Stroma derived from breast cancer tissue increases breast cancer stem cells population in vitro

Novi Silvia Hardiany,¹ Septelia Inawati Wanandi,¹ Kana Tachi,² Akira Shiraishi,² Toshiki Kato,² Osamu Ohneda²

¹Department of Biochemistry & Molecular Biology, Faculty of Medicine Universitas Indonesia, Indonesia
²Laboratory of Regenerative Medicine & Stem Cell Biology, Faculty of Medicine University of Tsukuba, Japan

Corresponding Author:
Dr. dr. Novi Silvia Hardiany, MBiomed
Dept. Biochemistry & Molecular Biology, Faculty of Medicine Universitas Indonesia
Telp. +6221-3910734
Email: novi.silvia@ui.ac.id

Abstract

Breast cancer is the most frequent cancer causing death in woman population in the world. Accumulating evidence found that cancer stem cells (CSCs) are responsible for high tumor recurrence, therapy resistance and metastasis. The behavior of breast CSCs are influenced by their microenvironment. It is remain unclear whether stroma derived from primary breast cancer tissue can increase breast CSCs population. Therefore, this research was a pilot study to analyze the effect of breast cancer stroma towards breast CSCs population and pluripotency characteristic in vitro. Stroma was identified from primary breast cancer tissues obtained from 2 patients, while breast cancer cells were isolated from pleural effusion of breast cancer patient (BC#1). The BC#1 cells were directly co-cultured with breast cancer stroma (BCS) and also treated by 50% normoxic and hypoxic conditioned medium (CM) of BCS for 48 hours. After that, breast CSCs population using aldefluor assay, mammosphere assay as well as pluripotency characteristic using qPCR were observed. The result exhibited that breast CSCs population was 2.9-fold up regulated after co-culture with BCS, 1.7-fold up regulated after treated by hypoxic CM and 1.5-fold up regulated by normoxic CM. Moreover pluripotency characteristic (OCT-4, SOX-2 and NANOG mRNA expression) were also increased. Hypoxic CM increased more mammosphere number of BC#1 cells compared to normoxic CM. We concluded that stroma derived from breast cancer tissue increased breast CSCs population in which cell to cell interaction confers more CSCs population compared to CM treatment. Meanwhile hypoxic CM also stimulates more breast CSCs compared to normoxic CM. Further research is needed to analyzed secreted molecule from stroma which is responsible in enhancement of CSCs population.

Key Words: breast cancer cells, stroma, cancer stem cells, co-culture, conditioned medium
Introduction

Breast cancer is the most frequent cancer causing death in woman population in the world. Accumulating evidence found that cancer stem cells (CSCs) are responsible for high tumor recurrence, therapy resistance and metastasis (Tang et al, 2007; Dalerba et al. 2007). Therefore, targeted breast cancer stem cells research is really crucial. Cancer stem cells are small-scale sub population of cancer cells which own stemness and tumorigenic. Breast CSCs arise from mammary multipotent stem cells research is really crucial. Cancer stem cells are small-scale sub population of cancer cells which own stemness and tumorigenic. Breast CSCs arise from mammary multipotent stem cells research is really crucial. Cancer stem cells are small-scale sub population of cancer cells which own stemness and tumorigenic. Breast CSCs arise from mammary multipotent stem cells due to genetic defects in pathways controlling self-renewal and differentiation (Shipitsin et al., 2007). Identification of breast CSC could be detected by its surface markers. Breast CSCs express the hyaluronan receptor CD44 (CD44+) and lack of the expression of CD24 (CD24-), an endogenous inhibitor of the chemokine receptor CXCR (Al-Hajj et al, 2003). Other marker for breast cancer stem cells is aldehyde dehydrogenase 1 (ALDH1+) activity. ALDH is an enzyme catalyzed intracellular retinoic acid that connected to cellular differentiation and stem cells protection. ALDH1 is better as a marker for breast CSCs compared to CD44+/CD24- (Tanei et al., 2009).

The behavior of breast cancer stem cells is influenced by their microenvironment. There are complex interactions between breast cancer stem cells with stromal as the component of microenvironment such fibroblasts, mesenchymal stem cells, adipocytes, endothelial cells, and immune cells through growth factor and cytokines networks. Previous study reported that mesenchymal stem cells (MSCs) were recruited from bone marrow and homing around the cancer cells to support tumor growth and metastasis (Chaturvedi et al, 2013). Liu et al elaborated the effect of human bone marrow MSCs toward breast cancer stem cells population in SUM-159 cell line. Those MSCs secrete chemokine which is responsible for increasing ALDH1 positive cells in SUM-159 cell line. They proposed that IL-6 and CXCL-7 loop serve as regulator pathway for breast cancer stem cells self renewal (Liu et al., 2011). Moreover MSCs were already isolated and identified from various cancers such as human gastric cancer, ovarian cancer, including breast cancer and those cells could stimulate proliferation of cancer cells (Cao et al, 2009; Lis et al, 2011; Zhang et al; 2013). However, it still remains unclear whether stromal derived from cancer tissues can induce the enhancement of cancer stem cells. Therefore, this research was a pilot study to analyze the effect of primary breast cancer stroma toward breast cancer stem cells population and pluripotency characteristic.

Material and Methods

Cell culture

Primary breast cancer cells (BC#1) were isolated from metastatic pleural effusion of breast cancer patient as described previously (Tachi et al, 2016). Those cells were cultured in DMEM high glucose medium (Gibco) supplemented with 10 % FBS (Thermo Fisher Scientific), L-glutamine, MEM-non essential amino acid and penicillin/streptomycin at 37°C in a humidified atmosphere of 5 % CO2 and 95 % air. Meanwhile stromal cells were isolated from breast cancer patients (n=2) who undergone mastectomies at University of Tsukuba hospital (Japan) with ethics approval from Tsukuba University. Fresh cancer tissues were cut into small pieces (< 1 mm) and washed with phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS). Afterward, they were treated by 0.1 % collagenase and incubated at 37°C for 1 hour. Dissociation of tissues was also assisted by syringe 20G followed by filtering it into cells strainer 100 µm-sized (BD Falcon) and centrifuged at 1000 rpm to get single cell suspensions. Those cells were floated in 24-well plate contains DMEM high
confer the highest ALDH1 activity). It was performed by diluting CM in freshly DMEM low glucose/10% FBS. Breast cancer cells were cultured with that freshly 50% CM for 48 hours.

**Analysis of pluripotency**

Pluripotency genes (OCT4, SOX2 and NANOG) as well as ALDHI mRNA were analyzed using quantitative Polymerase Chain Reaction/qPCR. First, RNA was extracted from breast cancer cells after treatment with conditioned media and co-culture using Sepasol RNA 1 Super G (Nacalai, Tesque, Kyoto, Japan). cDNA synthesis was performed using Revertra Ace qPCR RT kit (Toyobo). Then, cDNA was amplified using qPCR (Applied Biosystem, Foster City, CA, USA) with Thunderbird SYBR qPCR Mix (Toyobo). PCR cycles (40 cycles) for 10 seconds on 95°C; 30 seconds on 60°C; 30 seconds on 72°C, Melt curve analysis for 1 minutes on 95°C; 1 minutes on 55°C; 10 seconds on 55°C (80 cycles, increase 0.5°C every cycles). The primer sequences are shown in table 1.

**Table 1. Primer sequences (Tachi et al, 2016)**

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<th>Gene</th>
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<th>Antisense</th>
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<td><strong>Human OCT4</strong></td>
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<td><strong>Human SOX2</strong></td>
<td>5’-GATTGGAACCTTGGTCCGAG-3’</td>
<td>5’-CCGGTTATTATAATCCGGGTGCT-3’</td>
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<td><strong>Human NANOG</strong></td>
<td>5’-ACAGAAATACCTCACCTCATCAGGAGCAG-3’</td>
<td>5’-CTCCAGGTTGATTTGTCGAGTGTGC-3’</td>
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**Mammosphere assay**

Approximately 1 X 10⁴ breast cancer cells (BC#1) after normoxic and hypoxic CM-treatment were cultured in ultra low attachment of 6-well plate (Corning) using DMEM high glucose medium (Gibco) supplemented with 10 % FBS (Thermo Fisher Scientific), L-glutamine, MEM-non essential amino acid and penicillin/streptomycine. The mammosphere size ≥ 700 μm was counted under a microscope.

**Statistical Analysis**

All data were presented as means ± SD from triplicate experiments. Statistical analysis was performed using Student’s t test with p < 0.05 as a significant difference.
Results

Morphology of breast cancer cells (BC#1) and stromal cells

Morphology of primary breast cancer cells (BC#1) were epithelial-like cells which had adhered to the plastic surface (figure 1A), while stromal cells appear spindle shape or fibroblastic and also attached to the plastic surface as shown in figure 1B.

Figure 1. Morphology of breast cancer cells (A) and stromal cells (B) were different. Magnification X100.

Characteristics of Stromal Cells

Flow cytometry analysis exhibited that stromal cells expressed positive for CD90, CD73, CD105 but negative for CD31, CD45. Their expression same with mesenchymal stem cells from adipocyte tissue (AT-MSC) as a control (figure 2).

Figure 2. Flow cytometry analysis. A. Stromal cells demonstrated positive for CD90, CD73, CD105, but negative for CD31, CD45. AT-MSC as a control isolated from adipocyte tissue of healthy person.

Differentiation assay also proved that stromal cells were able to differentiate into both osteocytes and adipocytes as shown by positive staining of Oil Red O (figure 3). Therefore, stromal cells resemble mesenchymal stem cells.
Figure 3. Differentiation assay. Stromal cells were capable to differentiate into adipocyte and osteoblast. (AT-MSC: Adipocyte Tissue-Mesenchymal Stem Cells).

Generation of GFP-stromal cells
Stromal cells have been labeled by GFP (as shown in figure 4A) in order to distinguish them with breast cancer cells after co-culture. GFP-stromal cells were sorted (figure 4B) using FACS sorting and plated into 100-mm petri dishes (Sumimoto, Bakelite) for expansion.

Figure 4. A. GFP-stromal cells were seen under a fluorescence microscope. Magnification x200. B. FACS sorting was performed to obtain purified GFP-stromal cells.

Direct co-culture increased breast cancer stem cells population.
We did direct co-culture between GFP-stromal cells and breast cancer cells to evaluate the effect of stroma cells towards breast CSCs. Breast cancer cells were appear surrounded by stromal cells (figure 5A-B). FACS sorting was performed to sort breast cancer population as GFP-negative cells. Afterwards, ALDH1 activity was observed that representing the population of breast cancer stem cells. The results demonstrated that breast CSCs population significantly increased after co-culture as shown in figure 5C.
Figure 3. Differentiation assay. Stomal cells were capable to differentiate into adipocyte and osteoblast. (AT-MSC: Adipocyte Tissue-Mesenchymal Stem Cells).

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Figure 5. Direct co-culture. (A) Morphology of stromal and breast cancer cells during co-culture was seen under light microscope (Magnification X100). (B) Using fluorescens microscope, stromal cells could be seen as a green cell while breast cancer was not seen (Magnification x100). (C) Direct co-culture significantly increased breast cancer stem cells population (p < 0.05)

Direct co-culture increased relative expression of pluripotency gene.
Pluripotency characteristic were observed by detecting relative expression of ALDH1, OCT4, SOX2 and NANOG using qPCR. ALDH1 and OCT4 expression significantly up-regulated in breast cancer cells upon co-cultured with GFP-stromal cells (figure 6A-B). In addition, SOX2 and NANOG mRNA expression were also increased but not statistically significant (figure 6C-D).

Figure 6. Pluripotency characteristic ( * < 0.05; ** p < 0.01)
Discussion

This work was a pilot study to investigate the effect of stromal derived breast cancer tissue toward breast CSCs population. Although there were several researchers who already isolated MSCs from various cancers (Cao et al, 2009; Lis et al, 2011; Zhang et al, 2013), none of them explored the effect of those stromal cells into CSCs population. Nevertheless, Liu et al proved that breast CSCs population in SUM 159 was stimulated by MSCs derived from human bone marrow. They concluded that MSCs derived from bone marrow may promote human breast tumor growth by producing cytokine meshwork that stimulating CSCs population (Liu et al, 2011). Herein, we demonstrated that stromal derived from breast cancer tissues enhanced breast CSCs population detecting by an increase in ALDH positive cells.

ALDH activity was firstly measured by Cheung et al to isolate leukemia stem cells (Cheung et al, 2007). The function of ALDH is to convert retinal into retinoic acid (RA) in which RA play a role in stimulating RAR/RXR gene transcription for differentiation, survival and proliferation (Allahverdiyiev et al, 2012). Accumulating evidences found that ALDH was successfully detected in various cancers, therefore it is postulated as a universal CSC marker (Marcato et al, 2011). Stemness characters were regulated by transcription factor such as OCT4, SOX2 and NANOG that maintain pluripotency. We demonstrated that pluripotency characteristics were also up-regulated in breast cancer cells upon co-culture and conditioned medium treatment. It means that stromal cells support self renewal capacity of breast cancer cells leading to therapy resistance.

Characterization of our stromal cells exhibited that it seems to MSCs. Previous study proved that mesenchymal cells generate interleukin-6 (IL-6), interleukin-8 (IL-8), CCL5 and CXCL5. IL-6 already demonstrated as direct regulator of breast cancer stem cells self renewal through activation of STAT3 (Korkaya et al., 2011). Potter et al also proved that stromal cells secreted CCL2 which stimulate cancer cell migration, tumor growth and neo-angiogenesis (Potter et al, 2012). In our research, cell to cells interaction between stromal and breast cancer cells perhaps are important. According to our results, breast CSCs population increased higher in direct co-culture compared to conditioned medium (CM) treatment. Breast CSCs population were 2.9 fold up-regulated after direct co-culture; 1.7 fold up-regulated after hypoxic CM while only 1.5 fold up-regulated after normoxic CM treatment. Conditioned medium contain secreted factors which affect breast CSCs population. We consider that hypoxic condition in stromal cells may increase the production of secreted factors that can stimulate CSCs growth. In fact, our results exhibited that hypoxic CM treatment also enriched mammosphere number in breast cancer cells. Taken together we concluded that stromal derived breast cancer tissue could increase breast CSCs populations especially by facilitating cell to cell interactions. Further analysis is required to elucidate the mechanism of this process by exploring secreted molecules which is responsible for the enhancement of CSCs. It may provide new strategy for breast cancer therapy.

Acknowledgements

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References


**HASIL PENELITIAN SEJAWAT SEBIDANG ATAU PEER REVIEW**

**KARYA ILMIAH : PROSIDING**

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Novi Silvia Hardiany, Septelia Inawati W, Kana Tachi, Akira Shiraishi, Toshiki Kato, Osamu Ohneda

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Peer review

Prof.Dr.dr. Sri Widia A.Jusman, MS
NIP. 195201251978032002