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Smoking increases the premature associated senescence phenotype of circulating Endothelial Progenitor Cells

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Abstract

Smoking is a risk factor for cardiovascular disease. Notably, it is associated with endothelial progenitor cells (EPCs) dysfunction. It also influences the shift of the EPCs mobilisation from bone marrow. We hypothesized that smoking could induce a premature associated senescence phenotype on the circulating population of EPCs, which may contribute to the default of recovery endothelial injury. Peripheral Blood Mononuclear Cell (PBMC) samples were collected from 30 smokers at least for five years and 31 healthy subjects (non-smoker) as control. $CD117^+$ and $CD133^+$ cells were confirmed as the population of endothelial progenitor cells. Those marked cells with SA-β galactosidase were quantified as senescence phenotype. Then, FACS assessed the targeted cells. The average concentration of $CD133^+/CD117^+$ was 0.05% (±0.03) for smoker subjects and 0.03% (±0.02) for non-smoker ($p< 0.05$). Almost all of the EPCs population (98.33±3.53%) in the smoker group expressed SA-β gal positive cells ($p<0.001$). Thus, this study suggests that smoking is associated with a significant elevated premature senescence of EPCs, which may contribute to diminished bioavailability of mature EPCs of the smoker, reducing the potency of vascular maintenance and repair.

Keywords: premature senescence, endothelial progenitor cells, smoking, vascular repair

1. Introduction

Endothelial progenitor cells (EPCs) effluxed from bone marrow (BM) in response to various molecular signalling pathways. The EPCs population is around 0.0001 in the blood circulation (Tagawa *et al*., 2015; Zhao *et al*., 2016). EPCs are required for the repair of the endothelium when endothelial injury or dysfunction. Regenerative potential to repair endothelial injury is carried out only by mature EPCs. Mobilization of population endothelial progenitor cells from BM is the initial stage of maturation or differentiation of various cell types, including EPCs (Hur *et al*., 2004; Zhang *et al*., 2014; Tagawa *et al*., 2015; Zhao *et al*., 2016). Hematopoietic progenitor cells are characterized by CD117 and CD133 surface markers (Hur *et al*., 2004; Gargett *et al*., 2009). Changes in the number of hematopoietic progenitor cells in the blood circulation affect the availability of mature EPCs. Various factors that modulate endothelial dysfunction also induce dysfunction of EPCs and induce the reduction in circulating EPCs (Hur *et al*., 2004; Zhang *et al*., 2014; Tagawa *et al*., 2015; Zhao *et al*., 2016).

The senescence of the human cell is considered an essential hallmark of the ageing process (Honn *et al*., 2017). Ageing is a complex phenomenon associated with increasing age or the accumulation of long-term oxidative stress exposure. Cells characterised as senescence are recognised viable, but they cannot perform a functional role due to cell cycle arrest for a certain period; meanwhile, the disrupted factor exposure remains constant. Senescence markers were identified as intracellular specific changes, such as increased senescence-associated β-galactosidase activity (SA-β-Gal). The SA-β-ga is a marker widely used to identify senescence in cells and tissues (Young *et al*., 2009; Kurz *et al*., 2000; Lee *et al*., 2006).

Smoking is an established risk factor for cardiovascular disease (CVD) (Kaplan *et al*., 2017). Tobacco smoke contains more than 4720 compounds, including well-known harmful chemicals such as polycyclic aromatic hydrocarbons, free radicals, and oxidative gases (Yao *et al*., 2008; Rafacho *et al*., 2011). Smoking might become a habit due to the addictive property of nicotine (Kumboyono *et al*., 2020). Nicotine induces the mesenchymal (MSC) biological function because of its underlying cause of various diseases. But, researchers have argued this phenomenon due to the limited study (Huertas *et al*., 2010; Kumboyono *et al*., 2020). Several studies claim that smoking significantly inhibits the regenerative potential of MSC and has been implicated in the early degeneration of mesenchymal tissue (Greenberg *et al*., 2017). This study investigates the effect of smoking exposure on EPC's biological changes in vivo, further exploring the consequence of smoking-mediated premature senescence in the EPCs population.

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2. Methods

2.1. Research design

The study was a cross-sectional study with a simple random sampling technique—informed consent was given to collect peripheral blood from each participant. Participants were divided into two groups; current smokers are participants who smoked >10 cigarettes/day for five years ago (n=30), and participants who had never smoked were considered in the control group (n=31). The participant's inclusion criteria included; having no history of diabetes mellitus, hypertension, and coronary arterial disease, ideal weight, maintaining physical exercise with medium intensity three times a week. Laboratory analysts were blinded to smoking status during data collection and analysis.

2.2. Collection and Isolation of PBMC

Peripheral blood (5 mL/subject) was drawn by a heparinized venous puncture method at the forearm. PBMC isolated by density gradient centrifugation (catalogue#07801/07811, LympoperpTM, Germany) with a density of 1.077 g/mL) as previously reported (Shi *et al*., 1998; Masuda *et al*., 2014; Beyth *et al*., 2015). Mononuclear cells were collected, and the remaining erythrocytes lysed. $CD133^+$ and $CD117^+$ marked cells were purified by FACS auto-separator, using fluorescence anti-human CD133 APC-conjugated antibody monoclonal mouse IgG2A clone #170411 [FAB no.cat 11331A] and PE anti-human CD117/c-kit [Biolegend; no.cat. 323408].

2.3. Flow cytometry

Freshly isolated PBMC subjected to flow cytometry to detect surface antigen of EPC. The collected cell were resuspended with incubation buffer (0.5 g bovine serum albumin in 100 ml 1X PBS, stored at 4°C). Aliquot made in 1 x 106 cells/100 µl/tube. Suspended were cells incubated in 10 µl CD133 and 5 µl CD117 by 100 µl staining volume at room temperature for 20 minutes. The stained cells were washed with PBS/1% BSA three times, resuspended in 0.5 ml PBS/1% BSA/propidium iodide (PI; Sigma). Flow cytometric profiles were obtained using FACS BD flow cytometer and Cell Quest software (Becton Dickinson Immunocytometry Systems, Mountain View, CA).

2.1. SA-β galactosidase assay

Senescence-associated beta-galactosidase (SA-β gal) activity was detected using Cell EventTM Senescense Green Flow Cytometry Assay Kit (Invitrogen; C10841) following the manufacturer's protocol (Thermo Fisher Scientific Inc.).

2.2. FCM analysis

The scatter diagram of each PBMC population in an individual is gated into a cell-sized population of lymphocytes, monocytes, and the other larger cells. The percentage (%) of the marker positivity ($^+$) obtained from the EPCs population on each gate compared to the total cells in 3 gates.

2.3. Ethical Clearance

This experimental design has been fulfilled and approved by the Ethics Committee of Faculty of Medicine, Brawijaya University, Malang, Indonesia, by registered number: 1206-KEP-UB/2019.

2.4. Statistical Analysis

The percentage of targeted cells in each group was analysed using SPPS v.23 in value ± standard deviation. Student t-test was used to compare the means between groups. A p-value ≤ 0.05 is statistically significant and indicates strong evidence against the null hypothesis.

3. Result

3.1. Smoking induced the enrichment of circulating CD117+ cells

Cell population that was positively selected from PBMC consists of 0.33% – 3.25% $CD117^+$ cells and 0.15%-0.31% $CD113^+$ cells (Figure 1), indicating the efficacy of the isolation method. The smoking group showed the enrichment of $CD117^+$ cells compared to non-smoker (10 fold in $CD117^+$ cells; Table 1). The average concentration of $CD117^+$ cells in the current smoker group (3.24% ± 1.6) was significantly higher than in the non-smokers' group (0.33% ± 0.07) ($p<0.001$).

3.2. The decrease of CD113+ cells in the current smoker group compared to the non-smoker group

The proportion of $CD113^+$ cells of the current smoke group was 50% lower than the non-smoker group. Population of $CD133^+$ cells in non-smokers (0.31% ± 0.3) was significantly higher than in smokers (0.15% ± 0.01) ($p<0.05$) (Table 1).

3.3. Smoking increased the hematopoietic progenitor cell population compared to the non-smoker group

Based on fluorescent cell sorting, large hematopoietic progenitor cells were proportionally found in PBMC of the current smoker group (0.05% ± 0.03) than the non-smoker group (0.03% ± 0.02) ($p<0.05$) (Table 1).

3.4. The SA-β-galactosidase-expressed Hematopoietic Progenitor Cells elevation in the smoker group

In this study, the EPCs biological phenotype was evaluated by SA-β-galactosidase expression as a premature senescence hallmark. The senescence cells increased significantly in the current smoker group (98.33% ± 3.53). On the other hand, negative marked SA-β galactosidase cell in non-smoker group was significantly higher (55.51% ± 34.96) than in the current smoker group ($p<0.001$) (Table 1). Thus, it indicates that the premature senescence of EPCs increased due to the stress effect of smoking.

Table 1. Diversity of circulating progenitor cell populations in the smokers and non-smoker groups

Marker (% gate)	Circulating Progenitor Cell		
	Smokers [n=30]	Non-smokers [n=31]	t-test
$CD133^+$	0.15 ± 0.01	0.31 ± 0.3	0.011*
$CD117^+$	3.24 ± 1.63	0.32 ± 0.07	0.000**
$CD133^+$/ $CD117^+$	0.05 ± 0.03	0.03 ± 0.02	0.036*
$CD133^+$/ $CD117^+$ / SA-β gal.$^+$	98.33±3.52	55.51 ± 5.10	0.000**
$CD133^+$/ $CD117^+$ SA-β gal.$^-$	1.67±3.53	45.40 ± 3.35	0.000**

Data shown are the mean ± SD; Significant value p< 0.05*, p<0.001**

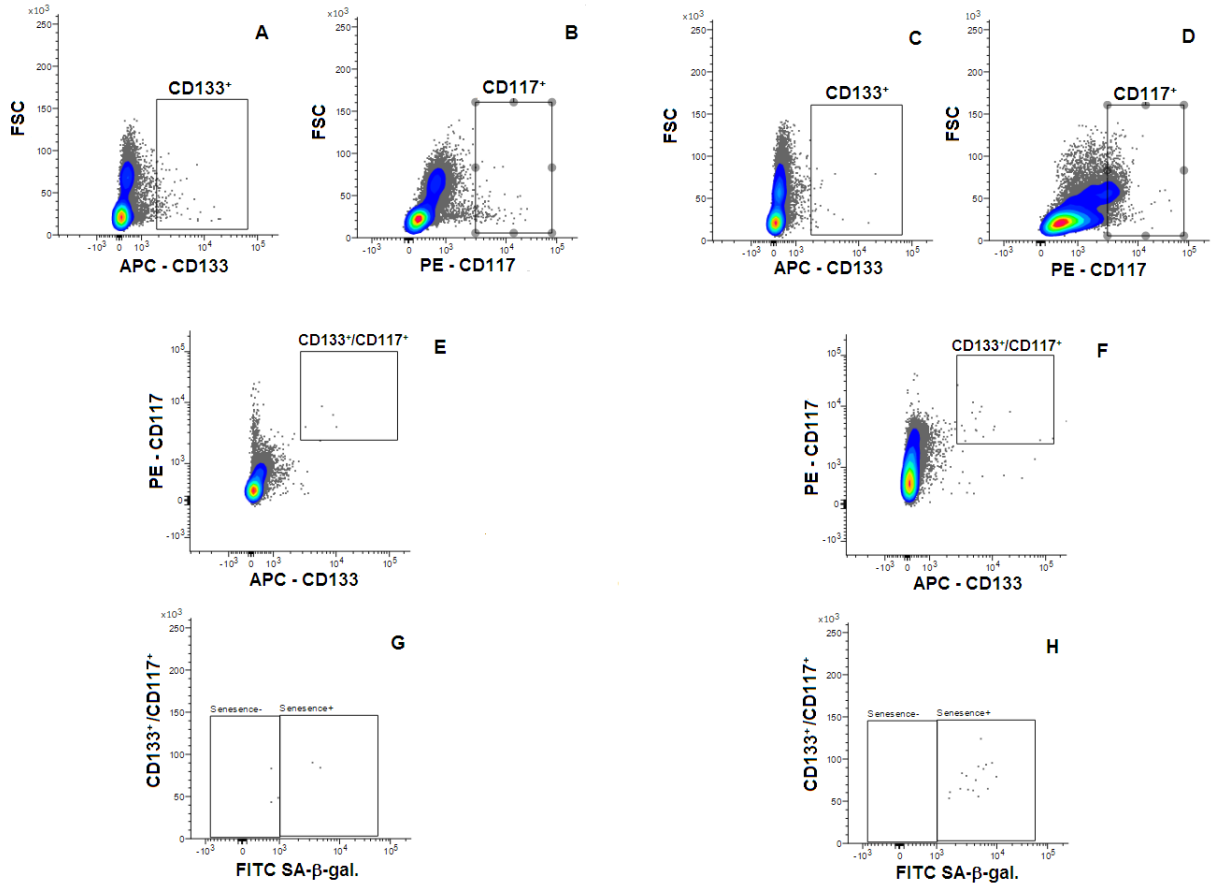

Figure 1. Cytometric analysis of cells that were positively CD133, CD117, and SA-β-galactosidase of each group. The investigated cell surface markers were hematopoietic progenitor cells (PE-CD117$^+$, APC-CD133$^+$). (A) CD133+ in non-smoker group; (B) CD117$^+$ in non-smoker group; (C) CD133$^+$ in the smoker group; (D) CD117$^+$ in the smoker group; (E) CD133$^+$/CD117$^+$ cells on non-smoker group; (F) CD133$^+$/CD117$^+$ cells on smoker group were higher than a non-smoker group; (G) senescence scatter plot gate which confirmed with SA-β-gal in non-smoker group; (H) senescence scatter plot visualization in the smoker group—representative analysis for one out of 61 PBMC samples.

4. Discussion

Reparative activity by EPCs has been declared in much research as a good advantage in both clinical approach and laboratory-based research. Still, it hasn't answered the remaining questions regarding the comprehensive framework of EPCs and their maturation in blood circulation. Based on their origin, bone marrow-derived EPCs biology is considered the source of EPCs and their differentiation through the vascular system. In this study, we used and compared different smoking habits that might exhibit different visualizing of biology EPCs.

The present study aimed to investigate EPCs viability through the premature senescence phenotype, confirmed by CD133$^+$ /CD117$^+$. Our finding showed that the premature senescence of EPCs and *increased SA-β-galactosidase-expressed hematopoietic progenitor cells population in the smoker group*. These results suggest that the elevation of EPCs mobilization from bone marrow modulated by smoking to compensate responses due to the increase of the senescence cells.

Nicotine is the primary addictive component of tobacco. It is a highly toxic compound that exerts its effects on almost every organ and system in the body. Nicotine exposure *in vitro* induced cell death (about 50%) in the MRC-5 cell line (Vajravelu *et al*., 2015). It also demonstrated that smoking also induces premature senescence in EPCs due to the effect of nicotine exposure. So, our result is in line with studies that investigate the cytotoxicity of tobacco compounds.

Analysis of aged stem cells in various tissues leads to common effectors and signalling pathways that contribute to stem cell dysfunction in response to toxic metabolites. Reactive oxygen species (ROS) that generated from electron 'leak' during mitochondrial oxidative phosphorylation plays fundamental roles in perturbed stem cell function leading to ageing (Takubo *et al*., 2013; Harris *et al*., 2013; Yu *et al*., 2013; Oh *et al*., 2014; Vlasceanu *et al*., 2018). In this study, the induction of actin filament reorganisation after a long time of nicotine exposure can be attributed to elevated cell senescence. Acrolein and other gas-phase oxidants in cigarette smoke remain stable in blood and thus are capable of acting directly on the vascular cells through impairs nitric oxide (NO)-mediated cell function (Bluhm *et al*., 1971; Barnova *et al*., 2005; Chung *et al*., 2005; Witschi *et al*., 2005; Mossman *et al*., 2006; Pervaiz *et al*., 2009; Lennartsson and Ronnstrand, 2012).

Smoking can trigger the ROS production enriched in both the gaseous and particulate components of smoking (Bluhm *et al*., 1971; Barnova *et al*., 2005; Kaplan *et al*., 2017). Alteration in ROS production due to cigarette smoking has a drastic effect on the host immunity through

increasing the production of pro-inflammatory cytokines such as interleukin-8 (IL-8) and tumour necrosis factor-alpha (TNF-α) (Chung *et al*., 2005; Witschi *et al*., 2005). The data demonstrate the elevated circulation of circulating $CD117^+$ cells in the smoker group, confirming the statement that expansion in $CD117^+$ cells considered to the process of lineage diminished ability to self-renew, occurs in synergy with other cytokines and risk-factor (Mossman *et al*., 2006)

Surface protein CD117 plays a vital role in regulating survival, maintenance, self-renewal, and endothelial stem cell re-endothelization (Mossman *et al*., 2006). In addition, our study reports that oxidative stress-mediated by smoking-induced imbalance redox signalling for cellular senescence (Höhn *et al*., 2017). Also, our results showed that environmental stimuli could regulate stem cell function (Lennartsson and Ronnstrand, 2012; Ren *et al*., 2017). High premature senescence EPCs in the smoker group of this study impacted the availability of mature EPCs in circulation. The maturation of EPCs in blood circulation is the result of the differentiation of bone marrow-derived EPCs marked by current-EPCs that gradually disappeared and were replaced by endothelial cell markers. This EPCs senescence might be considered EPCs dysfunction, which is the cause of repair endothelial dysfunction failure due to oxidative stress (Tousoulis *et al*., 2008; Cruciani *et al*., 2020).

The oxidative stress from smoking influences the cardiovascular system in two ways; by directly delivering free radicals to the vascular system and consuming antioxidants that would generally be available to protect against endogenous free radicals occurring from the respiratory process. DNA damage in mitochondria induced from oxidative stress in smoking might influence the checkpoint phase and result in the cell cycle arrest (Ambrose and Barua,2004; Lennartsson and Ronnstrand, 2012; Ren *et al*., 2017). The latest report showed that EPCs stem cells exhibit a series of these age-related changes that could trigger cell dysfunction/death and, in turn, a progressive decline in regeneration capacity (Koyuncu *et al*., 2015)

5. Conclusion

This research finding showed that smoking increases of EPCs premature senescence. This knowledge is essential for improving senescent cells' identification and characterization *in vivo* and developing rational strategies to modulate the senescence program for therapeutic in high-risk CVD populations.

Conflict OF Interest

The authors declare no conflict of interest.

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Authors Contributions

KK, TAW, and WN designed this study and prepared the manuscript.

TAW, WN, INC, and KK collected and analysed the clinical data.

FYC and INC significantly revised the manuscript.

TAW and KK are responsible and accountable for the accuracy or integrity of the work.

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