Dextran sulphate crowding and sodium deoxycholate lysis of primary breast fibroblast cells achieve extracellular matrix deposition and decellularization for breast cancer stem cell culture

Aroem Naroeni,[1] Aulia Ornella[2]

[1]Institute of Human Virology and Cancer Biology, Universitas Indonesia
[2]Magister Student at Molecular Medicine Universitat Tubingen, Stuttgart, Germany

Corresponding address: Aroem Naroeni
Email: anaroeni@yahoo.fr

Received: January 30, 2015; Revised: May 5, 2015; Accepted: May 24, 2015.

Abstrak

Latar belakang: Lingkungan mikro yaitu sel stromal dam matriks ekstraseluler saat ini dinyatakan sebagai kontributor dalam perkembangan tumor. Beberapa penelitian telah mengembangkan matriks ekstraseluler yang mendukung perkembangan sel *in vitro*. Matriks ekstraseluler adalah suatu kompleks susunan supramolekuler dari berbagai macam glycoprotein dan proteoglycan. Matriks ekstraseluler menyediakan integritas jaringan, bertindak sebagai scaffold alami tempat sel melekat dan berinteraksi serta berperan sebagai reservoir pertumbuhan sel. Penelitian ini bertujuan untuk mendapatkan deposisi dan deselularisasi yang optimal pada matriks ekstraseluler.

Metode: Dalam penelitian ini, kami mengembangkan *cells crowder* untuk meningkatkan deposit matriks ekstraseluler dari kultur sel primer fibroblast payudara yang diperoleh dari spesimen hasil operasi *mammoplasty*. Dextran 500 kDa ditambahkan dalam media kultur DMEM lengkap yang telah ditambahkan 0.5% FBS dan 100µM L-ascorbic acid 2-phosphate. Setelah tujuh hari, sel dilisis dengan menggunakan Sodium Deoxycolate (DOC).

Hasil: Deposisi matriks ekstraseluler dan proses deselulerisasi dari sel primer fibroblas payudara dapat terdeteksi dengan menggunakan antibodi Rabbit anti human fibronectin yang selanjutnya ditambahkan dengan anti rabbit IgG yang telah dikonjugasi dengan Alexa Fluor 488.

Kesimpulan: Penambahan *dextran* sulfat dan prosesing lysis dengan *sodium deoxycolate* dapat meningkatkan deposisi dan menghasilkan deselularisasi matriks ekstraseluler. ***(Health Science Journal of Indonesia 2015:1;43-7)***

Kata kunci: matriks ekstra selular, kanker mammae, stem cell, sel fibrob*last*

Abstract

Background: The microenvironment including stromal cells and extracellular matrix (ECM) is now considered an active contributor to tumor progression. Certain studies have developed ECM which supports a suitable cellular growth in vitro. The ECM is a complex supramolecular assembly of a variety of glycoproteins and proteoglycans. Extracellular matrix provides tissue integrity, acts as a native scaffold for cell attachment and interaction and also serves as reservoir for growth factors. The aim of this experiment w asto achieve the deposition and decellularization of ECM.

Methods: Cells crowder have been developed to increase ECM deposit in the primary breast fibroblast cells layer obtained from isolation of single cell from breast mammoplasty specimen. Five hundred kDa dextran was added into DMEM medium containing 0.5% fetal bovine serum (FBS) and 100 µm of L-ascorbic acid 2-phosphate. After seven days, cells were lysed by sodium deoxycholate (DOC). Results were observed in a fluorescence microscope.

Results: Extracellular matrix deposition and decellularization of primary breast fibroblast cells were detected by using extracellular matrix protein, fibronectin via rabbit anti human fibronectin and anti-rabbit IgG conjugated with Alexa Fluor 488.

Conclusion: Dextran sulphate increased extracellular matrix deposit in primary breast fibroblast cell layer and the treatment with sodium deoxycolate lysed cells resulted in extracellular matrix decellularization. ***(Health Science Journal of Indonesia 2015:1;43-7)***

Keywords: extracellular matrix, breast cancer stem cell, breast fibroblast cell

Until to date, it is not clear yet whether breast cancer stem cells could be the initiators of breast cancer cells and could be the results of stem cells/progenitor transformation or originate from certain tumor cells which lost their ability to differentiate and have similar characteristics as stem cells, e.g., their ability to self-renewal.[1] Breast milk gland consists of a branching ductal system that ends in terminal ducts with their associated acinar structure, the terminal ductal-lobular units together with interlobular fat and fibrous tissue. Most breast cancers arise in the terminal ductal-lobular units.[2] Recent data from La Barge *et al.*[3] suggest that progenitor cell fate decisions are dictated by a dynamic and reciprocal relationship existing between stem cell genomes and their environments. As the development of normal mammary glands is dictated by the microenvironment that includes the extracellular matrix and cellular components, such as immune and inflammatory cells, blood vessel cells and fibroblasts, it can also play a significant role in overall cancer development[4,5] It has been hypothesized that mutated mammary stem cells are the origin of breast cancer.[6,7] Carcinoma-associated fibroblasts and their corresponding adjacent counterparts have shown different genetic alterations that could play roles in breast carcinogenesis.[8]

Even though the presence of cancer stem cell subpopulations had been known, the clinical significance of this finding could not be described clearly yet. The major hypothesis of cancer stem cell models is that the clinical condition should strongly depend on the cancer stem cell population, either quantitatively (relative and absolute numbers of cancer stem cells) or qualitatively (associated with the biological characteristics of cancer stem cells). [9] Bao et al.[10] explained the functional implication of heterogenic tumor cell populations and the identification of functional therapy targets for eradication of severe brain cancer. The failure of radiotherapy is caused by the lack of ability to disseminate $CD133^+$ cell populations which initiate tumor.[10]

To date, growing cells in plastic flasks with liquid media for *in vitro* study is very common but far from physiological growth. The use of scaffold materials from synthetic to nature-derived materials as "collagen", fibrin, chitosan and glycosaminoglycans are more biocompatible but remain simplified copies of the complex extracellular milieu in living tissue. The extracellular matrix is a complex supramolecular assembly of a variety of glycoproteins and proteoglycans. Extracellular matrix provides tissue integrity, acts as a native scaffold for cell attachment and interaction, and also serves as a reservoir for growth factors[11]. Macromolecular crowding is a biophysical principle in which inert macromolecules in solution occupy a significant volume of the medium and hence impact on biochemical reactions both *in vivo* and *in vitro* [12].

In this research, we developed extracellular matrix deposition and decellularization to grow breast cancer stem cells and to study further the role of microenvironment in breast cancer stem cell initiation, tumor progression, protein implication into this process etc.

METHODS

Sample collection

Tissue samples were collected from reduction mammoplasty patients in Bina Estetika Clinic. With the patients' written informed consent. The study was approved by The Ethic Committee of Faculty of Medicine, Universitas Indonesia. Culture medium containing antibiotics was added into the tube containing samples prior to transportation and processing in the laboratory.

Isolation and culture of primary breast fibroblast cells

Tissues from reduction mammoplasty patients were chopped and digested with collagenase IV 0.1% (w/v) and incubated at 37°C. After 2.5- 3 hours of incubation, specimens were dissociated mechanically by using 10 ml serological pipets for 15-20 minutes. Afterwards, specimens were filtered by using a nylon filter 40 um and washed twice by using PBS. Cells obtained were cultured in DMEM High Glucose containing 10% FBS and 1% Penistrep. After more than two weeks, multiple types of epithelial cells and fibroblasts emerged; fibroblasts were removed by differential trypsinization (fibroblasts are loosely attached)

Fibroblast culture under mixed macromolecules crowding

Fibroblasts were seeded on 24-well plates at 50,000 cells per well in 10% FBS, 5% CO2 at 37°C. After 16 h, cells were cultured under crowded conditions by adding 500 kDa dextran sulphate (DxS) (100ug/ml) in Dulbecco's medium containing 0.5% FBS, 100um of L-ascorbic acid 2-phosphate. Cells were cultured under this condition for 72 hours.

Cell lysis and matrix decellularization

After culture under crowded condition, cells were lysed by using sodium deoxycholate (DOC). First, cells were washed three times with PBS and then incubated in 0.5% DOC solution + 0.5X protease inhibitor on ice for 10 minutes. This process was performed twice. Afterwards, cells were incubated in 0.5% DOC solution without 0.5X protease inhibitor and washed three times with PBS. Extracellular matrix was then detected by using immunofluorescence or applied for culturing breast cancer stem cells.

Immuofluorescence

Extracellular matrix formation was detected by using intracellular marker protein phalloidin for labelling F-actin, extracellular matrix marker protein, fibronectin, and DAPI to stain nuclei. After fixation with 4% paraformaldehyde for intracellular matrix, cells were permeabilized with 0.2% Triton and blocked with 3% bovine serum albumin in PBS. Phallodin and DAPI were incubated for 30 min and washed three times with PBS. For detecting extracellular matrix, cells were fixed with methanol and blocked with 3% bovine serum albumin in PBS. Primary antibody rabbit anti-human fibronection was incubated for 1.5 h followed by washing three times with PBS. Secondary antibody, chicken anti-rabbit IgG conjugated with Alexa Fluor 488 and 4',6-diamino-2 phenylindoldilactate (DAPI) were incubated 30 minutes and washed three times with PBS. Plates were observed in fluorescence microscope.

RESULTS

Primary breast fibroblasts were obtained after cell isolation. Cell growth was very slow and covered the T25 flask after 3 weeks of culture. Fibroblasts were selected by trypsinization and subcultured until confluent and reaching the number of cells needed for experiments (Fig. 1).

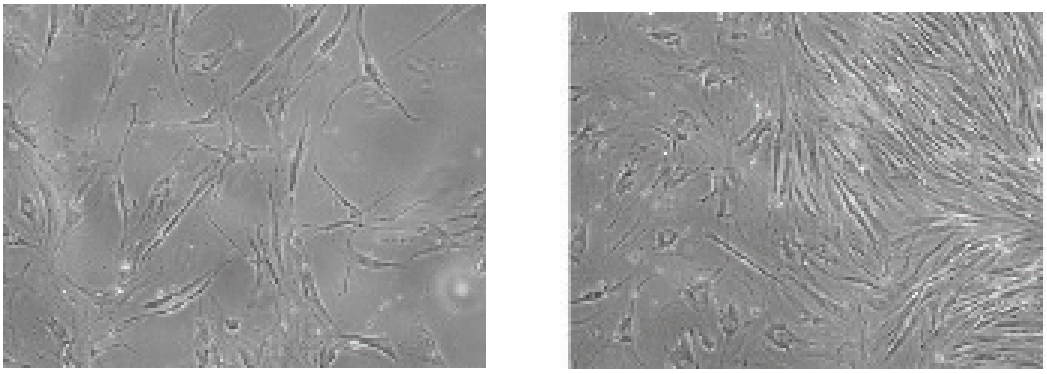

Figure 1. Culture of primary breast fibroblast cells after trypsinization; magnification 20x.

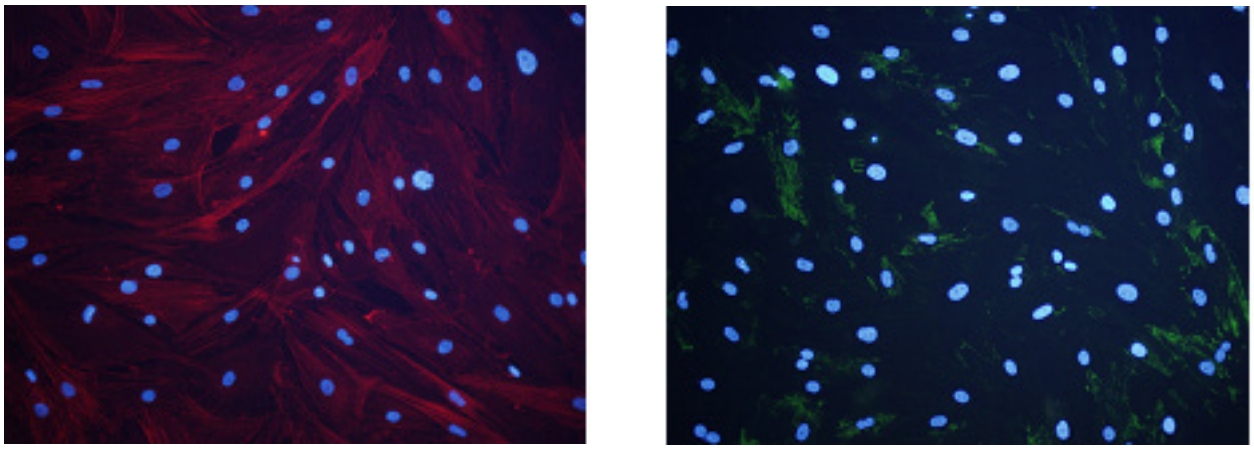

Figure 2. Primary breast fibroblast cell layer without crowder. Red: actin with phalloidin staining, green: fibronectin with primary antibody rabbit anti-human fibronectin and secondary antibody anti-rabbit conjugated with Alexa Fluor 488 staining, blue: nuclei with DAPI staining.

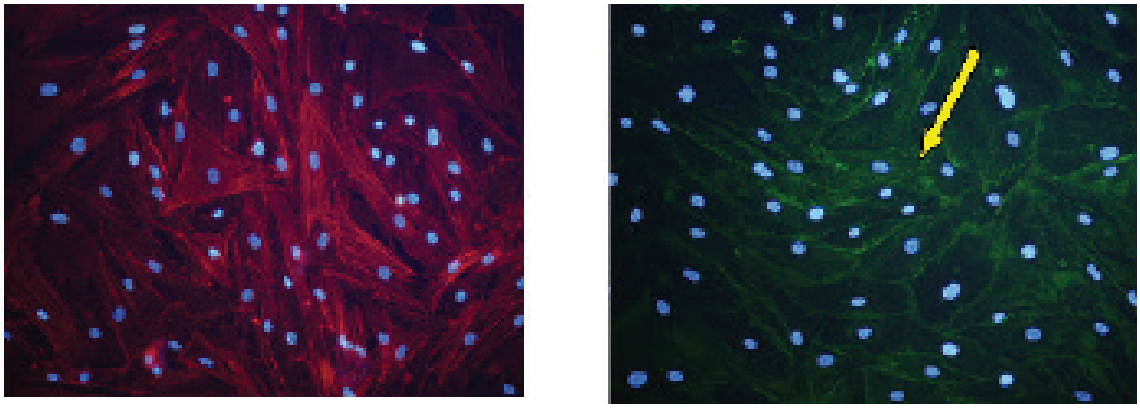

Figure 3. Primary breast fibroblast cell layer with dextran sulphate (DxS) crowder; red: actin with phalloidin staining; green: fibronectin with primary antibody rabbit anti-human fibronectin and secondary antibody anti-rabbit conjugated with Alexa Fluor 488 staining; blue: nuclei with DAPI staining; yellow arrow: deposition of extracellular matrix.

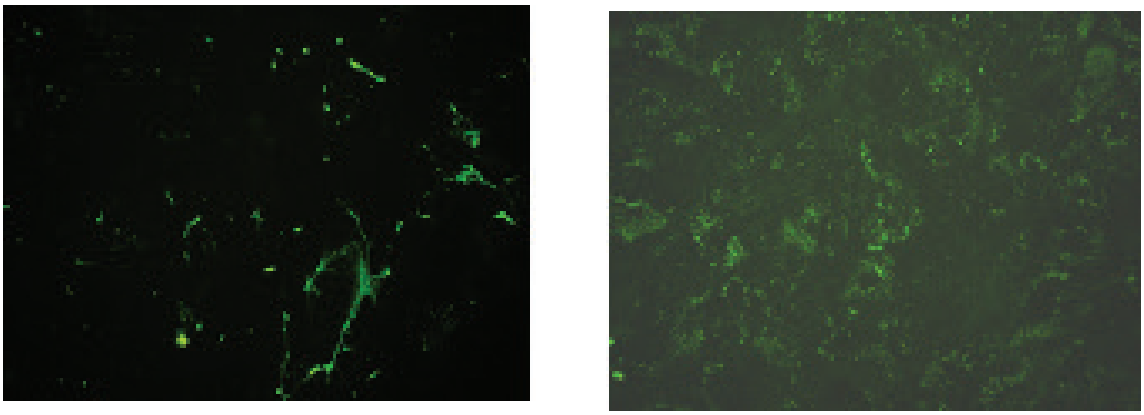

Figure 4. Extracellular matrix decellularization after sodium deoxycholate lysis (DOC) without DxS crowder (a) and with DxS crowder (b)

After being cultured in crowded condition by using 500 kDa dextran sulphate (DxS) 100 ug/ml, deposition of fibronectin was observed in extracellular matrix region (Fig. 3).

Cell layers were lysed by sodium DOC treatment. Extracellular matrix was detected by the presence of fibronectin protein and the absence of nuclei and actin via phalloidin marker (Fig.4).

DISCUSSION

Regenerative medicine brings new hope and new promise for regenerating damaged tissues and organs in the body by stimulating previously irreparable organs to heal themselves. This regenerative medicine empowers scientists to culture and propagate stem cells as ~~an~~ important resources for regenerative medicine. In addition, the new theory of the presence of cancer stem cells gives more research in the development of stem cell cultures.[13] Studies on stem cell cultures reported that various media and growth factors are under development to obtain suitable conditions for stem cells propagation and to maintain their pluripotence. Stem cells had been grown with various methods using cell feeder layers, extracellular matrices, conditioned media, various growth factors and many more.[14]

Primary breast fibroblast cells are senescent.They can grow for three to four passages or about 15-20 population doublings and then undergo senescence[2]. In this experiment, after 4 subcultures cells still continued to grow normally. The addition of 500 kD DxS our culture increased the deposition of extracellular matrix between cells. After decellularization using DOC, the natural scaffold had been formed, ready to be used as medium for culturing stem cells. It has been reported that

the addition of 500 kDa DxS, a negative-charged molecule reduced procollagen in the medium and increased dramatically the amount of collagen associated with the cell layer.[15]

This extracellular matrix decellularization will be useful for growing breast cancer stem cells because it is mimicking their natural microenvironment *in vivo*. It will be also useful for studying the role of microenvironment in breast cancer stem cell initiation, tumor progression, protein implicating in this process etc. This understanding will be useful to find the effective method to eliminate breast cancer stem cells and ~~agbvbvb void~~ the relapse of breast cancer after chemotherapy and radiotherapy. This method also provides a good microenvironment for developing media for stem cell propagation which is now needed for regenerative medicine.

avoid

In conclusion, Dextran sulphate (DxS) 100 ug/ml used as crowder increased extracellular matrix deposit in primary breast fibroblast cell layer. The treatment with sodium Deoxycolate (DOC) 0.5% lysed cells and resulted in extracellular matrix decellularization that could serve as native scaffold for cell attachment and interaction and also as reservoir for growth factors for culturing stem cells, cancer stem cells and studying the role of microenvironment in breast cancer stem cell initiation, tumor progression, protein implicating in this process, etc.

REFERENCES

1. Smalley M, Ashworth A. Stem cells and breast cancer: A field in transit. Nat Rev Cancer. 2003;3:832-44.
2. Dimri G, Band H, Band V. Mammary epithelial cell transformation : insight from cell culture and mouse models. Breast Cancer Research. 2005;7: 171-9.
3. La Barge MA, Nelson CM, Villadsen R, et.al. Human mammary progenitor cell fate decisions are products of interactions with combinatorial microenvironments. Integr Biol (Camb). 2009;1:70-9.
4. Mueller MM, Fusenig NE. Friends or foes - bipolar effects of the tumor stroma in cancer. Nat Rev Cancer. 2004;4**:**839-49.
5. Kalluri R, Zeisberg M. Fibroblasts in cancer. Nat Rev Cancer. 2006;6**:**392-401.
6. Al-Hajj M, Wicha MS, Benito-Hernandez A, et al. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA. 2003;100:3983-8.
7. Boulanger CA, Bruno RD, Rosu-Myles M, et al. The mouse mammary microenvironment redirects mesoderm-derived bone marrow cells to a mammary epithelial progenitor cell fate. Stem Cells Dev. 2012;21:948-54.
8. Hawsawi NM, Ghebeh H, Hendrayani SF, et.al. Breast carcinoma-associated fibroblasts and their counterparts display neoplastic-specific changes. Cancer Res. 2008;68**:**2717-25
9. Tang DG, Patrawala L, Calhoun T, et.al**.** Prostate cancer stem/progenitor cells: identification, characterization, and implications. Mol Carcinog. 2007;46:1-14.
10. Bao S, Wu Q, McLendon RE, et.al**.** Glioma stem cells promote radioresistance by preferential activation of DNA damage response. Nature. 2006;444:756-60.
11. Peng Y, Raghunath M. Learning from nature: emulating macromolecular crowding to drive extracellular matrix enhancement for the creation of connective tissue *in vitro.* Tissue Engineering. 2006;288:104-8.
12. Minton, AP. The influence of macromolecular crowding and macromolecular confinement on biochemical reactions in physiological media. Journal of Biological Chemistry. 2001;276:10577-80.
13. Jordan TJ, Guzman ML, Noble M. Mechanisms of disease: Cancer Stem Cells. The New England J Medicine. 2006;355:1253-61.
14. Ulloa**-**Montoya F, Verfailie CM, Hu WS. Culture System for Pluripotent Stem Cells. J Biosci and Bioengin. 2005;100:12-27.
15. Lareu, RR, Arsianti I, Subramhanya HK, et al**.** In vitro enhancement of collagen matrix formation and crosslinking for applications in tissue engineering: a preliminary study. Tissue Eng. 2007;13**:**385-91.