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Genistein Increase Intracellular Distribution of the High Motility Group Box-1 through p38 Pathway in HeLa culture cells induced by Tumor Necrosis Factor-α

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ABSTRACT

Cervical cancer is one kind of many cancers that cause death to women around the world. Many studies had support the statement that inflammation has a strong linkage with cancer development. Several factors like proinflammatory factor can influence tumor cell microenvironment, and induce a faster proliferation. TNF-α is suspected can induce proliferation. While cancer itself can induce inflammation, which is marked by several marker. One of them is HMGB1, released from the cell as active secretory lysosomes or passive diffusion. Genistein has demonstrated growth inhibitory effects of various types of cancer cells. It inhibits tyrosine kinase pathway, which can be activated by TNF-α. One of those pathways that have the link with proliferation is p38. This study tries to reveal about inhibitory effect of genistein toward p38 pathway that had been activated by TNF-α. This research was conducted by exposing cultured HeLa cells with various doses of genistein for 90 minutes, and then exposed to TNF-α 10 ng / mL for 20 minutes. Observations were made with a confocal microscope, by staining the cells with pp38-TRITC and HMGB1 antibody. The intensity was measured and analyzed by Fluoview software. The results suggest that there be significant differences between pp38 intranuclear intensity and HMGB1 extranuclear intensity of each dose of genistein (p = 0.000, ANOVA). pp38 and HMGB1 intensity were increased along with increasing genistein dose, but at high dose there were noted decreasing of pp38 and HMGB1 intensity. At apoptotic dose, pp38 and HMGB1 intensity were increased markedly, showing the effect of apoptosis. In general, increasing doses of genistein increase intranuclear p38 activation and HMGB1 extranuclear translocation. So there were a strong linkage between p38 activation and HMGB1 translocation in this study.

Keywords: *Genistein, TNF-a, Hela Cells, HMGB1, p38*

INTRODUCTION

Cervical cancer is one kind of many cancers that cause death to women around the world [1]. Until now, cervical cancer is treated by operative, chemotherapy and radiotherapy, depend on its stadium [2]. Several studies had support the statement that inflammation has a strong linkage with cancer development. Many pathways are involved, example like cyclooxygenase (COX-1 and -2), free radicals, TGF-β and TNF-α [3]. Several factors like proinflammatory factor can influence tumor cells microenvironment, and induce a faster proliferation [4, 5]. TNF-α is suspected can induce proliferation through NF-κB pathway [3]. In his review, Rakoff-Nahoum also writes cancer also induce inflammation, which marked by HMGB1 release. HMGB1 is an intranuclear protein, together histone they folded DNA and a parted when the DNA transcription process happen. It is a mobile molecule and can migrate from the nucleus to the cytoplasm through the nuclear pore [6]. It serves as a cytokine when released in the extracellular environment by inflamed and necrotic cells [7]. After translated, HMGB1 undergone various modifications to be

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able to function. One important modification that occurs on HMGB1 is acetylation, which causes HMGB1 out of the cell nucleus and can not return to the nucleus [8]. Acetylated HMGB1 will come out actively as secretory lysosomes [9]. Outside the cell, it bounded firmly to the RAGE receptor which is a potent mediator for inflammation [10]. HMGB1 overexpression inhibits apoptosis, which supports the argument that these molecules may act as antiapoptotic oncoprotein, and HMGB1 expression found increase in several different tumor types [11]. Thus, allegedly HMGB1 translocation from nucleus into the cytoplasm of the cancer cells can affect defense toward apoptosis. A model of the nature of cancer cells is HeLa cells culture. HeLa cell is a cell culture derived from a tissue biopsy of the cervix uteri Henrietta Lacks, a cervical cancer patients which taken in 1951 [12]. HeLa cells that induced by TNF-α can provide an illustration of the role of chronic inflammation in cancer. In a previous study, it was found that the induction of TNF-α in HeLa cell cultures can increase HMGB1 translocation from the nucleus to the cytoplasm, which may imply that the inflammatory pathway in this cell culture has been activated. From this preliminary research, allegedly that the pathways involved in the induction of HMGB1 translocation from the nucleus to the cytoplasm is the MAPK pathway. One group of protein kinases that are activated by inflammatory cytokines is p38. TNF and IL-1 activates p38 iso-forms to encourage the deployment of TRAF adapter proteins to the intracellular domain of the receptor origin. TRAF deployment activate various of MAPKKK involved in the activation of p38 [13]. Thus, it can be presumed that the induction of TNF - α activates the p38 pathway that encourages the release of HMGB1. The release of the intranuclear molecules will exacerbate the inflammatory process and promote the occurrence of metastasis. Genistein which is the major isoflavones found in soybeans, has demonstrated growth inhibitory effects of various types of cancer cells in vitro and vivo without toxicity to normal tissues [14]. It inhibits protein-tyrosine kinase and topoisomerase-II activity (DNA topoisomerase, type II), and is used as an antineoplastic and antitumor agent. Genistein can modify histone H3 with acetylate the histone proteins [15]. This study is trying to uncover the potential of genistein as the new hope of the cancer management, especially cervical cancer. In this study, genistein can inhibit the translocation of HMGB1 allegedly from the nucleus to the cytoplasm, which is activated by the p38 pathway in cultured HeLa cells induced by TNF - α. Thus, further research on the potential of genistein can be done.

MATERIALS AND METHODS

Cell Lines

Hela cells were obtained from ATCC. HeLa cells were cultured in Modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (Sigma), 100 IU/mL of penicillin and 1.25% penicillin-streptomycin, in 5% CO2 humidified atmosphere. The cells(105) were plated on coverslips in 15,6 mm diameter 24-well plate (Corning Costar, US). Cells were observed 48 h until confluent. When the cells have been confluent, Hela cells were given various dose of Genistein for 90 minutes [16]. After that, the cells activated with 10 ng/ml [17] TNF-α(#570102; R&D system, Minneapolis, US) for 20 minutes [18].

Immunofluoresence

The cells were fixed in situ by para-formaldehyde 4% (Sigma-Aldrich; Buchs, Switzerland) for 15 minutes, washed three times with PBS for five minutes, and then for five minutes with PBS containing 0.1% BSA (Sigma-Aldrich; Buchs, Switzerland). For the detection of HMGB1 and pp38, the cells were incubated with the HMGB1 IgG mouse monoclonal antibody (#MAB1690; R&D system, Minneapolis, US)diluted in 20 µg/mL in PBS-BSA for 45 min at room temperature (RT) in a humid atmosphere. This specificity has been proved by others [19]. To remove unbound primary antibodies, cells were washed three times for 5 minutes with PBS; subsequently, the secondary antibody, FITC secondary antibody(#F7250; Sigma-Aldrich, Buchs, Switzerland) were diluted in 1:2500 in PBS-BSA, was applied for 45 min at RT and unbound antibodies were removed as described above. For nuclear contrast, propidium iodide (#81845; Sigma-Aldrich, Missouri, US) were used in 1 mg/mL and applied for 5 minutes. In another plate, the cells were incubated with the pp38-TRITC conjugated IgM mouse mono-clonal antibodyfrom (#sc-7973 TRITC, Santa Cruz; Texas, US) diluted in 1:100 in PBS-BSA for 45 minutes. Unbound antibodies were removed, and

cells dissolved in PBS were immediately studied with the confocal microscope.

Confocal Imaging

Confocal fluorescent image were obtained by Olympus LV1000 confocal microscope scan head with 400x objective. Sequential excitation at 488 nm for FITC, 568 nm for propidium iodide and TRITC, and was provided by argon gas lasers, respectively. Emission filter 40x/1.30 Oil UPlan FLN were used for collecting green dye and 60x/1.35 Oil Uplansapowere used for collecting red dye. For thedetection of pp38, TRITC was used in channel one. For thedetection of HMGB1, FITC was used in channel one and PI were used in channel two. After sequential excitation, green and red fluorescent of the cells was saved and analyzed by Fluoview 1.7a version software.

RESULTS AND DISCUSSION

Intensity of intracellular phospho-p38

One of the possible causes of HMGB1 translocate from the cytosol to the intranuclearis caused by activation of p38 MAPK cascade. Activated p38 can be detected with antibody-phosphorylated p38 (pp38) which had been bound with fluorescent Rhodamine, and observed with a confocal microscope Olympus. Analysis result of thetest using One-Way ANOVA on the intensity of intracellular pp38 shows the results of homogeneity of variance with significant variance (p = 0:02). Results of One Way ANOVA showed average difference intensity pp38 significant (p = 0.000) between cell culture negative control, positive control and treatment.

Post Hoc Tukey HSD study showed that p38 activity is significantly increased at dose 10 nM, 100 nM, and 50 μM compared to the negative control (P <0.05). Meanwhile, when compared with the positive control, p38 activity was not increased significantly in all treatment doses. At dose 1 nM and 10 μM found decreased activity, though not statistically significant when compared with the positive control (Figure 1A and 1B). p38 activity significantly increased at a dose 10 nM, 100 nM and 50 μM compared to the negative control (P <0.05). Meanwhile, when compared with the positive control, p38 activity was not significantly increased in all treatment doses. At a dose of 1 nM and 10 μM found decreased activity, though not statistically significant when compared with the positive control. Other studies have suggested that genistein inhibits the growth of HeLa cells, but increased the phosphorylation of p38 [20].

From several previous studies, opposing effect have found. Huang said that genistein 50 μM can inhibit the activation of p38 when given before exposure to TGF - β [21]. Lakhsman support the statement, that genistein intake of 250 mg / kg in mice, resulted in plasma genistein concentration of 1.3 μM, can increase the expression of p38 but lowered it activation [22]. Both studies was conducted on cancer cells culture which are sensitive to estrogen receptors, namely PC3, PC3-M and prostate cancer cell cultures. It means that the effects of estrogen receptors on both study are still not taken into account. Another study mentioned that the genistein at dose 45 μM can increase the activity of p38 from the 15th minute until 120th [16]. Several studies support statement that p38 can activate estrogen receptors in endometrial cells [23, 24].

In cell culture with strong expression of estrogen receptors, genistein tends to act as estrogen agonists at low concentrations (<5 μM), and became estrogen antagonist at high concentrations (> 10 μM) [25]. At nanomolar concentrations, the effects of tyrosine kinase inhibition are difficult to achieve because it is too low [26]. Genistein itself has more potent selectivity toward estrogen receptor-β than estrogen receptor-α [26, 27]. While in cervix cancer cells, estrogen receptor type that more dominant is estrogen receptor-α. Decreased levels of estrogen receptor-α in cervical cancer cells can increase malignancy of cancer cells [28, 29, 30]. Thus, it can be presumed that there are other pathways exist in addition to the estrogen receptor-α and -β that can increase the activation of p38 in our study.

Xie et al. 2013 using the genistein as an apoptosis inducer agent at a dose 15 mg/mL [31]. This made clear by other studies stating that p38 is one of the apoptosis cell intermediate [32, 33]. It explains increasing in the intensity of p38 at dose 50 μM, where this dose as indicated on Szliszka, et al., 2008 is apoptotic dose of genistein [34]. The results of the studies

mentioned above, it can be concluded that genistein has different effects on different cell cultures, with different doses. The phenomenon can be affected by the estrogenic effects of genistein; receptor tendencies or genistein doses that given. Thus, it may explain the results of our study, which low doses exposure of genistein (1-100 nM) increases the effect of p38 activation, whereas at higher doses (1-10 µM or more) are inhibitors for p38. At apoptotic dose, p38 increased again due to the activation of apoptotic pathways.

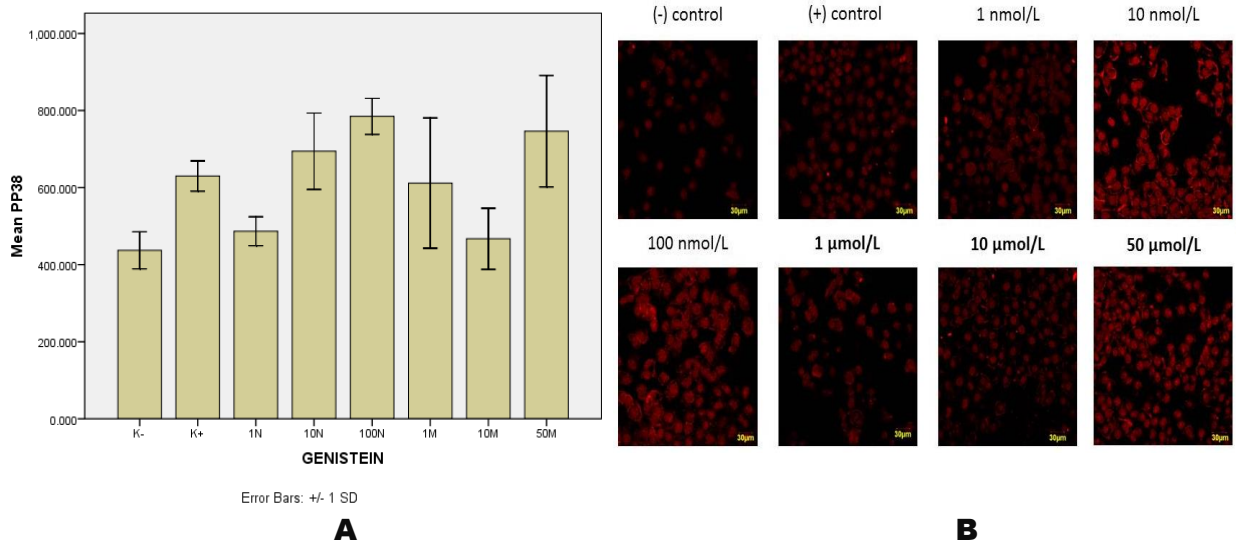

Figure1. **A**. Comparison of the mean intensity of intracellular pp38 in cultured HeLa cells without treatment (negative control) exposed to TNF-α treatment of 10 ng (positive control), and with various concentrations of genistein exposure (treatment). Note : K– (negative control), K+ (positive control), 1N (1 nM genistein), 10N (10 nM genistein), 100N (100 nM genistein), 1M (1 µM genistein), 10M (10 µM genistein), dan 50M (50 µM genistein). **B**. P38 antibody-TRITC staining and confocal microscopy observations. HeLa cell cultures were exposed to various concentrations of genistein for 90 minutes and exposed to TNF-α 10 ng / ml for 20 minutes.

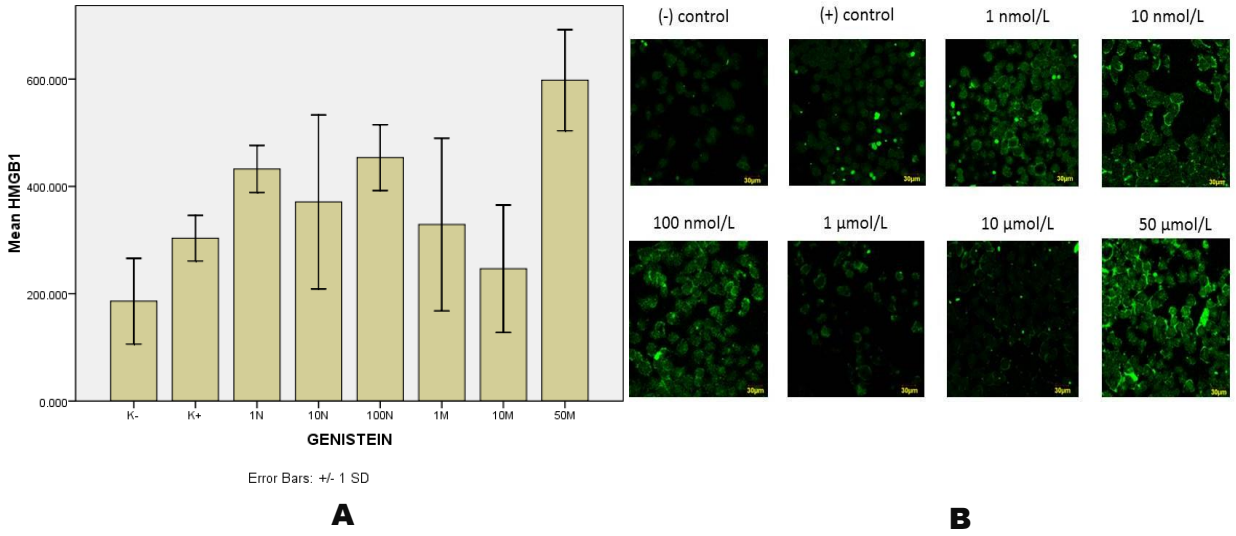

Figure 3. **A.** Comparison of the mean intensity of extranuclear HMGB1 in cultured HeLa cells without treatment (negative control), exposed to TNF-α treatment of 10 ng (positive control), and with various concentrations of genistein exposure (treatment). Note : K– (negative control), K+ (positive control), 1N (1 nM genistein), 10N (10 nM genistein), 100N (100 nM genistein), 1M (1 µM genistein), 10M (10 µM genistein), dan 50M (50 µM genistein). **B**. HMGB1/FITC staining and confocal microscopy observations. HeLa cell cultures were exposed to various concentrations of genistein for 90 minutes and exposed to TNF-α 10 ng / ml for 20 minutes.

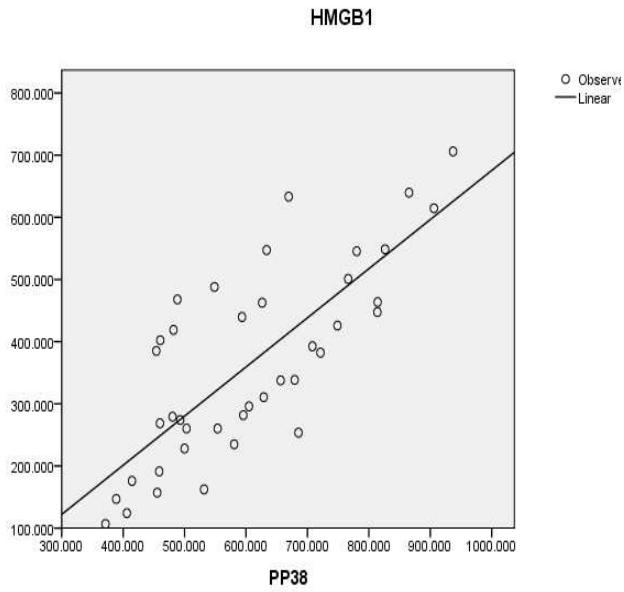

Figure **5.** Pearson test results obtained that the relationship between variables is significant and substantial.

Intensity of HMGB1 extranuclear

HMGB1 which stained with primary antibodies and fluorescent FITC is coming out from intranuclear. HMGB1 fluorescence intensity was measured using a confocal microscope Olympus. Analysis of test results using One-Way ANOVA on the intensity of HMGB1 extra-nuclear shows the results of homogeneity of variance with the variance was not significant (p = 0.52). Results of One Way ANOVA showed average difference intensity of HMGB1 were significantly (p = 0.000) between cell culture negative control, positive control and treatment.

The Post Hoc Tukey HSD analysis showed that HMGB1 significantly increased the number in the cytosol at dose 1 nM, 100 nM and 50 μM compared to the negative control (P <0.05). Meanwhile, when compared with the positive control, HMGB1 did not significantly increase in the cytosol in all treatment doses, except 50 μM (P <0.05). At dose 1 μM and 10 μM found decreased intensity, though not statistically significant. However compared to dose 100 nM, both dose can significantly reduce the intensity of HMGB1 in the cytosol (P <0.05) (Figure 3A and 3B).

The Post Hoc Tukey HSD analysis showed that HMGB1 significantly increased the amount in the cytosol in a dose of 1 nM, 100 nM and 50 μM when compared to the negative control (P <0.05). Meanwhile, when compared to the positive control, HMGB1 did not increase the amount in the cytosol significantly in all treatment doses, except 50 μM (P <0.05). At dose 1 μM and 10 μM found decreased activity, though not significant statistically. However toward dose 100 nM, both treatments can significantly reduce the intensity of HMGB1 in the cytosol (p <0.05). That is, in general, increasing doses of genistein also increase the amount of HMGB1 that translocated into the cytosol.

Induction of TNF-α can activate p38 pathway [14, 35], which finally activates MSK1 [36]. MSK1 activation would lead to activation of CREB (cAMP Response Element Binding Protein) [37]. This is confirmed by research Gustin (2004) [38], in which TNF-α can activate CREB through p38-MSK1 pathway via TNFR-1 receptor [38]. Activation of phosphorylated CREB binds to CREB-binding protein (CBP). After CBP activated, it will bind to p300 both with Histone Acetyltransferase activity [39]. Substrates of CBP/p300 is not only histones, but also non-histone proteins other [40], including HMGB1 [8]. Acetylated HMGB1 will be separated from DNA binding [8] because its domain of DNA on HMGB1 Nuclear Localization Sequence (NLS) experienced acetylation [39]. In addition, acetylated HMGB1 also will undergo translocation through CRM1 or exportin [41] and can not survive much longer in the nucleus [8]. This is consistent with the findings in our study where HMGB1 translocation to nucleus increased along with p38 activation. In conclusion, increasing doses of genistein increase intranuclear p38 activation and HMGB1 extra-nuclear translocation. Moreover, there were a strong linkage between p38 activation and HMGB1 translocation in this study.

Pearson test results obtained that the relationship between variables is significant, with p = 0.000, which means a significant relationship between the increase in the intensity of pp38 with an increase in the intensity of HMGB1. R = 0773 show an association with high strength. Positive value (+) means that between pp38 and HMGB1 has a unidirectional relationship, the higher the intensity of pp38, the higher the intensity of HMGB1 (Figure 5).

CONCLUSIONS

From the experiment, it can be concluded that exposure various doses of genistein increase intranuclear p38 activation and HMGB1

extranuclear translocations in a biphasic pattern. Genistein effects increase p38 phosphorylation and HMGB1 translocation in nanomolar doses, but decreased in micromolar doses. But overall, genistein increase p38 activation and HMGB1 acetylation. So there were a probable linkage between p38 activation and HMGB1 translocation in this study.

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