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Epidermal Growth Factor and Adenosine Triphosphate Induce Natrium Iodide Symporter Expression in Breast Cancer Cell Lines

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Abstract

AIM: This study aims to investigate the effect of ATP, EGF and combination of those two to the Natrium Iodide Symporter (NIS) expression in MCF7, SKBR3 and HaCaT cell lines.

METHODS: MCF7, SKBR3 and HaCaT cell lines were treated with ATP, EGF and combination of those two for 6, 12 and 24 hours. The expression of NIS mRNA was measured through quantitative-reverse transcription-polymerase chain reaction (qRT-PCR). The NIS protein expression was confirmed by immunocytofluorescence.

RESULTS: NIS mRNA was expressed in SKBR3 and HaCaT cell lines but not in MCF7. The levels of NIS mRNA expression, after treatment by epidermal growth factor (EGF), adenosine Tri-Phosphate (ATP) or the combination of both for 6 and 12 hours were not significantly different from those of untreated cells. However, the treatment by a combination of ATP and EGF for 24 hours increases the level of NIS mRNA expression by 1.6 fold higher than that of the untreated cells (1.6241 ± 0.3, $p < 0.05$) and protein NIS expression increase significantly by the treatment than untreated cells ($P < 0.05$).

CONCLUSION: The level of NIS expression varies among the different subtypes of breast cancer cell lines. MCF7 cell line is representing the luminal A subtype of breast cancer does not express NIS. Only SKBR3 cell line express NIS and this subtype might be suitable to receive radioiodine therapy as those cells expressing NIS. A combination treatment of EGF and ATP increases the expression of NIS mRNA and protein at the membrane in SKBR3 cells.

Introduction

Natrium Iodide Symporter is a plasma membrane glycoprotein. It transports two ions of sodium (Na^+) and one of iodide (I^-) and maintained by Na^+/K^+ ATPase [1], [2], [3]. In thyroid cells, NIS plays a pivotal role to accumulate iodine. Additionally, NIS is also expressed in breast cancer tissue and has been considered as a potential target for radioiodine therapy. However, NIS expression in breast cancer tissues had been reported to be varied between studies.

Furthermore, the molecular mechanism of NIS expression in breast cancer remains unclear [4]. Interestingly, it has been reported that NIS is highly expressed in invasive breast cancer tissues. A study by Tazebay *et al*. reported that indeed, there is a correlation between NIS expression and malignant transformation of human breast tissue [5], [6].

Other than the variation of NIS expression level between molecular subtypes of breast cancer, the location of NIS expression is another factor that may influence the uptake of radioiodine by breast cancer cells. Natrium Iodide Symporter supposed to be localised at membrane cell instead of in the cytoplasm to be fully functional as iodine co-transporter [7], [8]. Several agents were used to increasing NIS expression in breast cancer cells, and the effect of those agents had been proven could increase radioiodine uptake and cells susceptibility toward the treatment. A study reported, EGF

increased NIS expression at membrane cell in the T47D cell line [9]. Another study reported that a combination of ATP with all-trans-Retinoid Acid (tRA) and hydrocortisone had increased NIS protein expression and cell membrane targeting in MCF7 cells [10].

In this study, we examined the effect of EGF and ATP for NIS mRNA and protein expressions in SKBR3 and MCF7 breast cancer cell lines and HaCaT a normal cell line. MCF7 and SKBR3 cell lines are representing the luminal A and HER2 type of breast cancer, respectively [11]. HaCaT cell line consists of normal human keratinocyte cells. It is used as a control cell line.

Material and Methods

Cell lines and culture conditions

SKBR3, MCF7 and HaCaT cell lines were used in this study. SKBR3 was obtained from the American Type Culture Collection (ATCC). MCF7 and HaCaT cell lines from faculty of Medicine, Universitas Padjadjaran, Bandung-Indonesia. MCF7 and HaCaT cells were cultured in RPMI 1640 medium (Sigma-Aldrich). SKBR3 was cultured in McCoy's 5A medium (Sigma-Aldrich). Both cell culture mediums were supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1% Penicillin, 1% Streptomycin and 1% amphotericin B. The cells were incubated at 37° C and supplied with 5% of carbon dioxide (CO_2) until 80% confluence. The cells were incubated in serum-free medium overnight and followed by treatment of EGF 50 ng/ml (Abcam #ab9697), ATP 100μM (Sigma-Aldrich # 1388), and a combination of EGF and ATP, for 6, 12 and 24 hours. Each type of cell lines divided into four groups based on the type of treatments, such as ATP, EGF, combination ATP and EGF groups and without any treatment as a control group.

Quantitative real-time reverse transcriptase –PCR (qRT-PCR)

The cells were harvested at the appropriate time points and then followed by centrifuging at 1000 rpm for 4 minutes. The total RNA was isolated by using the RNeasy mini kit (Qiagen #74106) following the manufacturer's instructions. RNA was quantified using Nanodrop 2000, and 5 ng of RNA was reverse transcribed and analysed by one-step real-time quantitative PCR using Rotor-Gene Quantitect probe RT-PCR (Qiagen # 204443) using NIS and beta-actin primers. The mRNA NIS expression fold change of treated cells to those untreated was analysed using a method described by Livak et al., ($2^{-\Delta\Delta C}{}_T$) [12]. Three independent qPCR assays were conducted in triplicate.

Immunocytofluoresence

The cells were seeded on coverslips in a well of a 24-wells culture plate and fixated by 4% paraformaldehyde for 15 minutes. This was followed by incubation with protein blocking agent fluorescein-isothiocyanate (FITC) for 15 minutes. The cells were rinsed twice with ice-cold phosphate-buffered saline (PBS) and followed by overnight incubation with 2μg/ml rabbit polyclonal antibody anti-NIS (Abcam, #ab83816) at 4°C. The cells were rinsed three times with PBS and incubated at room temperature with Goat anti-Rabbit IgG secondary polyclonal antibody, with dilution 1:1500 (Abcam, #ab6716) for one hour. The cells were rinsed three times with PBS. The coverslips were placed over slides and mounted with fluoroshield containing 4', 6-diamidino-2-phenylindole (DAPI). The slides were inspected under immunofluorescence microscope (Olympus BX51) with 200 x magnification. Cells that only incubated with secondary antibody were used as a negative control.

Statistical analysis

Collected results are expressed as mean ± SD. Responses of treatments to NIS mRNA expression were calculated in numeric data. They were analysed by ANOVA method to compare means between groups and followed by the Turkey test. $P < 0.05$ was considered statistically significant when compared to controls.

NIS protein expression was analysed using the Her2/neu category. The results were divided based on the level of staining from 0 to 3. Level 0 was no staining, 1 was non-complete staining at the membrane and pale of > 10% tumour cells, 2 was complete staining around the membrane of ≤ 10% tumour cells, and 3 was complete staining at membrane of > 10% tumour cells. Level 0 and 1 were considered as negative, and level 2 to 3 were considered as positive [8], [13]. Mann-Whitney test was used to compare differences between treatment and control groups, $P < 0.05$ was considered statistically significant.

Results

Effect of EGF and ATP treatment on NIS mRNA expression by qRT-PCR

We treat all cell lines with ATP, EGF and the combination of both to induce the level of NIS mRNA expression. The data of NIS mRNA expression was calculated from the cycle threshold (C_T) target toward C_T of reference (beta-actin) and C_T of control genes. The NIS mRNA expression fold change of treated

cells to untreated was analysed.

The expression NIS mRNA is found in SKBR3 and HaCaT cells but not in treated or untreated of MCF7 cells. The levels of NIS mRNA expression in SKBR3 cells after treatment by EGF, ATP or the combination of both for 6 and 12 hours were not significantly different from those of untreated cells. However, the treatment by a combination of ATP and EGF for 24 hours increases the level of NIS mRNA expression by 1.6fold higher than that of the untreated cells (1.6241 ± 0.3, $p < 0.05$) (Figure 1). Furthermore, the treatments in the HaCaT cell line do not change the expression of NIS mRNA in treated and untreated cells.

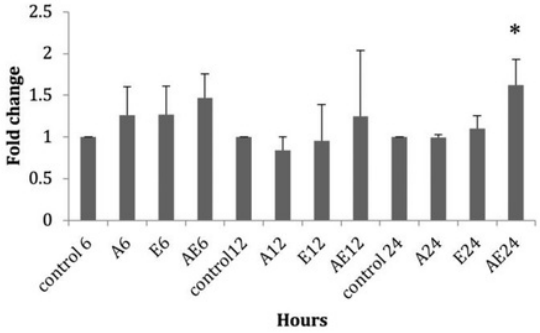

Figure 1: (A) represents the effect of ATP (A); EGF (E) and a combination of ATP and EGF (AE) for 6, 12, 24 hours treatment of SKBR3 cell; The combination of ATP and EGF (AE) for 24 hours treatment increases NIS mRNA expression of SKBR3 cell significantly when compared to untreated (control) cells, P < 0.05

Effect of EGF and ATP treatment on NIS protein expression by immunocytofluorescence

Results of immunocytofluorescence showed that NIS protein express in SKBR3 (Figure 2).

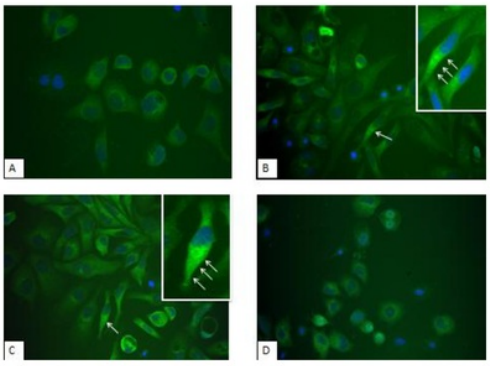

Figure 2: Immunocytofluorescence staining using primary antibody against NIS in the SKBR3 cell line (green) and merging with DAPI staining (blue) at 200 x magnification. The NIS protein expression after 24 hours of treatments with ATP (A), EGF (B) and the combination of both ATP + EGF (C) and untreated (D). The expression of NIS protein increase both in cytoplasm and membrane after 24 hours of treatment by EGF and combination ATP + EGF (arrows)

The NIS expression in those cells is mostly found in the cytoplasm. The treatments of EGF and the combination of both for 12 and 24 hours increase NIS protein expression at membrane cell twofold and threefold respectively, $p < 0.05$ (figure 2B-C). The cells that receive ATP only did not show increasing of NIS protein expression.

Our study shows, NIS protein expression in HaCaT cell line only detects in the cytoplasm. Treatment of ATP, EGF and the combination of both do not induce the NIS protein expression at membrane cell as well as do not increase staining level in the cytoplasm (Figure 3). Furthermore, we cannot detect NIS protein expression in MCF7 cell line in treated and untreated cells.

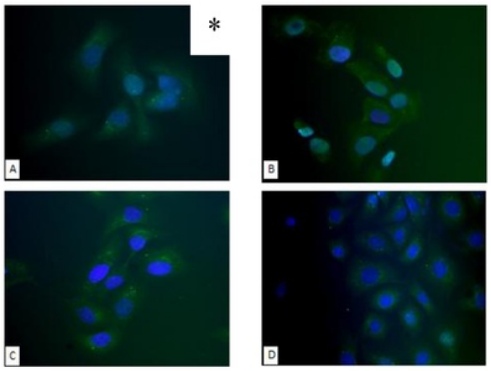

Figure 3: Immunocytofluorescence staining using primary antibody against NIS in HaCaT cell line (green) and merging with DAPI staining (blue) at 200 x magnification. NIS protein expression after 24 hours of treatment by ATP (A), EGF (B) and the combination of both ATP and EGF (C), untreated (D). NIS protein expressions show only in the cytoplasm. NIS protein expressions in a treat and untreated cells do not have a significant different p > 0.05

Discussion

The expression of NIS in extra-thyroid tissues has been reported in some previous studies with varying intensity. These findings have been considered as a potential for radioiodine therapy in the extra-thyroid tumour, including breast cancer [4], [14], [15]. A study by Tazebay et al. reported that NIS was expressed in more than 80% of invasive breast cancer tissues. Another study by Wapnir et al. reported that the expression was found in 76% of invasive breast cancer tissues. Studies by other groups reported that 34% out of 44 breast cancer tissues express NIS. Furthermore, they reported that 65.5% out of 23 triple-negative breast cancer tissues expressed NIS [16], [17], [18], [19].

In this study, we find that NIS mRNA and protein are expressed only in SKBR3 cells, and the expression is not detected in MCF7 cells. Previously published studies on MCF7 cells reported that adding

retinoid acid (RA) is mandatory for NIS expression [10], [19], [20]. Thus, it can safely be assumed that NIS expression in MCF7 is RA-pathway dependent. Till date, the molecular mechanisms of NIS expression in breast cancer are not clearly defined. Meanwhile, it is important to determine the molecular breast cancer subtypes which express NIS as a potential candidate to receive radioiodine.

MCF7 cell line has been established as a breast cancer model for luminal A subtype. It has estrogen and progesterone receptors positive (ER^+/PR^+), HER2 receptor negative (HER2-). Furthermore, the SKBR3 cell line as the HER2 subtype model with estrogen and progesterone receptors negative (ER^-/PR^-) and HER2 receptor-positive ($HER2^+$) [11], [21]. A study by Oh et al. reported that NIS expression is not correlated with the level of hormonal receptors, such as ER/PR. Hormones that regulate NIS expression in lactating breast stage may not play a role in breast cancer [22].

Moreover, a study by Dohan et al. reported that prolactin, oxytocin and estrogen did not induce NIS expression [10]. Instead, another study reported that NIS expresses significantly higher in ER-negative tumour compared to normal breast tissue [15]. Our finding also shows NIS expression is higher in SKBR3 cell compare to MCF7 cell. In this condition, the SKBR3 cell will be appropriate to receive radioiodine therapy. Further studies are needed to examine the effectiveness of radioiodine to breast cancer based on subtypes.

Besides the expression of NIS itself, membrane localisation of the NIS expression also influences radioiodine uptake by the breast cancer cell. The cell will be likely dependent on the level of functional NIS expression at the membrane cell [23]. In vitro study by Elliyanti et al., reported, SKBR3 cell showed higher uptake of radioiodine compare to MCF7 cell [24]. It seems, stimulating of the NIS expression at membrane cell is needed to increase radioiodine uptake. Several agents were used to increasing NIS expressions, such as retinoic acid, hormones and proliferative agents such as EGF and ATP [3], [9], [19], [25]. Binding of EGF with EGF receptor will stimulate the tyrosine kinase activity and will induce cell proliferation [26]. There is a correlation between NIS expression with cell proliferation and malignant transformation in human breast tissue [5], [6]. This leads us to hypothesise that inducing cell proliferation by EGF-EGFR pathway will increase NIS expression at the membrane. In this study, we observed that NIS mRNA expression and protein localisation at membrane cell increase by the combination of ATP and EGF treatment in SKBR3 cells (Figure 2). Treatment of ATP and EGF alone do not increase the level of NIS mRNA; but they increase NIS protein expression at membrane after 12, 24 hours of treatments. Another study reported closed to ours that EGF treatment augmented NIS mRNA and protein expression in T47D cell line, which transfected with NIS [9].

We are unable to detect NIS expression in treated and untreated MCF7 cell. This cell is negative or expresses a low level of EGF receptor [27]. This condition may explain the absence of NIS expression under EGF induction in MCF7 cells. However, EGF can augment NIS expression in MCF7 cells, which are transfected NIS, even though the cells do not have EGF receptors. Further studies are still required to be performed to elucidate the mechanism of NIS expression between breast cancer subtypes.

Additionally, NIS expression was detected in HaCaT cell, even though the expression is within the cytoplasm. Neither ATP nor EGF treatments increase NIS expression in HaCaT cell. Interestingly, radioiodine was taken up by HaCaT cell, but it showed very minimal toxic effect compare to SKBR3 and MCF7 cells [24].

In conclusion, the level of NIS expression varies among the different subtypes of breast cancer cell lines. MCF7 cell line is representing the luminal A subtype of breast cancer do not express NIS as well as SKBR3 cell line. Further studies are needed to cover the differences of NIS expression among breast cancer subtypes. It seems SKBR3 cell which represents the HER2 subtype of breast cancer might be the most suitable to receive radioiodine therapy. In vivo investigations are needed to prove it. Furthermore, a combination treatment of EGF and ATP in SKBR3 cell increases the expression of NIS mRNA and protein at membrane cells.

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