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Original Article

Activation of microvesicle peripheral blood mononuclear cells by mesenchymal stem cells secretome co-cultivated with osteosarcoma stem cell

[Activación de microvesículas de células mononucleares de sangre periférica por secretoma de células madre mesenquimales cocultivadas con células madre de osteosarcoma]

Fachrizal Arfani Prawiragara[1,2], Ferdiansyah[2*], Mouli Edward[2], Dwikora Novembri Utomo[2], Mohammad Hardian Basuki[2], Alexander Patera Nugraha[3], Fedik Abdul Rantam[4]

[1]Magister Clinical Medicine Program, Orthopedic and Traumatology Department, General Academic Dr. Soetomo Hospital/Faculty of Medicine, Airlangga, Surabaya, Indonesia.
[2]Department of Orthopaedic and Traumatology, General Academic Dr. Soetomo Hospital/Teaching Hospital, Faculty of Medicine, Airlangga University, Surabaya, Indonesia.
[3]Department of Orthodontics, Faculty of Dental Medicine, Airlangga University, Surabaya, Indonesia.
[4]Stem Cell Research Center and Development, Airlangga University, Surabaya, Indonesia.
*E-mail: ferdiansyah@fk.unair.ac.id

Abstract

Context: Microvesicle is a cell micro molecule that may play a role in the process of osteosarcoma stem cell apoptosis.

Aims: To investigate the activity of peripheral blood mononuclear cells (PBMCs) through the secretion of interleukin-2 (IL-2), interleukin-6 (IL-6), interleukin-10 (IL-10), C-X-C Motif Chemokine Ligand 13 (CXCL13) and tissue inhibitor of metalloproteinases-3 (TIMP-3) on co-cultivation of peripheral blood mononuclear cells (PBMCs) sensitized by mesenchymal stem cell secretome (MSCS) co-cultivated with osteosarcoma stem cells (OS-SCs).

Methods: This study was true experimental with a post-test only control group design. This was *in vitro* study PBMSCs sensitized by MSCS as then samples were divided into 4 treatment groups, respectively: Zero-day treatment (P0) PBMCs were co-cultivated with OS-SCs for 0 hours; First treatment (P1) PBMCs were co-cultivated with OS-SCs for 1 hour; Second treatment (P2) PBMCs were co-cultivated with OS-SCs for 2 days; Third treatment (P3) PBMCs were co-cultivated with OS-SCs for 4 days. The examination method used in this study was flow cytometry and indirect enzyme-linked immunosorbent assay (ELISA). The data were statistically analyzed with analysis of variance (ANOVA) with a $p \leq 0.05$ considered a significant difference.

Results: There was a tendency for a significant increase in extravesicular secretion in the secretion of IL-2, IL-6, IL-10, CXCL13, TIMP3, in the microvesicle PBMCs when sensitized by MSCs secretome co-cultivated with OS-SCs environment co-cultivated after the fourth day with significantly different between groups ($p \leq 0.05$).

Conclusions: PBMSCs' microvesicle such as IL-2, IL-6, IL-10, CXCL13, TIMP3 was significantly sensitized by MSCS and co-cultivated with OS-SCs after the fourth day of *in vitro*.

Keywords: medicine; non-communicable disease; non-infectious disease; osteosarcoma; stem cells.

Resumen

Contexto: La microvesícula es una micromolécula celular que puede desempeñar un papel en el proceso de apoptosis de las células madre del osteosarcoma.

Objetivos: Investigar la actividad de las células mononucleares de sangre periférica (PBMC) a través de la secreción de interleucina-2 (IL-2), interleucina-6 (IL-6), interleucina-10 (IL-10), C-X-C Motif Chemokine Ligand 13 (CXCL13) e inhibidor tisular de metaloproteinasas-3 (TIMP-3) en el cocultivo de células mononucleares de sangre periférica (PBMC) sensibilizadas por secretoma de células madre mesenquimales (MSCS) cocultivadas con células madre de osteosarcoma (OS-SC).

Métodos: Este estudio fue verdaderamente experimental con un diseño de grupo de control solo posterior a la prueba. Este fue un estudio *in vitro* de PBMSC sensibilizadas por MSCS, ya que luego las muestras se dividieron en 4 grupos de tratamiento, respectivamente: Tratamiento de día cero (P0) Las PBMC se cocultivaron con OS-SC durante 0 horas; Las PBMC del primer tratamiento (P1) se cocultivaron con OS-SC durante 1 hora; Las PBMC del segundo tratamiento (P2) se cocultivaron con OS-SC durante 2 días; Las PBMC del tercer tratamiento (P3) se cocultivaron con OS-SC durante 4 días. El método de examen utilizado en este estudio fue la citometría de flujo y el ensayo inmunoabsorbente ligado a enzimas indirecto (ELISA). Los datos fueron analizados estadísticamente con análisis de varianza (ANOVA) con $p \leq 0.05$ considerado como diferencia significativa.

Resultados: Hubo una tendencia a un aumento significativo en la secreción extravesicular en la secreción de IL-2, IL-6, IL-10, CXCL13, TIMP3, en las microvesículas de PBMC cuando se sensibilizan con el secretoma de MSC cocultivado con el entorno de OS-SC. cocultivados después del cuarto día con diferencias significativas entre grupos ($p \leq 0.05$).

Conclusiones: Las microvesículas de PBMSC como IL-2, IL-6, IL-10, CXCL13, TIMP3 fueron significativamente sensibilizadas por MSCS y cocultivadas con OS-SC después del cuarto día de *in vitro*.

Palabras Clave: células madre; enfermedad no transmisible; enfermedad no infecciosa; medicina; osteosarcoma.

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INTRODUCTION

Peripheral blood mononuclear cells (PBMCs) consist of T cells, B cells, natural killer (NK) cells, monocytes and dendritic cells. In PBMCs each contains about 70-85% lymphocytes with the composition of cluster of differentiation (CD), CD3+ consisting of CD4 and CD8, while B cells (5-10%), monocytes 5-20% while dendritic cells are not much around 1-2%. Once activated, they develop into several subsets of T-helper (Th) Th1, Th2, Th17, Th9, Th22 and follicle helper cells and regulatory cells (Akdis et al., 2012; Crotty, 2011; Tan and Gery, 2012; Sakaguchi et al., 2008; Hirahara et al., 2013). Therefore, if cultured *in vitro*, it contains many molecules that are secreted into the microenvironment, including extravesicle molecules that have a function and role in intercellular communication as well as maintaining homeostasis, including cytokines that play a role in non-specific immune responses in the microenvironment that can activate messenger protein p53. p53 is an extracellular vesicle molecule that is seen as a mediator in the induction of apoptosis through the secretion of several molecules (Pavlakis et al., 2020). This is also the case with the activation of the FAS-ligand (FasL) and the APO2 ligand (APO2L)/apoptotic-inducing ligand (TRAIL) of tumor necrosis factor (TNF)-related and stored in the cytoplasm of T cells (Monleón et al., 2001). Subsequently, there is a rapid release of bioactive molecules involved in cell death.

Meanwhile, p53 protein to limit malignant transformation via transcriptional activation of the program is considered primarily a cell autonomic response, and p53 regulatory activity through p53-mediated regulation of cell senescence (Pavlakis and Stiewe, 2020). In this process, the role of p53 works closely with nuclear factor-*kappa* B (NF-κB), a pro-inflammatory transcription factor that senescence-associated secretory phenotype (SASP). This reflects the upregulation of enzymes that degrade extracellular matrix (ECM) and secretion of inflammatory cytokines and immune modulators, such as insulin-like growth factor binding protein 7 (IGFBP-7), plasminogen activator inhibitor type-1 (PAI-1), interleukin (IL) IL-6, IL-8, IL-10, C-X-C motif chemokine ligand 13 (CXCL-3), Tissue inhibitor of metalloproteinases-3 (TIMP3) and C-X-C motif chemokine ligand-1 (CXCL-1), many of which control proliferation and motility in the microenvironment (Coppé et al., 2008). However, cells induced with wile type (WTp53) and mutation type (MTp53) were then analyzed by transcription factor p53, related to the G1 and G2 cell cycle, apoptosis, after DNA damage using H1299 cells at a temperature of 32 degrees Celsius caused an increase in protein p21 (WAF1) expression. Protein p21 and terminated G1 and G2 after DNA damage, but did not increase BAX expression or apoptosis (Cuddihy et al., 2008; Dwi Wibowo et al., 2017; Mastutik et al., 2021).

Another molecule that plays a role is CXCL13, an extravesicular molecule in both normal lymphoid tissue and transformed cells, and expresses CXCL13 from the monocyte/macrophage lineage, but also in the B cell assemblage (Legrer et al., 1998). Some of these cells expressing CXCL13 are CD14+, indicating a lineage from newly extravasated monocytes. Interestingly, monocytes stimulated *in vitro* with lipopolysaccharide increased CXCL13 secretion, and increased after *in vitro* maturation of monocytes to macrophages and decreased after maturation to dendritic cells. Therefore, newly recruited monocytes/macrophages play a role in lymphoid neogenesis in human inflammatory disease. Therefore, circulating monocytes are potential candidates for targeted chronic inflammation therapy in the future (Kazanietz et al., 2019).

In addition, TIMP-3 plays an important role in cancer by controlling cell death, angiogenesis, tumor inflammation, and tumor cell invasion and spread (Su et al., 2019). For example, restoration of TIMP-3 in cancer cells inhibits cell growth and promotes cell apoptosis. (Bian et al., 1996; Darnton et al., 2005). Furthermore, TIMP-3 overexpression increases the sensitivity of tumor cells such as osteosarcoma to clinical drug therapy by inhibiting IL-6 and IL-17. TIMP-3 also acts as a potential antiangiogenic agent by inhibiting endothelial cell formation. Thus, TIMP-3 can inhibit cancer cell migration, invasion, and metastasis *in vitro* and *in vivo* (Su et al., 2019). The decrease in TIMP-3 expression in some types of cancer compared to normal controls suggests that loss of TIMP-3 can lead to poor outcomes, including large tumor size, high tumor stage, and metastasis as well as decreased IL-6 expression (Bian et al., 1996; Han et al., 2018). The structure and function of TIMP-3 and discuss its contribution to carcinogenesis and its potential in cancer therapy. Therefore, this study aims to investigate the activation of PBMCs with MSCs increases microvesicle molecules that may act as anti-osteosarcoma stem cells (OS-SCs) *in vitro*.

MATERIAL AND METHODS

Chemicals and reagents

This study used chemicals, kits, and reagents as follows: alpha MEM medium (StableCell™ MEM, Merck, Sigma Aldrich, USA), Petridish (Sigma Aldrich, USA), phosphate buffer saline (PBS, Merck, Sigma Aldrich, USA), trypsin/collagenase IV (Stem cells Technologies, USA), Ficoll system (Merck, Sigma

Aldrich, USA), stem cell factor (SCF) (Stem cells Technologies, USA), CO_2 incubator (ThermoFisher, USA), ethylenediaminetetraacetic acid (EDTA) (Merck, Sigma Aldrich, USA), ELISA reader (Glo-Max® Discover Microplate Reader, Promega, USA) anti-CD4, CD8, CD332 (BioLegend, California, USA), BD-LSR II FACS Scan (FACSDiva, BD, USA) stem cell factor (SCF), IL-2, IL-6, IL-10, CXCL13, TIMP3 (Bioassay Technology Laboratory, Birmingham, UK and Elabscience, Texas, USA), 0.02% tween-X (Merck, Sigma Aldrich, USA), 1% bovine serum albumin (BSA) solution (Merck, Sigma Aldrich, USA), Statistical Package of Social Science (SPSS) (IBM corporation, Chicago, USA) version 20.0 software.

Study design

This scientific study was an *in vitro* experiment using a true experimental post-test only control group design. The sample was then randomly chosen and allocated to one of four groups. This research was conducted at Dr. Soetomo General Hospital and the Stem Cell Research and Development Center at Universitas Airlangga in Surabaya, Indonesia. Concerning the study protocol, the Dr. Soetomo General Hospital's ethical health council in Surabaya, East Java, Indonesia, has given research ethics authorization for this investigation.

Osteosarcoma stem cell isolation

Isolation of osteosarcoma stem cells was carried out by aseptically cutting 2-3 cm of osteosarcoma tissue from the Department of Orthopedics and Traumatology, which was inserted into a complete alpha-MEM growing medium and then washed in PBS three times to remove contaminants. The pieces were then cut into small pieces with sterile scissors until crushed in a Petri dish after that they were given complete medium and trypsin/collagenase IV in a ratio of 3:1 and immediately put in a 100 mL Erlenmeyer and placed on a magnetic stirrer and rotated with an output speed of 15 with a temperature of 37°C. Then wait for 30 minutes. Finally, the debris and osteosarcoma cells were separated by filtration (Mahyudin et al., 2021).

Isolation of PBMCs

Isolation of PBMCs using the Ficoll system with a gradient of 1.077: A sample of 10 mL of whole blood from healthy volunteers who agreed to participate in this study by filling out written informed consent was mixed with EDTA was then washed with PBS. After being centrifuged at 1600 rpm, the supernatant was removed, and then the blood was taken with a Pasteur pipette and put into a 15-mL tube containing 5 mL of Ficoll. Then centrifugation was carried out at 1600 rpm with a temperature of 20°C. Then the buffy coat is separated with a pipette slowly and put in sterile PBS. After centrifugation, the pellet was resuspended entirely with a growth medium added with secretome and SCF and finally incubated in an incubator at 37°C for 2 days (Rantam et al., 2015; Mahyudin et al., 2020).

Cultivation PBMCs and OS-SCs

Co-cultivation between sensitized PBMCs and mesenchymal stem cell osteosarcoma (OS-MSCs) at a ratio of 5:1 was then cultured in a 10 cm Petri dish or flash dish, and then given 10 mL of alpha MEM medium, finally, incubated in a 5% CO_2 incubator with a temperature of 37°C for 5 days (Mahyudin et al., 2021).

Flow cytometry

Isolated PBMCs from humans were centrifuged at a temperature of 4°C at a speed of 1600 rpm for 30 minutes. Finally, it was washed with medium without serum two times. After washing, it was reacted with each anti-CD4, CD8, and CD332 antibodies for 1 hour, finally analyzed using a BD-LSR II FACS Scan (Le Bert et al., 2020; Mahyudin et al., 2021).

Indirect enzyme-linked immunosorbent assay (ELISA)

ELISA analysis was performed to determine the secretion of IL-2, IL-6, IL-10, CXCL13, TIMP3 secreted by PBMCs after sensitization with secretome and CSF. The first rare 100 µL supernatant medium was coated on a 96 well microplate using a medium coating with a ratio of 1:10 for 24 hours at 4°C, after washing three times with 0.02% tween-X, then blocked with 1% BSA solution, and after washing, each antibody was added in a ratio of 1:500 and then incubated in an incubator at 37°C for 2 hours. After washing, it was reacted with human conjugate labeled with alkaline phosphatase, added PNpp, incubated in a dark room for 15 minutes, and then stopped with 1 N H_2O_2 or 1N HCl. Finally, the reading was carried out using an ELISA reader with a wavelength of 450 nm (Pucci et al., 2021; Mahyudin et al., 2021). Meanwhile, for validation was used the ELISA kit (Bioassay Technology Laboratory, Birmingham, UK and Elabscience, Texas, USA).

Statistical analysis

Statistical Package of Social Science (SPSS) (IBM Corporation, Chicago, USA) software was used in this study to analyze the data. Data were collected, processed, and statistically tested in several stages. The first stage was the assumption test in the form of a normality test to ensure that the data were normally distributed. Next, a comparison was made between

the treatment and control groups using analysis of variance (ANOVA) with significantly different at p≤0.05. This analysis was used to explain the effect of time (0 hours, 1 hour, 2 days, 4 days) and secretion of IL-2, IL-6, IL-10, CXCL13, TIMP3. The data were expressed as mean ± standard deviation.

RESULTS

After isolation of osteosarcoma stem cells and characterization using markers CD105 and CD45, these were labeled with fluorescein isothiocyanate (FITC), and 4′,6-diamidino-2-phenylindole (DAPI) (Fig. 1). OS-SCs were immediately co-cultivated with PMBCs sensitized with MSCS (Fig. 2). This study was focused on the activity of extravesicle molecules secreted by PBMCs through the activation of wild-p53 to induce the secretion of extravesicle molecules such as IL-2, IL-6, lL-10, CXCL13, and TIMP3, which inhibit the growth of OS-SC through the apoptotic pathway. Flow cytometry analysis showed that PBMCs were activated using MSCS secretomes (Fig. 3).

The observations and analysis results by ANOVA in all treatments, which were distinguished by the time between 0 hours, 1 hour, 2 days, and 4 days, showed a very significant difference of p≤0.05. The level of each variable was increasing day by day. Likewise, there were significant differences between the types of subjects being tested. Although there was no difference in the secretion of IL-2 and IL-6 molecules, when compared with the secretion of IL-10, CXCL13, and TIMP13, there was a significant increase in the OD value. During the 0 hours of treatment, there was no significant difference in the IL-2 and TIMP-3 tests but different in the other tests. Likewise, in the 1 h treatment, there was no significant difference in the secretion of IL-2, IL-6, and IL-10 but different in other tests. Meanwhile, the 2 days and 4 days of treatments differed significantly, with p≤0.05 in all types of tests (Fig. 4).

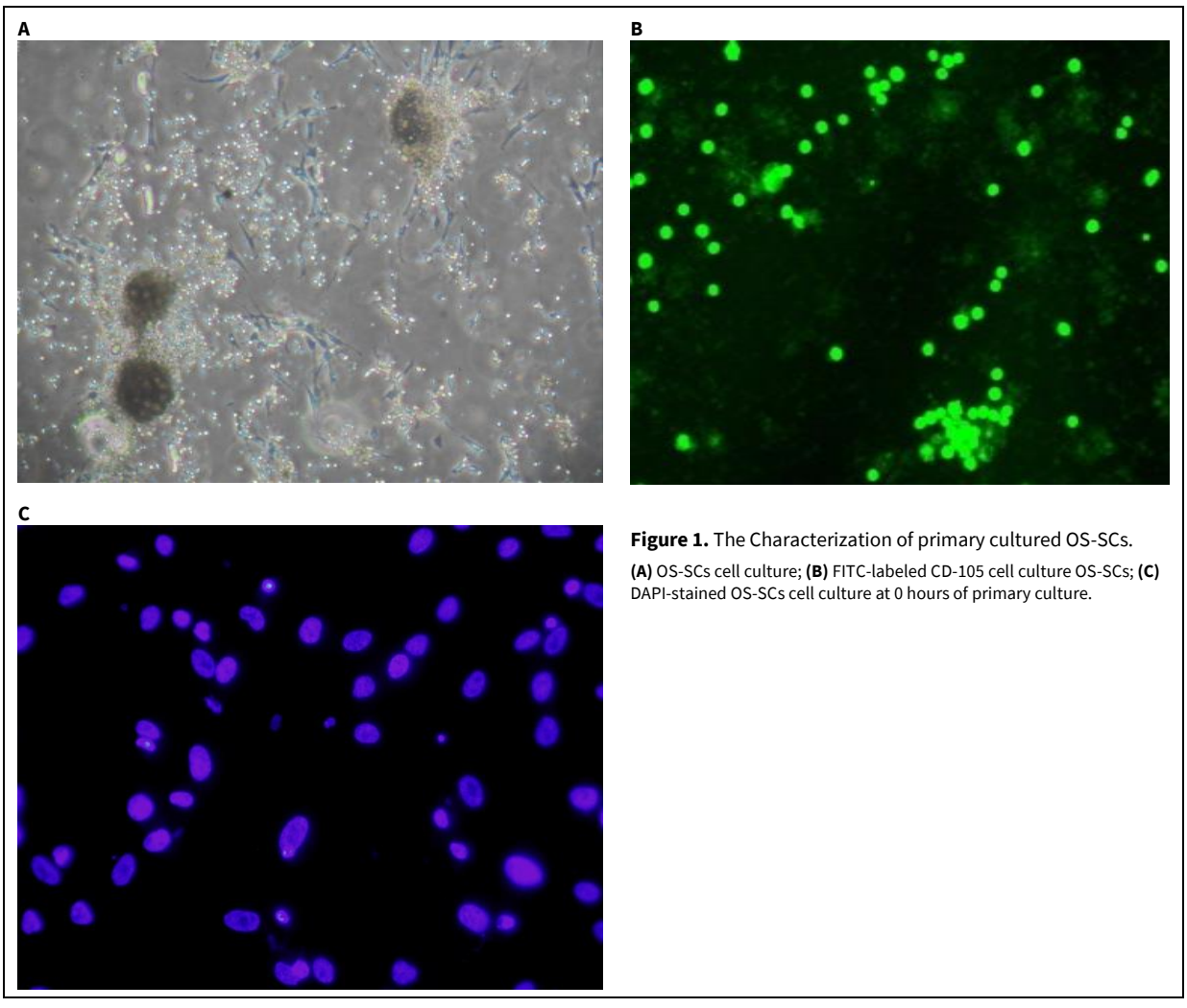

Figure 1. The Characterization of primary cultured OS-SCs. **(A)** OS-SCs cell culture; **(B)** FITC-labeled CD-105 cell culture OS-SCs; **(C)** DAPI-stained OS-SCs cell culture at 0 hours of primary culture.

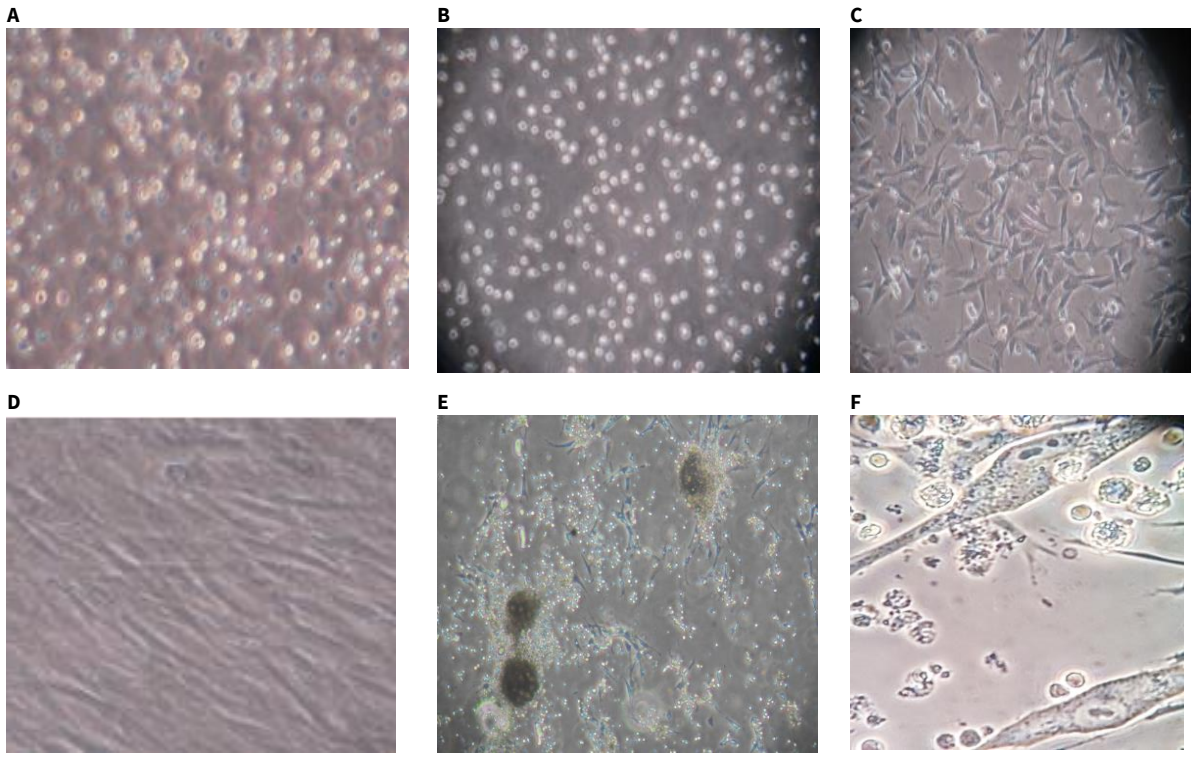

Figure 2. The culture of OS-SCs with or without co-cultivation with PBMC sensitized with secretome.

(A) OS-SCs culture alone at 0 hours; **(B)** OS-SCs culture alone at 1 hour; **(C)** OS-SCs culture alone at 2 days; **(D)** OS-SCs culture alone at 4 days; **(E)** OS-SCs co-cultivation with PBMC sensitized with secretome at 0 hours; **(F)** OS-SCs co-cultivation with PBMC sensitized with secretome at 1 hour; **(G)** OS-SCs co-cultivation with PBMC sensitized with secretome at 2 days; **(H)** OS-SCs co-cultivation with PBMC sensitized with secretome at 4 days.

DISCUSSION

Extravesicles have an important role in the microenvironment related to pro-inflammatory and inhibiting inflammation factors, especially supported by the association with cancer cells such as fibroblasts and immune cells that can inhibit cancer growth (Kalluri, 2016). There are many extravesicle products secreted by immune cells that can inhibit and stimulate the activity of wild-type-P53 so that it can secrete extravesicle molecules that play a role in inhibiting cancer cells because it acts as an extracellular messenger P53 (Pavlakis et al., 2020). As in previous studies (Mahyudin et al., 2021) in co-cultivation between activated PBMCs with secretome MSCs and OS-SCs, there was an increase in the expression of TNF-*alpha*, IL-6, which can stimulate apoptosis through extrinsic pathways played by macrophages, NK-cells and as well as other cells such as T-CD8 cells. In this study, we explored the role of extravesicle molecules in inhibiting tumor development through the mechanism of cell senescence, therefore through an exploratory approach to molecules in the microenvironment, which are the result of secretion of immune cells associated with cancer cells to solve problems in the case of osteosarcoma.

The investigation of extravesicle molecules that may be directly related to WT-P53 activity of secretome-stimulated PBMCs showed an increase in the secretion of IL-2, IL-6, IL-10, CXCL-13, and TIMP3. This molecule plays an important role in inhibiting tumor metastasis, especially in stem cells, by suppressing malignant tumor transformation (Pavlakis et al., 2020). The secretome contains about 200 thousand molecules consisting of small and large molecules. Extra vesicles include small molecules that can trigger

WT-P53 and force cells to secrete cytokines and CXCL13 and TIMP3.

CXCL-13 is a product of monocytes (M1) to macrophages and can also be stimulated *in vitro* with BAG6 and CBP/p300 dependent acetylation of p53 (Schulder et al., 2019). It turns out that it can also be activated by small and large molecules from the secretome to be able to secrete CXCL-13 with significant OD values. In addition, it can also induce the secretion of IL-6 and IL-10 with a significant value of $p<0.05$ and shows an increase in secretion over time, these results seem to be parallel with fibroblast-associated cancer (Sahai et al., 2020). On the other hand, IL-10 can also switch from pro-inflammation to anti-tumor immunity (Oft, 2014). Likewise, IL-6 is a cytokine that plays a role through paracrine effects, which can also suppress cells from becoming senescent. This happens because it is an extravesicle product secreted in the cell microenvironment that plays a role in the up and down-regulation of transcription factor genes (Coppé et al., 2008). Therefore, senescence-associated extravesicle (SA-EV) is very important as a homeostatic factor. It is proven that an increase in SA-EV secretion occurs due to DNA damage (Hitomi et al., 2020).

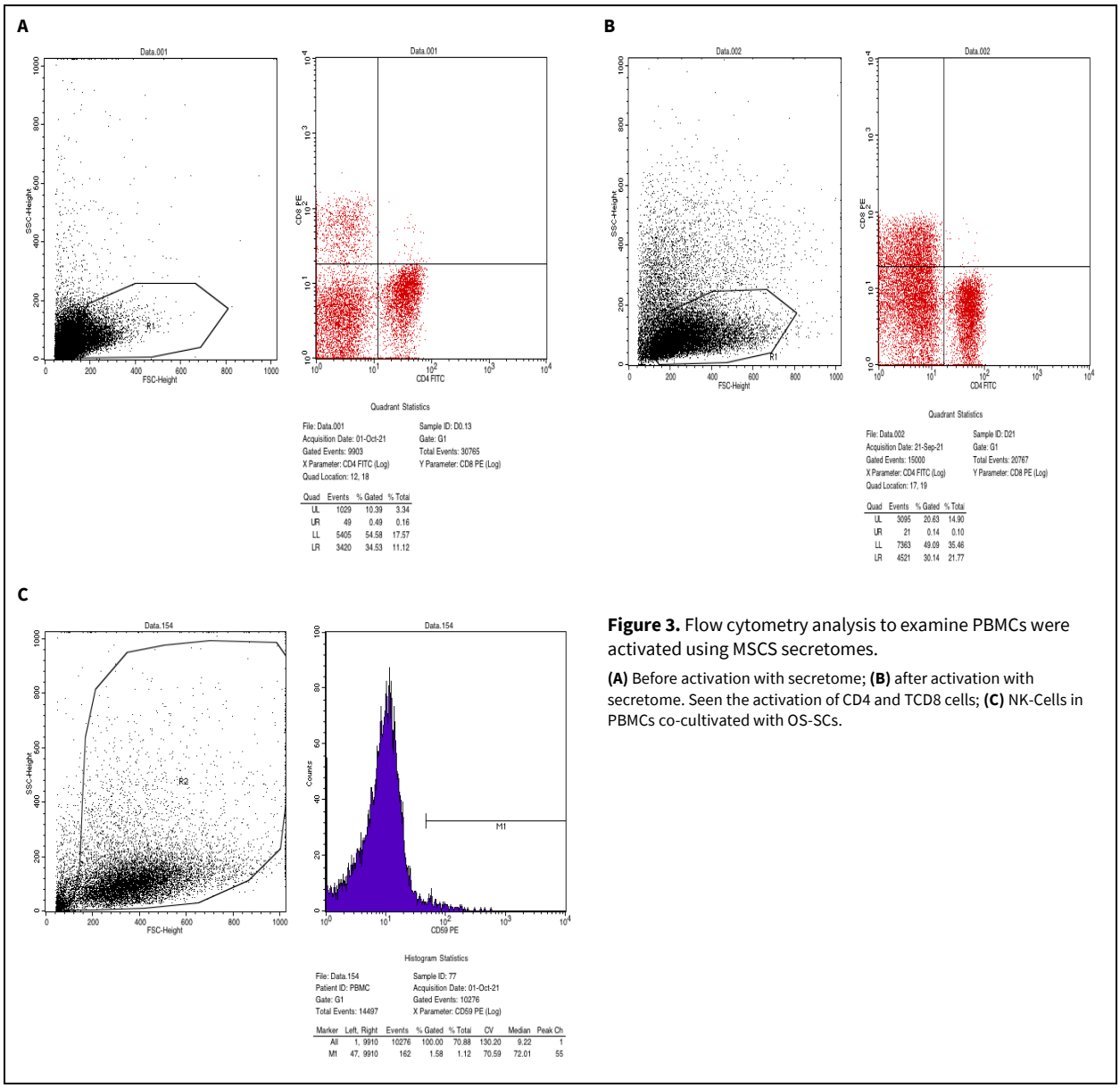

Figure 3. Flow cytometry analysis to examine PBMCs were activated using MSCS secretomes.

(A) Before activation with secretome; **(B)** after activation with secretome. Seen the activation of CD4 and TCD8 cells; **(C)** NK-Cells in PBMCs co-cultivated with OS-SCs.

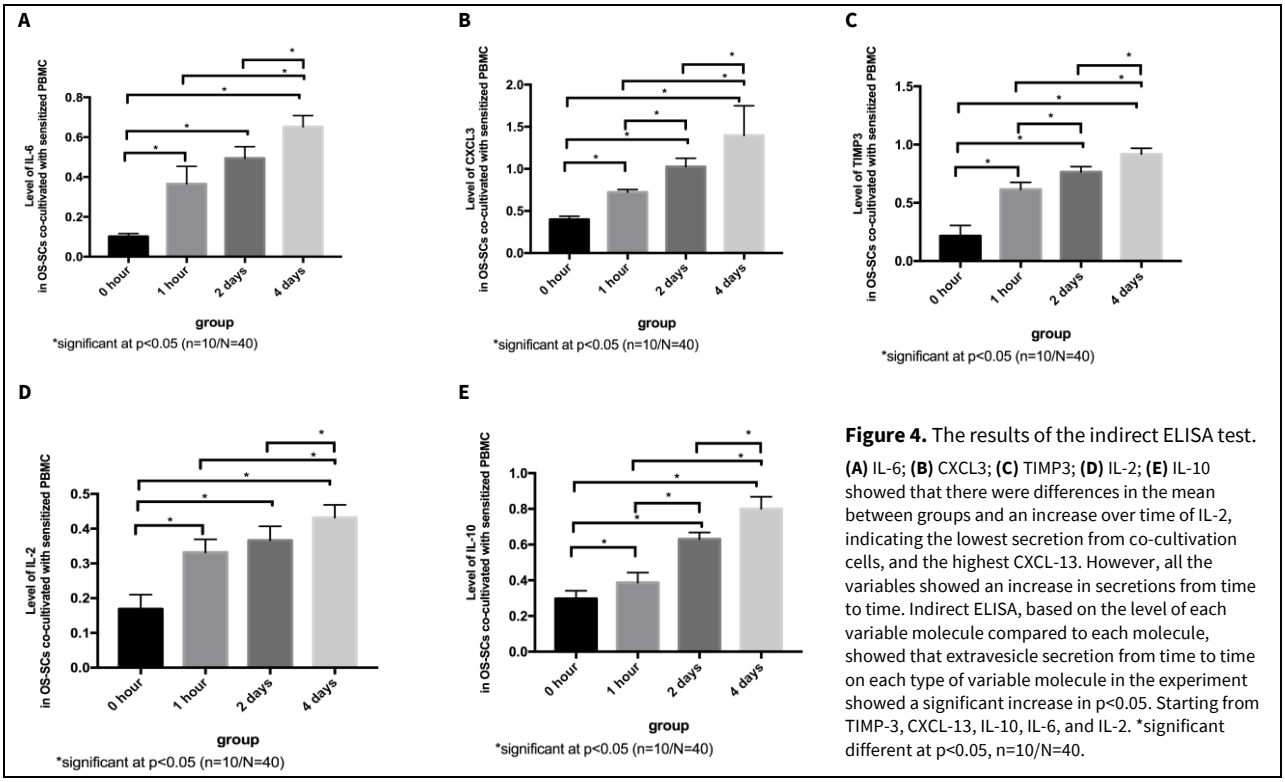

Figure 4. The results of the indirect ELISA test. **(A)** IL-6; **(B)** CXCL3; **(C)** TIMP3; **(D)** IL-2; **(E)** IL-10 showed that there were differences in the mean between groups and an increase over time of IL-2, indicating the lowest secretion from co-cultivation cells, and the highest CXCL-13. However, all the variables showed an increase in secretions from time to time. Indirect ELISA, based on the level of each variable molecule compared to each molecule, showed that extravesicle secretion from time to time on each type of variable molecule in the experiment showed a significant increase in p<0.05. Starting from TIMP-3, CXCL-13, IL-10, IL-6, and IL-2. *significant different at p<0.05, n=10/N=40.

Overexpression of IL-10 is used for cancer treatment. IL-10 secretion is also quite high in OD value, and there is an increase from time to time because it belongs to the pleiotropic cytokine group as an inflammatory, especially in cancer. It has anti-tumour effects through stimulation of T cells. In humans, the combination with PEGylated (PEGH-IL-10) can reject tumors and be resistant to tumors in the long term, as well as stimulation of CD8+ T-cells and the expression of IFN-gamma and granzymes at the tumor site of MHC molecules (Oft, 2014). In addition, IL-10 is a soluble molecule because it also acts as a mediator of communication between cells to modulate the immune response to cancer and has an apoptotic effect (Rallis et al., 2021). The increase in extravesicular activity due to the activation of WT-P53 through a secretome added to co-cultivation of PBMCs and OS-SCs can secrete extravesicles in the microenvironment, which may in the future be developed into a topical therapeutic strategy because extravesicles are a product of WT-P53 activation in inducing anti-inflammatory drugs. These metastatic agents can recruit tumor-repressive patrolling monocytes to metastatic sites associated with immune checkpoint inhibitors (Berraondo et al., 2019).

CONCLUSION

Mesenchymal stem cell secretome (MSCS) can activate the microvesicle of peripheral blood mononuclear stem cells (PBMSCs) such as IL-2, IL-6, IL-10, CXCL13, TIMP3 when co-cultivated with osteosarcoma stem cells (OS-SCs) after the fourth day *in vitro* significantly. However, further study is still needed to investigate the exact apoptosis mechanism of OS-SCs with various methods.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTION:

Contribution	Prawiragara FA	Ferdiansyah	Edward M	Utomo DN	Basuki MH	Nugraha AP	Rantam FA
Concepts or ideas		x	x	x			
Design	x	x	x	x	x		x
Definition of intellectual content	x	x	x	x	x	x	x
Literature search	x	x		x	x	x	x
Experimental studies	x	x	x	x	x	x	x
Data acquisition	x	x	x		x	x	x
Data analysis	x	x		x	x	x	x
Statistical analysis	x	x	x	x	x	x	x
Manuscript preparation	x	x	x	x	x	x	x
Manuscript editing	x	x	x	x		x	x
Manuscript review	x	x	x	x	x	x	x

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