Apoptosis and Necrosis on T47D Cells Induced by Shiga-Like Toxin from Local Isolates of Escherichia coli O157:H7

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Abstract

Background Apoptosis and cell cycle arrest induction are targeted in the strategy of cancer therapy. Furthermore, bacterial toxins such as Shiga-like toxin producing Escherichia coli have been suggested to be used as a novel therapeutic agent against tumor malignancies, either as independent anti-neoplastic agents, or in combination treatment with chemo or radiotherapy. The aim of study was to investigate the potency of Shiga-like toxin originating from local strains of E. coli O157:H7 which was known less toxic than ATCC 43894 as a new cancer therapy. Methods As many as 10 culture cells T47D cell line were subjected by crude extract Shiga-like toxin originating from five local isolates of E. coli O157:H7 with each codes KL-48(2), SM-25(1), SM-7(1), DS-21(4), and one isolate ATCC 43894 as a control with IC50 doses, respectively. The treatment was observed for 24 h, with two replications. An FITC-Annexin V and PI assay was used to observe apoptosis and necrosis effect, and simultaneously with cell cycle analysis using propidium iodide (PI) staining. Results The study shown that T47D cells treated with Shiga-like toxin from local strain KL-48 (2) show the lowest viable cell, followed by SM7(1), ATCC 43894, SM-25(1), DS-21(4) in contrary with the control cells with each percentages at 15.20, 16.36, 22.17, 22.64, 33.86, and 94.36%, respectively. The results were also confirmed by the induction of the cell cycle arrest in phase G0-G1 as inactive phase, i.e. 66.41, 63.37, 61.52, 55.36 and 47.28% for T47D cells treated with toxins of KL-48(2), ATCC 43894, SM 25(1), SM 7(1), and DS 21(4), respectively. Conclusions These results show tendency deleterious effect of Shiga-like toxin from local isolates on T47D cells, so It is concluded that they have potency as a good anticancer drug against Gb3-expressing breast cancer.

Background

Shiga toxin *Escherichia coli* (STEC) is a major public health concerns in developed and developing countries due to the severity of the diseases they cause. The infection caused by this bacterium may result in bloody diarrhea, and the subsequent life-threatening sequalae, including acute renal failure and neurological abnormalities [1]. On the other hand, STEC also has been exploited for medical purposes such as cancer therapy or imaging [2]. STEC has multifunctional capabilities to inactivate multiple cell stress signaling pathways which may result in apoptosis, autophagy or activation of the innate immune respone [3]. Apoptosis may be triggered by activation of c-Jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38MAPK) [4]

Scientific studies indicate that Shiga toxin (Stx)-induced apoptosis on specific cell is an important process in the pathophysiological response of humans to this bacterial toxin. Apoptosis has been reported in several different cell types as a results of Stx1 and Stx2 action associated with infection by this bacterium [5]. Apoptosis, or programmed cell death, is a multi step process that is important in eliminating damaged or abnormal cell [6]. Furthermore, apoptosis and cell cycle arrest induction are targeted in the strategy of cancer therapy [7].

Shiga-like toxins produced by *Escherichia coli* O157:H7 damages cellular nucleic acids by removing a specific adenine from 28S rRNA in ribosome [8]. All STEC contain a pentameric ring of identical B-

subunits with each subunit approximately 7.7 kDa, non-covalently associated with a single A-subunit of approximately 32 kDa [9]. The B-subunit of STEC specifically binds to sugar moiety of glycosphingolipid globotriaosylceramide (Gb3) in the plasma membrane of target cells, and mediates uptake and intracellular transport of the toxin, and is then transported to the endoplasmic reticulum following the retrograde route. The A subunit is cleaved in the trans-Golgi network, and the enzymatically active A1 part is translocated from the lumen of the endoplasmic reticulum to the cytosol. The A1 fragment irreversibly modifies ribosomal 28S RNA, leading to the inhibition of biosynthesis and cell death by apoptosis [10].

Shiga-like toxins efective against specific signaling pathways could reduce treatment side-effects to normal tissue and be an approach to generate specific anti tumour agents [8]. Shiga and Shiga-like toxin producing *Escherichia coli* have been proposed as an anti-cancer therapy due to its low general toxicity and high specificity against tumours expressing its receptor globotriasosylceramide (Gb3) [11]. Moreover, Gb3 has been reported to be increased on the surface of several tumour cells lines such as breast cancer [12]. Remarkably, many types of cancer cells overexpress Gb3 on their surface, and therefore, enable binding of toxins or the non-toxic pentameric Stx B-subunits coupled to anti-cancer agents [13].

The aim of this research was to evaluate the potential of Shiga-like toxin producing *Escherichia coli* O157:H7 local isolates which were known to have lower toxicity than control ATCC 43894 [14] as a novel agent for enhancing apoptosis and necrosis in T47D cells. We demonstrate that verotoxin has potency to be use as an anti-cancer drug in Gb3-expressing breast cancer.

Methods

Cultivation of *Escherichia coli* O157:H7 isolates

Cultivation of the five isolates of *E. coli* O157:H7 i.e. KL-48(2), SM-25(1), SM-7(1), DS-21(4), and control isolate ATCC 43894 was initiated by culturing on lactose broth medium (LB) at 37°C, and incubated aerobically overnight. Presumptive *E. coli* O157 isolates were re-confirmed using *E.coli* O157 latex agglutination test (Ovoid, DR120M) according to the previous method [15, 16].

Isolation of Shiga-like toxin

Isolation of Shiga-like toxin was performed by culturing of isolates on *Luria Bertani* / LB broth (Sigma, L3022) and incubated on 37^{o}C, 24 h, subsequently it was centrifugated at 2000 rpm for 40 min at 4^{o}C. 5.97 g of ammonium sulfate (Sigma, A4418)15 ml was added to 15 ml of the supernatant gradually in order to obtain 65% percentage of saturation. The solution was recentrifugated at 2000 rpm for 40 min. The supernatant was removed, and the precipitate was diluted with 3 ml of sterile physiological saline, and then dialyzed at 4^{o}C overnight. Furthermore, the toxin was sterilized by Millipore filtered with 0.22 µm

filters (Corning, 431 219). The concentration of the toxin was measured by calculation of optical dencity at a wavelength of 595 nm [17, 18].

Preparation of T47D cancer cells

T47D cancer cells as a collection from integrated research and testing laboratory, Gadjah Mada University were used in this study. One ml of T47D cell maintained under standard cell culture conditions was grown as a monolayer culture in Dulbecco's Modified Eagle Medium (DMEM) (Sigma, D6046) supplemented by 10% Newborn Calf Serum (Sigma N4887), 100 IU penicillin/ml, 100 mg/ml Streptomycin, and 50 µg fungizon (Fisher Scientific, BW17-745H). It was incubated at 37^{o}C, in a humidified atmosphere containing 5% CO_2.

Toxicity assay

The analysis of the toxicity effect in the form of cytophatic effect (CPE) among treatments and control was done according to the previous studies by measuring inhibitory concentration 50% (IC_{50}) value of the cells. 50 µL of T47D cells were implanted into 96 well micro plate (Merck) and incubated at 5% CO_2 for 24 hours to obtain confluent growth with density of 5 x 10^4 cells/well. Then the media were replaced with new ones, to which 50 µL of crude toxin with serial dilution was added. After 15 min of incubation at room temperature, the crude toxin was removed and monolayer cells were washed two times with Dulbecco's Modified Eagle Medium (DMEM). 100 µL complete growth medium (DMEM with 10% Newborn calf serum, 100 IU penicillin/ml, 100 mg/ml Streptomycin, and 50 µg fungizon) was then added to the cells before they were incubated at 37^{o}C, 5% CO_2 for 24h. A positive test was shown by the amount of T47D cell lyses after incubation. At the end of incubation, the media were removed and then the cells were washed with a solution of phosphate buffer saline (PBS). 100 µL of culture media and 10 µL of MTT reagent (3-(4, 5 dimetiltiazol-2-yl) -2.5-diphenyl tetrazolium bromide) 0.5% was added to each well. Cells were incubated again for 4-6 h in 5% CO_2 incubator at 37°C to form formazan. The reaction was stopped by 100 µL of MTT reagent stopper (sodium dodecyl sulfate). The cells were incubated overnight at room temperature, and then analyzed by ELISA reader at λ 550 nm [17, 18].

Cell apoptosis and necrosis assay

Apoptosis of T47D cells was determined according to the previous method with slight modification [19, 20]. An FITC-Annexin V and PI method (Invitrogen; Thermo Fisher Scientific, Inc.) was used to assess apoptosis. Briefly, 1×10^6 T47D cells were harvested, washed twice with cold PBS by centrifugation at 2000 rpm for 5 min, and resuspended in 100 µL binding buffer (Thermo Fisher Scientific, Inc.). A total of 100 µL Annexin V-fluorescein isothiocyanate and 2 µL PI was added to the solution. Following 10 min incubation in the dark at room temperture. 400 µL binding buffer was added to the solution and cells

were analyzed using the Accuri™ C6 Flow Cytometer. The results were analyzed using CellQuest™ software 1.0 (BD Biosciences). A quadrant dot plot was used to identify whether cells were in the early or late phase of apoptosis and whether they were living or necrotic.

Cell cycle analysis with propidium iodide staining

The method was according to the previous method with slight modification [21]. The T47D cells with density 7 x 10^5 cells upon completion of 24 h incubation with / without IC_{50} of each Shiga-like toxin local isolate and control ATCC 43894. The cell cultures were washed with PBS by centrifugation at 2000 rpm for 5 min and treated with 0.1% trypsin at 37^oC. The cell suspension was collected, washed once with PBS (2000 rpm, 5 min), and re-suspended for 30 min, 4^oC in PBS containing 70% cold absolute ethanol for fixation and permeabilization of the cell membrane. After that, the cells were washed twice with PBS by centrifugation at 2000 rpm for 5 min, and the cells were treated with 40 µg/mL Rnase in PBS (final volume 100 ml), for 15 min at 37^oC. Finally, 2 µl of PI staining solution was added to the cells, followed by 10 min incubation in the dark at room temperature. The cell cycle analysis was performed by a Fluorescence Activated Cell Sorter (FACSCalibur, Becton Dickinson, San Jose CA USA), and PI fluorescence (designated as Fl-2 Height in the histogram plots) was measured at 488 nm. Ten thousand cells were analyzed in each experiment. The percentage of cells arrest in the G0/G1, S, and G2/M phases of the cell cycle were then determined.

Results

Toxicity assay

Results of toxicity assay showed the differences in the viable or deleterious of T47D cells among each treatment and control (Additional file 1: Fig. S1). The percentage of deleterious cells was calculated as a cytophatic effect / CPE based on the OD at 550 nm λ and it is briefly summarized in Table 1.

The results of the toxicity test in Table 1 show that Shiga-like toxin producing *E. coli* O157:H7 strain KL-48(2) from human origin have IC_{50} values almost equal to control isolate ATCC 43894, and slightly different from others. The concentration of toxin that is required for 50% inhibition *in vitro* of ATCC 43894 is 0.92 ug / mL and KL-48(2) is 0.94 µg / mL, On the other hand, toxins of SM-25(1), SM-7(1), and DS 21(4) require higher concentrations to inhibit 50% of cells i.e. 1.08; 1.03; and 1.03 ug / mL, respectively.

Cell apoptosis assay

Detection of toxicity effects (apoptosis or necrosis) caused by each isolate of *E. coli* O157:H7 on T47D cell was analyzed by using FITC-Annexin V and PI methode (Fig. 1). Annexin V binding was assessed using bivariate FCM, and cell staining was evaluated with fluorescin isothiocyanate (FITC)-labelled Annexin V (green fluorescence), simultaneously with dye exclussion of propidium iodide (PI) (negative for red fluorescence). The test described, discriminates intact cells ($FITC^-/PI^-$), apoptotic cells (($FITC^+/PI^-$) and necrotic cells ($FITC^+/PI^+$) [19].

The results of T47D cells treated with Shiga-like toxin isolated from various strain (Fig. 1) show various viable cells, apoptosis, late apoptosis, and necrosis that are summarised in Table 2.

T47D cells treated with Shiga-like toxin isolated from local strains show various percentages of effects on the T47D cells cycle after 24 h of incubation (Table 2). The result is in contrary to T47D cells control. T47D cells treated with Shiga-like toxin KL-48(2) show the lowest percentage of viable cells, followed by SM 7(1), ATCC 43894, SM-25(1), DS-21(4) and control with each percentages as 15.20, 16.36, 22.17, 22.64, 33.86, and 94.36%, respectively. These results show the tendency towards deleterious effects of Shiga-like toxin treatment on T47D cells. The equal effect of Shiga-like toxin on breast cancer tissue has been reported in previous studies [21, 22].

Cell cycle analysis

Flow cytometry as a method for differentiating of the DNA content in various of cell cycle phases. The ploidy of cells in G1, S, G2 and M is 2N, 2-4N, 4N and 4N, respectively. However, G0 and G1 phase, G2 and M phase, which both have an identical DNA content, could not be discriminated based on their differences in DNA content. The result of the study which analyzed T47D cells cycle with and without treatment of Shiga-like toxin is presented in Fig 2, and the various cycle cell phase i.e. G0-G1, S, and G2-M phases with each percentage are summarised on Table 3.

The treatments of T47D cells with different Shiga-like toxins (Table 3) show various effects on the cell cycle arrest. The cells treated with KL-48(2) toxin show the highest effect on the cell cycle arrest in G0-G1 phase compared to others. The G0-G1 phase is known as a resting phase, as the cell has left the cycle and has stopped dividing. The percentage of cells in G0-G1 phase are 66.41, 63.37, 61.52, 55.36 and 47.28% for T47D cells treated with toxins of KL-48(2), ATCC 43894, SM 25(1), SM 7(1), and DS 21(4), respectively. The results are different from T47D cells control without toxin treatment which show the highest percentage on G2-M phase on the active phase for the preparation of cells to divide (mitosis).

Discussion

Results of the study indicate the treatment of Shiga-like toxin originating from local strains of *E. coli* O157:H7 is effective in decreasing the number of T47D viable cells compared to the control. Application of Shiga-like toxin is proven to trigger T47D cells to enter apoptosis, late apoptosis, and necrosis stages. This study reinforces the statement of the previous study [21] which states that the bacterial toxins such as verotoxin are known to be used as a therapeutic agent against malignant tumors including the breast cancer cells, either used alone or in combination with medication such as chemo or radiotherapy. The study also shows that apoptosis was induced rapidly (60%) in HeLa cells after exposure to Shiga toxin within 4 hours [5]. Cancer therapy with toxins is known to be very effective and can reduce the side effects on normal tissue due to the mechanism of action through very specific signaling pathways [8].

All members of the Stx family are composed of 1A and 5B subunit proteins. Each B subunit (StxB) binds with high affinity to the glycosphingolipid globotriaosylceramide, Gb3 (CD77) present on select eukaryotic cells [23, 24]. The A subunit is an N-glycosidase that removes adenine 4342 of 28S RNA of the 60S ribosomal subunit [8], rendering ribosomes inactive for protein synthesis [25]. The Stx1B induces apoptosis with accompanying DNA fragmentation, whereas the Stx1A is found to be necrotic and no DNA fragmentation occurred [26, 27]

Generally, exposure of cancer cells to Stx activates caspase 3, 6, 8, and 9. Caspase 8 is known to active Bid, an endogenous protein known to permeabilize mitochondrial membranes. The cleavage of Bid will convert from inactive 26 kDa form to an active 15 kDa capable of disrupting the mitochondrial outer membrane. This activity will induce release of cytochrome C from the mitochondria and trigger activation of caspase-9, which then accelerates apoptosis by activating caspase-3 [5]. Furthermore, the previous study also showed that apoptosis involving caspase-3 activation is induced after Stx is transported to the Golgi apparatus, which is similar to the inhibition of protein synthesis caused by Stx [27].

The ability of Shiga-like toxin to trigger T47D cells to enter apoptosis, late apoptosis, and necrosis stages compared to the control cells is also proven by the results of cell cycle analysis (Table 2). The results of the study show that treatment of T47D cells with Shiga-like toxin of ATCC 43894, KL-48(2), SM-25(1), and SM-7(1) was more effective in arresting the cell cycle in G0-G1 phase, except for DS-21(4), with the lowest effect. Propidium iodide (PI) is a fluorescent dye that binds specifically to double stranded nucleic acids [28, 29]. In the flow cytometry assay employed, PI fluorescence is indicative of the DNA content of the cells. Cells in the G2/M phase are preparing to divide and they contain double the amount of DNA (4n) compared to cells in the G1 phase that have not yet replicated their DNA (2n DNA content) [21].

Cell cycle analysis (Table 3) show a simultaneous effect with cytotoxic assay. The higher amount of the viable cells in the control is correlated with the higher cell cycle on G2-M phase as an active phase, and the lower percentages of viable cells are correlated with the higher cell cycle on S-phase or G0-M phase. The result is strengthened by several articles which have presented correlations between DNA ploidy classification and cell cycle variables, and clinical pathologic variables [30]. The reability of the flow

cytometry method to detect cell cycle phase and equal phenomena has also been found by several researchers. The researchers showed a decrease of leukemic cells in the S, G2 and M phase was followed by an increase of G1 phase [31]. Another result also showed that in respons to genotoxic induced lymphocyte, that an accumulation of the cell in G2+M phase was accompanied by a decrease in the G0 + G1 population [32].~

Conclusions

Apoptosis and necrosis have been reported in several different cell types as a result of Stx1 and Stx2 action associated with infection by this bacterium. Cells treated with local Shiga-like toxin show higher apoptosis and necrosis effect than control cell, and also higher cell arrest on G0-G1 phase of cell cycle. The results confirm local strains to be a novel candidate for anticancer therapy in Gb3-expressing breast cancer, although further research was still needed in order to investigate its potency as anticancer treatment completely.

Abbreviations

KL-48 (2): Klinis-48(2); SM 7(1): Sapi Samuan 7(1); ATCC 43894: American Type Culture Collection 43894; SM-25(1): Sapi Samuan 25(1); DS-21(4): Daging Sapi 21(4); G0: Gap 0; G1: Gap 1; S: Synthesis; G2: Gap 2; M: Mitosis; IC_{50}: Inhibitory concentration 50.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publications

Not applicable

Availability of data and materials

The authors declare (the/all other) data supporting the findings of this study are available within the article and additional file.

Competing Interests

The authors declare that they have no competing interests.

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Author’s Contributions

Conceived and designed experiments: IWS. Performed the experiments: IWS, IGNS, KJPP, DAW. Analyzed data: IWS. Contributed reagents / materials: KJPP. Wrote the paper: IWS, IGNS, KJPP, and DAW. All the authors have read and approved the final manuscript.

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Tables

Table 1. The inhibitory concentration 50% (IC_{50}) of shiga-like toxin on the formation of cytophatic effect (CPE) on T47D cells after 24 h of observation

Shiga-like toxin strains	Source of strains	IC_{50} Concentration (µg/µL)
ATCC 43894	Human, America	0.92
KL-48(2)	Human, Indonesia	0.94
SM 25(1)	Cattle feces, Indonesia	1.08
SM 7(1)	Cattle feces, Indonesia	1.03
DS 21(4)	Beef, Indonesia	1.03

Note: IC_{50} (Inhibitory Concentration 50%) is the concentration of drug that is require for 50% inhibition *in vitro*

Table 2. Percentages of T47D cells with apoptosis, late apoptosis, and necrosis as well as viable cells after 24 h treated Shiga-like toxin with inhibitory concentration 50 (IC_{50}).

Treatments	Percentages of T47D cells after treatment			
	Viable cells	Apoptosis	Late apoptosis	Necrosis
T47D control cell (without toxin)	94.36	0.14	0.01	5.51
T47D cell + ATCC 4389 toxin	22.17	3.43	63.68	10.84
T47D cell + KL-48(2) toxin	15.20	4.52	67.66	12.73
T47D cell + SM 25(1) toxin	22.64	3.02	63.90	10.53
T47D cell + SM 7(1) toxin	16.36	2.12	62.60	19.13
T47D cell + DS 21(4) toxin	33.86	6.61	54.74	4.89

Table 3. Percentages of T47D cells with each cell cycles arrest after 24 h treated /without Shiga-like toxin with inhibitory concentration 50 (IC_{50}).

Treatments	Percentages of T47D cell cycle after treatments		
	GO-G1	S-phase	G2-M
T47D control cell (without toxin)	40.69	22.56	42.30
T47D cell + ATCC 4389 toxin	63.37	17.58	19.06
T47D cell + KL-48(2) toxin	66.41	17.10	16.61
T47D cell + SM 25(1) toxin	61.52	20.14	18.35
T47D cell + SM 7(1) toxin	55.36	25.33	19.25
T47D cell + DS 21(4) toxin	47.28	22.95	29.71

Supplementary Files

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