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We have reached a decision regarding your submission to Journal of Mathematical and Fundamental Sciences, "Conditioned Medium of Co-Culture IL2-Induced NK Cells and human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) toward Apoptotic Genes Expression in Breast Cancer Cells Line (MCF7)" (MSID:9081) - ICAMBBE 2017 #17.

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Contribution to science::
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Language style is difficult to change and still some sentences is very short. However, regarding the substance overall this paper quality is good and contribution to science is also good.

Wahyu Widowati <wahyu_w60@yahoo.com>
Kepada:Journal of Mathematical and Fundamental Sciences
Kam, 25 Okt 2018 jam 19.08
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Effects of Conditioned Medium of Co-Culture IL2-Induced NK Cells and human Wharton's Jelly Mesenchymal Stem Cells (hWJMSCs) on Apoptotic Genes Expression in a Breast Cancer Cells Line (MCF-7)

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Abstract. Background: Breast cancer (BC) is the most known cancer among women and one of the major causes of cancer mortality prevalence in woman. The mortality of most patients with solid tumors is mainly due to metastatic spread to other organs. Metastasis in breast cancer (BC) occurs due to immunosurveillance deficiency including impairment of natural killer (NK) cell maturation. Therefore, the focus of recent cancer therapy is to promote developed-NK cells as drugs. Conditioned Medium (CM) from human Warton's Jelly Mesenchymal Stem Cells (hWJMSCs-CM) is known to possess anticancer activity. The CM of co-culture NK cells and hWJMSCs is expected to boost anticancer toward BC cells.

Objective: To analyse the effect of CM of IL2-treated hWJMSCs towards secretion of effector molecules, BC cells' apoptotic index and related genes expression, and cytotoxic granules in IL2-treated NK and hWJMSCs.

Materials and Methods: Human recombinant IL-2 was used to induce NK cells (IL2-NK) and hWJMSCs (IL2-hWJMSCs). TNF-α, IFN-γ, perforin, granzyme were measured by ELISA. The inhibition of cell proliferation was measured by MTS. The BC cells apoptosis was measured by flow cytometry and apoptotic gene expression.

Results: CM from IL2-treated hWJMSCs inhibit NK proliferation and BC cells. Co-culture of IL2-NK cells and hWJMSCs induced apoptosis, as well as increased expression of Bax and p53, and decreased Bcl-2 in BC cells.
Conclusion: CM of co-culture IL2-treated NK cells and hWJMSCs induce apoptosis in BC cells, as indicated by increased Bax and p53, and decreased Bcl-2 expression.

Keywords: *human Wharton's jelly mesenchymal stem cells - interleukin-2 - conditioned medium - natural killer cells - breast cancer*

1. Introduction

Breast cancer (BC) is one of the most regularly recognised cancer type, which is the first cause of female cancer death [1]. Tumors metastatic to the organs is mainly reason of the patiens mortality [1]. An ideal therapeutic strategy is therefore needed to target metastatic and non-metastatic tumor cells directly, which has to show local activity over the time of treatment [2]. Metastatic tumor cells require invasive and escaping ability from immune cells for spreading to other organs [3,4,5]. BC occurrence and its metastatis may also be supported by antitumor immunity [6], immunosurveillance deficiency, natural killer (NK) cell maturation damage, low number of NK cell and its decreased cytotoxic activity in peripheral blood mononuclear cells (PBMCs) [7,8,9,10], NK abnormalities [11], low tumor infiltrate [12-13], abnormal expression of activating receptors, and overexpression of inhibitory receptors CD158a, CD158b, and NKG2A [10].

Stimulating NK cell functions in combination with other anticancer agents to improve their anticancer activity is one of the promising cancer therapies [14]. Interleukin-2 (IL-2) can be used to trigger NK cells [15]. Cytoplasmic granules, perforin (Prf1) and granzymes (Gzm) are released by NK cells to suppress cancer, leading to apoptosis [16]. Secrotome such as TNF-α and different effector molecules such as IFN-γ can lead to the apoptosis of cancer cells. [17].

Another promising anticancer agent is the secretome of mesenchymal stem cells (MSCs) found in conditioned medium (CM), which contains microparticles that mediate therapeutic effects against cancer [18]. MSCs release various factors which can inhibit glioma, melanoma, lung cancer, hepatoma, and breast cancer cells proliferation [19 - 22]. Furthermore, MSCs-CM inhibited mammary carcinoma, osteosarcoma, pancreatic, and lung tumor growth [23].

To date, hWJMSCs-CM has been known to exhibit anticancer activities, yet further development to enhance its potential is still required. An approach suggesting the increase of cytokines treatment as anticancer such as IL-2, may directly induce NK cells to target cancer cells

and hWJMSCs-CM indirectly. In this research, we aimed to elucidate the properties of IL2-hWJMSCs-CM against BC and NK cell viability, the effect of IL2-NK cells in BC cell inhibition, NK cell proliferation, and the level of TNF-α, IFN-γ, perforin, and granzyme, and cytotoxic activity of IL2-NK against BC cells.

2. Materials and Methods

2.1. Interleukin 2-induced human Wharton's Jelly Mesenchymal Stem Cells (IL2-hWJMSCs)

Human Wharton's Jelly mesenchymal stem cells (hWJMSCs) at passage 4 (P4) were obtained from our previous study [23]. Surface markers expression and differentiation ability into three different lineages had been performed in order to characterize hWJMSCs [23, 24]. The informed consent from all donors and protocol approval of the Institutional Ethics Committee at the Stem Cell and Cancer Institute, Jakarta, Indonesia and the Institutional Ethics Committee collaboration between Maranatha Christian University, Bandung, Indonesia and Immanuel Hospital Bandung, Bandung, Indonesia were obtained to conduct this research. 5×10^5 cells of hWJMSCs were maintained in minimum essential medium-α (MEM-α) (Gibco, 12561056), supplemented with 20% fetal bovine serum (FBS, Gibco 10270106) and 1% of Antibiotic-Antimycotic (Gibco, 1772653) and incubated in 5% CO_2 at 37 ˚C for 24 h. Then, the medium was replaced, and the cells were washed with 1 x PBS sterile and supplemented with new growth medium. Next, the cells were treated with 125 and 250 ng/ml of IL-2 (Biolegend, 589106) and incubated in 5% CO_2, at 37 ˚C for 24 h. The IL2-hWJMSCs were harvested, and the medium supernatant was collected, centrifuged at 500 g for four mins at room temperature, and filtered through a 0.22 µm MillexeGV Filter Unit with Durapore (Millipore, SLGV 033 RS). The filtered medium, which was the conditioned medium of IL2-hWJMSCs, was stored at -80 ˚C [23 - 25].

2.2. Cytotoxic Activity of IL2-hWJMSCs-CM toward Breast Cancer Cells

The breast cancer cell lines, which was MCF-7 (ATCC® HTB22™), was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia. Around 5×10^3 cells/well of MCF-7 were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Gibco 11995065) supplemented with 10% FBS and 1% Antibiotic-Antimycotic. Then, the cells were incubated at 5% CO_2, 37 ˚C for 24 h [23, 26, 27]. Moreover, various concentration of IL2-hWJMSCs-CM (30% and 60%) had been used to treat cultured MCF-7 incubated around 24 h. The viability of the cells was counted based on an optimized reagent containing resazurin,

which was converted to fluorescent resorufin by viable cells absorbed the light at 490 nm in the 3-(4,5- dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). The absorbance of the cells was measured at 490 nm using spectrophotometer (Multiskan Go, Thermo Scientific) [23, 24, 26].

2.3. Cytotoxic Activity of IL2-hWJMSCs-CM toward NK Cells

NK92MI cell line (ATCC® CRL2408™) was obtained from Aretha Medika Utama Biomolecular and Biomedical Research Center, Bandung, Indonesia. 1×10^4 cells/well of NK92MI were plated in 96 well-plate and maintained in RPMI 1640 medium (Gibco 22400089) supplemented with 10% FBS, 1% Antibiotic-Antimycotic. Next, the cells were treated with various concentrations of IL2-hWJMSCs-CM (30% and 60%) and then incubated for 24 h. The cell viability in each well was measured by the MTS assay, as described in section 2.2. [23, 26].

2.4. Cytoplasmic Granules and Effector Molecules Measurement of hWJMSCs and IL2-NK Cells in Co-Culture Conditon

NK92MI cells were grown at density $2x10^6$ cells/well in NK medium containing RPMI 1640, 20% FBS, 1% Antibiotic-Antimycotic. Then, the cells were treated with 10 ng/ml of human recombinant IL-2 and incubated at 5% CO_2, 37 °C for 96 h, which resulted in IL2-induced NK (IL2-NK) cells.

The hWJMSCs were cultured at density of $1x10^6$ cells/well in MEM-α, supplemented with 10% FBS, 1% Antibiotic-Antimycotic and incubated in 5% CO_2, 37 °C for 24 h. Furthermore, NK cells at density $5x10^6$ cells/well were plated in 24-well plate to achieve the ratio 1:5 (hWJMSCs : NK = 1:5; hWJMSCs : IL2-NK = 1:5), containing hWJMSCs in NK medium (RPMI 1640) and MEM-α medium at the ratio 1:1 (50%:50%) corresponding to the optimized conditions (data not shown). IL2-NK cells and hWJMSCs co-culture were then placed at 37 °C, 5% CO_2 for 24 h. Medium resulted from this co-culture condition was preserved in -80 °C for the next assays to measure IFN- γ, TNF- α, perforin (Prf1), and granzyme B (GzmB) [25, 28, 29].

2.5. Cytotoxic activity of CM from co-culture of hWJMSCs and IL2-NK toward MCF-7

MCF-7 cells were cultured at density $5x10^3$/well in DMEM medium added with 10% FBS and 1 % Antibiotic-Antimycotic and then placed at 37 °C, 5% CO_2 for 24 h [23, 26, 27]. Moreover, the cells were treated with (hWJMSCs+NK)-CM, (hWJMSCs+IL2-NK)-CM, in 30% or 60%

concentrations, and incubated for 24 h. The viability of the cells was calculated using MTS assay according to manufacturer procedure (Section 2.2) [23, 26].

2.6. Apoptosis Assay of CM from co-culture of hWJMSCs and IL2-NK toward MCF-7 using Flowcytometer

MCF-7 at density $1x10^6$ cells/disk were maintained in DMEM medium added with 10% FBS and 1% Antibiotic-Antimycotic and incubated at 37 °C, 5% CO_2 for 24 h. Moreover, the cells were treated using 50% CM of coculture hWJMSCs and IL2-induced NK (hWJMSCs-CM, (hWJMSCs+NK)-CM, (hWJMSCs+IL12-NK)-CM)) and incubated for 24 h. Next, the cells were washed using Phosphate Buffered Saline (PBS), harvested using 0.25% trypsin EDTA (Gibco, 25200072) and counted using hemocytometer. Around $5 \times 10^5 - 1 \times 10^6$ counted cells were added with 500 µl of Annexin V binding buffer (Miltenyi Biotec, 130-092-820), 5 µl of Anti-FITC (Miltenyi Biotec, 130-048-701), and 5 µl of propidium iodide (Miltenyi Biotec, 130-093-233), and the cells were incubated in darkness at 4 °C. MACSquant Analyzer 10 (Miltenyi Biotec) was used to analyse the apoptotic percentage of MCF-7 [26].

2.7. Real-time PCR assay for the apoptotic induction of MCF-7 cell line by TNF-α, IFN-γ

Recombinant human TNF-α (Biolegend 570106) and recombinant IFN-γ (Biolegend 570206) were used to determine the apoptosis induction ability toward MCF-7. MCF-7 cells at density $2x10^6$/well were maintained in 500 µl of DMEM supplemented with 10% FBS, 1% Antibiotic-Antimycotic, TNF-α (175, 350 ng/ml), IFN-γ (175, 350 ng/ml) and incubated at 37º C, 5% CO_2 for 24 h [30].

MCF-7 cells total RNA was isolated (AurumTM Total RNA Mini Kit, Bio-Rad 732-6820) based on the manufacturer's instructions. The total RNA yield was estimated spectrophotometrically at 260/280 nm (Table 1). The RNA quality was confirmed via electrophoresis, the RNA purity was verified, then the RNA was reverse-transcribed into cDNA (iScript cDNA Synthesis Kit, 170-8841; Bio-Rad). PCR amplification was carried out using a PikoReal™ Real- Time PCR System (Thermo Scientific Inc.). The qPCR conditions were pre-denaturation at 95°C for 30 seconds, then 40 cycles of qPCR with denaturation for 5 seconds at 95°C, annealing for 20 seconds at 58°C, then elongation for 30 seconds at 72°C using Sso7d-fusion Polymerase and EvaGreen Dye as detection

system (SsoFast™ EvaGreen®Supermix, 172-5201AP). The data analysis was calculated using Livak formula :

ΔCT_1 → target gene expression

= CT target gene – CT house-keeping gene

ΔCT_2 → ΔCT control

= CT control – CT house-keeping gene

$\Delta\Delta CT \rightarrow \Delta CT_1 - \Delta CT_2$

$2\text{-}\Delta\Delta^{CT}$ → comparison of expression level

As an internal control, β-actin was included as the house-keeping gene. The primers used for RT-PCR are summarized as : β-actin (forward : 5'-TCT GGC ACC ACA CCT TCT ACA ATG-3', reverse : 5'-AGC ACA GCC TGG ATA GCA ACG-3'), p53(forward : 5' AGA GTC TAT AGG CCC ACC CC 3', reverse : 5'-GCT CGA CGC TAG GAT CTG AC 3'), Bax (forward :5'-TGC TTC AGG GTT TCA TCC AG 3', reverse : 5'-GGC GGC AAT CAT CCT CTG 3'), Bcl-2 (forward : 5'-GGT CAT GTG TGT GGA GAG CG -3', reverse : 5'-GGT GCC GGT TCA GGT ACT CA-3') [31].

3. Results

3.1.MSCs markers and multipotent differentiation

Passage 4 (P4) of hWJMSCs strongly expressed CD105, CD73 and CD90 (>95%), and showed poor expression of CD34, CD45, CD14, CD19 and HLA-II (<2%) [23]. The cells showed multipotent differentiation into adipocytes, chondrocytes, and osteocytes (data not shown). Therefore, hWJMSCs were confirmed based on the surface marker and differentiation capability [23, 24, 30].

3.2. Cytotoxic activity of IL2-hWJMSCs-CM toward BC cells

To determine the effect of CM from hWJMSCs treated with IL2 at concentrations of 125 ng/ml and 250 ng/ml IL2-hWJMSCs-CM against BC cell proliferation, we evaluated the inhibition of IL2-hWJMSCs-CM on MCF-7 at different concentrations of CM (30% and 60%) (Figure 1).

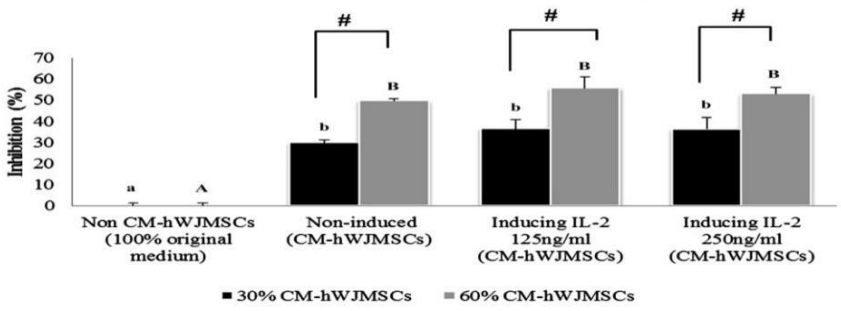

Figure 1 The effect of IL2-hWJMSCs-CM on BC cells growth inhibition

*The data are presented as histogram among treatments, this research was conducted in triplicate for each treatment. Small letter (a,b) presents significant difference between hWJMSCs-CM (noninduced, inducing IL-2 125 ng/ml, inducing IL-2 250 ng/ml) and non-hWJMSCs-CM (100% medium of MCF-7) in 30% of hWJMSCs-CM, capital letter (A,B) in 60% of hWJMSCs-CM based on Tukey HSD post hoc test ($p < 0.05$), symbol # presents significant difference between concentrations 30% and 60% of hWJMSCs-CM based on the student's t-test ($p<0.05$). Figure 1 shows that hWJMSCs-CM and IL2-hWJMSCs-CM inhibited cell proliferation and had anticancer activities against MCF-7 cells. The higher concentration (60%) of hWJMSCs-CM and IL2-hWJMSCs-CM increased the inhibition of BC cells (MCF-7) compared to the lower concentration (30%) based on the student's t-test ($p<0.05$).

3.3.Effect of IL2-hWJMSCs-CM on NK cell inhibition

IL-2 can activate and improve NK cells. Therefore we performed IL2-hWJMSCs-CM effect with different concentrations (125 and 250 ng/ml) on inhibition of NK cells growth. It can be seen in Figure 2.

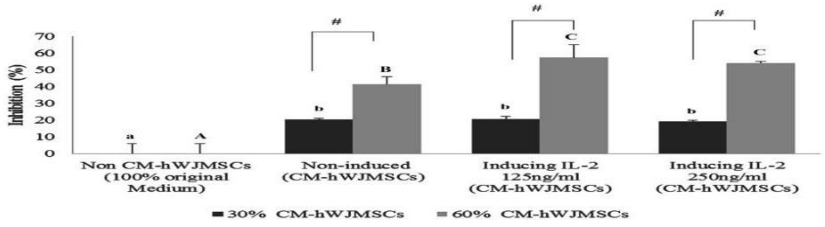

Figure 2 The effect of (IL2-hWJMSCs)-CM on NK cells growth inhibition

*The data are presented as histograms comparing treatments, this research was conducted in triplicate for each treatment. Small letter (a,b) presents significant difference between hWJMSCs-CM (noninduced, IL-2 125 ng/ml inducing , and IL-2 250 ng/ml inducing) and non-hWJMSCs-CM (100% medium of NK cells) in 30% of hWJMSCs-CM, capital letter (A,B,C) in 60% of hWJMSCs-CM based on the Tukey HSD post hoc test ($p < 0.05$), symbol # presents significant difference between concentrations 30% and 60% of CM-hWJMSCs based on t-test ($p<0.05$). Figure 2 shows that both hWJMSCs-CM and (IL2-hWJMSCs)-CM were toxic against NK cells. Effect of (IL2-hWJMSCs)-CM were significantly more toxic at a higher concentration (60% CM) than a lower concentration (30% CM) of CM, with no significant differences between concentrations of IL-2.

3.4. Effect of IL2-NK cells on the Level of TNF-α, IFN-γ, perforin, and granzyme in hWJMSCs and NK cells co-culture condition

Repairing and degrading damaged tissue is the function of NK cells, in addition to preventing pathogens [32]. Other study suggested that MSCs have ability to inhibit the proliferation and improve immune cells function, showing that MSCs influence the proliferation and cytolytic activities of NK cells [33 - 35] and IFN-γ production [36]. NK cells was activated using IL-2 (10 ng/ml) and non-induced-NK cells, which was co-cultured with hWJMSCs. The cells (hWJMSCs:NK cell ratio = 1:5) were assessed for the ability to secrete TNF-α, IFN-γ, perforin, and granzyme B and the effect of IL-2 inducer and NK cells number can be seen in Table 1.

Table 1 The effect IL-2 inducer and NK cell number on IFN-γ, TNF-α, perforin, and granzyme levels in co-cultured hWJMSCs and NK cells

Co-culture hWJMSCs and (IL2-NK)	Concentrations of effector molecules, cytoplasmic granules			
	IFN-γ (pg/ml)	TNF-α (pg/ml)	Perforin (pg/ml)	Granzyme B (pg/ml)
hWJMSCs-CM	0.94±0.19 [a]	2.64±0.47 [a]	0.00±0.00 [a]	0.00±0.00 [a]
hWJMSCs-CM +NK(1:5)	22.6±1.9 [b]	8.58±0.07 [b]	90±30 [b]	16.3±3.2 [b]
hWJMSCs-CM + IL2-NK (1:5)	23.9±1.0 [b]	28.1± 3.0 [c]	180±10 [c]	57.33±0.58 [c]

*This experiment was performed with 3 replications. The data of concentartions of IFN-γ, TNF-α, perforin, and granzyme (pg/ml) are presented as mean ±standard deviation,. Different small letters in the same column (a,b in IFN-γ; a, b, c in TNF-α; a,b,c in perforin; a,b,c in granzyme) indicate significant differences among treatments based on Tukey HSD post-hoc test ($p < 0.05$).

3.5. Effect CM from co-cultured hWJMSCs and NK cells towards cytotoxic effect of MCF-7

Complex factors which can reduce cancer cells proliferation, are secreted by MSCs [21-23]. The hWJMSCs-CM can suppress the immunomodulatory system, NK cells, and their cytotoxic effect. To increase cytotoxic effect of hWJMSCs and NK cells toward cancer cells, activation of them are required . Cytokines, such as interleukins can probably be used to boost directly the anticancer property of NK cells and indirectly that of hWJMSCs-CM.

The hWJMSCs-CM has been known to exhibit anticancer activities containing microparticles that mediate therapeutic effects against cancer [18]. The MCF-7 cells were treated with (hWJMSCs+NK)-CM, (hWJMSCs+IL2-NK)-CM in 30% or 60% concentrations and incubated for 24 h (Table 2).

Table 2 The effect of CM from co-culture of hWJMSCs and IL2-NK on MCF-7 proliferation

CM of coculture of hWJMSCs and IL2-activated NK cells	Proliferation inhibition toward MCF-7 cells (%)	
	Concentration 30% of CM coculture (hWJMSCs+ IL2-NK)	Concentration 60% of CM coculture (hWJMSCs+ IL2-NK)
Non-CM (MCF-7 cells only)	0.00±6.6 [a]	0.00±6.6 [a]
hWJMSCs-CM	32.66±6.6 [b]	48.0±8.7 [b]
hWJMSCs-CM + NK (1:5)	42.1±3.6 [bc]	54.6±8.0 [bc]
hWJMSCs-CM + IL2-NK (1:5)	61.5±1.3 [c]	68.3±3.8 [c]

*The data are presented as mean±standard deviation. The proliferation inhibition were measured in triplicate for each sample. Different superscripts letter in the same column (a, b, bc, c) of among 30% and 60% CM concentration of coculture hWJMSCs and NK cells (comparison cell number of hWJMSCs : NK = 1:5) indicated significant differences based on Tukey HSD post-hoc comparisons (P< 0.05)

Based on cytotoxic data (Table 2), hWJMSCs-CM inhibited MCF-7 proliferation and its combination with NK and activated-NK cells increased cytotoxic activity of hWJMSCs-CM toward MCF-7.

3.6. Effect of CM from co-cultured hWJMSCs and IL2-NK toward MCF-7 apoptosis

Secretome components secreted by MSCs such as chemokines, cytokines, and growth factors are crucial to maintain the homeostasis and signal transduction with stromal cells (fibroblast, endothel cell, macrophage) [37]. These soluble factors in CM determined stem cells fate in *in vitro* assays [38]. We measured apoptosis ability of CM from co-cultured hWJMSCs and IL2-NK at 50% concentration in normal media towards MCF-7. The (hWJMSCs+IL2-NK)-CM induced apoptosis of MCF-7 (Table 3, Figure 3).

Table 3 The effect CM from co-cultured hWJMSCs and IL2-NK toward apoptosis of MCF-7

CM-(hWJMSCs + IL2-NK)	Early Apoptosis (%)	Live cells (%)	Necrosis (%)	Dead cells (%) (late apoptosis)
Non-CM (MCF-7 cells only)	4.51±0.78 [a]	87.1±1.6 [c]	6.74±0.29 [b]	1.65±0.78 [a]
hWJMSCs-CM	33.11±0.92 [b]	49.3±1.0 [b]	6.70±0.28 [b]	10.88±0.15 [b]
(hWJMSCs+NK)-CM	34.7±1.9 [b]	53.2±3.8 [b]	4.87±0.22 [a]	7.3±1.7 [b]
(hWJMSCs+IL2-NK)-CM	36.5±2.7 [b]	42.47±0.65 [a]	5.01±0.87 [a]	15.9±1.7 [c]

*The data are presented as mean±standard deviation. The early apoptosis, live cells, necrosis, late apoptosis were measured in triplicate for each sample. Different superscripts letter in the same column (a, b) of early apoptosis among CM of co-culture hWJMSCs and IL2-NK cells and different superscript letter in the same column (a, b, c) of live cells among CM of co-culture hWJMSCs and NK cells, different superscript letter in the same column (a, b) of necrosis cells among CM of co-culture hWJMSCs and NK cells, different superscript letter in the same column (a, b, c) of late apoptosis among CM of co-culture hWJMSCs and NK cells indicated significant differences based on Tukey HSD post-hoc comparisons (P< 0.05)

Table 3 shows that CM from hWJMSCs, co-cultured hWJMSCs and NK, and co-cultured hWJMSCs and NK activated by IL2 (10 ng/ml) increased early and late apoptotic number of cells, and reduced the number of live cells and necrosis of MCF-7 cells. The CM from the cocultured

hWJMSCs and IL-2 activated-NK cells was more active to induce early apoptosis and kill MCF-7 (late apoptosis) compared to that from coculture with NK cells without activation or hWJMSCs-CM alone.

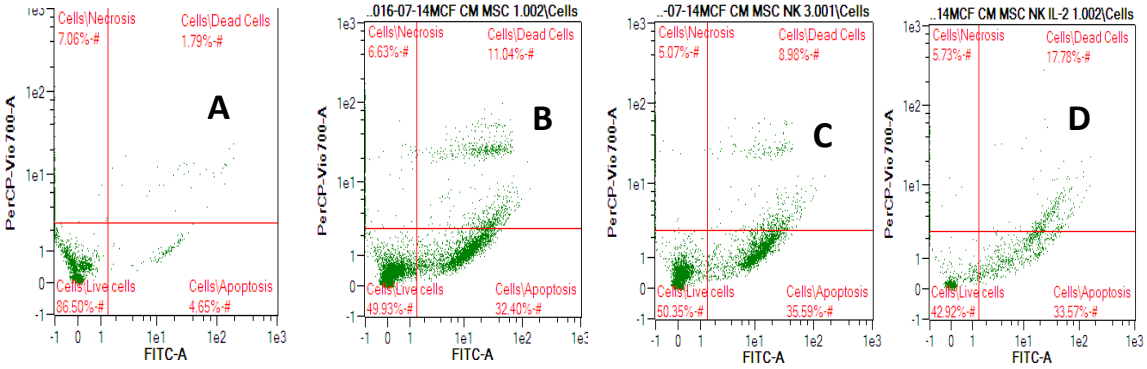

Figure 3 The effect CM from cocultured hWJMSCs and IL2-activated NK cells on apoptosis of MCF-7 cells

Apoptosis Percentage of BC cells treated with CM from co-culture of hWJMSCs and IL2-activated NK cells were determined by flow cytometry.
A : Control (MCF-7 cell untreated)
B : MCF7 treated with hWJMSCs-CM
C : MCF7 treated with (hWJMSCs+NK)-CM,
D : MCF7 treated with (hWJMSCs+IL-12-NK)-CM

Immunotherapy using activated-NK cells has been developed recently [6]. hWJMSCs-CM induced apoptosis by upregulating Casp3 and Casp9 as the apoptotic genes and down-regulating the antiapoptotic genes in the human U251 cell line [39]. The present research was performed to measure the proapoptotic and antiapoptotic gene expression namely Bax, p53, and Bcl-2 (Table 4).

Table 4 Effect CM from co-cultured hWJMSCs and IL2-NK toward apoptotic genes on BC cells

Condtioned Medium (hWJMSCs + IL2-NK)-CM	Bax	p53	Bcl-2
Non-CM (MCF-7 cells only)	1.00±0.00 [a]	1.00±0.00 [a]	1.00±0.00
hWJMSCs-CM	1.36±0.36 [ab]	1.23±0.25 [a]	0.93±0.09

(hWJMSCs+NK)-CM	1.52±0.17 [b]	1.47±0.15 [ab]	0.96±0.19
(hWJMSCs+IL2-NK)-CM	1.46±0.16 [b]	1.94±0.44 [b]	0.88±0.19

*The data is mean±standard deviation. The proapoptotic (Bax, p53) and antiapoptotic genes (Bcl-2) were measured in triplicate for each sample. Different superscript letters in the same column (a, ab,) among 50% CM of coculture hWJMSCs and ILs-NK cells indicated significant differences based on Tukey HSD post-hoc comparisons (P< 0.05).

Based on the data (Table 4) NK and activated-NK cells increased apoptotic gene expression including Bax and p53. CM-hWJMSCs and CM from co-culture hWJMSCs and NK cells did not significantly affect antiapoptotic gene (Bcl-2).

4. Discussion

CM contains many cytokines, growth factors, putative microvesicles including (micro) RNA, IL-6, IL-8 [25], tumor growth factor-β1 (TGF-β1), monocyte chemoattractant protein-1 (MCP-1), regulated on activation normal T cell expressed and secreted (RANTES), collagen type I, fibronectin, secreted protein acidic and rich in cysteine (SPARC), and insulin-like growth factor binding protein-7 (IGFBP-7), which can be used as anticancer [36, 40].

The ability of IL-2 to improve the anticancer activity of hWJMSCs-CM both in CM concentration of 30% and 60% against MCF-7 did not show significant differences (Figure 1). However, the hWJMSCs-CM could suppress MCF-7 viability with an inhibition value around 29-49%. A previous study showed that both CM from WJMSCs-normoxic and WJMSCs-hypoxic could inhibit cancer cells proliferation, including HeLa, SKOV3, PC3, HSC3, and HepG2 with IC_{50} values of 51-74% [24], which was in line with these findings. Another research suggested that 50% concentration of CM from hWJMSCs repressed breast adenocarcinoma (MDA-MB-231), ovarian carcinoma (TOV-112D), and osteosarcoma (MG-63) cells with 30-60% inhibition [40]. Even though the recombinant IL-2 was unable to improve the anticancer activity of hWJMSCs-CM, the cytotoxicity of hWJMSCs against cancer cells can be enhanced using engineering methods such as transfection and transduction. Thus, engineered hWJMSCs expressing IL-2 can be used as anticancer, since hWJMSCs expressed IL-2 to regulate inflammation and possess anticancer activity via direct killing effects of tumors or positive modulating of the endogenous immune system [41, 42].

Furthermore, hWJMSCs-CM and IL2-hWJMSCs-CM inhibited NK cell proliferation (Figure 2). The higher CM concentration (60% CM), the more toxic CM against NK cells was. The cytotoxic activity of hWJMSCs-CM and (IL2-hWJMSCs)-CM on NK cells was not significantly different, which indicated that IL-2 induction did not have a major effect on the cytotoxic activity

of hWJMSCs-CM. A previous study revealed that the hWJMSCs were toxic against NK cells, as MSCs produced PGE2 and TGF-β, which efficiently inhibited to activate and suppress cytotoxicity effect of downstream resting NK cells toward cancer cells. [43]. Several studies stated the interaction between NK cells and MSCs, revealing that NK cells killed MSCs and the MSCs strongly inhibited IL-2-induced NK cell proliferation and NK cell cytotoxicity, while CD244 and NKG2D were down-regulated under cell-to-cell contact in the interaction between NK and MSCs in the presence of IL-2 or IL-15 or following stimulation by combinations of IL-12/IL-15 and IL-12/1L-18 [44, 45, 33, 46]. Also, the function of NK cells is impaired by the combination of human adult stem cells (hASCs) and bone marrow mesenchymal stem cells (hBM-MSCs) [47].

There are four mechanisms of activated NK cells to kill targeted cancer cells: releasing cytoplasmic granules (perforin, granzyme), death receptor-induced apoptosis (FasL, TRAIL, TNF-α), effector molecule production (IFN-γ) or antibody-dependent cellular cytotoxicity (ADCC), a result of Fc-γ receptor (FcγR) mediated interaction with NK cells [16, 48]. The activation of granzyme and perforin release from NK cells is triggered by binding of FcγR to the Fc domain, causing target tumor cell lysis and Fc-dependent tumor cell phagocytosis [48, 49]. NK cells contributed to tumor death process by monoclonal antibodies (mAb) therapy by directing ADCC through Fcg RIIIA (CD16) [50].

The hWJMSCs-CM has potential anti-cancer activity toward various cancer cells, such as HeLa, SKOV3, HepG2, PC3, HSC3, A549, HT29, MCF-7 [23, 39]. MSCs inhibit the immune system cells proliferation and function such as NK cells cytotoxicity by suppressing TNF-α, IFN-γ secretion [33, 34, 35, 51]. However, this research revealed that IL2 could improve cytotoxicity of NK cells, co-culture IL2-NK and hWJMSCs by increasing TNF-α, IFN-γ, GzmB and Prf1 secretion (Table 1). ILs (IL-2, IL-12, IL-15, IL-18) and IFNs control NK cells cytotoxic activities [52]. This finding was relevant to another study suggesting that co-cultured NK cells and human *Adipocyte Stem Cells* (hASCs) secreted 40±32.5 pg/ml IFN-γ but co-culture of uninduced NK cells and hASCs did not secrete IFN-γ [47], which was relevant to hWJMSCs-CM cytotoxic effect to kill cancer cells using apoptosis pathway (Table 3, 4).

Production of TNF-α, IFN-γ, Prf1, GzmB increased when the ratio of NK cells were high (Table 1), meaning that the cytotoxic effect of NK cells was also increased. This result was relevant to a previous study that there was interaction between MSCs with both innate and adaptive immune systems, leading to the activation of few effector functions [53]. The phenotype, cytokine

secretion, and proliferation of NK cells were suppressed at lower NK-to-MSCs ratios, which affected the innate immune responses and regulation of adaptive immunity [34]. Moreover, the secretion of IFN-γ was improved when co-culture hASCs, hBM-MSCs, and NK cells were at the ratio 1:1 for 72 h, as well as co-culture of hASCs and IL-2-expanded NK cells [54,46].

The co-culturd NK cells and AML (K562) cancer cells secreted the highest IFN-γ compared to co-cultures of NK+hASCs and NK+hBM-MSCs and the lowest IFN-γ was produced by NK control [46]. The MSCs inhibit the activated NK cells and effector of inducing function such as cytotoxic activity and cytokines production[46]. This was in line with the result research (Figure 2).

Prf1 mediates cytotoxicity with direct killing by NK cells and indirect killing by secreting death-inducing ligands [55]. Cell growth inhibition and cell death, apoptosis, chromosome condensation and DNA fragmentation, increased Casp-3 activity, and the release of apoptosis-inducing factor (AIF) and cytochrome c from the mitochondria toward cancer cells (HepG2, SK-BR-3, and HeLa) were activated by Prf1 secretion [56].

The surface of target cells is bound by Gzm in the concentration-dependent and saturable manners and transported into the cells via endocytosis [57]. The internalization of GzmB into a target cells was determined by Prf1 [57]. Apoptosis through intrinsic mitochondrial pathway was mainly induced by GzmB either by cleaving Bid or activating Bim, leading to Bak/Bax activation and subsequent signal transduction of Casp-3 activation [58]. GzmB secreted in CM of co-cultured hWJMSCs and NK cells activated apoptosis (Table 1) and also induced the expression of Bax gene (Table 4). Activation of p53 tumor suppressor gene as one of the apoptosis pathway was induced by GzmB to kill cancer cells [59].

Apoptosis in human carcinoma cell lines such as HCT116-3 [60] and MCF-7 [61] was induced using TNF-α, including PKR activation in U937 [62].

IFNγ modulates the pro-apoptotic genes but not pro-necrotic genes in HT-29 as cell death program through sensitizing the cells [63]. Treatment of IFNγ on a human conjunctival cell line resulted in overexpression of Bax, whereas Bcl-2 and p53 protein levels were not modified. Protecting (Bcl-2, Bcl-Xl) or sensitizing (Bax, bak, bad) the cell to undergo apoptosis was controlled by Bcl-2 protein family [64]. IFNγ-inducible protein 10 (IP10) upregulated p53 and induced Bax expression on HeLa cells [65]. IFN-γ enhanced the apoptosis by decreasing

mitochodrial membrane potential, upregulating p53 and Bax, down-regulating Bcl-xL, and improving caspase-3 activity [66].

In conclusion, the IL2-hWJMSCs-CM and hWJMSCs-CM had anticancer activities against BC cells and higher CM concentrations increased the inhibition potency. The IL2-hWJMSCs-CM and hWJMSCs-CM had a toxic activity against NK cells, and higher CM concentrations increased the inhibition potency. High cell number of NK and IL-2 inducer increased the secretion of TNF-α, IFN-γ, Prf1, and GzmB which play role as potent anticancers. (hWJMSCs+IL2-NK)-CM was toxic against BC cells, higher concentration of (hWJMSCs+IL2-NK)-CM increase cytotoxic activities. The (hWJMSCs+IL2-NK)-CM induced apoptosis in BC cells through increasing apoptotic genes expression (Bax, p53). Further research, preclinical and clinical studies should be pursued prior to therapy application.

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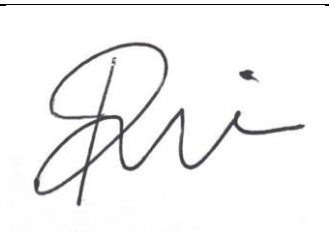
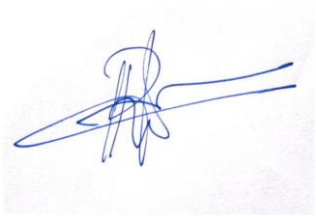
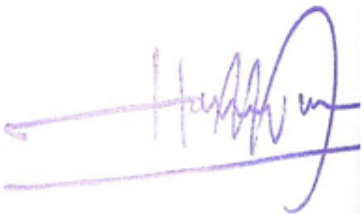
Dwi Davidson Rihibiha ----------	 ----------	October 18, 2018 ----------
Rizal Rizal ----------	 ----------	October 18, 2018 ----------
Harry Murti ----------	 ----------	October 18, 2018 ----------
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