shiga, Japan) on gelatin-coated culture dish. Subcul-
turing was always performed at 80% to 90% confluence, and 20% of the cells were regularly replated. Cells were given fresh growth medium every 2 days. For all of the experiments, cells at $\approx$80% confluence were used. Senescence-associated $\beta$-galactosidase activity was analyzed as described previously. In some experiments, cells were pretreated with 2 $\mu$mol/L of YC137 (Bel-2 inhibitor) in the presence or absence of 1 mmol/L of N-acetylcysteine for 15 to 18 hours. For the treatment with hydrogen peroxide, cells were incubated with 1 mmol/L of hydrogen peroxide for 40 hours in the growth medium. Only migration of senescent HUVECs infected with retrovirus was examined by wound healing assay. Otherwise, cell migration was analyzed by modified Boyden chamber assay using 50 ng/mL of vascular endothelial growth factor as a chemoattractant. Please see the online Data Supplement for the detailed procedures. Endothelial apoptosis was induced by serum and growth supplement depletion for 18 to 24 hours. Nuclei were stained with Hoechst 33342, and the number of apoptotic cells demonstrating condensed nuclei was counted and divided by the total number of cells to calculate the percentage of apoptotic cells. Cell proliferation was analyzed by CellTiter 96 aqueous 1 solution cell proliferation assay kit (Promega, Madison, WI) by culturing cells in the growth medium for 3 days after plating exactly the same number of cells. Mitochondrial superoxide was quantitatively detected by using MitoSOX Red mitochondrial superoxide indicator (Invitrogen) as the manufacturer recommended. Mitochondrial membrane potential was evaluated by using JC-1 (Invitrogen). NO production was detected by using a NO detection kit for microscopy (Enzo Life Sciences, Plymouth Meeting, PA) as the manufacturer recommended. The experiments were repeated $\geq$2 times, in which 1 to 2 independent cultures were used.

### Immunoblotting and Immunocytochemistry

Immunoblotting and immunocytochemistry were performed as described previously with minor modifications. Please see the online Data Supplement for the detailed procedures.

### Analysis of the Mitochondrial Glutathione Pool

Analysis of the mitochondrial glutathione (GSH) pool was performed as described previously. Please see the online Data Supplement for the detailed procedures.

### In Vitro and In Vivo Angiogenesis Analysis Using Matrigel

In vitro and in vivo angiogenesis analysis using Matrigel was performed as described previously with minor modifications. Please see the online Data Supplement for the detailed procedures.

### Statistical Analysis

Differences between groups were analyzed by Student $t$ test or 1-way ANOVA with post hoc multiple comparison by Bonferroni/Dunn test. $P<0.05$ was considered statistically significant. Data are presented as mean±SD as indicated.

### Results

#### Preparation of Senescent ECs

Normal somatic cells enter a state of irreversible growth arrest and altered function after finite number of divisions, and this process is termed “replicative senescence.” To prepare senescent ECs, we cultured primary human umbilical vein ECs (HUVECs) for an extended period until passage 22, as described in the Method section. We termed HUVECs in passage 4 to 5 as “young cells” and HUVECs in passage 21 to 22 as “senescent cells,” as reported previously. We also isolated aortic ECs from aged (20-month–old) and young (2-month–old) mice and used them for ex vivo experiments.

Senescent HUVECs, as well as aortic ECs isolated from aged mice, demonstrated flat and enlarged morphology and significant senescence-associated $\beta$-galactosidase activity, indicating that they were senescent (Figure 1A and 1D). Furthermore, expressions of p16$^{ink4a}$ and p21$^{Waf1/Cip1}$ were significantly increased in senescent HUVECs as compared with those in young cells (Figure 1B). Both p16$^{ink4a}$ and p21$^{Waf1/Cip1}$ are cyclin-dependent kinase inhibitors, which accumulate in ageing cells to induce cell cycle arrest. Significant expression of CD31 was detected in senescent HUVECs, although the expression level was slightly but significantly reduced compared with that in young cells (Figure 1C; 92.5±2.4%; $P<0.05$; $n=4$). Immunostaining of CD31 in senescent HUVECs demonstrated a similar staining pattern to that in young cells (Figure 1C). These collectively exclude the possibility that other type of cells dominated the cell population during the extended culture period. Consistent with the cellular senescence, proliferation was markedly reduced in the aortic ECs isolated from aged mice compared with that in ECs isolated from young mice (Figure 1E).

#### Cellular Senescence Leads to Impaired Endothelial Angiogenic Functions

To investigate an impact of cellular senescence on endothelial angiogenic functions, we examined the angiogenic capacities of senescent HUVECs and aortic ECs isolated from aged mice. ECs migration and tube formation on Matrigel were significantly attenuated, whereas apoptosis was significantly enhanced in both senescent ECs as compared with those in young control cells (Figure 2A and 2B). Thus, cellular senescence not only induces the higher susceptibility to apoptosis but also impairs angiogenic functions in ECs, which potentially attenuates the angiogenesis in association with ageing.

Because downregulation of eNOS was reported to play a role in the higher susceptibility to apoptosis in senescent ECs, and eNOS plays an important role in the regulation of endothelial functions, we examined eNOS expression in young and senescent HUVECs. We observed a modest reduction of total eNOS expression in senescent HUVECs (52.6±12.9%; $P<0.005$; $n=3$), whereas no significant reduction of phosphorylated eNOS expression was detected, despite the substantially impaired angiogenic functions in senescent ECs (Figure 2C). These results urged us to seek the molecular mechanism(s) leading to the impaired angiogenic capacity in the senescent ECs other than the eNOS reduction.

#### Expression of Bel-2 Is Downregulated in Senescent ECs

To identify the underlying mechanism(s) responsible for the impaired angiogenic functions in senescent ECs, we examined the gene expressions and intracellular signaling pathways that regulate the ECs functions. Unexpectedly, phosphorylation of extracellular signal–regulated kinase and Akt and expression of Tie-2 were significantly increased in senescent HUVECs (Figure 3A; phospho-extracellular sig-
nucleolar–regulated kinase: 165.2±23.9%, \( P<0.005 \); phospho-Akt: 155.7±35.9%, \( P<0.05 \); Tie-2: 147.4±16.3%, \( P<0.05 \); \( n=4 \) each) compared with those in young cells, whereas the expression of vascular endothelial growth factor receptor 2 was not altered during the senescence (Figure 3A; 93.1±26.1%; \( P=0.63 \); \( n=4 \)). Consistently, vascular endothelial growth factor and basic fibroblast growth factor induced extracellular signal–regulated kinase activation in senescent HUVECs to the extent similar to that in young cells, whereas the expression of a dominant-negative form of TRF2 mediates the senescence-like state, presumably by inducing end-to-end chromosome fusions without reducing the telomere length.24 Overexpression of dominant-negative TRF2 led to significantly reduced Bcl-2 expression in ECs compared with that in young control cells (Figure 3E). These strongly suggest that the loss of Bcl-2 is indeed associated with the ECs senescence. In addition, eNOS expression was significantly reduced, whereas the Bax expression was not changed in HUVECs overexpressing the dominant-negative TRF2 (Figure S3D).

**Bcl-2 in the Regulation of ECs Functions**

We then investigated a role of Bcl-2 in the regulation of ECs functions. As expected, pharmacological inhibition of Bcl-2 degradation is unlikely to be the major cause of the Bcl-2 depletion. In contrast, mRNA expression of Bcl-2 was significantly reduced in senescent HUVECs (Figure 3E).

To further confirm whether the loss of Bcl-2 is associated with cellular senescence in ECs, we prepared another model for senescent ECs. Telomere repeat factor 2 (TRF2) plays a key role in the protective activity of telomeres, and overexpression of a dominant-negative form of TRF2 mediates the cellular senescence-like state, presumably by inducing end-to-end chromosome fusions without reducing the telomere length.24 Overexpression of dominant-negative TRF2 led to substantial senescence-associated \( \beta \)-galactosidase activity without shortening the telomere in young HUVECs, as reported previously (Figure S3A through S3C). Bcl-2 expression was significantly reduced in the HUVECs overexpressing the dominant-negative TRF2 as compared with that in control cells (Figure 3F). These strongly suggest that the loss of Bcl-2 is indeed associated with the ECs senescence. In addition, eNOS expression was significantly reduced, whereas the Bax expression was not changed in HUVECs overexpressing the dominant-negative TRF2 (Figure S3D).

**Figure 1. Preparation of senescent endothelial cells (ECs).** A, Replicative senescent human umbilical vein ECs (HUVECs) were flat and enlarged in morphology and demonstrated significant senescent-associated \( \beta \)-galactosidase activity (SA-\( \beta \)-gal). Bar=200 \( \mu \)m. B, Cyclin-dependent kinase inhibitors, such as p21 and p16, were significantly upregulated in senescent HUVECs as compared with those in young cells. \( P<0.05 \) and \( **P<0.0001 \) vs young HUVECs (\( n=5 \) each). C, Senescent HUVECs expressed CD31. Bar=200 \( \mu \)m. D, ECs were isolated from aortas of young (2-month–old) and aged (20-month–old) mice. Aortic ECs of aged mice demonstrated flat and enlarged morphology and showed significant SA-\( \beta \)-gal activity. Bar=100 \( \mu \)m. E, In support of their senescence, proliferation was significantly reduced in ECs of aged mice as compared with that in aortic ECs of young mice. \( **P<0.0001 \) vs aortic ECs of young mice (\( n=5 \) each).

\[ \text{Relative protein expression}\]

\[ \text{Young Senescent}\]

\[ \text{Aged Senescent}\]

\[ \text{Preparation of senescent endothelial cells (ECs).}\]

**Young**

**Senescent**

**A**

**B**

**C**

**D**

**E**

\[ \text{CD31}\]

\[ \text{GAPDH}\]

\[ \text{Young mice aortic ECs}\]

\[ \text{Aged mice aortic ECs}\]

\[ \text{Phase contrast SA-\( \beta \)-gal}\]

\[ \text{Proliferation}\]

\[ \% \text{of control}\]

\[ \text{Young Senescent Aged}\]
YC13712 significantly enhanced the serum and growth factor deprivation-mediated apoptosis in young ECs (Figure 3G). YC137 did not induce the endothelial apoptosis in the absence of apoptotic stimuli at this concentration (data not shown), indicating that YC137 itself is not cytotoxic. Interestingly, both EC migration and tube formation were significantly reduced by the treatment with YC137 in young HUVECs, as well as in aortic ECs of young mice (Figure 3G). Similarly, small interfering RNA–mediated gene knockdown of Bcl-2 (Figure S4) significantly attenuated EC migration and tube formation, whereas it enhanced the endothelial apoptosis in young HUVECs (Figure 3H). In contrast, overexpression of Bcl-2 in young HUVECs significantly enhanced the migration and tube formation, whereas it reduced endothelial apoptosis (Figure S5). These results strongly suggest that Bcl-2 plays an important role in the regulation of EC functions, in addition to governing the endothelial apoptosis.

It has been reported that overexpression of Bcl-2 in ECs enhances angiogenesis by increasing the expressions of angiogenic cytokines, such as CXCL-1 and CXCL-8.25 Therefore, we examined the expression of CXCL-1 and CXCL-8 in the senescent HUVECs. Despite the decrease in Bcl-2 level, mRNA expressions of both CXCL-1 and CXCL-8 were rather increased in the senescent HUVECs (Figure S6), suggesting that CXCL-1 and CXCL-8 do not provide a mechanism by which loss of Bcl-2 attenuates the endothelial functions during the cellular senescence.

Augmented Mitochondrial Oxidative Stress in Senescent ECs Impairs the Endothelial Angiogenic Functions

In addition to its antiapoptotic function, Bcl-2 has been reported to have an antioxidative property by locating GSH at mitochondria where reactive oxygen species are constantly generated for respiration and energy production.16 Furthermore, the accumulation of mitochondrial damage is one of the most widely accepted causes of ageing.26,27 Therefore, we analyzed the mitochondrial oxidative stress by using MitoSOX and found that excessive mitochondrial superoxide was accumulated in senescent HUVECs (Figure 4A). In addition, mitochondrial membrane potential assessed by JC-1 staining was significantly attenuated in senescent HUVECs.
Figure 3. Bcl-2 expression was markedly reduced in senescent endothelial cells (ECs). A, Expression of vascular endothelial growth factor (VEGF) receptor 2 (KDR), Tie-2, phospho-Akt and total-Akt, and phospho- and total-extracellular signal–regulated kinase (ERK) was examined in young and senescent human umbilical vein ECs (HUVECs). B, Expression of Bcl-xL was modestly increased, whereas
Mitochondrial oxidative stress is augmented in senescent human umbilical vein endothelial cells (ECs; HUVECs). **A**, Mitochondrial superoxide detected by MitoSOX was significantly increased in senescent HUVECs. \(^*P<0.005\) vs young HUVECs (n=8 each). Mitochondrial membrane potential assessed by the red/green ratio of JC-1 staining was significantly attenuated in senescent HUVECs. \(^*P<0.05\) vs young HUVECs (n=5 each). **B**, Treatment with 1 mmol/L of N-acetylcysteine (NAC) significantly reduced the mitochondrial oxidative stress and restored mitochondrial membrane potential in senescent HUVECs. \(^*P<0.01\) and \(^*P<0.05\) vs vehicle treatment (n=6 each). Bars=100 \(\mu\)m for MitoSOX and 10 \(\mu\)m for JC-1. **C**, Treatment with NAC restored the EC functions in senescent HUVECs. \(^*P<0.05\) vs vehicle treatment (n=4 each). Bar=200 \(\mu\)m.

We also examined the expression of manganese superoxide dismutase (MnSOD) that catalyzes the dismutation of superoxide into oxygen and hydrogen peroxide at mitochondria. \(^28\) MnSOD expression was significantly increased in the senescent HUVECs compared with that in young cells (Figure S7A; 248.3±9.7%; \(P<0.0001\); n=4), which might be because of compensation for the excess superoxide production. Because mitochondrial uncoupling proteins (UCPs) play a role in the protection of mitochondria from oxidative damage, we examined the UCP-2 expression as well. We found that UCP-2 expression was also enhanced in senescent HUVECs compared with that in young cells (Figure S7A; 261.7±15.4%; \(P<0.0001\); n=4). The expression of either MnSOD or UCP-2 was not affected by Bcl-2-silencing in young HUVECs, suggesting that the enhanced expression of MnSOD and UCP-2 in senescent ECs is not associated with cellular senescence.
the loss of Bcl-2 during the senescence (Figure S7A). On the other hand, overexpression of Bcl-2 (Figure S7B) in young HUVECs significantly increased the expression of MnSOD (Figure S7A; 158.7±30.4%; \( P<0.05; n=4 \)) and UCP-2 (Figure S7A; 145.6±19.1%; \( P<0.05; n=4 \)), suggesting a possible role for Bcl-2 in the regulation of MnSOD and UCP-2 expressions in ECs. We then examined the expressions of catalase and GSH peroxidase 1 that catalyze the decomposition of hydrogen peroxide to water and oxygen. Catalase mRNA expression was significantly increased (228.4±9.5%; \( P<0.001; n=6 \)), whereas GSH peroxidase 1 expression was not changed in the senescent HUVECs (100.0±7.4%; \( P=0.46; n=6 \)).

We next investigated the morphology of mitochondria by electron microscopy and found that mitochondria appeared to be irregular in morphology in senescent HUVECs (Figure S8A). Mitochondrial DNA copy number was significantly increased in senescent HUVECs (254.3±55.2%; \( P<0.05; n=4 \)), whereas expression of mitochondrially encoded protein cytochrome c oxidase subunit II was significantly reduced in senescent cells (Figure S8B; 38.8±15.7%; \( P<0.005; n=4 \)). These findings are consistent with the previous reports showing that mitochondrial DNA is increased, whereas mitochondrial mRNA levels were reduced in tissues of aged human and rats.\(^{29–31}\) Knockdown of Bcl-2 in young HUVECs did not affect the cytochrome c oxidase subunit II expression (Figure S8B), whereas the overexpression of Bcl-2 tended to increase cytochrome c oxidase subunit II expression in young HUVECs (Figure S8B; 138.5±46.2%; \( P=0.1590; n=4 \)).

Figure 5. Retrovirus-mediated transfection of Bcl-2 in senescent human umbilical vein endothelial cells (ECs; HUVECs) reduced the mitochondrial oxidative stress and restored the EC functions. A, Biotinylated glutathione (GSH) was detected by fluorescence-labeled avidin, and mitochondria was detected by MitoTracker. GSH was well colocalized with mitochondria in young HUVECs, whereas it was dispersed throughout the cytosol and accumulated in nucleus in senescent HUVECs. Transfection of Bcl-2 appeared to partly restore the colocalization of GSH with mitochondria. Bar=10 \( \mu \)m. B, Transfection of Bcl-2 significantly reduced the mitochondrial oxidative stress and restored mitochondrial membrane potential in senescent HUVECs. **\( P<0.005 \) and ***\( P<0.01 \) vs mock control (n=11 each for MitoSOX and n=6 each for JC-1). Bars=100 \( \mu \)m for MitoSOX and 10 \( \mu \)m for JC-1. C, Transfection of Bcl-2 significantly restored the EC functions in senescent HUVECs. *\( P<0.05 \) vs mock control (n=4 each). D, Treatment with 1 mmol/L of N-acetylcysteine (NAC) significantly increased the Bcl-2 expression in senescent HUVECs. *\( P<0.05 \) vs vehicle control (n=3 each). E, Bcl-2 expression was significantly reduced by the treatment with 1 mmol/L of hydrogen peroxide in young HUVECs. *\( P<0.005 \) vs vehicle control (n=4 each).
We then examined the subcellular localization of GSH in young and senescent HUVECs to investigate whether loss of Bcl-2 affects the mitochondrial localization of GSH, as reported previously. Biotin-labeled GSH was largely colocalized with mitochondria in young HUVECs (Figure 5A). In contrast, GSH was dispersed throughout the cytosol and accumulated in nucleus in senescent cells, and only minimal colocalization with mitochondria was observed (Figure 5A). Retrovirus-mediated transfection of Bcl-2 (Figure S9) appeared to partly restore the colocalization of GSH with mitochondria in senescent HUVECs (Figure 5A). These results strongly suggest that the loss of Bcl-2 reduces the mitochondria-associated GSH, which might deteriorate the mitochondrial redox environment in senescent HUVECs.

Of note, transfection of Bcl-2 significantly reduced the mitochondrial superoxide and restored the mitochondrial membrane potential in young HUVECs (Figure 5B). Furthermore, Bcl-2 transfection significantly improved the endothelial functions, including not only apoptosis but also migration in senescent HUVECs (Figure 5C). Consistently, inhibition of Bcl-2 in young HUVECs significantly enhanced the mitochondrial oxidative stress and reduced the mitochondrial membrane potential (Figure S10A). Moreover, treatment with antioxidant significantly attenuated the impaired angiogenic functions induced by Bcl-2 inhibition in young HUVECs (Figure S10B). These collectively indicate that the loss of Bcl-2 exacerbates the impaired angiogenic functions in senescent ECs at least partly by augmenting the mitochondrial oxidative stress.

Interestingly, antioxidant treatment also increased Bcl-2 expression in senescent HUVECs (Figure 5D), whereas treatment with hydrogen peroxide significantly reduced Bcl-2 expression in young HUVECs (Figure 5E). These indicate a crucial role of oxidative stress in the senescence-associated Bcl-2 depletion in ECs.

We also studied a association between Bcl-2 and NO bioactivity in senescent ECs. Transfection of Bcl-2 in senescent HUVECs enhanced the total eNOS expression and led to the higher NO production, but phosphorylated eNOS levels normalized with the levels of total eNOS was not altered (Figure S11). These suggest a possible role for Bcl-2 in the regulation of eNOS expression but not in the control of eNOS phosphorylation during the senescence.

**Bcl-2 Gene Transfer Improves Angiogenesis in Aged Mice In Vivo**

We finally investigated a role for Bcl-2 in the impaired angiogenesis associated with ageing in vivo. Matrigel con-
taining adenovirus carrying either Bcl-2 or LacZ gene was subcutaneously implanted into flanks of aged mice (24 months old). Gene transfer was confirmed by X-gal staining of Matrigel plugs containing LacZ adenovirus (Figure 6A). Bcl-2 gene transfer markedly improved the in vivo angiogenesis in Matrigel plugs implanted into aged mice (Figure 6B). In contrast, Bcl-2 inhibitor significantly attenuated the in vivo angiogenesis in Matrigel plugs implanted into young mice (2 months old; Figure 6C). Taken together, our data demonstrate that the loss of Bcl-2 during the senescence exacerbates the impaired endothelial angiogenic functions and reduces angiogenesis in association with ageing at least partly by deteriorating the mitochondrial redox state.

Discussion

Ischemic cardiovascular and cerebrovascular diseases are the leading causes of death and disability in elderly people. Because angiogenesis is reduced in aged animals, restoring the angiogenesis could be a promising approach to treat and/or prevent ischemic diseases in the elderly population. Recently, a link between cellular senescence and age-related tissue dysfunction has been shed light.32 Nevertheless, a direct link between cellular senescence in ECs and angiogenesis is not fully elucidated. In the current study, we showed that cellular senescence in ECs led to significantly attenuated angiogenic functions, in which the loss of Bcl-2 during the senescence plays an important role by deteriorating the mitochondrial redox state.

We have analyzed the expression change for a variety of molecules that would affect the angiogenic functions during the senescence. Unexpectedly, many of these molecules demonstrated compensatory activation or deactivation, for example, enhanced expression of phospho-Akt, phospho-extracellular signal–regulated kinase, Tie-2, CXCL-1, and CXCL-8, as well as reduced expression of thrombospondin 1, thrombospondin 2, and vasoohbin 1 observed in senescent ECs. Also, the expression of antioxidative molecules such as MnSOD, UCP-2, and catalase was enhanced in senescent HUVECs, presumably aimed at compensation for the excess superoxide production during the senescence. Nevertheless, mitochondrial oxidative stress was significantly augmented in senescent ECs, and this enhanced mitochondrial oxidative stress would play a predominant causative role in the impaired angiogenic functions in senescent ECs.

Mitochondria play an important role in ECs, not only as the powerhouse but also as an important regulator of endothelial functions.33 Also, mitochondria are the major source of endogenous oxidants,34 and the rate of respiration is responsible for the generation of reactive oxygen species. Given that oxidative stress is crucially involved in the ageing process in ECs,55,56 mitochondria are highly prone to oxidative damage during the senescence.57 In fact, we observed reduced mitochondrial membrane potential, as well as irregular mitochondrial morphology, in senescent HUVECs. Mitochondrial GSH is one of the main lines of defense for the maintenance of the appropriate mitochondrial redox environment to avoid oxidative modification leading to mitochondrial dysfunction.57 Because Bcl-2 plays a key role in the association between mitochondria and GSH,46 loss of Bcl-2 would exacerbate mitochondrial oxidative stress by creating a state of redox imbalance, and, thus, loss of Bcl-2 during the senescence should have a significant impact on the senescence-associated impairment of ECs functions. The present study collectively demonstrates that cellular senescence reduces Bcl-2 expression at least partly through the oxidative stress, and this loss of Bcl-2 further augments mitochondrial oxidative stress, which consequently impairs the angiogenic functions in ECs (Figure 6D).

Perspectives

Impaired angiogenesis plays a crucial role in the higher incidence and morbidity of ischemic diseases in an elderly population. This study demonstrates that cellular senescence in ECs impairs endothelial angiogenic functions, at least partly because of the excessive mitochondrial oxidative stress, in which the loss of Bcl-2 plays an important role by deteriorating the mitochondrial redox state. Therefore, regulating Bcl-2 function in ECs could be a potential pharmacotherapeutic target to treat and/or prevent ischemic diseases in an elderly population. We found that, in addition to reducing the oxidative stress, treatment with antioxidant enhanced the Bcl-2 expression in senescent ECs as well, which would further improve the mitochondrial redox environment. Our present data collectively suggest that antioxidant therapy would be beneficial to restore the impaired endothelial angiogenic functions associated with ageing, although the effect of antioxidant vitamin supplementation on clinical events was still inconclusive.

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Disclosures

None.

References


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In the *Hypertension* article by Uraoka et al (Uraoka M, Ikeda K, Kurimoto-Nakano R, Nakagawa Y, Koide M, Akakabe Y, Kitamura Y, Ueyama T, Matoba S, Yamada H, Okigaki M, Matsubara H. Loss of Bcl-2 During the Senescence Exacerbates the Impaired Angiogenic Functions in Endothelial Cells by Deteriorating the Mitochondrial Redox State. *Hypertension*. 2011;58:254–263), a correction has been made to Figure 3G. An incorrect picture of tube formation for aortic endothelial cells of young mice treated with vehicle was used.

The authors regret the error.

These corrections have been made to the current online version of the article, which is available at http://hyper.ahajournals.org/content/58/2/254.full.
ONLINE SUPPLEMENT

LOSS OF BCL-2 DURING THE SENESCENCE EXACERBATES THE IMPAIRED ANGIOGENIC FUNCTIONS IN ENDOTHELIAL CELLS BY DETERIORATING THE MITOCHONDRIAL REDOX STATE

Maki Uraoka, Koji Ikeda*, Ritsuko Kurimoto-Nakano, Yusuke Nakagawa, Masahiro Koide, Yoshiki Akakabe, Youhei Kitamura, Tomomi Ueyama, Satoaki Matoba, Hiroyuki Yamada, Mitsuhiko Okigaki and Hiroaki Matsubara

Department of Cardiovascular Medicine, Kyoto Prefectural University School of Medicine, 465 Kajii, Kawaramachi-Hirokoji, Kamigyo, Kyoto 602-8566, Japan.
Materials

Antibodies for CD31, COX-2, UCP-2 and p21<sup>Waf1/Cip1</sup> were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for Bcl-2 and Bax were obtained from BD Biosciences Pharmingen (San Jose, CA). Antibody for HA-tag was obtained from Roche Applied Science (Germany). Antibodies for p16, total-ERK, phospho-ERK, total-Akt, phospho-Akt, total-eNOS, phospho-eNOS, VEGF-R2 (KDR), Tie-2, and Bcl-xL were obtained from Cell Signaling Technology (Danvers, MN). Antibody for MnSOD was obtained from Abcam (Cambridge, UK). For immunoblotting the antibodies were used in a dilution of 1:200 (CD31, COX-2 and UCP-2), 1:400 (COX-2), 1:500 (Bcl-2), 1:1000 (p16, Bax, HA, total-ERK, phospho-ERK, total-Akt, phospho-Akt, total-eNOS, phospho-eNOS, VEGF-R2, Tie-2, and Bcl-xL), and 1:2000 (MnSOD).

A retroviral backboned plasmid for a dominant negative form of TRF2 was obtained from Addgene (Cambridge, MA).

Quantitative PCR

Total RNA was isolated from cells by using TRIzol (Invitrogen, Carlsbad, CA). Complementary DNA was then synthesized by using the First Strand cDNA synthesis kit (Invitrogen). Quantitative PCR was performed with LightCycler (Roche Applied Science) by using FastStart DNA Master plus SYBR green I kit (Roche Applied Science). Expression levels of target genes were all corrected by GAPDH expression. The sequence of primers used in the experiments was shown in Table-S1.

Immunoblotting and Immunocytochemistry

Immunoblotting and immunocytochemistry were performed as previously described with minor modifications<sup>1</sup>. Briefly, cells were lysed in RIPA buffer containing protease inhibitor cocktail (Sigma, ST Louis, MO) and phosphatase inhibitors. Samples were prepared by repeating the experiments 2-3 times, in which 1-2 independent cultures were used. After measurement of protein concentration using DC protein assay kit (Bio-Rad Laboratories, Hercules, CA), crude cell lysates in the same amount were run on 10-15% SDS-PAGE gel, followed by immunoblotting. For immunocytochemistry, cells were fixed with 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100. Cells were incubated with anti-CD31 antibody
followed by detection using fluorescence-labeled secondary antibody (Invitrogen).

**Cell culture and transfection**

Short interfering RNA (siRNA) for Bcl-2 was obtained from Dharmacon (Lafayette, CO). Negative control siRNA (scramble) was obtained from Ambion (Austin, TX). Transfection of siRNA was performed by using RNAiMAX regent (Invitrogen) as previously described. For transfection of Bcl-2, cells were incubated with retrovirus carrying either Bcl-2 gene or empty vector for 36 h, followed by incubation with fresh growth medium for 24 h. Cells were then incubated with retroviruses again for 36 h before experiments. For transfection of a dominant negative form of telomere repeat factor 2 (TRF2), cells were incubated with retrovirus carrying either dominant negative TRF2 gene or GFP for 36 h, followed by additional incubation with fresh retroviruses for 36 h. Cells were then incubated with fresh growth medium for 24 h before experiments. The experiments were repeated at least 2 times, in which 1-2 independent cultures were used.

**Endothelial migration assay**

Migration capacity was analyzed using a modified Boyden chamber assay or wound-healing assay as described previously. For the wound healing assay, a longitudinal scrape was made with a pipette-tip in growth-arrested confluent ECs and washed with serum free medium. Cells were incubated for 12 h in serum-depleted medium before measuring the area of migration. A modified Boyden chamber assay was performed using a Transwell Permeable Support 8.0 µm Polycarbonate membrane (Coster) coated with 0.5% gelatin. In some experiments, cells were pre-treated with 2 µM YC137 (Bcl-2 inhibitor) in the presence or absence of 1mM N-acetylcysteine for 15-18 h. The lower chamber contained 50 ng/ml VEGF as a chemoattractant. Endothelial cells were prepared in serum-free medium, and $8.5 \times 10^4$ cells were added to the upper chamber in migration buffer (DMEM containing 0.1% BSA). After 4 h of incubation, migrated cells were counted under a microscope. All experiments were performed at least 2 times in which 1-2 independent cultures were used.

**Isolation of endothelial cells from mouse aorta**

Thoracic aorta was extracted from young (2-month old) and aged (20-month old)
old) C57BL/6 mice, and immediately cut into 1-mm-thick rings and placed in tissue culture plates to culture. Explants were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Endothelial cells sprouting out of the explants were subcultured, and then used for experiments within 1-2 passages. We confirmed that more than 90% of cells were positive for CD31. All animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee of the Kyoto Prefectural University of Medicine.

**Analysis of the mitochondrial glutathione (GSH) pool**

Analysis of the mitochondrial GSH pool was performed as previously described. Young or senescent HUVECs were preincubated with biotinylated GSH monoethyl ester (Invitrogen) for 1h. Mitochondria was labeled by incubating cells with MitoTracker (Invitrogen) for 30 min. Cells were then fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100, followed by incubation with streptavidin conjugated with fluorescence. Subcellular localization of GSH was analyzed under confocal fluorescence microscopy (FluoView FV1000, Olympus, Japan).

**Measurement of mitochondrial oxidative stress and membrane potential**

For measurement of mitochondrial superoxide, cells were incubated with MitoSOX dye for 10 min, and then fixed with 4% paraformaldehyde. The cells were analyzed under fluorescence confocal microscopy and fluorescence intensity was measured in 4-5 independent fields.

To assess the mitochondrial membrane potential, cells were incubated with 200 nM JC-1 for 30 min, and then analyzed under fluorescence confocal microscopy. Mitochondrial membrane potential was evaluated by the ratio of red and green fluorescence.

**In vitro and in vivo angiogenesis analysis using Matrigel**

*In vitro* and *in vivo* angiogenesis analysis using Matrigel was performed as previously described with minor modifications. Briefly, *in vitro* angiogenesis analysis was performed in 96-well plates coated with 50 µL of Matrigel (BD Biosciences). Two × 10⁴ HUVECs were plated on Matrigel and incubated for 6 h. Length of tubes was measured in 2-4 independent fields. For *in vivo* Matrigel-plug
assay, Matrigel was mixed with 50 ng/mL VEGF, 50 ng/mL basic FGF, and 50 units/mL heparin before injection. To investigate the in vivo association between Bcl-2 depletion and impaired angiogenesis in aged mice, Matrigels was further mixed with the adenovirus carrying either LacZ or Bcl-2 gene at 5 × 10⁹ pfu. Adenoviruses carrying the LacZ or Bcl-2 gene were obtained from Vector Biolabs (Philadelphia, PA), and purified by using Add-N-Pure Adenovirus purification kit (Applied Biological Materials, Belgium) before use. To examine the effect of Bcl-2 inhibition on in vivo angiogenesis in young mice, either 4 µM YC137 or vehicle was added in the Matrigel. Matrigel were injected subcutaneously into the bilateral flank of young (2-month old) or aged (24-month old) C57BL/6 mice on day 0, and the Matrigel plugs were extracted at day 7. Matrigel-plugs containing adenovirus (injected into aged mice) was embedded in Tissue-tek OCT compound (Sakura Finetechanical, Japan) without fixation in order to avoid the reduction of LacZ enzymatic activity, and snap frozen followed by sectioning. X-gal staining of Matrigel-plugs containing LacZ adenovirus was performed as previously described. Matrigel plugs containing vehicle or YC137 (injected into young mice) were fixed with 4% paraformaldehyde and embedded in paraffin followed by sectioning. ECs were detected by using fluorescent-labeled isolectin GS-IB4 (Invitrogen). Images were captured by the fluorescence microscopy under exactly the same condition.

Measurement of mitochondrial DNA copy

Mitochondrial DNA copy was measured as previously reported. Briefly, DNA was collected by using FastPure DNA kit (Takara Bio, Japan). Real-time PCR was performed for cytochrome c oxidase subunit 1 (COX1) and cytokine-like protein C17. COX1 DNA copies were normalized to nuclear gene, C17.
Reference for the online supplement


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Figure S1. A, Growth factor induced ERK activation was similar between young and senescent HUVECs. B, mRNA expressions of anti-angiogenic factors such as thrombospondin (TSP)-1, TSP-2 and vasohibin-1 were reduced despite the attenuated angiogenic capacity in senescent HUVECs. *P<0.05 versus young HUVECs (n=5 each).
figure S2. Inhibition of either lysosome or proteasome degradation did not restore the Bcl-2 expression in senescent HUVECs.
**figure S3.** A, Transfection of target genes into HUVECs by using retrovirus was confirmed by the induction of GFP. B, Telomere length was analyzed by Southern analysis of terminal restriction fragments obtained by digestion of genomic DNA through the hybridization with labeled oligonucleotides complementary to the telomeric repeat sequence. C, Overexpression of dominant negative TRF2 (TRF2-DN) led to significant SA-β-gal activity in young HUVECs. Bar; 500 µm. D, Overexpression of TRF2-DN in young HUVECs reduced the eNOS expression, while Bax expression was not changed. *P<0.001 versus young HUVECs (n=4 each).
**figure S4.** Knockdown of Bcl-2 in young HUVECs was confirmed by quantitative PCR. *P*<0.05 versus scramble control (n=4 each).
Figure S5. Overexpression of Bcl-2 significantly enhanced endothelial migration and tube-formation, while it reduced endothelial apoptosis in young HUVECs. ***P<0.0001, **P<0.005 and *P<0.05 versus GFP control cells (n=4 each). Bars; 500 µm for tube-formation and 100 µm for apoptosis.
Figure S6. mRNA expressions of both CXCL-1 and CXCL-8 were significantly enhanced in senescent HUVECs. **P<0.01 and *P<0.05 versus young HUVECs (n=4 each).
Figure S7. A, Expressions of MnSOD and UCP-2 were enhanced in senescent HUVECs as compared with those in young cells. Knockdown of Bcl-2 (Bcl2-KD) did not affect the expression of MnSOD and UCP-2, whereas overexpression of Bcl-2 (Bcl2-HA) increased those expressions in young HUVECs. B, Retrovirus-mediated overexpression of Bcl-2 was confirmed.
**Figure S8.**

**A,** Mitochondria (arrows) in senescent HUVECs demonstrated irregular morphology. Autophagosomal vacuoles and/or secondary lysosome (arrowheads) were frequently observed in senescent HUVECs. Original magnification is x10,000.

**B,** Protein expression of cytochrome c oxidase subunit II (COX-2) was significantly reduced in senescent HUVECs as compared with that in young cells. Knockdown of Bcl-2 (Bcl2-KD) did not reduce the COX-2 expression, while overexpression of Bcl-2 tended to increase the COX-2 expression in young HUVECs.
**Figure S9.** Transfection of Bcl-2 in senescent HUVECs was performed by using retrovirus carrying Bcl-2 gene.
Figure S10. A, Bel-2 inhibitor (YC137) significantly enhanced the mitochondrial oxidative stress as well as attenuated the mitochondrial membrane potential in young HUVECs. **P<0.01 versus vehicle control. (n=5 each). B, Treatment with NAC attenuated the impaired endothelial functions induced by YC137 in young HUVECs. *P<0.05 versus vehicle control (n=4 each).
**Figure S11.** Transfection of Bcl-2 significantly increased the total eNOS expressions, and enhanced NO production in senescent HUVECs. In contrast, phospho-eNOS levels normalized with the total eNOS levels were not altered by Bcl-2 transfection. **P<0.01 and *P<0.05 versus mock control (n=3-6 each). Bar; 200 µm.**