Update on a tumor-associated NADH oxidase in gastric cancer cell growth

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Abstract

Gastric cancer is one of the most common human malignancies, and its prevalence has been shown to be well-correlated with cancer-related deaths worldwide. Regrettably, the poor prognosis of this disease is mainly due to its late diagnosis at advanced stages after the cancer has already metastasized. Recent research has emphasized the identification of cancer biomarkers in the hope of diagnosing cancer early and designing targeted therapies to reverse cancer progression. One member of a family of growth-related nicotinamide adenine dinucleotide (NADH or hydroquinone) oxidases is tumor-associated NADH oxidase (tNOX; ENOX2). Unlike its counterpart CNOX (ENOX1), identified in normal rat liver plasma membranes and shown to be stimulated by growth factors and hormones, tNOX activity purified from rat hepatoma cells is constitutively active. Its activity is detectable in the sera of cancer patients but not in those of healthy volunteers, suggesting its clinical relevance. Interestingly, tNOX expression was shown to be present in an array of cancer cell lines. More importantly, inhibition of tNOX was well correlated with reduced cancer cell growth and induction of apoptosis. RNA interference targeting tNOX expression in cancer cells effectively restored non-cancerous phenotypes, further supporting the vital role of tNOX in cancer cells. Here, we review the regulatory role of tNOX in gastric cancer cell growth.
INTRODUCTION

According to Global Cancer Statistics, nearly 1 million new gastric cancer cases and more than 700000 gastric cancer-associated death were reported in 2012[1]. Unfortunately, despite progression in the diagnosis and treatment of advanced gastric cancer, many patients suffer from metastasis and later recurrence of this disease. Thus, their poor prognosis reflects the fact that gastric cancers are often diagnosed at an advanced stage. Current research efforts have focused on available diagnostic and prognostic biomarkers of gastric cancer. For example, carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9) have been used as standard biomarkers for gastric cancer diagnosis[2]. A method for detecting circulating tumor cells in gastric cancer patients, which could provide important targets for treatment and critical surrogate markers, is also being developed[3]. Moreover, polymorphisms of certain genes, such as epidermal growth factor receptor (EGFR), have been used as risk markers for gastric cancer[4]. Serum level of interleukin-18 (IL-18), which plays a role in the pathogenesis of malignancies and is a determinant of clinical outcome in gastric cancer patients, is another risk marker[5]. Recent studies have revealed that several microRNAs, when abnormally expressed, are potential biomarkers of gastric cancer[6]. Although intensive efforts to identify biomarkers in this field are ongoing, universal biomarkers for gastric cancer are scarce because of the heterogeneous properties of this cancer[2]. Thus, new and specific gastric cancer markers for diagnoses and therapeutic purpose are urgently needed.

We have previously described tumor-associated NADH oxidase (tNOX, also known as ENOX2), a member of a family of growth-related NADH (or hydroquinone) oxidases[7-10]. Unlike its counterpart CNOX (ENOX1), identified in normal rat liver plasma membranes and shown to be stimulated by growth factors and hormones, tNOX activity purified from rat hepatoma cells is unresponsive to growth stimuli and is instead constitutively active[7]. Subsequent studies have confirmed that tNOX is present in an array of cancer cell lines, including those derived from breast, cervix, colon, and lung cancer as well as leukemias[11-14]. Its activity is also observed in the sera of cancer patients but not in those of healthy volunteers, suggesting its clinical relevance[15-17]. tNOX cDNA was subsequently cloned from a HeLa cell cDNA library[9], and functional motifs of tNOX protein have been identified. The important role of tNOX in cell growth regulation is supported by a study that showed that the growth rate of mouse embryo fibroblasts (MEFs) from tNOX-overexpressing transgenic mice is approximately twice that of wild-type cells[18]. Given its expression in an array of cancer cell lines and presence in sera of cancer patients, taken together with other characteristics discussed above, tNOX possesses potential as a biomarker. Here, we briefly review the regulatory role of tNOX in gastric cancer cell growth.

EXPRESSIONS OF tNOX PROTEIN IN GASTRIC CANCER CELLS

Although tNOX expression has been demonstrated in an array of cancer cell lines, there is relatively less information regarding tNOX protein expression in gastric cancer cells. To demonstrate the expression of tNOX protein in gastric cancer cells, we utilized cell lines derived from human stomach cancers, including AGS (gastric adenocarcinoma), TMC-1 (derived from the lymph node of a moderately differentiated stomach adenocarcinoma), MKN45 (from the lymph node of a moderately differentiated stomach adenocarcinoma), SNU-1 (from a poorly differentiated primary carcinoma of the stomach), TMK-1 (from a poorly differentiated adenocarcinoma), and SCM (human gastric carcinoma) cells. Western blot analyses showed that tNOX protein was expressed to varying degrees in all gastric cancer lines (Figure 1). tNOX expression was higher in TMC-1, SCM, and TMK-1 cells than in SNU-1, MKN45, and AGS cells. Antisera used in western blot analyses were raised against bacterial tNOX, as described previously[12], and they recognized the same tNOX protein band as that identified by a commercially available anti-tNOX polyclonal antibody[14] (Protein Tech Group, Inc. Chicago, IL, United States).

We also continuously monitored cell growth dynamics, measured as cell impedance and displayed in the form of cell index (CI) values[19-21]. In this application of the cell impedance assay, the existence of cells on top of the electrodes creates an increase in electrode impedance,
Recent progress has focused on the chemopreventive effects of capsaicin, reflecting its anti-growth activity against various human cancer cell systems, including prostate[30-32], colon[33,34], hepatoma[35,36], breast[11,37] cancer, as well as leukemic[38-40]. However, there are few studies available reporting on the cytotoxicity of capsaicin in gastric cancer cells[41-43].

We have investigated the effects of capsaicin on different gastric cancer cell lines, including SCM, SNU-1, and TMC-1. In these studies, which measured metabolic activity by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction assays, we verified that capsaicin exerted a concentration-dependent inhibitory effect on SCM cell proliferation. After 24 h exposure, 100 µmol/L and 200 µmol/L capsaicin decreased SCM cell viability to less than 70% and 50% of control groups, respectively. Cell proliferation, measured by counting SCM cell number, was also significantly decreased in a concentration and time-dependent manner by capsaicin exposure[44]. We studied the anticancer activity of capsaicin on the proliferation of SNU-1 cells, which were derived from a poorly differentiated human gastric carcinoma, and TMC-1 cells, a metastatic gastric carcinoma line[45]. These assays showed that capsaicin induced significant cytotoxicity in SNU-1 cells at 100 µmol/L, diminishing cell numbers to smaller than 40% and 30% of control groups after 48 and 72 h exposure, respectively. To our surprise, 100 µmol/L capsaicin did not decrease the number of TMC-1 cells, even after a 72 h exposure[45]. These results imply that capsaicin exerted differential cytotoxic effects on gastric cancer lines derived from different stages of cancer progression.

**DUEL EFFECTS OF CAPSAICIN ON CELL GROWTH AND tNOX IN TWO GASTRIC CANCER LINES**

To further investigate whether the differential inhibitory effects of capsaicin on cell growth inhibition involves cell death, specifically apoptosis, we analyzed cells for apoptotic subpopulations using flow cytometry. Interestingly, capsaicin provoked cytotoxicity in SCM cells concurrently with caspase 3-mediated poly (ADP-ribose) polymerase (PARP) cleavage and apoptosis induction[44]. Using the pan-caspase inhibitor Z-VAD-FMK, we confirmed that capsaicin-induced apoptosis in these cells was dependent on caspase activity[44]. Consistent with results obtained in other gastric cancer cell lines, capsaicin was found to induce cytotoxicity and apoptosis in SNU-1 cells, possibly through up-regulation of p53[40]. We also demonstrated that 100 and 200 µmol/L capsaicin triggered apoptosis in 16.1% and 26.2% of SNU-1 cells, respectively[46]. In contrast, TMC-1 cells were fundamentally unresponsive to the apoptotic effect of capsaicin; exhibiting very little apoptosis in response to capsaicin exposure. The greater cytotoxicity of capsaicin toward SCM and SNU-1 cells was also reflected in the apoptotic activity
To examine whether tNOX expression level is crucial for the survival of gastric cancer cells, we knocked down tNOX expression in TMC-1 cells using small interfering (hairpin) RNA (shRNA). These tNOX-depleted TMC-1 cells were more responsive to capsaicin treatments, as evidenced by enhanced caspase 3-mediated PARP cleavage, greater loss of mitochondrial membrane potential, and higher intracellular oxidative stress level compared with control groups. Thus, decreased tNOX expression in TMC-1 cells resulted in greater sensitivity to the effects of capsaicin. Interestingly, reduced expression of tNOX appeared to affect cell-cycle progression such that capsaicin-induced G1 accumulation was enhanced and proliferation was reduced in tNOX-depleted TMC-1 cells, supporting a significant role for tNOX in TMC-1 cell growth.

We also examined the possibility that decreasing tNOX protein levels in cancer cells is sufficient to diminish tumor growth in animals. To this end, mice were injected with parental (wild type) HCT116 human colon cancer and either control (scrambled RNAi) HCT116 cells or tNOX-knockdown HCT116 cells, and xenograft tumors were examined after 60 d. We found that the growth of HCT116 xenograft tumors was significantly reduced in tNOX-knockdown groups, indicating that tNOX depletion in cancer cells reduces their capacity to form tumors in vivo.

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The regulatory role of tNOX in cell growth is not limited to gastric cancer cells. Utilizing a loss-of-function approach, Chueh et al. reduced tNOX expression in HeLa cervical cancer cells using antisense...
oligonucleotides and found that tNOX deficiency reduced cell proliferation, as determined by colony-formation assays. A subsequent study utilizing shRNA to specifically and effectively inhibit tNOX expression in HeLa cells showed that tNOX knockdown attenuated cell proliferation and migration by interfering with the Rac pathway\cite{14}. In another study, tNOX-knockdown was shown to sensitize cells to stress-induced apoptosis in human HEK293 cells derived from human embryonic tissues\cite{13}, which share properties with cancer. Notably, we found that tNOX is abundantly expressed in these embryonic cells. Conversely, a gain-of-function approach showed that tNOX over-expression in non-cancerous MCF-10A cells gave rise to enhanced invasiveness, an aggressive feature of cancer cells, confirming a vital role for tNOX in cancer progression\cite{47}. Moreover, transient up-regulation of tNOX in HCT116 cells augmented cell proliferation and migration in vitro and in vivo\cite{46}. These phenomena were also observed in A549 human lung cancer cells, demonstrating that an epithelial-to-mesenchymal transition mechanism may be involved in the enhanced cell migration associated with tNOX up-regulation\cite{22}.

**CONCLUSION**

In this review, we summarized the recent literature on the biological function of tNOX in gastric cancer cells. We also considered the paradoxical effect of capsaicin on cancer growth and tNOX expression, which results in differential cellular outcomes. Collectively, these various lines of evidence establish a significant regulatory role for tNOX in cancer cell proliferation, survival, and migration. This information may provide a reasonable framework for the future development of tNOX-targeting agents as a new class of anti-tumor therapeutics.

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Capsaicin can alter the expression of tumor forming-related genes and sufficient as a cellular target for the anticancer actions of capsaicin and the green tea catechin (-)-epigallocatechin-3-gallate. and is predicted to be associated with the promotion of capsaicin-induced apoptosis of gastric cancer cells.

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