Effect of TNFα and IFNγ Toward Apoptosis in Breast Cancer Cells

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Direct and Indirect Effect of TNFα and IFNγ Toward Apoptosis in Breast Cancer Cells

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Background: Breast cancer (BC) is the leading cause of death in women. Cancer therapies using TNFα and IFNγ have been recently developed by direct effects and activation of immune responses. This study was performed to evaluate the effects of TNFα and IFNγ directly, and TNFα and IFNγ secreted by Conditioned Medium-human Wharton’s Jelly Mesenchymal Stem Cells (CM-hWJMSCs) toward apoptosis of BC cells (MCF7).

Materials and Methods: BC cells were induced by TNFα and IFNγ in 175 and 350 ng/mL, respectively. CM-hWJMSCs were produced by co-culture hWJMSCs and NK cells that secreted TNFα, IFNγ, perforin (Prf1), granzyme B (GzmB) for treating BC cells. The BC cells were treated with CM-hWJMSCs in 50%. The expression of apoptotic genes Bax, p53, and the anti-apoptotic gene Bcl-2 were determined using RT-PCR.

Results: TNFα and IFNγ at concentration of 350 ng/mL induced higher Bax expression compared to 175 ng/mL. TNFα and IFNγ 350 ng/mL, 175 ng/mL induced p53 expression, whilst TNFα and IFNγ at 350 ng/mL decreased Bcl-2 expression. Perf1, GzmB, TNFα and IFNγ-containing CM-hWJMSCs induced significantly apoptosis percentage, induced Bax expression, but did not effect p53, Bcl-2 expression.

Conclusion: TNFα and IFNγ directly induce Bax, p53, decrease Bcl-2 gene expression. The Prf1, GzmB, TNFα, IFNγ-containing CM-hWJMSCs induce apoptosis and Bax expression.

Keywords: breast cancer, Wharton’s Jelly mesenchymal stem cells, TNFα, IFNγ

Introduction

Breast cancer (BC) is one of the most commonly oncologic diseases worldwide, the leading cause of cancer death among women, with 882,900 cases diagnosed and 324,300 deaths in 2012, approximately 25% of cancer cases and 15% of cancer deaths among women. The incidence BC worldwide will reach 3.2 million new cases per year by 2050. BC is
the most diagnosed cancer and the leading cause of cancer deaths among women in the ASEAN region. The highest incidence rate per 100,000 is found in Singapore (59.9) and the lowest in Vietnam (15.6), Indonesia exhibits the highest mortality rate of 36.2 per 100,000 patients while the lowest is Singapore around 13.6 per 100,000 patients.4

BC treatments are chemotherapy, surgery, endocrinotherapy, radiotherapy and molecular-targeted therapy.3 All therapies are expensive and low effectiveness.4 The development of efficient and effective therapy is highly required, which can directly target both primary and metastatic side. Metastatic side that features invasives and escapes antitumor immunity.6-9 Metastasis in BC is caused by an immunosurveillance deficiency, including an impairment of NK cell maturation, low NK cell count in peripheral blood mononuclear cells (PBMCs), low NK activity10, decreased cytotoxic functions11-13, NK abnormalities12, poor tumor infiltrate14-15, inefficient homing to malignant tissues.12 The promising cancer therapies are to stimulate NK cell functions, the combination NK cells with other anticancer agents16, NK cells respond to various cytokines, such as interleukin-15 (IL15), IL1817-19. NK cells suppress cancer by releasing cytoplasmic granules, perforin (Prf) and granzyme (Gzm), to trigger cells apoptosis.20 Activated-NK cells secrete tumor necrosis factor (TNFα), various effector molecules, such as interferon (IFNγ) that can induce cancer cell apoptosis.21

Mesenchymal Stem Cells (MSCs) have ability the homing to tumors, these stem cells have been engineered as drug delivery system. The condition medium of human MSCs (hMSCs) have been proved to inhibit bladder cancer cells viability by secreting soluble factor which is involved in PTEN/PI3K/Akt.22 Wharton’s Jelly (WJ) is part of umbilical cord as one source of hMSCs and has many advantages including low risk infection, non-carcinogenesis, multipotency and low immunogenicity.23 The hWJMSCs have anticancer activity which mediate via cell-to-cell and/or non-cellular contact mechanism. The conditioned medium of hWJMSCs inhibit cancer proliferation.24 Anticancer agents produced by engineered MSCs are: a). Immunostimulation such as chemokine C-X3-C motive ligand 1 (CX3CL1), IFN and interleukins (IL2, IL7, IL12), b). Pro-drug conversion such as cluster of differentiation (CD) and herpes simplex virus thymidine kinase (HSV-tk), c). Apoptosis induction such as IL-8, Natural Killer 4 (NK4) and Tumour Necrosis Factor-related apoptosis inducing ligand (TRAIL).21,25,26

TNFα can induce apoptotic (caspase-dependent) or necrotic (caspase-independent) cell death in vitro, depending on the cell type used.27 The IFNs mediate anticancer effect directly by modulating immunomodulatory response or directly by regulating tumour cell proliferation and differentiation28 and inhibition of tumour angiogenesis29. My previous research resulted that TNFα and IFNγ have anticancer activities toward BC cells (T47D, MCF7), but it was non toxic toward hWJMSCs.30

This research was the continuing study to evaluate the directly effects of TNFα and IFNγ which indirectly effect of TNFα and IFNγ which secreted in CM co-culture hWJMSCs and NK cells toward apotosis of BC cells (MCF7) and to increase the cytoxic of NK cells was induced by interleukins (IL15, IL18).

Materials and methods

**Real-time PCR assay for the apoptotic induction of MCF7 cell line by TNFα, IFNγ**

To determine the apoptosis induction ability of recombinant human TNFα (Biologend 570106), recombinant human IFNγ (Biologend 570206) toward MCF7 (ATCC®HB22TM) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia. The MCF7 cancer cells were cultured in density 2x10⁶/well, 500 μL RPMI (1640 Gibco 22400089), 10% FBS (Gibco 10270106), 1% Antibiotic Antimycotic (Gibco 1772653) and supplemented with TNFα (175, 350 ng/mL), IFNγ (175, 350 ng/mL) incubated in 37°C, 5% CO₂ for 24 h.30

Total RNA was isolated from MCF7 cells (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 were confirmed via electrophoresis and measured its purity then RNA was reverse-transcribed into cDNA (iScript cDNA Synthesis Kit, 170-8841; Bio-Rad), for which the mixture was firstly incubated at 25°C for 5 minutes, then 42°C for 30 minutes, and finally at 85°C for 5 minutes. PCR amplification was performed using a PikoRealTM 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. As an internal control, β-actin was included as the house-keeping gene. The primers used for RT-PCR are summarized are : β-actin...
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Co-culture of hWJMSCs and ILs-NK cells for measuring the cytoplasmic granules and effector molecules

NK92MI cells (ATCC® CRL2408™) from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia at density 2x10⁶ well were grown and maintained in NK medium RPMI 1640 (Gibco 22400089), 20% FBS (Gibco 10270106), 1% Antibiotic Antimycotic (Gibco 1772653). The cells were treated with 5, 10 ng/mL of recombinant human IL15 (Biolegend 715902) or IL18 (GenScript Z031189) per 24 h and incubated at 5% CO₂, 37°C for 96 h, which resulted in IL15-induced NK (IL15-NK), IL18-induced NK (IL18-NK) cells.

The hWJMSCs were obtained from Aretha Medika Utama, Biomolecular and Biomedical Research Center, Bandung, Indonesia at density 2x10⁶ were cultured in minimum essential medium-α (α-MEM, Gibco 12561056), 10% FBS, 1% Antibiotic Antimycotic. The hWJMSCs were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. NK cells were adjusted to 1x10⁵ for the ratio 1:1 (hWJMSCs : NK = 1:1; hWJMSCs : IL15-NK = 1:1; hWJMSCs : IL18-NK = 1:1). NK cells were resuspended in 24-well plates containing hWJMSCs in NK medium (RPMI 1640, Gibco 22400089) and α-MEM medium at the ratio 1:1 (50%:50%) according the optimized research (data are not shown). The hWJMSCs and hWJMScs+ILs-NK cells and hWJMSCs were incubated in a humidified atmosphere with 5% CO₂ at 37°C for 24 h. Cells and medium were centrifuged at 500 g for 4 mins, and the medium was preserved in -80 °C for the next assays such as for IFNγ, TNFa, Prf1, and GzmB. 31-32

Cytotoxic activity of CM from co-culture of hWJMSCs and ILs-NK toward MCF7

The BC cells with density 1x10⁶ cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic). The cells were washed using PBS and the cells were harvested and inactivated using 0.25% trypsin EDTA (Gibco, 25200072). The cells were centrifuged in 1600 rpm, and cells were counted using hemocytometer. The accounted cells 5x10⁵ - 1x10⁶ were added 500 μL Annexin V binding buffer (Miltenyi Biotec, 130-092-820), 5 μL Anti-FITC (Miltenyi Biotec, 130-048-701), 5 μL Propidium Iodide (Miltenyi Biotec, 130-093-233) furthermore the cells were incubated in darkness, 4°C and the apoptotic percentage of MCF7 cells were analyzed using MACSquant Analyzer 10 (Miltenyi Biotec).

Apoptotic gene expression of CM from co-culture of hWJMSCs and ILs-NK toward MCF7 using RT-PCR

The BC cells with density 1x10⁶ cells/disk were cultured in complete medium (DMEM + 10% FBS + 1% Antibiotic Antimycotic). The cells were incubated in humidified atmosphere, 5% CO₂, 37°C for 24 h. Furthermore, the cells were treated with CM-(hWJMSCs+IK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK) in 30% or 60% concentrations and were incubated for 24 h. The cell viability was assayed based on an optimized reagent containing resazurin, which was converted to fluorescent resorufin by viable cells that absorbed the light at 490 nm using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). 36,24,30

Apoptotic activity of CM from co-culture of hWJMSCs and ILs-NK toward MCF7 using flowcytometer

The BC cells lines MCF7 (5x10³/well) were cultured in complete medium which consisted of DMEM (Gibco 11995065), supplemented with 10% FBS and 1% Antibiotic Antimycotic. The cells were incubated in a humidified atmosphere, 5% CO₂, 37°C for 24 h. 33-35 Furthermore, the cells were treated with CM-(hWJMSCs+IK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK) in 30% or 60% concentrations and were incubated for 24 h. The cell viability was assayed based on an optimized reagent containing resazurin, which was converted to fluorescent resorufin by viable cells that absorbed the light at 490 nm using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium (MTS) assay (Promega, Madison, WI, USA). 36,24,30
Total RNA Mini Kit, Bio-Rad 732-6820) based on the manufacturer’s instructions. The Bax, p53, Bcl-2 genes expression along with the constitutively expressed β-actin gene was analyzed using real-time quantitative polymerase chain reaction (qPCR).37

**Statistical analysis**

Statistical analysis was analyzed with Statistical Package for the Social Sciences (SPSS) statistics version 20.0 software. One-way analysis of variance (ANOVA) was conducted, followed by Tukey HSD post-hoc test and \( p<0.05 \) was considered to be significant.

**Results**

**The effect directly TNFα, IFNγ toward apoptotic and antiapoptotic genes expression**

The previous research showed that TNFα, IFNγ inhibited breast cancer proliferation (MCF7) with median inhibitory concentration (IC\(_{50}\)) 0.36 μg/mL, 0.34 μg/mL.30 This study was the continued-research to elucidate the apoptosis mechanism of TNFα, IFNγ in MCF7 cells. In order to determine the apoptotic inducing activity of TNFα, IFNγ toward MCF7 cells, the expression of apoptotic genes was determined by RT-PCR. We measured the expression of proapoptotic genes, specifically p53, B-cell CLL/lymphoma 2 (Bcl-2), Bcl2-associated X protein (Bax). TNFα induced significantly the apoptotic genes both low and high concentrations (175, 350 ng/mL) toward Bax, p53 genes expression. TNFα 350 ng/mL was more active than 175 ng/mL to reduce Bcl-2 and induce Bax (Table 1). IFNγ in both concentrations (175, 350 ng/mL) induced apoptotic genes, IFNγ 350 ng/mL was able to down regulate Bcl-2 (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RNA Purity (260/280 nm)</th>
<th>Bax</th>
<th>p53</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 TNFα on MCF7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (MCF7 untreated)</td>
<td>2.4288±0.4768</td>
<td>1.00±0.00  a</td>
<td>1.00±0.00 a</td>
<td>1.00±0.00 b</td>
</tr>
<tr>
<td>TNFα 350 ng/mL</td>
<td>2.3493±0.2650</td>
<td>23.62±1.43 c</td>
<td>2.94±0.31 b</td>
<td>0.33±0.08 a</td>
</tr>
<tr>
<td>TNFα 175 ng/mL</td>
<td>2.5902±0.3898</td>
<td>5.29±0.93 b</td>
<td>2.17±0.56 b</td>
<td>1.28±0.31 b</td>
</tr>
<tr>
<td>2 IFNγ on MCF7</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (MCF7 untreated)</td>
<td>2.4288±0.4768</td>
<td>1.00±0.00  b</td>
<td>1.00±0.00 b</td>
<td>1.00±0.00 b</td>
</tr>
<tr>
<td>IFNγ 350 ng/mL</td>
<td>2.6019±0.4678</td>
<td>34.79±4.35 b</td>
<td>4.17±1.06 b</td>
<td>0.18±0.04 a</td>
</tr>
<tr>
<td>IFNγ 175 ng/mL</td>
<td>2.2870±0.3335</td>
<td>3.49±0.35 a</td>
<td>2.83±0.62 b</td>
<td>1.07±0.16 b</td>
</tr>
</tbody>
</table>

The data are presented as mean±standard deviation. The gene expression were measured in triplicate for each sample. Different superscripts in the same column (a,b,c) for each effect of TNFα or IFNγ on MCF7 indicate significant differences among the means of groups (concentrations of TNFα or IFNγ) based on Tukey HSD post-hoc comparisons (\( p<0.05 \)).
Tabel 2. Effect interleukins (IL-15, IL18)-induced NK cells toward TNFα, IFNγ, perforin, granzyme level in co-culture hWJMSCs and NK cells.

<table>
<thead>
<tr>
<th>Conditioned Medium of Co-culture hWJMSCs and ILs-induced NK Cells</th>
<th>Level of CM Secretome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IFNγ (pg/mL)</td>
</tr>
<tr>
<td>CM-hWJMSCs</td>
<td>0.94±0.19 a</td>
</tr>
<tr>
<td>CM-NK cells</td>
<td>8.06±1.90 a</td>
</tr>
<tr>
<td>CM co-culture hWJMSCs + NK (1:1)</td>
<td>7.72±2.52 a</td>
</tr>
<tr>
<td>CM co-culture hWJMSCs + IL15-NK (1:1)</td>
<td>8.17±2.60 a</td>
</tr>
<tr>
<td>CM co-culture hWJMSCs + IL18-NK (1:1)</td>
<td>19.72±5.52 b</td>
</tr>
</tbody>
</table>

The data are presented as mean±standard deviation. The level of secretome were measured in triplicate for each sample. Different superscripts in the same column (a,b) of TNFα, IFNγ, granzyme and perforin among single cells (hWJMSCs, NK cells) and co-culture cells (hWJMSCs and ILs-induced NK cells, hWJMSCs and NK cells) indicated significant differences based on Tukey HSD post-hoc comparisons (p<0.05).

Complex factor which are able to reduce the proliferation of glioma, melanoma, lung cancer, hepatoma, and breast cancer cells.46-49 CM-hWJMSCs can weaken the immuno-modulatory system, NK cells, lower cytotoxic effect. To activate hWJMSCs and NK cells are required to increase cytotoxic effect toward cancer cells. To activate the anticancer potential by utilizing cytokines, such as interleukins, may directly boost the anticancer property of NK cells and indirectly CM-hWJMSCs. Conditioned medium of coculture hWJMSCs and ILs-induced NK cells for treatment toward MCF7 cells. The cells were treated CM-(hWJMSCs+NK), CM-(hWJMSCs+IL15-NK), CM-(hWJMSCs+IL18-NK) in 30% or 60% concentrations and were incubated for 24 h (Table 3).

Effect CM from co-culture of hWJMSCs and ILs-NK towards Apoptotic activity of MCF7

MSCs secrete secretome such as chemokins, cytokines, growth factor as bioactive and nutrition50, these secretome are important to preserve the homeostasis, cross talk with stromal cells (fibroblast, endothel cell, macrophage). The soluble factor of CM affect stem cells fate by in vitro assay.51 The released soluble factor of MSCs affect brain cancer cells, melanoma, lung cancer by in vivo assay46, CM-MSCs reduce NFkB secretion of hepatome, breast cancer cells and inhibit cells proliferation by in vitro assay49.

The activated-NK cells significantly increase secretion of IFNγ, TNFα, chemokins, cytokin playing role immune respond, eliminate cancer cells.46,52 We measured

Table 3. Effect cytotoxic of CM co-culture hWJMSCs and NK cells toward MCF7.

<table>
<thead>
<tr>
<th>CM of Co-culture of hWJMSCs and ILs-activated NK Cells</th>
<th>Proliferation Inhibition Toward MCF7 Cells (%)</th>
<th>Concentration 30% of CM Co-culture (hWJMSCs+ILs-NK)</th>
<th>Concentration 60% of CM Co-culture (hWJMSCs+ILs-NK)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CM (MCF7 cells only)</td>
<td></td>
<td>0.00±6.61 a</td>
<td>0.00±6.61 a</td>
</tr>
<tr>
<td>CM-hWJMSCs</td>
<td></td>
<td>32.55±6.57 b</td>
<td>48.00±8.70 b</td>
</tr>
<tr>
<td>CM-hWJMSCs + NK (1:1)</td>
<td></td>
<td>40.44±4.02 b</td>
<td>51.86±3.17 bc</td>
</tr>
<tr>
<td>CM-hWJMSCs + IL15-NK (1:1)</td>
<td></td>
<td>56.82±7.66 c</td>
<td>60.55±4.91 bc</td>
</tr>
<tr>
<td>CM-hWJMSCs + IL18-NK (1:1)</td>
<td></td>
<td>58.91±3.17 c</td>
<td>64.49±1.25 c</td>
</tr>
</tbody>
</table>

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,c) of among 30% CM concentration of coculture hWJMSCs, NK cells and different superscript letter culture in the same column (a,b,bc,c) of among 60% CM concentration of coculture hWJMSCs, NK cells indicated significant differences based on Tukey HSD post-hoc comparisons (p<0.05).
the CM from co-culture of hWJMSCs and ILs-NK (with concentrations 50%) towards apoptotic activity in MCF7 cells. CM-(hWJMSCs+ILs-NK) induced apoptosis of BC cells (Table 4, Figure 2).

Table 4 showed that CM from coculture hWJMSCs and NK cells which were activated by ILs 10 ng/mL (IL15, IL18) induced apoptosis, reduced live cells, necrosis in MCF7 cells. The activated NK cells were more active to induce apoptosis and kill BC cells compared to NK cells without activating or CM-hWJMSCs. IL15 and IL18 activate NK cells to induce apoptosis MCF7 cells, IL18 was more active to induce NK cells compared to IL15.

Table 4. Effect CM from co-culture of hWJMSCs and ILs-NK towards Apoptotic activity of MCF7.

<table>
<thead>
<tr>
<th>CM-(hWJMSCs + ILs-NK)</th>
<th>Early Apoptosis (%)</th>
<th>Live cells (%)</th>
<th>Necrosis (%)</th>
<th>Dead cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CM (MCF7 cells only)</td>
<td>4.51±0.78 a</td>
<td>87.10±1.56 c</td>
<td>6.74±0.29 b</td>
<td>1.65±0.78 a</td>
</tr>
<tr>
<td>CM-hWJMSCs</td>
<td>33.11±0.92 b</td>
<td>49.30±1.01 b</td>
<td>6.70±0.28 b</td>
<td>10.88±0.15 d</td>
</tr>
<tr>
<td>CM-(hWJMSCs+IL15-NK)</td>
<td>34.66±1.93 b</td>
<td>53.15±3.75 b</td>
<td>4.87±0.22 a</td>
<td>7.32±1.74 c</td>
</tr>
<tr>
<td>CM-(hWJMSCs+IL18-NK)</td>
<td>38.16±2.14 c</td>
<td>38.99±1.48 a</td>
<td>15.29±0.89 d</td>
<td>7.55±0.42 c</td>
</tr>
<tr>
<td>CM-(hWJMSCs+IL18-NK)</td>
<td>47.03±0.54 d</td>
<td>42.95±1.12 b</td>
<td>4.96±0.33 a</td>
<td>5.06±0.80 b</td>
</tr>
</tbody>
</table>

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

**Effect CM from co-culture of hWJMSCs and ILs-NK towards pro- and anti-apoptotic gene**

The progressive BC cells involved disfunction NK cells, furthermore cancer therapy needs to improve NK cells cytotoxicity, inhibit losing antitumor immune system. CM-hMSCs significantly inhibited proliferation, induced apoptosis, significantly upregulated the apoptotic genes of both Casp3 and Casp9, significantly downregulated the antiapoptotic genes such as SURVIVIN and XIAP, induced and completed differentiation in human U251 cell line. We measured the proapoptotic and antiapoptotic gen expression namely Bax, p53 and Bcl-2 (Table 5.)
Tabel 5. Effect CM from co-culture of hWJMSCs and ILs-NK towards pro-apoptotic and anti-apoptotic genes on MCF7 cells.

<table>
<thead>
<tr>
<th>Conditioned Medium</th>
<th>RNA Purity (260/280 nm)</th>
<th>Bax</th>
<th>p53</th>
<th>Bcl-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-CM (MCF7 cells only)</td>
<td>2.2368±0.2639</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
<td>1.00±0.00</td>
</tr>
<tr>
<td>CM-hWJMSCs</td>
<td>2.6868±0.4326</td>
<td>1.36±0.36</td>
<td>1.23±0.25</td>
<td>0.93±0.09</td>
</tr>
<tr>
<td>CM-(hWJMSCs+NK)</td>
<td>2.4541±0.3385</td>
<td>1.52±0.17</td>
<td>1.47±0.15</td>
<td>0.96±0.19</td>
</tr>
<tr>
<td>CM-(hWJMSCs+IL15-NK)</td>
<td>2.7321±0.1301</td>
<td>1.61±0.27</td>
<td>2.03±0.36</td>
<td>0.98±0.10</td>
</tr>
<tr>
<td>CM-(hWJMSCs+IL18-NK)</td>
<td>2.3336±0.3337</td>
<td>2.56±0.40</td>
<td>3.70±0.95</td>
<td>0.89±0.24</td>
</tr>
</tbody>
</table>

The data are presented as mean±standard deviation. The pro-apoptotic (Bax, p53) and antiapoptotic genes (BCL2) were measured in triplicate for each sample. Different superscripts letter in the same column of BAX, TP53 gene expression (a,b) among CM concentration of co-culture hWJMSCs and ILs-NK cells indicated significant differences based on Tukey HSD post-hoc comparisons (p<0.05).

Discussions

The effect of TNFα and IFNγ against MCF7 cells exhibited that the cytokines decreased the cell viability in a dose dependent manner. The IC_{50} value of TNFα and IFNγ against BC cell lines were found 242.77-266.88 ng/mL for T47D and 295-03-364.78 ng/mL for MCF7. Concentration 350 ng/mL of TNF-α and IFN-γ was more active to increase Bax expression compared to 175 ng/mL but there was not significant difference between 350 ng/mL and 175 ng/mL in p53 gene expression. Concentration 350 ng/mL of TNFα, IFNγ reduced Bcl-2 expression, 175 ng/mL of TNFα, IFNγ could not reduce Bcl-2 expression. TNFα-induced apoptosis in BC cells through Bcl-2 in low expression. IFNγ increased Bax level. p53 and Bax may be the targets for the IFNγ based chemo-immunotherapy of the chemotherapy-resistant cancers. This study was contradictory with previous research that Bcl-2 expression inhibited TNFα-induced apoptosis in MCF7 cells, overexpression of Bcl-2 and Bcl-XL was correlated with an increased resistance to TNFα-induced apoptosis. Insulin-Like Growth Factor- Binding Protein-3 (IGFBP-3) expression is up-regulated in response to TNFα, with some evidence that it may mediate the inhibitory effects of TNFα in TNF-sensitive BC cell lines. IGFBP-3 and -5 translocate to the nucleus in BC cells, they can transcriptionally modulate the expression of apoptotic genes such as bax and Bcl-2. It has been reported that p53 can induce apoptosis through a transcription-independent pathway, promote cell death by binding to Bcl-XL, contribute to TNFα-induced apoptosis in retinoblastoma fibroblasts. IFNγ inhibits growth human pancreatic carcinoma cell lines (AsPc-1, Capan-1, and Capan-2), induced DNA fragmentation and poly (ADP ribose) polymerase (PARP) cleavage and increase anti proliferative activity in pancreatic cancer cells due to apoptotic induction. ILs-activated NK cells release cytoplasmic granules (Prf1, Gzm), detah receptor (FASL, TRAIL, TNFα), effector molecules (IFNγ, NO) which kill target tumor cells.

CM-hWJMSCs contains various growth factors, cytokines, chemokines, and tissue regenerative agents, therapeutic potency, CM-hWJMSCs inhibit proliferation of cancer cell lines including HeLa, SKOV3, HepG2, PC3, HSC3\textsuperscript{24}, A549, HT29, MCF7\textsuperscript{66}. MSCs have side effect to suppress the proliferation and function of immune system cells, influence cytotoxic activities of NK cells\textsuperscript{38,40}, suppress TNFα, IFNγ secretion\textsuperscript{41}. Our research to improve NK cells cytotoxicity using ILs (IL15, IL18), the result showed that ILs increased TNFα, IFNγ secretion by NK cells or co-culture ILs-NK and hWJMSCs. ILs increased GzmB and Prf1 secretion by co-culture NK cells and hWJMSCs (Table 2). NK cells activity were controlled by cytokine and ILs (IL2, IL12, IL15, IL18) and IFNs. IL-15 induces NK cells viability and proliferations, inhibits antiapoptosis through inhibition of gene expression Bim, Noxa and induces Mcl-1. IL15 triggers NK cells activity and proliferation. IL12 induced IFNγ secretion by NK cells dose dependent manner. Co-culture of NK cells and human Adipocyte Stem Cells (hASCs) secretes 40±32.5 pg/mL IFNγ but co-culture NK cells uninduced IL2 and hASCs did not secrete IFNγ (DellaRosa, 2012). hMSCs increase IFNγ secretion by NK cells induced IL12/IL18. MSCs modulate the cells...
cross talk the IL12R/STAT4 pathway. IL12, IL18 induce NK cells to release IFNγ. Co-culture hWJMSCs and NK cells could help NK cells to release TNFα, IFNγ, Prf1, Gzm (Table 2) and IL15, IL18-induced NK cells increased the secretion of secretome (Table 2). This results were in line with cytotoxic effect of hWJMScs-CM through apoptosis pathway to kill cancer cells (Table 3, 4). IL18 was most active to induce NK cells in producing TNFα, IFNγ, Prf1, Gzm and furthermore CM-( hWJMScs+IL18-NK) was the most active to induce apoptosis in BC cells (Table 3, 4). This result was validated with previous research that IL2 is capable to restore the cytotoxicity and granular content of exhausted NK cells. Cytotoxicity of NK cells is executed mainly through the granule exocytosis pathway by releasing Prf1 and GzmB into the immunological synapse after the conjugate formation with targets. Strategies to improve NK cell activity, growth, development and differentiation for tumor immunotherapy are actively using IL2, IL15. Prf-mediated cytotoxicity used for direct killing by NK cells is more important than indirect killing by secretion of death-inducing ligands by NK cells. GzmB binds to the target cell surface in the concentration-dependent and saturable manners and enters the cells via endocytosis. GzmB primarily induces apoptosis via the intrinsic mitochondrial pathway by either cleaving Bid or activating Bim leading to the activation of Bak/Bax and subsequent generation of active Casp-3. CM of co-culture hWJMScs and NK cells secreted GzmB which induced apoptosis (Table 4), induced Bax gene expression (Table 5), these results was consistent with previous research that the several proteins that are involved in gzmB-induced apoptosis, including casp-9 and -3, Bim, Bid, Bak, Bax, and XIAP and Bcl-2 was not detected. Gzm B induced apoptosis cancer cells by involving induction of p53 tumor suppressor gene. Prf1 induced 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 (Hep G2, SK-BR-3, HeLa).

Conclusion

TNFα and IFNγ induce Bax, p53, decrease Bcl-2 gene expression directly. CM of coculture hWJMScs and ILs-activated NK increase secretion of TNFα, IFNγ, Prf1, GzmB furthermore inhibit breast cancer cells proliferation, induce apoptosis and increase Bax expression.

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