Antimetastatic Potential of N-Acetylcysteine on Human Prostate Cancer Cells

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Objective: *N-acetylcysteine (NAC), is one of the cheapest, safest and widely used over-the-counter-drugs in Thailand. Here the authors examine the antimetastatic potential of NAC on the metastasis of human prostate cancer cells.*
Material and Method: *Cytotoxicity of NAC to human prostate cancer cells, DU145 and PC3, were determined by proliferation assay using the 3-(4, 5-dimethylthiazol, 2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent. Cell migration and invasion were assessed by using a chemotaxis chamber containing membrane pre-coated with collagen IV and Matrigel®, respectively. Cell attachment onto the surface of the membrane coated with collagen IV was tested for its adhesion potentiality.*
Results: *NAC could inhibit the growth of DU145 and PC3 cells. Suppression of migration and invasion of both human prostrate cancer cells were observed. Cell attachment to the collagen IV-coated surface was obviously reduced. All inhibitions occurred in a dose-dependent fashion in both cell lines.*
Conclusion: *NAC could have a high potential in attenuating the migration of the human prostate cancer cells from their primary site and their adhesion and invasion to the remote locations. Hence, NAC might suppress the growth of the primary and the secondary tumors. Our findings suggest that NAC had a high possibility to become an antimetastatic agent for testing in clinical trials. Then, NAC might be used clinically as an optional adjuvant therapeutic drug in addition to the conventional standard treatment of human prostate cancer, obtaining a better outcome with the least toxic and affordable substance.*

Keywords: *Postate cancer, Metastasis, N-acetylcysteine (NAC), Cell migration, Cell proliferation*

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Prostate cancer represents the most common noncutaneous cancer among the male populations. It has been found to be the third and second leading cause of elderly male cancer-related mortality in the United States during 2008 and 2010, respectively. During the past two decades prostate cancer is becoming a major malignancy in many Asian countries[1]. In Thailand, the incidence rate of prostate cancer was 3.5 per 100,000 male population. It was ranked as 7th of most common cancer in men[2]. Many scientists and clinicians are putting strong efforts on searching new therapeutic strategies, *i.e.* natural products, medicinal herbs, etc., preferentially targeting cancer cells without affecting the normal cells and also eliminating the resistant cancer cells[3].

During the last two decades, oxidative stress was found to be an important key in the development and progression of many human cancers including the prostate cancer. Signaling pathways of oxidative stress have been implicated in the processes of carcinogenesis, metastasis and therapeutic resistance. Oxidative stress was reported to be involved in the development of human prostate cancer[4,5]. Many reactive oxygen species (ROS) regulators have been examined to be used in chemoprevention of human prostate cancer. Several compounds, especially those containing antioxidant activity, isolated from medicinal plants have been tried with the hope of being new drugs with mild degree of side effects[2,3].

Antioxidants are quite popular for their capability to minimize the oxidative stress, which is believed to be the cause of several pathological conditions. N-acetylcysteine (NAC) or N-acetyl-L-cysteine, $C_5H_9NO_3S$, is a pharmaceutical drug primarily used as a mucolytic agent and as an antidote of acetaminophen toxicity[6,7]. It is a glutathione precursor that plays an important role in increasing the glutathione levels and also known as an exogenous antioxidant or

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cell detoxifier. Glutathione is a small size intracellular protein containing antioxidant activity due to its crucial role in the free radical neutralization and antioxidant defense system. NAC is a thiol-containing antioxidant or cysteine prodrug which supplies cysteine for glutathione synthesis. For this reason NAC is considered a powerful antioxidant[8,9]. NAC is a highly effective drug in the treatment of the diseases associated with the oxidative stress due to its capabilities to scavenge and quench ROS, the harmful free radicals that can cause oxidative damage to organs and to DNA in the body[9,10].

The purpose of the present studies is to examine the potential and possibility of using N-acetylcysteine as an alternative drug or adjuvant therapy for chemoprevention of human prostate cancer.

Material and Method

Cell culture

Human prostate adenocarcinoma cell lines, DU145 and PC3, were grown as a monolayer in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 mg/mL streptomycin in 10-cm diameter tissue culture dishes. Both cell cultures were maintained at 37°C in a tissue culture CO_2 incubator within humidified atmosphere containing 5% CO_2 until 80% confluency and then subcultered twice a week. All chemicals for cell culture were purchased from Gibco BRL.

Cell proliferation assay

The cytotoxic effects of NAC (Sigma) on the proliferative capability of the human prostate adenocarcinoma cell lines, DU145 and PC3, were determined by using a modification of the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay[11]. Briefly, the prostate cancer cells at a density of 3.5 x 10^4 cells/well in serum-containing medium were seeded in 24-well microplates and grown to 80% confluency. Then, they were treated with NAC at various concentrations in a humidified atmosphere of 5% CO_2 at 37°C for 24 h. After the incubation, cells were washed twice with phosphate-buffered saline (PBS) and 150 µl of culture medium containing 1 mg/ml of MTT dye was added to each well and incubated further for 4 h. The medium containing MTT dye was then replaced with 150 µl of DMSO. The blue color of the oxidized MTT (formazan) was determined by measuring the absorbance at 570 nm using ELISA microplate reader (BIO-RAD 550). The percentage of proliferation was calculated and compared to that of the untreated cells. All chemicals for the cell proliferation assay were obtained from Sigma.

Cell migration assay

Migration of prostate cancer cells was assessed by using 48-well chemotaxis chamber (Neuroprobe Inc. Gaithersburg, MD) and a polycarbonate membrane of 8 µm pore size coated with collagen IV as described previously[11] with some modifications. Briefly, the cancer cells at a concentration of 5 x 10^5 cells/ml in cultured medium containing NAC at various concentrations, were seeded on the upper compartments and allowed to migrate toward the chemoattractant, 10 ng/ml of epidermal growth factor (EGF) (Sigma), in the lower compartments. After incubation for 4 h at 37°C in a humidified atmosphere of 5% CO_2, the nonmigrated cancer cells on the upper surface of the membrane filter were wiped off with a cotton swab. The migrated cancer cells adhering to the lower surface of the filter were fixed with methanol and stained by using a Diff-Quick Stain Kit (Baxter). A quantitative assessment of the migrated prostate cancer cells attached to the lower side of the membrane was quantitative assessed by counting the number of cells in five random fields (40x) under a light microscope. Migration of the untreated cancer cells, being suspended on the upper compartment with DMEM without NAC, served as the reference value (control) and is referred to as 100% migration. Percentage of inhibition at 50% of reference value was used to compare with the migration at each dose of NAC.

Cell invasion assay

The invasiveness of prostate cancer cells was determined by using 48-well chemotaxis chamber, a membrane invasion culture system (Neuroprobe Inc. Gaithersburg, MD) as described previously[11] with slightly modifications. The membrane filters were coated with Matrigel (Becton Dickinson) and the assays were subsequently performed similar to those of the migration assay.

Cell adhesion assay

The adhesion assay was performed as described previously[11] with some modifications. Briefly, 96-well culture plates (Corning) was pre-coated with 1 µg/ml collagen IV (Sigma) at 4°C overnight. The cultured cells were washed with serum-free medium, trypsinized, incubated with various concentrations of NAC for 6 h and reseeded at a density of 5 x 10^4 cells/

well on the pre-coated plates. After the cells were allowed to adhere for 30 min at 37°C in a humidified atmosphere of 5% CO_2, the wells were washed three times with PBS to remove the non-adherent cells, while the adherent cells were stained with 0.1% crystal violet (Merck) for 30 min, washed with tap water and air-dried. The stained cells were lysed with 10% acetic acid (Merck) to release the dye. The intensity of the stain, in direct proportion to the number of adherent cells, was quantitated by measuring the absorbance at 595 nm using an ELISA microplate reader.

Statistical analysis

All data are presented as mean ± standard deviation (SD) of at least three separate experiments with different cell preparations performed in triplicate. The difference between the values for each treatment concentration and the respective controls was analyzed using the two-tailed Student's t-test. Differences were considered statistically significant when $p < 0.05$.

Results

Effect of NAC on cancer cell proliferation

The inhibitory effect of NAC on cancer cell proliferation was determined in two human prostate cancer cell lines, DU145 and PC3. The cells were cultured in several concentrations of NAC for 24 h and analyzed using the MTT assay. As shown in Fig. 1, the survival of DU145 and PC3 was inhibited by NAC with different 50% inhibitory concentration (IC_{50}) of 14.58 ± 0.85 and 32.28 ± 0.71 mM, respectively. All concentrations of NAC significantly inhibited cell growth in a dose-dependent fashion in both cell lines ($p < 0.05$). The percentage of cell survival was calculated based on the untreated cells.

Effects of NAC on the migration of human prostate cancer cells

Metastatic process of cancer cells is dependent on the ability to migrate or spread from their original site to the new remote locations. The chemotaxis assay was used to evaluate the cell migration from the upper compartment towards the lower chamber through porous filters of 8 μm pore size. Two human prostate cancer cell lines, DU145 and PC3, were treated with NAC at various concentrations (0, 10, 20, 30, 40 and 50 mM) and then seeded on the surface of the membrane filters pre-coated with collagen IV. The percentage of the cancer cells migrating through the collagen IV-coated membrane after exposure to NAC at various concentrations was significantly decreased in a dose-dependent manner comparing with those cells without NAC, $p < 0.05$, (Fig. 2). A concentration of at 20 mM NAC could dramatically inhibit the cancer cell migration, and 50 mM completely abolished it. The IC_{50} values of NAC for DU145 and PC3 migration were 16.01 ± 0.63 and 23.77 ± 0.58 mM, respectively.

Effects of NAC on the invasion of human prostate cancer cells

The capability of the cancer cells to invade the underlying basement membrane is another crucial step determining the severity of the disease. Invasiveness of the prostate cancer cells was assessed using the chemotaxis assay and was performed as in the migration assay, except that the porous polycarbonate filters was pre-coated with the basement membrane matrix, Matrigel®. Invasiveness of the two human prostate cancer cell lines, DU145 and PC3, treated with NAC at various concentrations (0, 5, 10, 15, 20 and 25 mM) was significantly decreased in a dose-dependent manner comparing with those cells without NAC, $p < 0.05$, (Fig. 3). The IC_{50} values of DU 145 and PC3 cells were 5.40 ± 0.42 and 8.89 ± 0.55 mM, respectively.

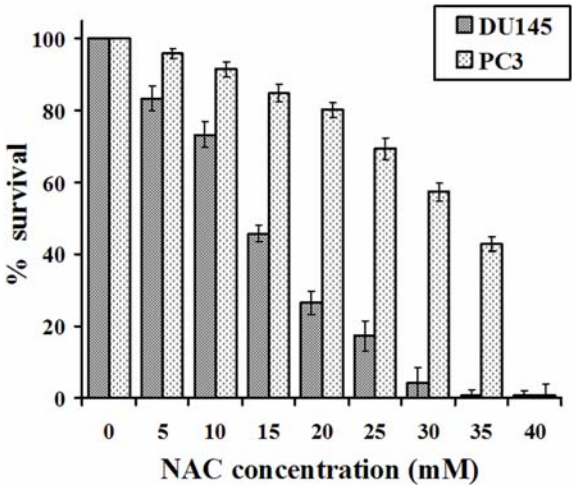

Fig. 1 Effect of NAC on proliferation of the human prostate cancer cells, DU145 and PC3. The cancer cells were treated with NAC at the concentrations of 0, 5, 10, 15, 20, 25, 30, 35 and 40 mM for 24 hours. All concentrations of NAC significantly inhibited cell growth in both cell lines ($p < 0.05$) Percentage of survival was calculated and compared to control (untreated cells). Each data point represents mean ± SD from three independent experiments. Each of the experiment was done in triplicate

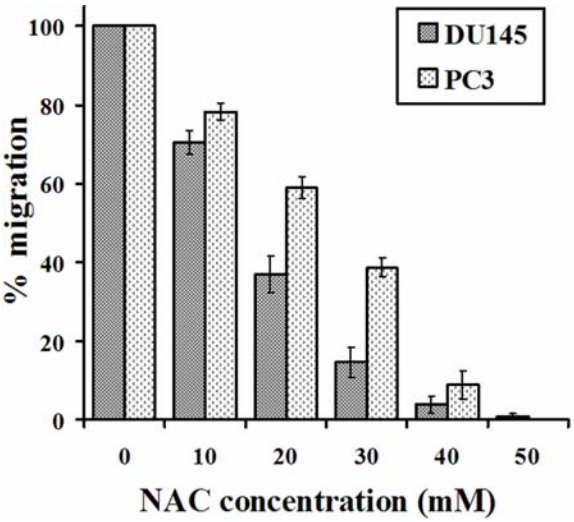

Fig. 2 Effects of NAC on the migratory ability of human prostate cancer cells, DU145 and PC3. The cancer cells were treated with NAC at the concentrations of 0, 10, 20, 30, 40 and 50 mM and then seeded on the collagen IV-coated membrane filters of the chemotaxis chamber. The percentage of the cancer cells migrating through membrane after exposure to NAC at various concentrations was significantly decreased comparing with untreated cells, $p < 0.05$. Each data point represents mean $\pm$ SD from three independent experiments. Each of the experiments was done in triplicate

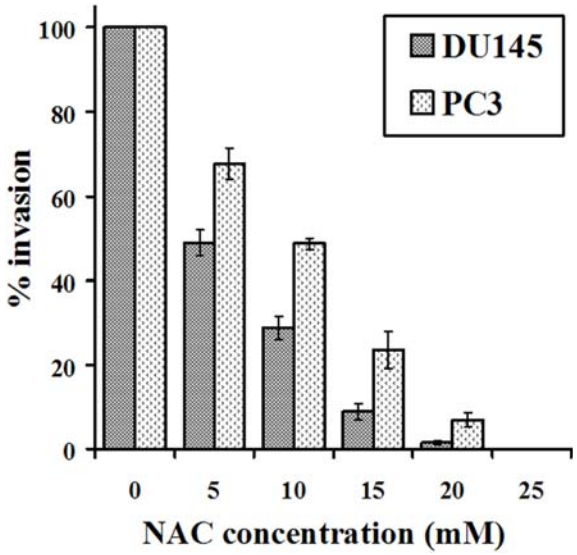

Fig. 3 Effects of NAC on the invasiveness property of human prostate cancer cells, DU145 and PC3. The cancer cells were treated with NAC at the concentrations of 0, 5, 10, 15, 20, and 25 mM and then seeded on the Matrigel-coated membrane filters of the chemotaxis chamber. Invasiveness of DU145 and PC3 treated with NAC at all concentrations was significantly decreased comparing with those cells without NAC. Each data point represents mean $\pm$ SD from three independent experiments. Each of the experiments was done in triplicate

Effects of NAC on the adhesion properties of human prostate cancer cells

Adhesion of cancer cells to remote environments, *e.g.*, epithelial, endothelial cells, basement membrane, or cellular matrix proteins, is one of the inevitably steps in cancer metastasis. Fig. 4 show anti-adhesion effect of NAC on the two human prostate cancer cell lines DU145 and PC3, treated with various concentrations of NAC for 6 h before seeding and allowing the cells to adhere for 30 min. NAC could reduce the number of adherent cells in a dose-dependent manner compared to the control ($p < 0.05$). NAC at the concentration of $\geq$ 6 mM could markedly inhibit the prostate cancer cell adhesion. The IC_{50} of NAC for DU145 and PC3 adhesion were 5.84 $\pm$ 0.54 and 7.96 $\pm$ 0.62 mM, respectively.

Discussion

Spreading of a disease from one tissue where it first started to nearby or another non-adjacent tissue in the body can occur as metastasis. Most cancers, including prostate cancer, have the ability to penetrate or infiltrate many different parts of the body. The most common sites of cancer metastasis are lungs, bones, and liver. Metastasis is a major problem for clinicians handling cancer treatment as it causes suffering and mortality in cancer patients. The most deadly feature of cancer is its capability to metastasize. Metastatic process is still not completely understood due to complication involving various biological steps[12,13]. The adhesion between cancer cells must be firstly deregulated to prevent cancer cells breaking away from the primary tumor and migrating to invade adjacent organs including blood and or lymphatic vessels before establishing a secondary tumor at a remote location. To prevent these events, it is necessary to reduce or inhibit the invasion of the basement membrane by the primary tumor, the migration through the extracellular matrix around the tumor and the adhesion and proliferation of the cancer cells at the nearby or remote tissues[14,15].

The ability to invade the extracellular matrix is a prerequisite for cancer metastasis. It is necessary to overcome the forces that maintain the cell-to-cell adhesion within a primary tumor. Our results showed

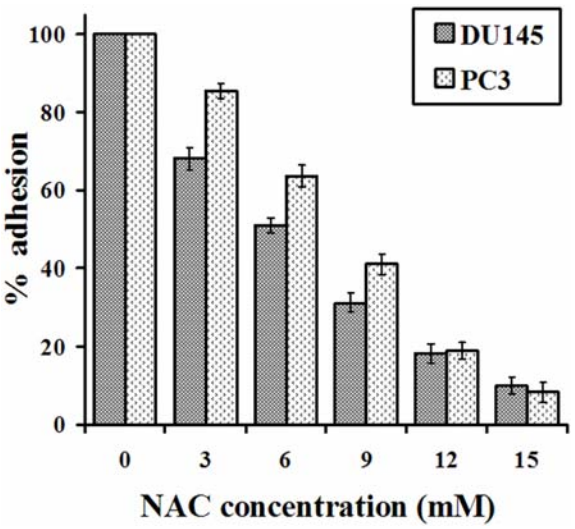

Fig. 4 Effects of NAC on the adhesive property of human prostate cancer cells, DU145 and PC3. The cancer cells were pre-treated with NAC for 6 hr at the concentrations of 0, 3, 6, 9, 12 and 15 mM for 6 h and then seeded on the collagen IV-coated 96-well plate. All concentrations of NAC could reduce the number of adherent cells compared to the control ($p < 0.05$). Each data point represents mean $\pm$ SD from three independent experiments. Each of the experiments was done in triplicate

that NAC could inhibit the migration of DU145 and PC3 with 50% inhibitory concentration (IC_{50}) of 16.01 $\pm$ 0.63 and 23.77 $\pm$ 0.58 mM, respectively. The invasion of DU145 and PC3 through the basement membrane were also inhibited by NAC with low IC_{50} at 5.40 $\pm$ 0.42 and 8.89 $\pm$ 0.55 mM, respectively. The uniform and significant decrease in the motility of both prostate cancer cells that treated with NAC occured in a dose-dependent fashion. These results could indicate a low probability of prostate cancer cells to detach from their primary tumor mass with NAC treatment reducing prostate cancer metastasis.

Obviously the authors found that NAC at low concentrations could inhibit the adhesion of DU145 and PC3 to collagen IV with IC_{50} at 5.84 $\pm$ 0.44 and 7.96 $\pm$ 0.52 mM, respectively. This opens the possibility that NAC could inhibit the attachment of prostate cancer cells to the surface membrane of remote tissues or organs allowing the immune system in the present body to kill the majority of the metastatic cancer cells, hence, reducing the chances of the cancer cells to grow as a secondary tumor.

The authors' results also indicate that NAC could decrease the survival rate of prostate cancer cells and inhibit the growth of both primary and secondary tumors by suppressing the ability of cancer cell to proliferate. Comparing the IC_{50} values of all the experiments, NAC could inhibit the mechanisms of metastasis, namely the migration, invasion and adhesion more than the tumor growth. Moreover, NAC could markedly suppress the invasion and adhesion processes with low IC_{50} value. In all experiments, NAC showed higher inhibitory effects to DU145 than those of PC3. This might be due to the higher metastatic potential of PC3 comparing to that of DU145[15].

The IC_{50} in all experiments of proliferation, migration, invasion and adhesion might be reduced if the period of exposure to NAC is extended. Normally, oral administration of NAC in repeated doses are required for the human body to maintain the serum concentration of NAC more constant, allowing the cancer cells to be regularly exposed to NAC for a longer period. In addition, the human immune system plays a crucial role in fighting cancer cells. So, both processes can complement work each other attenuating the metastasis of prostate cancer and thus the NAC concentration needed for antimetastatic effect is reduced.

Conclusion

The authors' data showed that NAC could significantly suppress the proliferation, migration, adhesion and invasion of both human prostate cancer cells. This might imply that NAC is a potential adjuvant drug for the prevention and treatment of prostate cancer metastasis complementing the conventional treatment modalities. Further investigations to determine the molecular mechanism are worthwhile.

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Potential conflicts of interest

None.

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ศักยภาพของ N-Acetylcysteine ในการต้านการแพร่กระจายของเซลล์มะเร็งต่อมลูกหมากคน

อธิคม สุภาพผล, รุ้งตะวัน สุภาพผล

วัตถุประสงค์: *ศึกษาศักยภาพของ N-Acetylcysteine (NAC) ซึ่งเป็นยาที่ปลอดภัยและมีใช้กันอย่างแพร่หลายในท้องตลาดในการต้านการแพร่กระจายของเซลล์มะเร็งต่อมลูกหมากคน*

วัสดุและวิธีการ: *ทดสอบความสามารถของ NAC ในการยับยั้งการเจริญเติบโตของเซลล์มะเร็งต่อมลูกหมากคนชนิด DU145 และ PC3 โดยใช้สารเอ็มทีที การเคลื่อนย้ายที่และบุกรุกของเซลล์มะเร็งด้วย chemotaxis chamber ที่มีเยื่อเมมเบรน ซึ่งเคลือบด้วยคอลลาเจนโฟ และแมททริเจล รวมทั้งการเกาะตัวของเซลล์มะเร็งด้วยการศึกษาความสามารถในการยึดเกาะบนผิวที่เคลือบด้วยคอลลาเจนโฟ*

ผลการศึกษา: *NAC มีผลต่อเซลล์มะเร็งต่อมลูกหมากคนทั้งชนิด DU145 และ PC3 โดยสามารถยับยั้งการเจริญเติบโต ลดการเคลื่อนย้ายที่ต้านการบุกรุก และการเกาะตัวบนผิวที่เคลือบด้วยคอลลาเจนโฟ ได้อย่างชัดเจนซึ่งการยับยั้งทั้งหมดเป็นลักษณะที่แปรตามขนาดความเข้มข้น*

สรุป: *NAC น่าจะมีศักยภาพสูงในการลดความรุนแรงของเซลล์มะเร็งต่อมลูกหมากคนในการเคลื่อนย้ายที่ และบุกรุกจากก้อนเนื้อมะเร็งปฐมภูมิไปเกิดเป็นก้อนเนื้อมะเร็งทุติยภูมิ ก่อให้เกิดแนวความคิดในการนำ NAC ที่น่าจะมีศักยภาพสูงในการต้านการแพร่กระจายของเซลล์มะเร็งต่อมลูกหมากมาประยุกต์ใช้ร่วมกับรักษามะเร็งต่อมลูกหมากคนตามวิธีมาตรฐานในปัจจุบันเพื่อให้ได้ผลการรักษาที่ดีขึ้น*