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Enhancement of the Inflammatory Response of Aging Vascular Endothelial Cells by Gram-Negative Bacterial LPS and Antimicrobial Peptide LL-37

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Abstract

Cellular senescence is associated with the induction of a proinflammatory phenotype. Previous studies revealed that senescent endothelial cells are localized at the sites of atherosclerotic lesions, suggesting the involvement of endothelial cell senescence in atherogenesis. Importantly, bacterial infection has been speculated to contribute to the pathogenesis of atherosclerosis. However, the effect of bacterial components or host-derived antimicrobial substances on senescent endothelial cells is not fully understood. In this paper, we investigated the effects of gram-negative bacterial lipopolysaccharide (LPS) and antimicrobial peptide LL-37, which is deposited at the atherosclerotic loci, on senescent endothelial cells using serially passaged human umbilical vein endothelial cells (HUVECs). The results indicated that senescent endothelial cells basally exhibited proinflammatory phenotype, as evidenced by increased expression of intercellular adhesion molecule-1 (ICAM-1) and activation of NF-κB (phosphorylation of p65), compared with non-senescent cells. Of note, senescent endothelial cells more potently responded to the stimulation of LPS or LL-37, as evidenced by further enhanced ICAM-1 expression and NF-κB activation, compared with non-senescent cells. Moreover, expression levels of the receptors for LPS (TLR4) and LL-37 (purinergic receptor P2X7) were upregulated in senescent endothelial cells, suggesting that both LPS and LL-37 enhance the ICAM-1 expression and NF-κB activation in senescent endothelial cells possibly via the upregulated TLR4 and P2X7. These observations indicate that senescent endothelial cells may contribute to the pathogenesis of atherosclerosis via the basal proinflammatory phenotype and the enhanced inflammatory responses against atherogenic factors including bacterial LPS and host-derived antimicrobial LL-37.

Keywords

LL-37

Cellular senescence

Senescence-associated secretory phenotype (SASP)

NF-κB

1. Introduction

Cellular senescence has been identified as a phenomenon in which cells cease to divide and proliferate irreversibly in *in vitro* culture systems [1]. Senescent cells are found in lesions of age-related diseases and are thought to partly reflect aging *in vivo*. Recently, senescence-associated secretory phenotypes (SASPs) have been identified in senescent cells and are thought to be involved in the pathogenesis of chronic inflammation such as atherosclerosis [2]. On the other hand, bacterial infection has been suggested as a factor that exacerbates atherosclerosis, but the mechanism has not yet been fully elucidated. LL-37, an antimicrobial peptide released from host cells upon infection, has also attracted attention as an aggravating factor in atherosclerosis. We focused on three risk factors in atherosclerosis, which are cellular senescence, bacterial infection, and host-derived antimicrobial peptides, and investigated the effects of lipopolysaccharide (LPS) and LL-37, a bacterial constituent and an antimicrobial peptide, respectively. We found that LPS and LL-37 induced an enhanced inflammatory response against aging vascular endothelial cells. In this article, we outline the enhancement of inflammatory responses in aging vascular endothelial cells, focusing on the effects of NF-κB, a signaling molecule that plays a central role in the induction of SASP, LPS, and LL-37, and their respective receptor expression.

2. Cellular aging

Cellular senescence was first reported by Hayflick *et al.* in 1961 as a phenomenon in which cell proliferation in primary cultured human cells irreversibly ceases after a proliferative phase [1]. It is now known that cellular senescence is induced by oxidative stress, DNA damage, inflammatory factors, as well as long-term culture. In addition to a decrease in proliferative capacity, the characteristics of senescent cells include a flattened and enlarged morphology, increased expression of proteins involved in cell cycle arrest, and increased acidic β-galactosidase activity. Cellular senescence was previously thought to be a defense mechanism that inhibits cancer development, but in recent years, it has become clear that senescent cells have inflammatory traits (SASPs), which have attracted attention as being involved in the pathogenesis of age-related chronic inflammation [2].

Atherosclerosis is an age-related disease caused by chronic inflammation of blood vessels. Aged vascular endothelial cells and vascular smooth muscle cells have been observed in atherosclerotic lesions, and these cells are thought to be involved in the pathogenesis and progression of atherosclerosis. However, the mechanisms by which cellular senescence is involved in the pathogenesis of atherosclerosis and its relationship with other aggravating factors in the pathogenesis of the disease are not fully understood.

3. Cellular senescence induced by long-term culture of vascular endothelial cells

We first attempted to induce cellular senescence by long-term culture of human umbilical vein endothelial cells (HUVECs) [3]. HUVECs purchased from Lonza were cultured in culture dishes (100 mm diameter) in an EGM-2 vascular endothelial cell culture medium. The cells were exfoliated with trypsin/EDTA solution before reaching confluence (every 3–4 days), collected, and passaged. After about 80 days of passaging, cell proliferation slowed down when the population doubling level (PDL), which indicates the number of cell divisions, passed 30, and the cells became flattened, enlarged, and heterogeneous in morphology, as displayed in **Figure 1A**. The senescence-associated beta-galactosidase (SA-β-gal) assay is one of the methods commonly used to assess cellular senescence, in which the senescent cells are stained blue. As shown in **Figure 1B**, our PDL32 cells stained blue more strongly than PDL4 cells with fewer passages. We also examined changes in the expression of senescence-associated proteins. p21 (Waf1/Cip1) is an inhibitory

protein of cyclin-dependent kinases, which suppresses the cell cycle and slows down proliferation. Western blot results in **Figure 1C** showed that p21 expression was upregulated in PDL32 cells. These results indicate that PDL32 cells have the characteristics of senescent cells. Therefore, in the present study, PDL32 and above were used in the following experiments as senescent cells, and PDL4 and below were non-senescent cells used as controls.

Senescent cells produce increased amounts of adhesion factors, cytokines, and proteases (induction of the inflammatory trait SASP), which have been shown to be involved in enhancing inflammation [2]. Intercellular adhesion molecule-1 (ICAM-1) is an adhesion factor produced by vascular endothelial cells, which plays an important role in the adhesion and infiltration of monocytes to vascular endothelial cells in various inflammatory settings, including atherosclerosis. Therefore, we investigated changes in ICAM-1 expression in senescent cells and found that its expression was upregulated in PDL32 cells compared with PDL4 cells as presented in **Figure 1C**, confirming the induction of SASP.

4. Activation of NF-κB in aging vascular endothelial cells

Next, the activation state of NF-κB in aging vascular endothelial cells was examined. Among NF-κB molecules, p65 (RelA) is involved in a wide range of inflammatory responses, including ICAM-1 expression. Furthermore, p65 is reported to be involved in the homeostatic induction of SASP in senescent cells [4]. Therefore, we compared the expression levels of p65 and its activation (phosphorylation) in senescent and non-aging cells. Based on the results shown in **Figures 2A** and **2B**, the expressions of p65 and phosphorylated p65 were increased in senescent cells. The activity of NF-κB is regulated by the balance between activating and inhibitory factors. A20 is an inhibitory factor that suppresses excessive activation of NF-κB signaling [5]. Interestingly, A20 expression was down-regulated in aging cells, as displayed in **Figure 2C**. These results suggest that the induction of homeostatic SASP in aging vascular endothelial cells involves increased expression of p65 and its phosphorylation, as well as activation of NF-κB through decreased expression of A20.

5. Inflammatory response of aging vascular endothelial cells to LPS

Bacterial infection has been reported to be involved in the exacerbation of atherosclerosis. Therefore, we compared the response of LPS, a bacterial virulence factor, on aged vascular endothelial cells with that of non-aged cells. 24 hours after exposure to LPS, the induction of ICAM-1 expression was observed in both aged and non-aged cells. Interestingly, the induction of

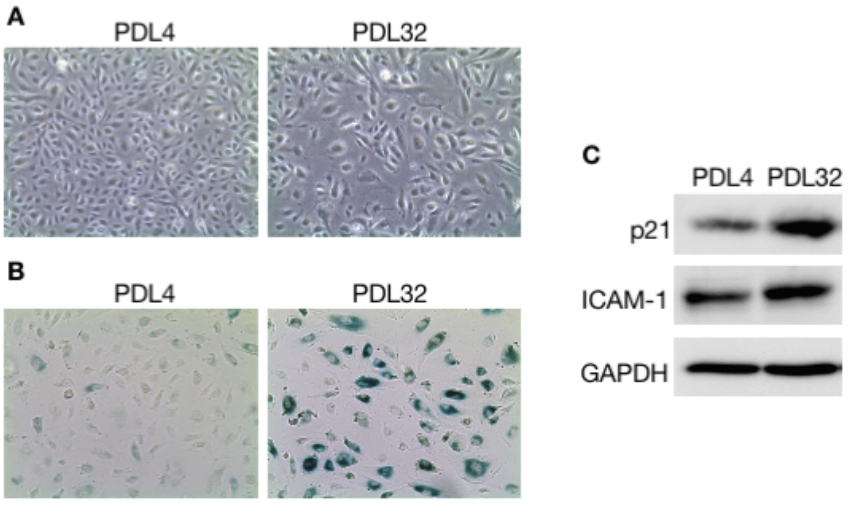

Figure 1. Characteristics of senescent cells regulated by long-term passage culture of HUVECs. HUVECs were cultured in an EGM-2 medium for vascular endothelial cells and passaged every 3–4 days. Comparison of population doubling level (PDL) 4 (equivalent to passage 4) and PDL32 (equivalent to passage 12) cells. (A) Phase-contrast microscopic image: PDL32 cells were flattened, enlarged, and had a heterogeneous morphology; (B) PDL32 cells were positive for SA-β-Gal staining; (C) the expression of p21 and ICAM-1 was enhanced in PDL32 cells compared to PDL4. In this study, PDL32 and above were used in experiments as senescent cells, and PDL4 and below as non-senescent cells.

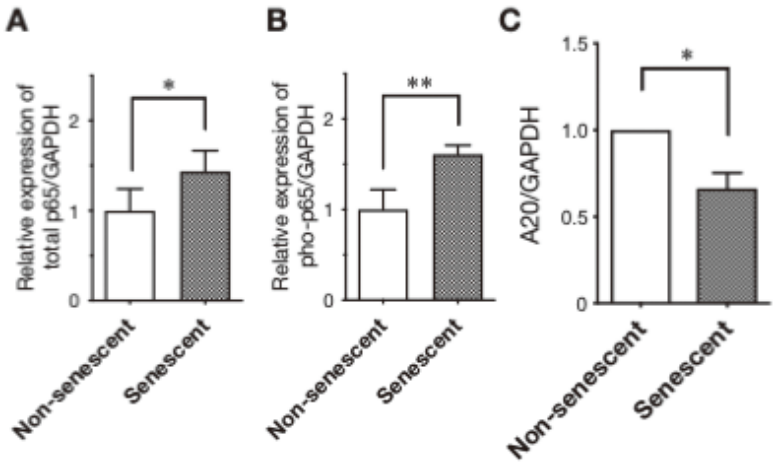

Figure 2. Activation of NF-κB in senescent cells when unstimulated. Expression of total p65 (A), phosphorylated p65 (B), and A20 (C) in senescent and non-aging cells was analyzed by Western blotting and expressed relative to non-aging cells. p65 expression and phosphorylated p65 (pho-p65/GAPDH) were higher in senescent cells compared to non-aging cells. In contrast, the expression of A20, a repressor of NF-κB signaling, was decreased in the senescent cells. (*$P < 0.05$, **$P < 0.01$)

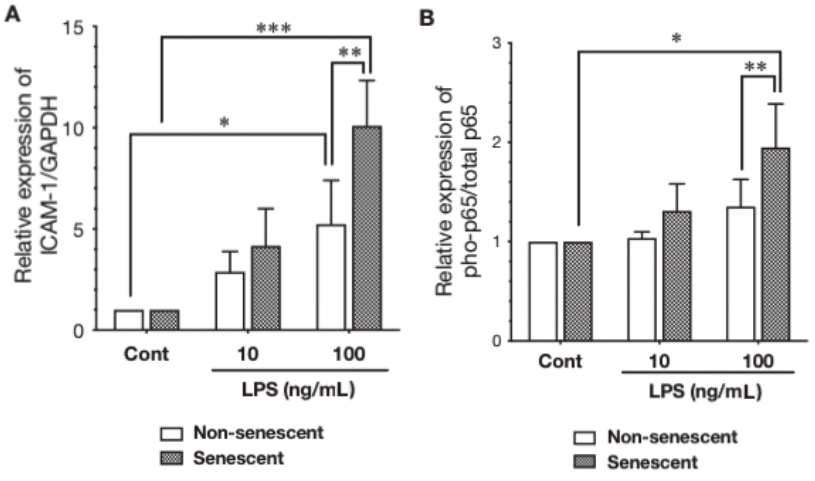

Figure 3. Induction of ICAM-1 expression and activation of NF-κB in senescent cells during LPS stimulation. Senescent and non-senescent cells were stimulated with LPS (*E. coli* O111:B4, 10 or 100 ng/mL) and ICAM-1 expression (A, after 24 hours) and p65 phosphorylation (pho-65/total p65, B, after 1 hour) Western blot analysis was performed. LPS (100 ng/mL) induced ICAM-1 expression and p65 phosphorylation in both aging and non-aging cells, but the effect was stronger in aging cells. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

ICAM-1 expression by LPS was enhanced in senescent cells, as presented in **Figure 3A**. ICAM-1 expression by LPS is induced by the NF-κB pathway [6]. When p65 activation was compared between aging and non-aging cells, LPS induced phosphorylation of p65 in aging and non-aging cells, but the degree of induction was stronger in aging cells, as shown in **Figure 3B**. These results suggest that the induction of ICAM-1 expression by LPS is enhanced in aging vascular endothelial cells, which may involve activation of NF-κB p65.

6. Inflammatory response of aging vascular endothelial cells to LL-37

LL-37 is an antimicrobial peptide produced by human neutrophils, monocytes, and intestinal epithelial cells, and released by these cells upon infection stimulation. Interestingly, LL-37 is highly expressed in atherosclerotic lesions and deletion of CRAMP (mouse homolog of human LL-37) in a mouse model of atherosclerosis improved the pathological state [7,8], LL-37 attracts attention as an aggravating factor in atherosclerosis. In this study, LL-37 was applied to aging vascular endothelial cells and the responses of aging and non-aging cells were compared. Although LL-37 is known to act on vascular endothelial cells to induce ICAM-1 expression [9], LL-37 was found to be a potent aggravating factor for atherosclerosis, and it induced ICAM-1 expression in both aging and non-aging cells. Interestingly, the induction of ICAM-1 expression by LL-37 was enhanced in senescent cells, as displayed in **Figure 4A**. Next, to investigate the signaling pathway of LL-37 stimulation, we examined whether LL-37 activates the NF-κB pathway by immunocytochemistry using p65 nuclear migration as

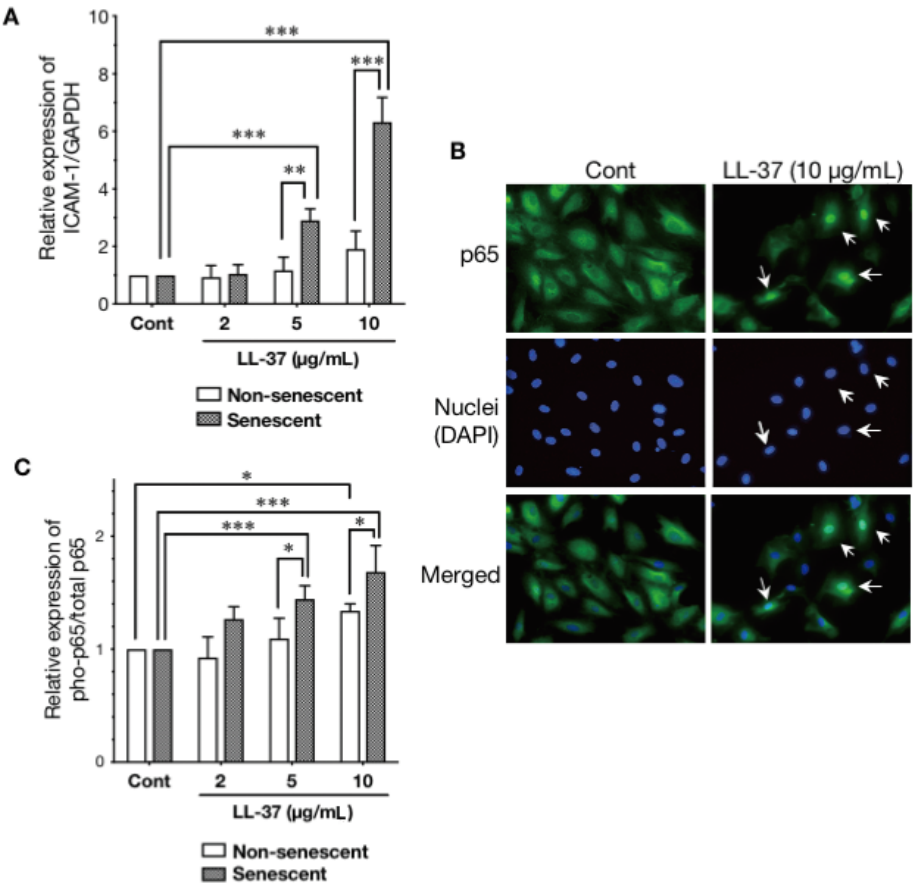

Figure 4. Induction of ICAM-1 expression and activation of NF-κB in senescent cells upon LL-37 stimulation. Senescent and non-senescent cells were stimulated with LL-37 (2, 5 or 10 μg/mL) and ICAM-1 expression (A, after 24 hours) and p65 phosphorylation (pho-65/total p65, C, after 4 hours) Western blot analysis was performed. Results are shown relative to unstimulated in both aging and non-aging cells. LL-37 induced ICAM-1 expression and p65 phosphorylation in aging and non-aging cells, but the effect was stronger in aging cells (LL-37 5 and 10 μg/mL). B) Non-aged cells were stimulated with LL-37 (10 μg/mL, 4 hours), and the localization of p65 was observed by immunocytochemistry. Arrows indicate cells in which p65 is nuclear localized. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

an indicator [10]. The results in **Figure 4B** showed that LL-37 induced nuclear migration of p65, indicating that LL-37 activates NF-κB. Therefore, we compared p65 activation in aging and non-aging cells and found that LL-37 induced phosphorylation of p65 in aging and non-aging cells, but the degree of induction was stronger in aging cells (**Figure 4C**). These results suggest that LL-37 activates NF-κB p65 more in aging vascular endothelial cells, with a concomitant enhancement of ICAM-1 expression.

7. Changes in LPS and LL-37 receptor expression in aging vascular endothelial cells

The inflammatory response (induction of ICAM-1 expression and activation of NF-κB p65) by LPS and LL-37 was enhanced in aging cells (**Figures 3** and **4**). To clarify this mechanism, the expression of LPS and LL-37 receptors was examined by flow cytometry. Based on **Figure 5A**, the results showed that the receptor for LPS, TLR4, was more strongly expressed in senescent cells than in non-aging cells. In contrast, the expression of CD14, a co-receptor for LPS, was down-regulated in aging cells, as shown in **Figure 5B**.

Several molecules have been reported as receptors for LL-37, including the formyl peptide receptor 2 (FPR2) and the purine receptor P2X7 in vascular endothelial cells. When the expression of these receptors was examined in aging and non-aging cells, it was found that FPR2 expression did not differ between aging and non-aging cells (**Figure 5C**), whereas P2X7 was strongly expressed in aging cells (**Figure 5D**).

8. Conclusion

Bacterial infection is considered an aggravating factor in atherosclerosis, but few details have been elucidated. We investigated the effects of LPS, a membrane component of Gram-negative bacteria, and LL-37, an

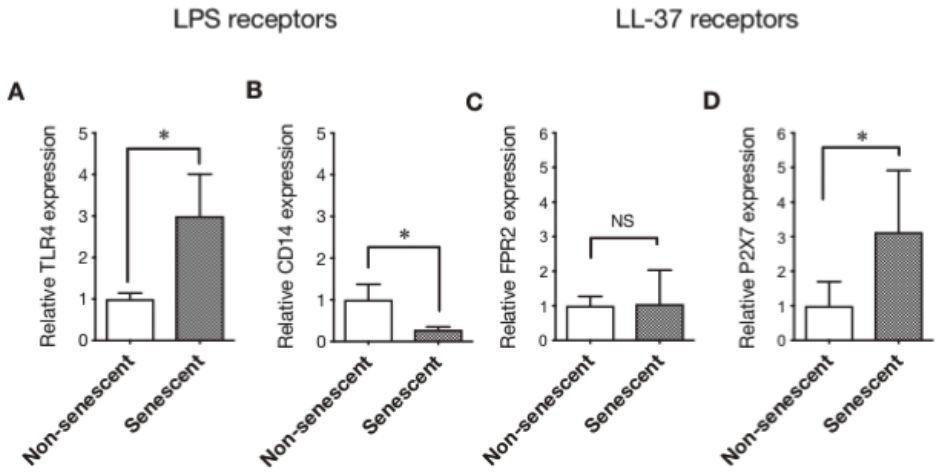

Figure 5. Altered expression of LPS and LL-37 receptors in senescent cells. The expression of TLR4 (A), CD14 (B), FPR2 (C), and P2X7 (D) in aging and non-aging cells was analyzed by flow cytometry. Results were expressed relative to non-aging cells. The expression of the LPS receptor TLR4 was increased in aging cells, whereas the expression of CD14 was decreased. The expression of the LL-37 receptor P2X7 was increased in aging cells, whereas the expression of FPR2 was unchanged. (*$P < 0.05$, NS not significant)

antimicrobial peptide produced by host cells, on aging vascular endothelial cells, and found that both LPS and LL-37 acted on aging endothelial cells and enhanced their inflammatory response. As a possible mechanism, NF-κB is homeostatically activated in aging vascular endothelial cells via phosphorylation of p65, which is further activated upon stimulation by LPS and LL-37. The expression of homeostatic (unstimulated) inflammatory traits in senescent cells and the enhanced inflammatory response to LPS and LL-37 stimulation are both mediated by the activation of the NF-κB signaling pathway. This is of interest for understanding the mechanisms by which the inflammatory response is enhanced in senescent cells.

The fact that the expression of the LPS receptor, TLR4, and the LL-37 receptor, P2X7, was up-regulated in the aging vascular endothelial cells suggests that the enhanced expression of these receptors (TLR4 and P2X7) is involved in the enhancement of the inflammatory response to LPS and LL-37. This suggests that increased expression of these receptors (TLR4, P2X7) may be involved in the enhanced inflammatory response to LPS and LL-37. In studies using mouse models of atherosclerosis, it has been reported that deletion of TLR4 or P2X7 improves the pathophysiology of atherosclerosis [11,12], suggesting that downstream signaling pathways of these receptors are important in the pathogenesis of atherosclerosis. It has been reported that TLR4 and P2X7 are strongly expressed in vascular endothelial cells at plaque sites in atherosclerosis patients or mice models [13,14]. Therefore, it is possible that in the pathogenesis of atherosclerosis, bacterial-derived LPS, and host-derived LL-37 stimulate aging vascular endothelial cells via TLR4 and P2X7, respectively, and exacerbate inflammation via the NF-κB signaling pathway. Furthermore, this suggests that one of the mechanisms by which cellular senescence enhances inflammatory responses may be the induction of receptor expression for inflammation-related molecules in aging cells. The involvement of cellular senescence, bacterial infection, and host-derived molecules in the pathogenesis of atherosclerosis is still in the process of being elucidated, and future research is expected.

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Disclosure statement

The authors declare no conflict of interest.

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