APPENDIX

DEPLETION OF SUPEROXIDE ANION RADICALS BY INCREASING MANGANESE SUPEROXIDE DISMUTASE AFFECTED THE GROWTH PATTERN OF NORMAL AND CANCER GASTRIC MUCOSAL CELLS

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การลดระดับสารอนุมูลอิสระซุปเปอร์ออกไซด์แอนไอออนโดยการเพิ่มระดับแมงกานีสซุปเปอร์ออกไซด์ดีสมูวเตสส่งผลต่อแบบแผนการเจริญเติบโตของเซลล์ปกติและมะเร็งเยื่อบุกระเพาะอาหาร

DEPLETION OF SUPEROXIDE ANION RADICALS BY INCREASING MANGANESE SUPEROXIDE DISMUTASE AFFECTED THE GROWTH PATTERN OF NORMAL AND CANCER GASTRIC MUCOSAL CELLS

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บทคัดย่อ: การศึกษาชีววิทยาของเซลล์เยื่อบุกระเพาะอาหารปกติเปรียบเทียบกับเซลล์มะเร็งที่ถูกชักนำด้วย N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) ทำให้ได้ฐานข้อมูลที่สำคัญเกี่ยวกระบวนการเกิดมะเร็ง โดยหนึ่งในออร์กาเนลที่มีความสำคัญต่อการมีชีวิตที่มีกระทบในระหว่างที่เซลล์ปกติพัฒนาไปเป็นเซลล์มะเร็งคือไมโตคอนเดรีย วัตถุประสงค์ของการศึกษานี้เพื่อศึกษาถึงการบกพร่องของระบบต้านสารอนุมูลอิสระแมงกานีสซุปเปอร์ออกไซด์ดีสมูวเตสน่ามีบทบาทหลักในการทำให้เซลล์อยู่ในสภาวะเครียดเนื่องสารออกซิเดชันในไมโตคอนเดรีย ส่งให้อัตราการเจริญเติบโตจำเพาะของเซลล์มะเร็งช้าลงเมื่อเทียบกับเซลล์ปกติ เพื่อวัตถุประสงค์ดังกล่าว จึงได้ทำการชักนำให้เซลล์ปกติและมะเร็งมีการแสดงออกของแมงกานีสซุปเปอร์ออกไซด์ดีสมูวเตสสูงขึ้นโดยวิธียีนทรานเฟคชัน การเพิ่มขึ้นของระดับแมงกานีสซุปเปอร์ออกไซด์ดีสมูวเตสส่งผลให้มีการเพิ่มอัตราการเจริญเติบโตจำเพาะของเซลล์มะเร็งแต่ลดอัตราการเจริญเติบโตจำเพาะของเซลล์ปกติตามลำดับ ผลการทดลองสื่อถึงระดับสารอนุมูลอิสระ ซุปเปอร์ออกไซด์แอนไอออนในไมโตคอนเดรียของเซลล์มะเร็งน่าสูงกว่าในเซลล์ปกติ และในสภาวะที่เพิ่มระดับของแมงกานีสซุปเปอร์ออกไซด์ดีสมูว เตสมีผลทำให้เกิดเปลี่ยนสารอนุมูลอิสระไปเป็นออกซิเจนและไฮโดรเจนเปอร์ออกไซด์ การเติมสารอนุมูลอิสระจากภายนอกเช่นควอซีตินมีผลทำให้ระดับสารอนุมูลอิสระภายในเซลล์ลดลง ทั้งเซลล์มะเร็งและเซลล์มะเร็งที่ทรานเฟคยกเว้นในกลุ่มเซลล์ปกติ อย่างไรก็ตามควอซิตินมีผลลดอัตราการเจริญเติบโตจำเพาะในทุกเซลล์ที่ศึกษา ผลการศึกษาชี้ให้เห็นว่าระบบ

ต้านสารอนุมูลอิสระแมงกานีสซุปเปอร์ออกไซด์ดีสมูวเตสเสียไประหว่างที่เซลล์ปกติพัฒนากลายเป็นเซลล์มะเร็ง

Abstract: The biology of normal gastric mucosal compared with its corresponding cancer cells induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) is crucial provided the data base of pathogenesis. One of the pivotal intracellular organelle affected during the normal cell development to be cancer cells should be mitochondria. This study aimed to investigate whether the defects of Mn-SOD antioxidant system might predominantly cause an increase in mitochondrial oxidative stress by which leading a slower specific growth rate of cancer compared with normal cells. For these purposes, the normal and cancer gastric mucosal cells were transfected with the pCR3.1-Uni plasmids containing a sense human Mn-SOD cDNA insert using the Lipofectamine 2000 reagent. The overexpression of Mn-SOD caused an increase while decrease in specific growth rate of cancer and normal cells, respectively. The results signified that the mitochondrial $O_2^{-\bullet}$ level of cancer cell might be higher amounts than the normal cells and the presence of additional Mn-SOD should catalyze the breakdown of the radicals to oxygen and H_2O_2. An additional exogenous antioxidant such as quercetin caused a change of the intracellular reactive oxygen species content of both cancer and cancer transfected exception their corresponding normal cells. However, quercetin decreased in specific growth rate of all cell lines. The results suggested the Mn-SOD antioxidant system was impaired during normal cell development to be cancer cells.

Introduction: Free radicals are important intermediates constantly produced in vivo through a variety of normal metabolic processes as well as being common intermediates generated after exposure to drugs, xenobiotics or ionizing radiation (Curtin et al., 2002). The last decade has seen a huge surge of interest in free radicals in biology, and the pathogenesis of many diseases has been associated with intracellular reactive oxygen species (ROS_i) (Halliwell and Gutteridge, 1999). Furthermore, the uncontrolled generation of ROS_i may lead to aging, inflammation, and neurodegenerative disorders (Sun and Chen, 1998). ROS_i are generated by mitochondria via the release of electrons from the electron transport chain and the reduction of oxygen molecules to superoxide anion radicals ($O_2^{-\bullet}$). Superoxide anion radicals, through the reaction catalyzed by superoxide dismutase (SOD), are transformed into the much less reactive hydrogen peroxide moiety (H_2O_2). Production of ROS_i is essential for a number of biochemical reactions involved in the synthesis of prostaglandins, hydroxylation of proline and lysine, oxidation of xanthine and other oxidative processes (Halliwell and Gutteridge, 1999). Excessive oxidation leads to impairment of cell functions and development of morbid conditions (Halliwell and Cutteridge, 1999, Amer et al 1993). The cells maintain the ROS_i pool to the tolerable levels via both endo- and exogenous antioxidant, particularly the manganese-superoxide dismutase (Mn-SOD) that present in the mitochondria catalyzes the breakdown of the superoxide anion into oxygen and H_2O_2. The molecular yield, H_2O_2 can freely diffuse throughout the cells thus protect the mitochondrial damage from $O_2^{-\bullet}$. Moreover, H_2O_2 is an important mediator for various cellular activities including detoxification, death and growth. We have previously reported that the mitochondrial of multidrug resistant cancer cells have defects of electron transport chain and

antioxidant system compared with their corresponding drug sensitive cells (Reungpatthanaphong et al., 2003). Indeed the mitochondrial dysfunction should be caused by the exceeded of $O_2^{-\bullet}$ concentration (Kothan, 2004). However, an elimination of the $O_2^{-\bullet}$ by increasing the Mn-SOD at the matrix compartment affected the redox state thus growth pattern of cells did not study.The results reported here in this study showed that, when the normal and cancer gastric mucosal cells were transfected with Mn-SOD gene and the transfected cells presented the high level of Mn-SOD, locally accumulated in the mitochondria. These resulted in a decrease in ROS_i and delayed the growth of normal but not cancer gastric mucosal cells.

Methodology: A rat normal gastric mucosal cell line (RGM1) and an N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) induced gastric cancer cell line (RGK1) were use in this study. RGM1 and RGK1 cells were stably transfected with the pCR3.1-Uni plasmids only or the pCR3.1-Uni plasmids containing a sense human Mn-SOD cDNA insert using the Lipofectamine 2000 reagent according to the manufacturer's instructions. Cells were cultured in Dulbecco's modified Eagle's medium/ Nutrient F-12 (DMEM /F-12) supplemented with 10% fetal bovine serum (FBS) at 37 ^{o}C in a humidified atmosphere 5 % CO_2 in air. A nondenatured gel assay for SOD activity was performed according to the method of Beauchamp and Fridovich (Anal Biochem. 1971) with slight modification. **Determination of cell growth pattern and ROS_i**: cells were seed at density of 5×10^4 per dish and incubated in the absence or in the presence of quercetin 30, 100 and 200 μM. After 24, 48, 72, 96 and 120 h, cells were counted using a Particle Counter PA-2000. Hydroxyphenyl fluorescein (HPF), a fluorescence dye for selectively detecting $OH^{\bullet}$ radicals, was used in the present study. Cells were treated with quercetin for 24 h and then 10 μM HPF was added to the cells before incubation for 15 min at 37°C. Bioimages of HPF were obtained using a CSU-10 confocal laser scanning unit. HPF was excited at 488 nm and the emissions were filtered using a 515-nm barrier filter. Statistic Analysis: descriptive data and *t*-test analyses were performed with SPSS software version 10.

Results, Discussion and Conclusion: Normal gastric mucosal cell line (RGM1) and its cancer cells (RGK1) were stably transfected with the pCR3.1-Uni plasmids only or the pCR3.1-Uni plasmids containing a sense human Mn-SOD cDNA insert. The expression of MnSOD was characterized by measuring its activity using a nondenatured gel assay. Figure 1 demonstrated that the human Mn-SOD activity in the transfected cells were clearly detectable and higher level compared with those of parental and control plasmid transfected cells. The results also indicated that the human Mn-SOD was slightly found in RGM1 and RGK1 cells.

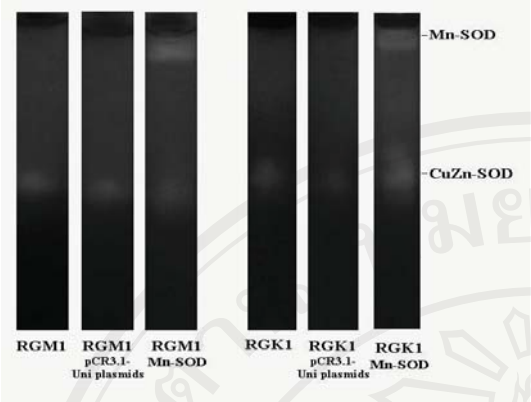

Figure.1. The human Mn-SOD activity. Cells were lysed and the supernatant containing 50 mg of protein was loaded and separated by electrophoresis through a 12% polyacrylamide gel at 4°C. Native polyacrylamide gel was stained for SOD.

The effects of an overexpression of Mn-SOD on cell growth pattern were clearly shown in Figure 2a. The specific growth rate (γ) was determined as indicated in Figure 2b. Contrary to cancer cells, an increase in Mn-SOD level resulted in slower specific growth rate of normal gastric mucosal cells although the Mn-SOD was found to up regulate in the both transfected cells. The results signified that the $O_2^{-\bullet}$ level of cancer cell might be higher amounts than the normal cells and the presence of additional Mn-SOD should catalyze the breakdown of the radicals to oxygen and H_2O_2 resulted in a depletion of $O_2^{-\bullet}$ of normal cells but in the balance level of cancer cells. In order to verify whether a depletion of ROS_i other than originated from mitochondrial can also be affected the growth pattern an exogenous antioxidant quercetin was used. The cell growth assays were performed in the absence or presence of quercetin (30, 100 and 200 μM) and the cell numbers were counted at 24, 48, 72, 96 and 120 h. Figure 2b also showed that quercetin inhibited cell growth in RGM1, RGK1, Mn-SOD transfected and control plasmid transfected cells in dose dependent manner.

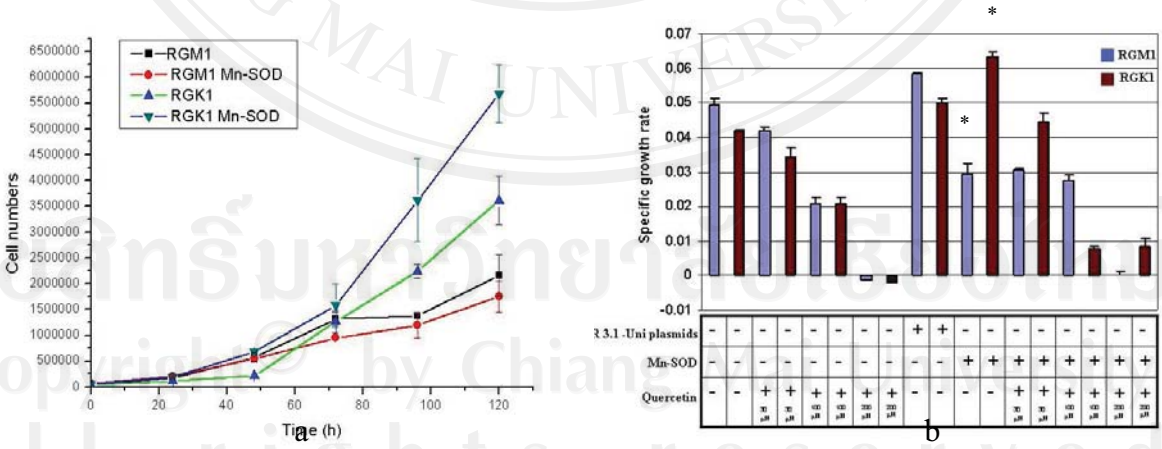

Figure 2 Growth curves (a) and variation of specific growth rate (b) of RGM1, RGK1, Mn-SOD-transfected cells. * Represents $P<0.05$ compared to parental cells.

The endogenous ROS_i levels of both normal and cancer cells and their corresponding transfected cells were measured as indicated in Figure 3. The results demonstrated that the ROS_i of cancer cells was higher amount than those of normal

cells. It should be noted that up to 200 μM quercetin caused gradually decreased in ROS_i of cancer cells but did not for normal cells. The similar results were observed for normal and cancer transfected cells. However, the similar series of experiments performed using erythromyelogenous leukemic cell and small cell lung carcinoma found that quercetin is an efficient antioxidant molecule and it passively diffused through the plasma membrane and immediately depleted the ROS_i (Kothan 2004).

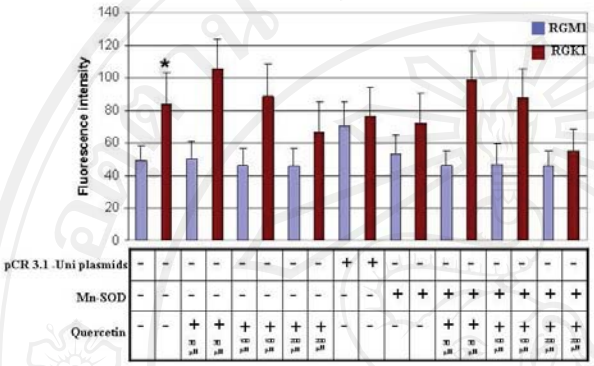

Figure 3. Effects of Mn-SOD and quercetin on the intracellular reactive oxygen species of normal and cancer gastric mucosal cells and their corresponding Mn-SOD transfected cells. * Represents $P<0.05$ compared normal cells.

This study was clearly shown that cancer gastric mucosal cell consisted of ROSi levels higher than the normal cells. In addition, an exogeneous antioxidant quercetin did not deplete the ROS_i in normal gastric mucosal cell but done in cancer cells. We would like to stress here that the cancer cells have defects of antioxidant system probably the Mn-SOD by which leading an impairment of mitochondria compared with normal cells. The finding suggested that the pivotal intracellular organelle changed during normal cell development to be cancer cells should be mitochondria**.**

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Keywords: Normal gastric mucosal cells, mitochondrial defect, intracellular reactive oxygen species, Mn-SOD activity, Cancer gastric mucosal cells

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