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Doxorubicin Induces Lamellipodia Formation and Cell Migration

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Abstract

Breast cancer is the main cause of cancer death among women, especially breast cancer metastasis. Metastasis process begins with the ability of cell cancer invasion. Doxorubicin, a antracycline chemotheraphy, is known to induce TGFβ1, thus promote invasion. The aim of this study is to optimize doxorubicin doses to induce lamellipodia formation in 4T1 and MCF-7/HER2 cells. Lamellipodia formation was observed by morphological changes using microscope inverted. The effect of doxorubicin on cell viability was analyzed using MTT assay. Rac1 expression after doxorubicin exposure was determined by western blotting. Lamellipodia formation was observed by morphological change of the cell at the dose 10, 25, 50 and 100 nM. Doxorubicin at the dose of 10 nM could induced lamellipodia formation without affect cell viability in both 4T1 and MCF-7/HER2 cells. Doxorubicin induced cell cycle arrest at G2/M phase at all doses. Doxorubicin 10 nM also decrease Rac1 expression compared to control.

Keywords: Doxorubicin, lamellipodia, Rac1, migration.

INTRODUCTION

Metastasis, one of hallmark of cancer, is spreading of cancer cell from primary site to distant site. Therapy of cancer metastasis becomes challenging and metastasis is the main cause of cancer death among patient. Metastasis process begin with local invasion, cells detached from primary site, increase motility and release proteolytic enzyme to degrade extracellular matrix, such as serine, cysteine, and metalloprotease (Verma and Hansch, 2007). Metastasis was characterized by loss of E-cadherin, a molecule involved in cell adhesion (Hanahan and Weinberg, 2011). Cell migration depend on cell polarization and lamellipodia formation via Rac1 cascade. Rac1 activation induce actin polymerization and increase cell motility (Arpaia, *et al*., 2012).

Doxorubicin is widely used as chemotherapy in breast cancer. Long-term use of doxorubicin cause some side effects leading to reduce chemotherapy effectivity. In addition, doxorubicin also induced TGFβ1 signaling activity in triple negative receptor breast cancer cell type and MDA-MB 231. Doxorubicin induced epithelial-mesenchymal transition (EMT) and initiate invasion via TGFβ1 signaling. Thus, doxorubicin could induce cell migration to distant site. On the other hand, activation of TGFβ1 signaling also generate stem-like breast cancer cell and increase drug resistance (Bandyopadhyay, *et al*., 2010). The aim of this study is to optimize doxorubicin doses to induce lamellipodia formation on 4T1 and MCF-7/HER2 cells.

METHODS

Cell Culture

Breast cancer cell line culture type 4T1 (originally from ATCC^{R}-CRL-2539^{TM}) and MCF7/HER2 were obtained from Prof. Masashi Kawaichi (Nara Institute of Science and Technology, NAIST, Japan). The cells were maintained in Dulbecco's Modifies Eagles medium (DMEM) high glucose (Sigma) supplemented with 10% FBS (Sigma), HEPES, sodium bicarbonate, 1.5% Penicilin-Streptomycin and 0.5% Fungizone (Gibco). Cells were cultured with 5% CO_2 in 37^{o}C.

Doxorubicin Sample Preparation

Doxorubicin was dissolved in DMSO (Sigma), and freshly diluted in culture medium in several concentrations before used.

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Morphologycal Lamellipodia Formation

Murine 4T1 cells were treated with doxorubicin (10, 25, 50, and 100 nM) for 96 hours. The medium with doxorubicin was changed every alternate day. Representative pictures of lamellipodia formation detected by inverted microscopy (x100 magnification) (Bandyopadhyay, *et al*., 2010).

Proliferation Assay

Approximately $2x10^3$ 4T1 cells/well and MCF-7/HER2 $2.5x10^3$ 4T1 cells/well were seeded in 96-well plates and incubated for 24 hours. Cells were treated with increasing concentration of doxorubicin (10, 25, 20, and 100 nM) for 24, 48, and 72 hours. Cultured medium was removed and cells were washed with PBS (Sigma). MTT 0.5 mg/mL in medium were added into each well and incubated for 3-4 hours. MTT reaction was stopped by the addition of SDS 10% in HCl 0.01 N, and incubated overnight in the dark room. The absorbance was measured using ELISA reader at λ 595 nm (Merck ELISA reader). Each treatment were carried out in triplicate, and the absorbance data were provided as percent viability compared to control cells (untreated) (Mosmann, 1983).

Cell Cycle Analysis

The 4T1 cells ($7.5x10^4$ cells) were seeded in 6-well plate and incubated for 24 hours. Cell were treated with doxorubicin 10, 25, 50 and 100 nM for 24 hours After the treatment, cells were harvested by trypsinization and stained with the staining solution contains propidium iodide (PI) 1 mg/mL protease inhibitor, 10 mg/mL RNAse and 0,1% (v/v) Triton X-100 (Merck). Cells were incubated for 5 minutes in the dark room, transferred into a flow cytometric tube and analyzed by BD FACS Calibur (BD Bioscience, USA). Cell cycle distribution was acquired by using Flowing software.

Western Blot

Approximately $4x10^3$ 4T1 cells and $3x10^3$ MCF-7/HER2 cells were seeded in 6-well plates, and incubated for 24 hours. Cell were treated with doxorubicin 10, 25, 50 and 100 nM for 48 hours. Protein was extracted using radio immune precipitation assay (RIPA) buffer (Tris HClpH 7.6, NP 40, Na-deoxycholate, NaCl, SDS, phenyl methyl sulfonyl flouride (PMSF), NaF, and cocktail inhibitor protease, then separated in 14% acrylamide gel by SDS-PAGE electrophoresis. After transferring to polyvinylidene fluoride (PVDF) membrane, the membrane was incubated overnight at 4°C with either the mouse monoclonal antibody against Rac1 (Santa Cruz cs22475) or β-actin (Santa Cruz sc-47778). After incubation with secondary antibody anti-mouse (Santa Cruz sc-516102) for 1 hour, the protein bands were visualized using ECL (Amersham) and detected using Luminograph. The relative protein levels were calculated in reference to the amount of β-actin protein.

RESULTS

The Effect of Doxorubicin in Cells Proliferation

The cytotoxic drug doxorubicin is a well-known chemotherapeutic agent which is used in treatment of a wide variety of cancers inducing intracellular ROS accumulation, cell cycle arrest and apoptosis. However, doxorubicin treatment is often hampered by severe side effects and resistance of cancer cells to therapy. To determine the effect of doxorubicin in inducting lamellipodia formation on 4T1 and MCF-7/HER2 cells, firstly we examined the cytotoxic effect of doxorubicin at different concentrations (0, 10, 25, 50 and 100 nM) for 0, 24, 48 and 72 hours by MTT assay. As shown in Fig. 1, doxorubicin demonstrated a cytotoxic effect on 4T1 and MCF-7/HER2 cells. Doxorubicin reduced the viability on both of cells in a dose-dependent and a time-dependent manner.

Effect of Doxorubicin on Cell Cycle Modulation

In order to investigate the mechanism of physicological changes focusing on cell cycle, the cells were processed for flowcytometry by PI staining. Our study showed that cell proliferation was affected by doxorubicin treatment with concentration dependent manner. Furthermore, cell cycle analysis was elaborated to understand the modulation of cell proliferation through cell cycle alteration. Here, we found that doxorubicin treatment conducted cell to perform G2/M arrest and increase accumulation of sub-G1 phase cells (Fig. 2). The highest effect of cell cycle alteration showed in 25 nM doxorubicin treatment in both phase. Interestingly, the cell percentage of each phase was different. Whereas, the 10 nM of doxorubicin gave similar proliferation effect, similar to the control. Hence, it needed to explore further about the morphological alteration due to different concentration of doxorubicin.

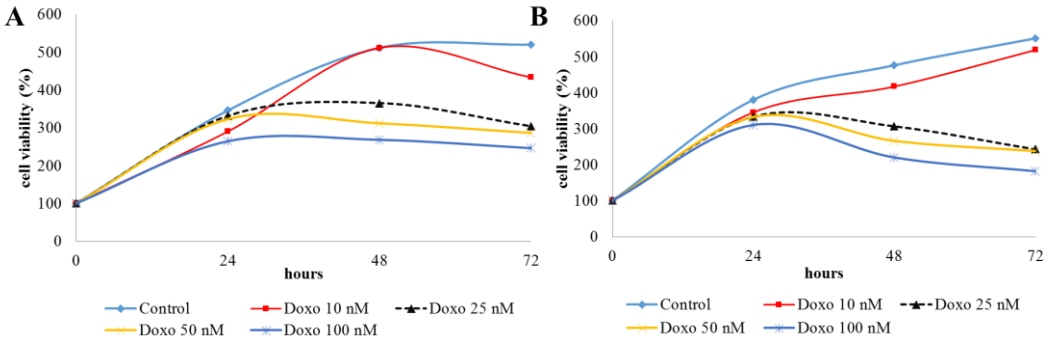

Figure 1. Proliferation effect of Doxorubicin (Doxo) on cell viability. A. Proliferative assay of Doxo 10, 25, 50, and 100 nM on 4T1 cells. B. Proliferative assay of Doxo 10, 25, 50, and 100 nM on MCF-7/HER2 cells. Proliferative assay was determined by MTT assay as described in the methods. Cell was treated with various concentration of Doxo for 24, 48, and 72 hours. Cell viability after treatment was compared to baseline. Each experiment was conducted in triplicate. Mean values from the three experiments ± standard error of mean are shown.

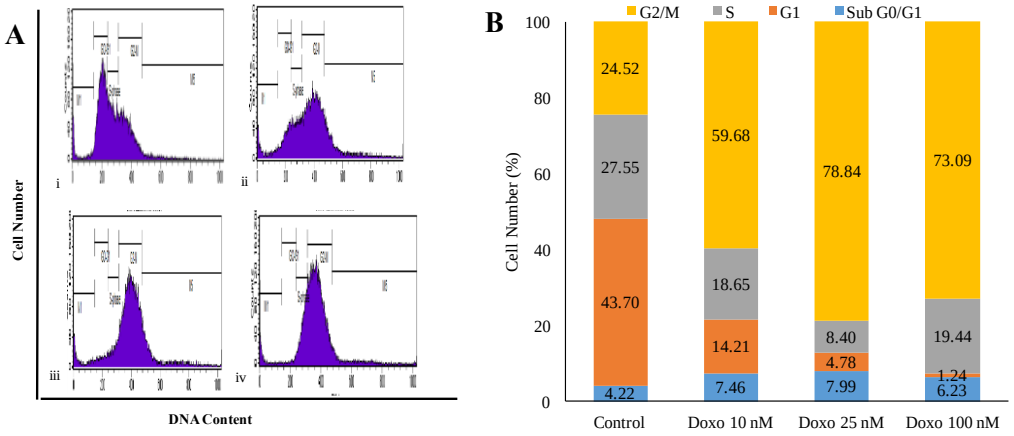

Figure 2. Effects of Doxorubicin on Cell Cycle Modulation on 4T1 Cells. Cells were harvested after 24 h of treatment, stained with propidium iodide and DNA content were analyzed by using flowcytometry. (A) The profiles of cells in the phases of sub-G1, G1, S and G2-M (i) control cells, (ii) doxorubicin 10 nM, (iii) doxorubicin 25 nM, (iv) doxorubicin 100 nM. X-axis showed the relative content of DNA and the Y axis showed the relative cell numbers. (B) Quantification of cells distribution in each phase (Sub G0/G1, G1, S, and G2/M phase) of the various treatments. The percentage of sub-G1 phase cells showed apoptotic cell population.

Effect of Doxorubicin on Lamellipodia Formation

Lamellipodia formation is beginning step of cell migration, thus effect of doxorubicin on lamellipodia formation was observed. The cells were treated using doxorubicin at the dose of 10, 25, 50, and 100 nM. Lamellipodia formation was observed at 0, 24, 48, 72, and 96 hours after treatment (Fig. 3). Change of cell morphology also observed. Lamellipodia of 4T1 cells was observed even at lowest doses (10 nM) after 24 hours treatment. Meanwhile lamellipodia of MCF-7/HER2 cells was observed at the doses of 25 nM after 24 hours treatment. Prolong exposure of doxorubicin on both cells after 96 hours treatment affect cell morphology leading to cell death.

Effect of Doxorubicin on Rac1 Expression

Rac1 is protein involved in rearrangemnet of actin and lamellipodia formation. To understanding whether lamellipodia formation were affected by Rac1 expression, thus Rac1 expression was observed. Rac1 expressions of 4T1 and MCF-7/HER2 after treatment with doxorubicin 10 nM was examined using western blot. doxorubicin 10 nM reduced Rac1 expression in both 4T1 and MCF-7/HER2 cells (Fig. 4). Due to this result, we understood that the formation of lamellipodia was different on both cell yet the treatment of 10 nM doxorubicin brought similar effect on Rac1 expression.

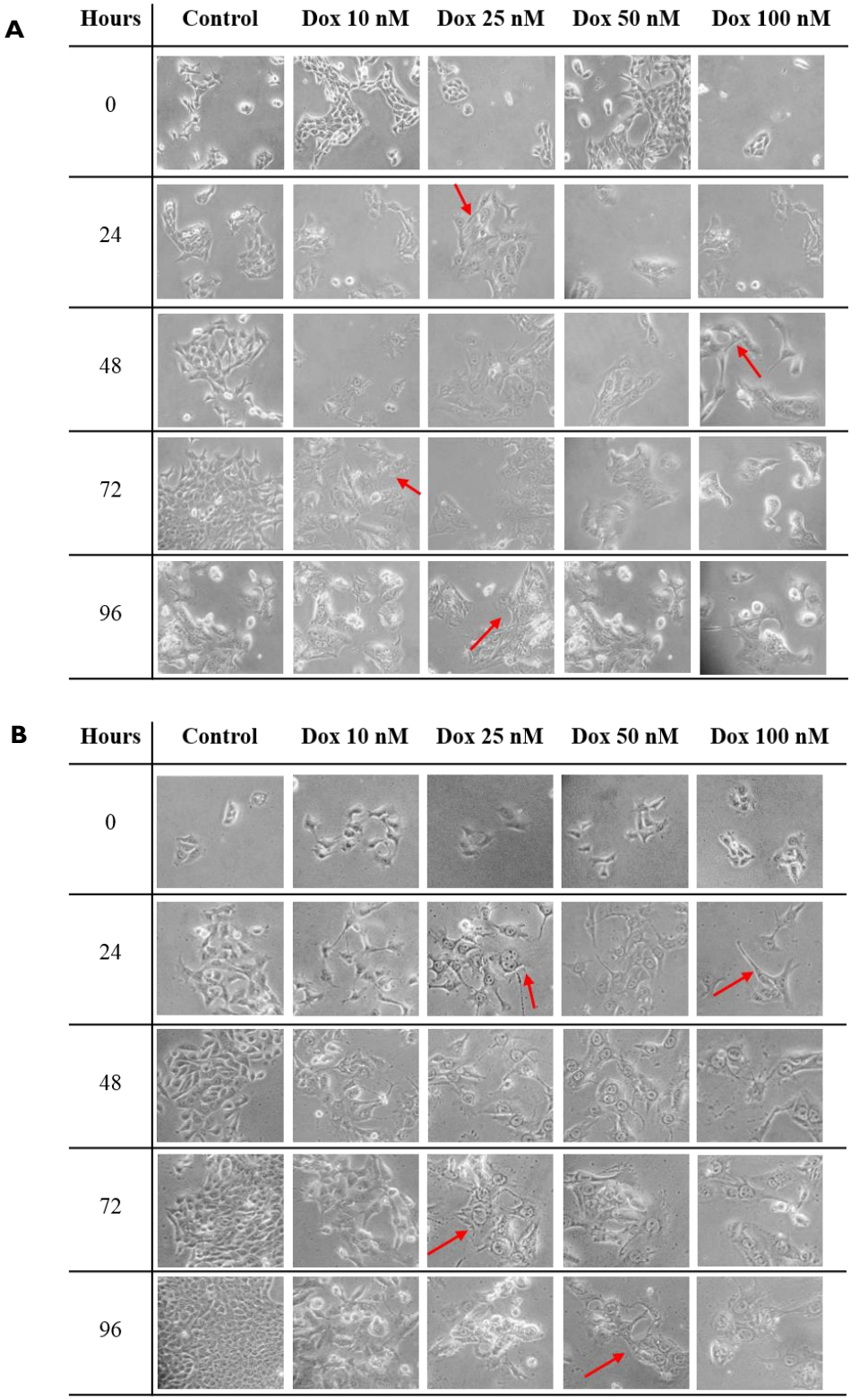

Figure 3. Induction of lamellipodia formation by Doxorubicin (Dox). (A) on 4T1 cells, (B) on MCF-7/HER2 cells. Representative pictures of 4T1 cells with mesenchymal morphology after treatment with untreated cells, Dox 10 nM, Dox 25 nM, Dox 50 nM and Dox 100 nM. Shown is confocal microscopy of the lamellipodia (100x magnification). Arrows indicate cells morphological changes.

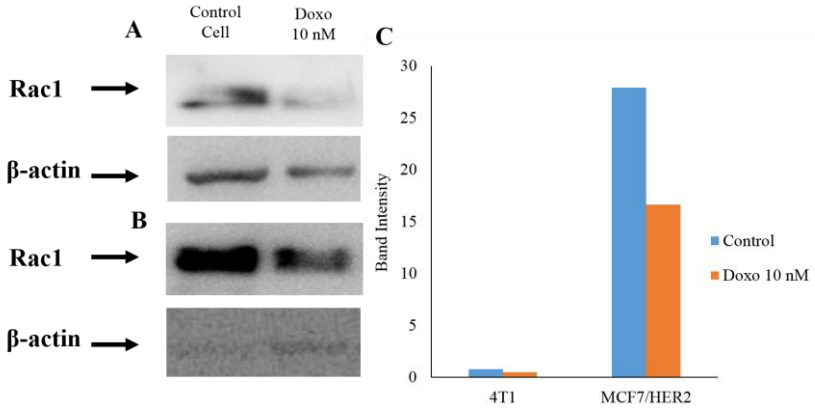

Figure 4. Rac-1 Protein Expression under Doxorubicin (Doxo). (A) 4T1 cell and (B) MCF-7/HER2 after treatment with Doxo 10 nM. Expression was compared between control (untreated) and Doxo treatment. Rac1 expression was observed with western blot shown different expression between groups. The levels of Expression were normalized by comparing with β-actin expression level

DISCUSSION

Doxorubicin is usually for first-line treatment of several type cancer. Drug chemosensitivity have been investigated in the several models of breast cancer cell such as T47D, WiDr colon cancer cells, MCF-7, MCF-7/Dox and showed various cytotoxic effects (Febriansah, *et al.*, 2014; Hermawan, *et al.*, 2010; Putri, *et al.*, 2016). This study explored the effect of doxorubicin on triple negative breast cancer cell, 4T1, that is highly metastasis in breast and HER2-overexpresed cell, MCF-7/HER2. The results of our cytotoxicity assay, doxorubicin inhibited cell growth in a dose-dependent and a time-dependent manner on 4T1 and MCF-7/HER2. Previous study reported the cytotoxic mechanism of doxorubicin was through disruptions topoisomerase II, resulting in DNA damage and cell death (Rowan, 1979).

Based on the result of cytotoxic effect of doxorubicin. This present study was continued to investigate possibility pathway in growth cells inhibition through cell cycle modulation. This study showed that doxorubicin inhibited 4T1 breast cancer cell growth through cell cycle modulation. Three concentration of doxorubicin caused cell accumulation in G2/M phase. Similar results were performed by previous studies that had been reported Putri, *et al* (2017) which showed doxorubicin could inhibit the growth of T47D cells by an accumulation of cells at the G2/M phase.

In this present study inhibition of cancer metastasis after treatment of doxorubicin was also observed. Cell migration is part of the process of metastasis. Interestingly, prolong use of doxorubicin exhibit several limitation such as toxicity and cancer cell resistance. On the other hand, doxorubicin activates TGF-β signalling in breast cancer cells which is increasing of TGF-β involved in mallignancies, such as metastatic breast cancer progression and survival. These also induce cancer cell migration and invation. Activation of TGF-β also induce Ephitelial-Mesencymal transition that increase cell motility and invasiveness (Bandyopadhyay, *et al*., 2010). The ability of cell to migrate involved formation of lamellipodia. The result of this study showed that low doses of doxorubicin could induced lamellipodia formation. Doxorubicin at low doses induced lamellipodia formation both in 4T1 and MCF-7/HER2 cell observed begin at 24 hours after treatment. This phenomenon was caused by doxorubicin ability to activate TGF-β leading to activation of Smad2 and Smad3. Increasing of Smad2 and Smad3 induce EMT, thus enhanced cell migration. In addition, activation of Erk MAP kinase, Rho GTPase and PI3 kinase/Akt pathway followed by TGF-β also induced cell growth, migration, and invasion (Xu, *et al*., 2009). Extension of lamellipodia was needed by cell to move on certain direction. Thus, observation of lamellipodia formation can be used as parameter to observed cell migration.

Rac1 is family of Rho GTPase protein that involved in cellular dynamic, particularly lamellipodia that initiate migration of cell (Ehrlich,

et al., 2002). Nevertheless, Rac1 is not the only protein that regulate actin structures, RhoG or Cdc42 also play role in these structure (Steffen, *et al.*, 2013). In this study, doxorubicin reduce Rac1 expression of 4T1 and MCF-7/HER2 cells. These result indicate that lamellipodia formation on those cell did not dependent to Rac1 expression. Rab5, a member of Rab family of GTPase, triggered lamellipodia formation independent with receptor tyrosine kinase/Ras/PI3-K/Rac pathways (Spaargaren and Bos, 1999). However, further study need to be conducted to confirm whether this phenomenon involved Rab5 activity.

CONCLUSION

Doxorubicin could inhibits cells growth by induced G2/M cell cycle arrest and the other hand doxorubicin induce epithelial mesenchymal transition through Rac1 independent-lamellipodia formation.

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