N-Acetylcysteine Inhibits Proliferation, Adhesion, Migration and Invasion of Human Bladder Cancer Cells

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Objective: *Bladder cancer is not only a major public health and economically burden for the patients but also a major clinical impact for Thai urologists. The authors' aim was to study the anti-metastatic effect of N-acetylcysteine (NAC), one of the cheap, safe and widely used over-the-counter-drugs in Thailand, on the human bladder cancer cells.*
Material and Method: *Effects of NAC at various concentrations on the growth, adhesion, migration, and invasion of the human bladder cancer cell line were assessed in vitro.*
Results: *NAC at the concentrations of 5, 10, 20 and 30 mM could directly and significantly inhibit the growth, adhesion, migration, and invasion of the human bladder cancer cells in a dose-dependent manner. The 50% inhibitory concentration (IC_{50}) value for cell viability was 33.33 $\pm$ 0.78 mM. The inhibitory effects on migration, invasion and adhesion properties of the cancer cells were dramatically observed at the concentrations of $\geq$ 10, $\geq$ 20, and $\geq$ 30 mM respectively.*
Conclusion: *NAC has an anti-metastatic effect on the human bladder cancer cells by inhibiting their growth, adhesion, migration, and invasion properties. This implies the high possibility that the urologists may apply the results to use it intravesically before, during and after the transurethral resection of bladder tumour, in addition to its conventional usage by oral and parenteral routes.*

Keywords: *N-acetylcysteine, Cell adhesion, Cell migration, Cell proliferation, Cell invasion, Urinary bladder neoplasms*

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Cancer is second only to cardiovascular disease as a cause of death in the United States[1]. In Thailand, cancer is also a major health problem accounting for the second most common cause of death after accidents[2]. Urinary bladder cancer is the second most common urological malignancy and representing for 2.46% among the ten leading sites of all cancers in the human body in the Thai populations with the male-to-female ratio about 3:1 (age-specific incidence rate of 4.2 and 1.3 per 10^5 population in men and women respectively)[3,4]. The clinical course of bladder cancer can be divided into 2 categories. The first group, approximately 70% of bladder cancer at presentation, is superficial non-muscle-invasive bladder cancer. It is defined as tumor confined to the mucosa (stage Ta, carcinoma in situ, Tis) and submucosal layers of the bladder (stage T1; TNM classification). This which have minimal risk of progression to death, but the recurrence rate in this group of tumors is as high as

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80%. About 10 to 20% of them may progress to muscle-invasive lesions[5]. Another group is high-grade, muscle-invasive cancer (stage T2-T4) and often lethal due to the high risk of progression. Transurethral resection or fulguration with following immunological or chemotherapeutic agent instillations into the bladder is preferred for superficial bladder cancer. Radical cystectomy with urinary diversion is mandatory for high-grade, muscle-invasive bladder cancer. However, the results of the treatments, apart from the health and economic burdens, are mostly unsatisfactory due to various factors, *e.g.*, high recurrence, and late or invasive stage at the first time that the patients were consulted or came to see the urologists. Morbidity and mortality of the cancer patients are not only caused by the growth of the primary tumor mass itself but also resulted from its invasive property to spread to other sites away from the original location. Even that invasive and metastatic bladder cancer still have both clinical and scientific challenges for urologists and their colleagues.

N-acetylcysteine (NAC), $C_5H_9NO_3S$, is a pharmacological agent widely and mainly used in various cough preparations as a mucolytic agent and in the management of paracetamol (acetaminophen) overdose. NAC also has powerful antioxidant effects by scavenging and quenching the reactive oxygen species (ROS), the harmful free radicals that can cause oxidative damage to muscles, organs, and DNA in the body. It acts intracellularly as a precursor of glutathione (GSH) and cysteine and extracellularly as an analogue of GSH. As a glutathione precursor, it may also boost the immune system function. These properties together with other mechanisms make NAC as one of the chemopreventive agents in the treatment of cancer metastasis[6-8].

The goal of the present study was to examine and evaluate whether NAC, one of the safe and widely used over-the-counter-drugs, could inhibit the processes of adhesion, invasion and migration of the bladder cancer cells. This could be applied to use clinically as another option for chemoprevention of the bladder cancer metastasis and thus minimize the morbidity and mortality of the patients.

Material and Method

Cell line and cell culture conditions

The human T24 bladder cancer cell line was a generous gift provided by Professor Akihiko Okuyama. (Department of Urology, Osaka University Graduate School of Medicine, Japan.) The cancer cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) (Gibco BRL), 100 U/ml penicillin (Gibco BRL), and 100 mg/ml streptomycin (Gibco BRL). The cells were grown in monolayers, maintained in a tissue culture incubator at 37°C in humidified air containing 5% CO_2 until 80% confluency. and then subcultured as described previously elsewhere[9].

Cell proliferation assay

The cytotoxic effects of NAC (Sigma) on the proliferative capability of the human bladder cancer cells were determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described previously[10] with some modifications. Briefly, the cancer cells at a density of 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, the cells were washed twice with phosphate-buffered saline (PBS) and 150 ml of culture medium containing 1 mg/ml of MTT dye (Sigma) was added to each well and incubated further for 4 h. The medium containing MTT dye was then replaced with 150 ml of dimethyl sulfoxide (DMSO). The spectrophotometric absorbancy of the blue color of the oxidized MTT (formazan) was measured at 570 nm using ELISA microplate reader (BIO-RAD 550). The percentage of proliferation was calculated and based on the untreated cells.

Cell adhesion assay

The adhesion assay was performed as described previously[11] with some modifications. Briefly, 96-well culture plates (Corning) were coated with 1 μg/ml collagen IV (Sigma) at 4^OC overnight. Then the solution was decanted and blocked for 1 h at 37°C with 1% bovine serum albumin (BSA) (Sigma). The wells were washed twice with PBS and once with DMEM to remove the excess of BSA prior to the addition of the bladder cancer cells. Cultured cells were washed with serum-free medium, trypsinized, incubated with various concentrations of NAC for 0, 1 and 3 h and reseeded at a density of 5 x 10^4 cells/ well on the pre-coated plates. After allowing the cells to adhere for 3 h 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 absorbance at 595 nm using an ELISA microplate reader.

Cell migration assay

The migratory capability of bladder cancer cells was assessed by using 48-well chemotaxis chamber, (Neuroprobe Inc. Gaithersburg, MD) as described previously[12] with some modifications. Briefly, the cancer cells, adjusted at 1 x 10^6 cells/ml in cultured medium containing NAC at various concentrations, were seeded on the collagen IV-coated surface of the polycarbonate membrane of 8 μm pore size in the upper compartments. The corresponding lower compartments were filled with culture medium alone (control) or 10 ng/ ml of epidermal growth factor (EGF) (Sigma) as the chemoattractant. After incubation for 6 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 on the lower surface of the filter were fixed and stained using a Diff-Quick Stain Kit (Baxter) and counted under a light microscope in five random fields (40x).

Cell invasion assay

The invasiveness of bladder cancer cells was assessed by using 48-well chemotaxis chamber, a membrane invasion culture system (Neuroprobe Inc. Gaithersburg, MD) as described previously[13] with some modifications. The membrane filters were coated with Matrigel (Becton Dickinson) and the assays were subsequently performed similar to those of the cell migration assays.

Statistics

All data are presented as mean ± standard deviation (SD) of at least three separate experiments with different preparations of the cells that 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. Statistical significance was considered when $p < 0.05$.

Results

Effects of NAC on the cell viability

The MTT assay was used to determine the effect of NAC at various concentrations, on the viability or proliferative capability of T24 bladder cancer cells. After exposure of the cancer cells to NAC for 24 h, the proliferation of T24 cell growth was inhibited significantly in a dose dependent manner, $p < 0.05$, (Fig. 1). The 50% inhibitory concentration (IC_{50}) value was 33.33 ± 0.78 mM. The percentage of cell survival was calculated based on the untreated cells.

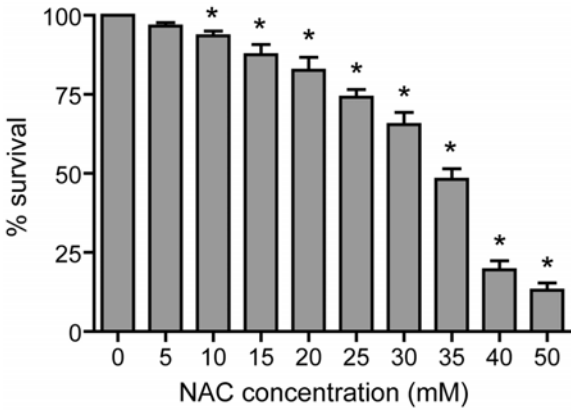

Fig. 1 Effects of NAC on the viability of the human T24 bladder cancer cells. The cancer cells were treated with NAC at the concentrations of 0, 5, 10, 15, 20, 25, 30, 35, 40 and 50 mM for 24 hours. Each data point represents mean ± SD from three independent experiments. Each of the experiments was done in triplicate

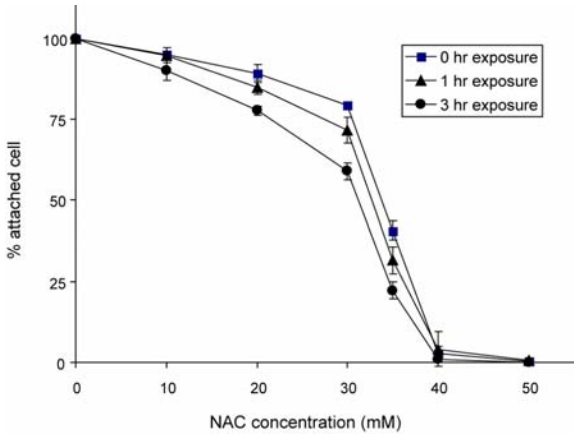

Fig. 2 Effects of NAC on the adhesive property of the human T24 bladder cancer cells. The cancer cells were pre-treated with NAC at the concentrations of 0, 10, 20, 30, 35, 40 and 50 mM for 1 and 3 h. Each data point represents mean ± SD from three independent experiments. Each of the experiments was done in triplicate

Effects of NAC on the adhesion of human T24 bladder cancer cells on the extracellular matrix proteins

The adhesion of the cancer cells to the epithelial or endothelial cells, basement membrane, or cellular matrix proteins is one of the important and earlier steps in the cancer metastatic processes. The authors assessed the anti-adhesion effect of NAC on the human T24 bladder cancer cells by treating the cells with various concentrations of NAC for 0, 1, and 3 h before seeding and allowing the cells to adhere for 3 hrs. As seen in the Fig. 2, NAC inhibited the cancer cell adhesion by reducing the number of attached cells in a dose and time dependent manner comparing with the control ($p < 0.05$). At the concentration of $\geq$ 30 mM it could inhibit the cell adhesion dramatically and with complete inhibition at 40 and 50 mM. The inhibition of cell adhesion could be observed from 0, 1, and 3 h and the differences were statistically significant particularly between 0 and 3 h.

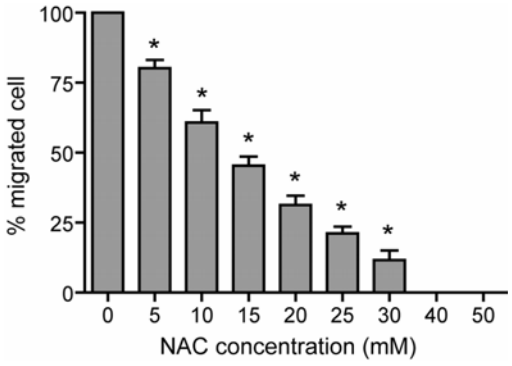

Fig. 3 Effects of NAC on the migratory property of human T24 bladder cancer cells. The cancer cells were treated with NAC at the concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 mM and then seeded on the collagen IV-coated membrane filters of the chemotaxis chamber. Each data point represents mean $\pm$ SD from three independent experiments. Each of the experiments was done in triplicate

Effects of NAC on the migration of human T24 bladder cancer cells

Another acquisition of a metastatic or invasive phenotype by the cancer cells is the capability to migrate or spread from their original site to the new remote locations. It is important among other various complex processes of cancer metastasis. The authors assessed the effect of NAC on the motility of the human T24 bladder cancer cells by treating the cells with NAC at various concentrations (0, 5, 10, 15, 20, 25, 30, 40 and 50 mM) and seeding them 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 pores 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. 3). As seen in Fig. 3 and 4, the numbers of migrated cancer cells on the under surface of the membrane was markedly and significantly decreased after they had been treated with NAC, $p < 0.05$. At the concentration of $\geq$ 10 mM it could dramatically inhibit the cancer cell migration and completely at 40 and 50 mM. The IC_{50} value was 12.82 ± 0.20 mM.

Effects of NAC on the invasion of human T24 bladder cancer cells

Capability to invade the underlying basement membrane, the cellular and intercellular matrix proteins and finally the blood and lymphatic vessels is another important step in the cancer metastatic processes. The modified Boyden transwell chamber (48-well chemotaxis transwell chamber, Neuroprobe) with the polycarbonate membrane filter which was pre-coated with Matrigel were used to determine the effects of NAC on the invasiveness of the human T24 bladder cancer cells. The authors observed uniform decrease of invasiveness of the cancer cells.The percentage of the invaded cancer cells after exposure to NAC at various concentrations was significantly decreased in a dose dependent manner comparing with those cells

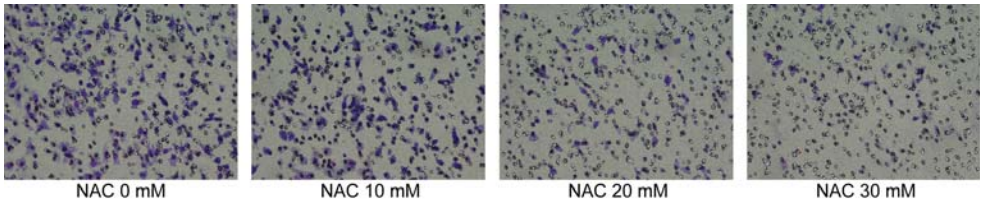

Fig. 4 Effects of NAC on the migration of human T24 bladder cancer cells seen under a light microscope (40x)

without NAC, $p < 0.05$, (Fig. 5). At the concentration of $\geq$ 20 mM it could dramatically inhibit the cancer cell invasion and completely at 40 and 50 mM. The number of the invaded cancer cells on the under surface of the membrane was markedly and significantly decreased after they had been treated with NAC, $p < 0.05$, (Fig. 6). The IC_{50} value was 22.20 $\pm$ 0.85 mM.

Discussion

Metastasis is a major problem for clinicians in handling cancer treatment. It remains a significant contributor to morbidity and mortality in cancer patients. It is a complex process involving various biological steps such as dysregulation of adhesion between cell to cell, detachment of cancer cells from the primary tumor, migration from the extracellular matrix around the primary tumor, invasion through the basement membrane of the primary tumor and adhesion of cancer cells to the basement membrane and to components of the extracellular matrix.

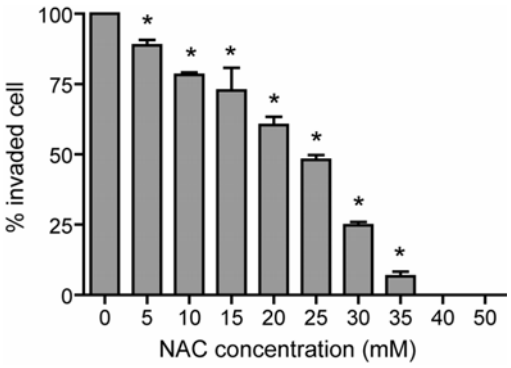

Fig. 5 Effects of NAC on the invasiveness property of human T24 bladder cancer cells. The cancer cells were treated with NAC at the concentrations of 0, 5, 10, 15, 20, 25, 30, 40 and 50 mM and then seeded on the Matrigel-coated membrane filters of the chemotaxis chamber. Each data point represents mean $\pm$ SD from three independent experiments. Each of the experiments was done in triplicate

Degradation of basement membrane and extracellular matrix, cell migration, invasion into stroma, intravasation, adhesion to endothelia and epithelia at remote sites and extravasation are also contributed[14-16]. The present results showed that NAC could inhibit the proliferation of T24 cells significantly after 24 h of treatment. The estimated 50% inhibitory concentration was 33.33 $\pm$ 0.78 mM which could be reduced if the longer period of exposure of the cells to NAC[17], corresponding to the principle of maintaining of drug level. The immune system in the human body kills the majority of the metastatic cancer cells. So, the fewer number of cancer cells at the original site, the less chance of the cancer cells to propagate to the remote location. Hence, the reduction in the number of the cancer cells by NAC may be an index indicating that the drug in some degree can inhibit the progression of the cancer cells.

Cell adhesion to extracellular matrix proteins and to the surfaces of other cells are mediated by various cell surface receptors and cell adhesion molecules including integrins and cadherins. Alterations in the expression and function of cell adhesion molecules contribute to many disease states including cancer[18,19]. In a previous report it showed the invasiveness properties of T24 cells, both high- and low-invasive subpopulations, on the matrigel[20]. The presented experiments demonstrated that NAC significantly inhibited adhesion capability of T24 cancer cells to the collagen IV which was of the important components of the extracellular matrix including membrane proteins. To the best of the authors' knowledge, this has not been studied before. The inhibitory effect was significantly both dose and time dependent. This would imply that NAC can inhibit the attachment or seeding of the circulating T24 cancer cells to the surface membrane of the remote tissues or organs and thus inhibiting or reduction in the metastasis of cancer.

Many biological processes such as embryonic morphogenesis, immune surveillance, and wound

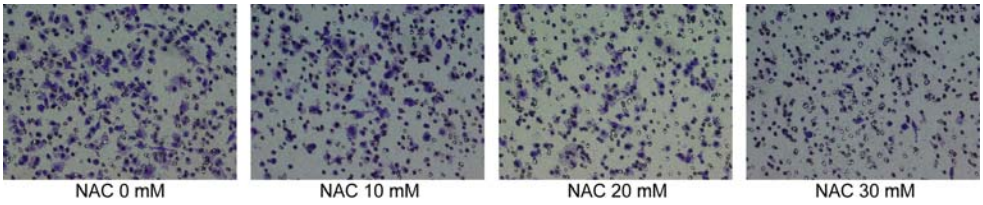

Fig. 6 Effects of NAC on the invasion of human T24 bladder cancer cells seen under a light microscope (40x)

healing are driven by cell migration. Abnormal cell proliferation and dysregulation of cell migration are also involved in many diseases, including cancer invasion and metastasis[21,22]. The authors observed the uniform decrease in motility of T24 cancer cells with NAC treatment. The effect was dose dependent with low IC_{50} value (12.82 ± 0.20 mM). This indicates that with NAC treatment, the less probability of T24 cancer cell movement from the primary tumor mass to a new location and thus inhibiting or reduction in the metastasis.

Invasion is another devastating process that is necessary for tumor metastasis formation and its sequels will ultimately lead to more than 90% of cancer deaths with no effective therapeutic option currently available for them[23]. If the authors could inhibit or reduce the metastasis, it could lessen the mortality or at least the morbidity of cancer patients. The results of the presented cell invasion assays demonstrated that NAC significantly inhibited the invasiveness of T24 cancer cells. The effect was dose dependent with IC_{50} value about 22.20 ± 0.85 mM. The presented data suggested that NAC might attenuate the process of T24 cancer cell invasion to the underlying basement membrane, intercellular matrix or remote tissues and organs. Hence, the drug may effectively inhibit or reduce the processes of bladder cancer metastasis.

Conclusion

The reduction in T24 cancer cell proliferation or growth of the tumor, cell adhesiveness, motility and invasiveness with NAC treatment confirmed the authors' hypothesis that NAC could inhibit T24 human bladder cancer cell metastasis more than its cytotoxic effect. The presented work demonstrated that NAC, one of the cheap and widely used over-the-counter drugs, might be worthwhile in clinical application particularly for urologists to use either orally and or intravesically along with the conventional management of the human bladder cancer in order to prevent the establishment of metastatic disease and tumor recurrences. These warrant further investigations with the concept for the utmost health care and lowest budget particularly during the recent world hamburger economic crisis.

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ความสามารถของ N-acetylcysteine ในการยับยั้งการเพิ่มจำนวนเซลล์ การเกาะตัว การเคลื่อนตัว และการบุกรุกของเซลล์มะเร็งกระเพาะปัสสาวะคน

อธิคม สุภาพผล, วีระสิงห์ เมืองมั่น, วรินทร ชวศิริ, รุ้งตะวัน สุภาพผล, วันดี กฤษณพันธ์

วัตถุประสงค์: *เพื่อศึกษาความสามารถของ N-acetylcysteineที่ใช้อยู่แพร่หลายในท้องตลาดและมีราคาถูกในการยับยั้งการกระจายของเซลล์มะเร็งกระเพาะปัสสาวะคนซึ่งเป็นปัญหาสำคัญทางสาธารณสุขต่อตัวผู้ป่วยและศัลยแพทย์ระบบทางเดินปัสสาวะในประเทศไทย*

วัสดุและวิธีการ: *ทดสอบความสามารถของ NAC ที่ความเข้มข้นต่าง ๆ ในการยับยั้งการเจริญเติบโต การเกาะตัว การเคลื่อนตัว และการบุกรุกของเซลล์มะเร็งกระเพาะปัสสาวะคน*

ผลการศึกษา: *NAC ที่ความเข้มข้น 5, 10, 20 และ 30 มิลลิโมล่าร์ สามารถยับยั้งการเจริญเติบโต การเกาะตัว การเคลื่อนตัว และการบุกรุกของเซลล์มะเร็งกระเพาะปัสสาวะคนได้อย่างมีนัยสำคัญทางสถิติ ความเข้มข้นของ NAC ที่ 33.33 $\pm$ 0.78 มิลลิโมล่าร์ สามารถยับยั้งการเจริญเติบโตของเซลล์มะเร็งได้ 50% (ค่า IC_{50}) นอกจากนี้ NAC ยังสามารถยับยั้งคุณสมบัติในการเคลื่อนตัว การบุกรุก และการเกาะตัวของเซลล์มะเร็งได้มากอย่างชัดเจนที่ความเข้มข้น $\geq$10, $\geq$20, และ $\geq$30 มิลลิโมล่าร์ ตามลำดับ*

สรุป: *NAC มีฤทธิ์ต้านการกระจายของเซลล์มะเร็งกระเพาะปัสสาวะคน โดยยับยั้งความสามารถในการเพิ่มจำนวนเซลล์ การเกาะตัว การเคลื่อนตัว และการบุกรุกของเซลล์มะเร็ง ซึ่งเป็นสิ่งบ่งบอกถึงความเป็นไปได้ที่ศัลยแพทย์ระบบทางเดินปัสสาวะจะนำไปประยุกต์ใช้ใส่ในกระเพาะปัสสาวะก่อน ระหว่าง หรือ หลังการผ่าตัดส่องกล้อง*