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Secretion of a Nucleoside Diphosphate Kinase (Nm23-H2) By Cells From Human Breast, Colon, Pancreas And Lung Tumors

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Cancer is the second leading cause of death in the US, with one in four deaths attributed to the disease. Patients who succumb to breast cancer do so from the formation of metastatic tumors. Deaths due to colon, pancreatic, lung and other cancers are more often the result of metastatic disease rather than the consequence of the primary tumor *per se* [1]. Metastasis is a complex set of biological events involving proteolysis, cell motility, intravasation and extravasation, cellular communication, angiogenesis, and tumor growth. A great deal of interest has developed surrounding the need for metastatic tumors to develop a blood supply from the host in order to grow and that on this basis, new cancer therapy can be developed [2]. We propose that the Nm23 gene product may have a central role in some of these events. The description of the first human Nm23 gene [3] as a homologue of a *Drosophila* gene involved in the formation of the wing disk [4] indicated a potential for metastasis inhibition. Several studies have supported this claim. However, because we have investigated the role of nucleotides, particularly ATP derived from endothelial cells [5], as regulators of regional blood flow [6] and as there is a role for Nm23 in this process [7], we favor the notion that a role exists for Nm23 in the events supporting metastasis, particularly those of intravasation and extravasation of tumor cells that would be supported by the extracellular actions of nucleotides.

Our hypothesis is supported by others who have now questioned the original claims of the 'tumor-suppressor' nature of the Nm23 association [8-10]. Four Nm23 genes are encoded in human cells, with the two most highly studied being Nm23-H1 and Nm23-H2. These genes encode NDPK-A and NDPK-B, respectively. Each of these enzymes, known to form homo-hexamers of 17.5 kDa (Nm23-H1) and 21.5 kDa (Nm23-H2) monomers, functions primarily as a nucleoside diphosphate kinase (NDPK) in maintaining intracellular "housekeeping" by nonspecific trans-phosphorylation. They have later been found to have DNA binding activity and other non-NDPK activities [11-14]. The enzymatic properties of the NDPK are curious, as these enzymes will bind a wide variety of both purine and pyrimidine triphosphates as phosphoryl donor and substrate. These enzymes, in the presence of divalent cations, covalently transfer the terminal γ-phosphate of a nucleoside triphosphate (NTP) to a nucleoside diphosphate (NDP) *via* a high-energy phosphohistidine intermediate:

$$N_1DP + N_2TP \Leftrightarrow N_1TP + N_2DP$$

The promiscuous nature of the enzyme may subserve its ability to act as a transphosphorylase, transferring ATP from one cellular compartment to another.

In a variety of human tumors (breast, lung, colon, and prostate), we have found the presence of NDPK-B to be elaborated as a phosphoprotein in the extracellular environment. We suggest here that NDPK-B, in addition to maintaining intracellular nucleotide concentrations, acts in accordance with our proposed Nucleotide Axis Hypothesis Model, whereby extracellular ATP acts at P2Y receptors on endothelial cells to promote vasodilation and vascular permeability [5,6]. A localized production of extracellular ATP, abluminally by tumor-derived NDPK-B, may induce tumor cell intravasation into capillary vessels, transit and extravasation at a distant site and as such, these events may be altered by blockade of this enzyme.

We show here that aggressive human tumor cells from disparate tumors secrete Nm23-H2 and that a series of structurally related non-nucleotide compounds, ellagic acid (EA), epigallocatechin gallate (EGCG), and epicatechin gallate (ECG), that have been proposed as anticancer agents inhibit angiogenesis; and are shown to inhibit the secreted NDPK-B (Malmquist, *et al. this volume*).

METHODS:

Production of NDPK-B. The human tumors MDA-MB-435s (breast ductal carcinoma), Calu-1 (metastatic lung carcinoma), WiDr (colorectal carcinoma), and PC-3 (prostatic carcinoma) were grown to confluence in DMEM with 10% heat-inactivated FBS and antibiotics. Media was replaced with Krebs buffer (25 mM Hepes, pH 7.4) and incubated at 37^{O}C with gentle rocking for 4 h. The supernatant was removed and concentrated by spin concentrators. NDPK activity of the retentate was assayed for activity and stored at –20^{O}C.

Western blot. Human breast cancer cells were grown to confluence, scraped from dishes and homogenized in standard buffers containing protease inhibitors. Membranes were prepared by differential centrifugation first at 10,000 x g for 5 min to remove nuclei and then at 48,000 x g for 30 min to recover cell soluble and particulate fractions. Samples were boiled in SDS sample buffer, separated by

PAGE on 12% gels, blotted to PDVF and probed for Nm23 isoforms using commercially available antibodies (US Biological, MA).

Determination of Nm23-H2 phosphoprotein. Breast cancer cells (435s) were grown to confluence and labeled with [^{32}P]-orthophosphate (100 μCi/10^7 cells) overnight. Radioactivity was then determined using scintillation in total protein by acid precipitation and in the concentrated Nm23 fraction following concentration. Counts associated with Nm23-H2 were confirmed using autoradiography. Nm23-H2 phosphoprotein was then incubated with ADP in the absence of a phosphoryl donor.

NDPK-B Kinase activity assay. Conversion of ADP to ATP, using GTP as the phosphoryl donor, was quantified using the Luciferin-Luciferase ATP assay as previously described [5]. All necessary steps were taken to eliminate and account for interference between potential NDPK-B inhibitors and the luciferase in the assay.

Angiogenesis assay. Fluorescence activated cell sorting (FACS) was used to select cardiac endothelial cells positive for the PECAM marker CD31+. Endothelial cells were plated on a matrix of basement proteins from the Engelbreth Holm-Swarm (EHS) mouse tumor. A complete DME media containing 10% FBS was used to maintain endothelial cultures. Differentiation of the cells into endothelial tubes was monitored in the presence and absence of the compounds of interest.

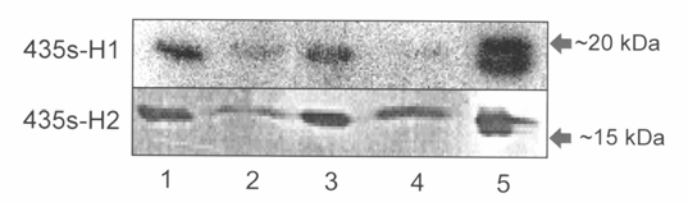
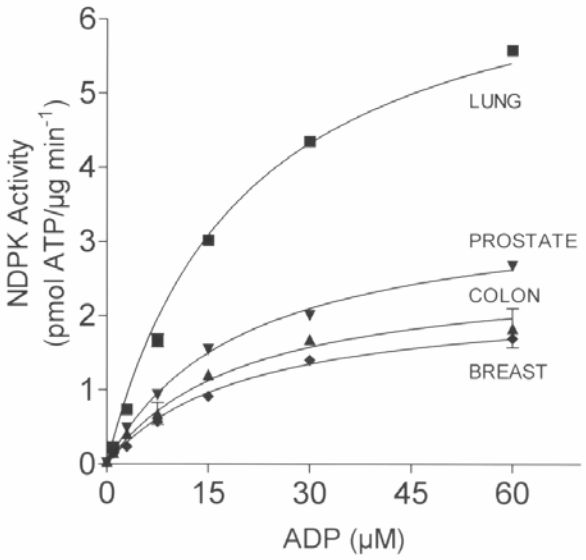


Figure 1. Western Blot analysis of Nm23 isoforms of breast cancer cells. Cell homogenates and conditioned incubation buffer were prepared and fractionated as described and separated by gel electrophoresis followed by blotting proteins to membranes. Westerns were probed with antibodies specific for Nm23-H1 and Nm23-H2 isoforms and developed using the alkaline phosphatase method. Lane 1, whole cell homogenate; Lane 2, membrane fraction; Lane 3, cytosolic fraction; Lane 4, secreted fraction; Lane 5, standard NDPK from rat liver (Sigma). Data are representative of numerous experiments with these and other tumor cell lines.

RESULTS: The NDPK secreted from MDA-MB-435s cells is the Nm23-H2 isoform (Figure 1). While both isoforms, H1 and H2, are readily detected with specific antibodies, only the H2 protein is found in the incubation medium surrounding cells. In order to explore our hypothesis that Nm23-H2 is secreted from cancer cells that are highly metastatic and form solid tumors at distant sites, we measured the ability of other cultures of cancer cells to secrete Nm23-H2 also. All metastatic human tumors studied expressed the same secreted NDPK-B isoform activity with similar affinities for the substrate ADP (21.59 ± 2.49 μM SEM) suggesting that the ability to secrete NDPK (Figure 2) is a property of transformed cells. Control experiments with normal cells did not result in the secretion of Nm23 proteins (not shown).

Because Nm23-H2 is secreted from tumor cells in culture, we wondered if it would require the presence of a phosphoryl donor outside the cell in order to generate an extracellular NTP, or if the protein is secreted in a phosphorylated form. We find that Nm23-H2 is secreted as a phosphoprotein (Figure 3) and that the phosphate is transferred to substrate (ADP) as expected for an NDPK. The loss radioactivity from Nm23-H2 was time-dependent in the presence of phosphate acceptor, consistent with the ping-pong transphosphorylase activity previously described for this enzyme.



	PC-3	WiDr	Calu-1	435s
KM	20.47 μM	27.02 μM	15.28 μM	23.6 μM

Figure 2. Nm23-H2 is a secreted NDPK. The incubation buffer was sampled from four different tumor cell types and the NDPK activity was assessed enzymatically in the presence of excess GTP (1 mM) by measuring the production of ATP production using the luciferin-luciferase assay. Relative luminescence units (RLU) were corrected for background and converted to ATP by comparison to a standard curve. Data are presented as mean ± SEM, n=3. Nm23-H2 substrate affinities (K_M) for ADP in disparate tumors are not significantly different while the apparent Vmax suggests distinctions. The average Nm23-H2 K_M from all sources is (21.59 ± 2.49 μM SEM).

The polyphenolic compounds epicatechin gallate (ECG), epigallocatechin gallate (EGCG), and ellagic acid (EA) were found in our lab to be potent inhibitors of NDPK-B transphosphorylation activity (Malmquist *et al. this volume*). Each of these compounds was used at 30 and 100 μM to determine the effect of these inhibitors of NDPK activity on angiogenesis. Each of the NDPK inhibitors tested produced a dose-dependent inhibition of angiogenesis consistent with our hypothesis and with a role for ATP in supporting angiogenesis.

CONCLUSIONS: Both Nm23-H1 and -H2 genes are expressed in breast cancer cells. The Nm23-H2 isoform appears to be abundant. Breast cancer cells may translate Nm23-H2 as both an *ecto*- and *exo*-enzyme, NDPK-B.

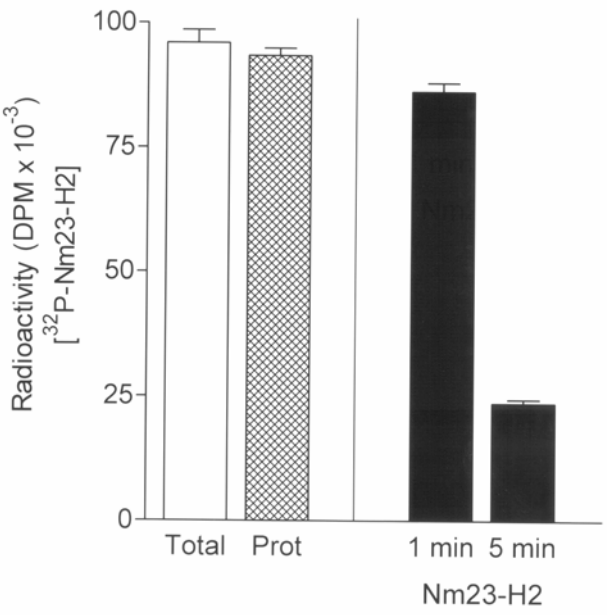


Figure 3. Detection of secreted Nm23-H2 as a phosphoprotein. [^{32}P]-Labeled cells were allowed to condition incubation buffer, which was collected, concentrated and used to determine [^{32}P] incorporation by radioactive scintillation counting. Radioactivity in the buffer collected (Total) and in total protein (Prot) were determined by acid precipitating protein and followed by scintillation counting. Nm23-H2 radioactivity was confirmed by autoradiography and found to account for the majority of radioactivity in secreted protein. ADP was added as a phosphoryl acceptor at 100 μM for 1 and 5 min. Data are Mean ± SEM of duplicate determinations in three experiments.

Western blots show that the -H2 isoform is found in the membrane of the breast cancer cell, but this may be due to its staging there for secretion. Cells secrete Nm23-H2 as a phosphoprotein. This suggests that the enzyme is able to transfer a phosphoryl group from a nucleoside triphosphate donor within the cell to a histidine in the protein and retain this phosphorylation until outside the cell where the enzyme can phosphorylate a nucleoside diphosphate. The phosphohistidine (ping-pong) transphosphorylase mechanism is well known for NDPKs. While such a reaction scheme can seem awkward as one nucleoside triphosphate is hydrolyzed in favor of another, the net gain is a clear benefit to tumor biology as it puts the nucleoside triphosphate outside the cell. As the enzyme is secreted as a phosphoprotein, it is capable of transphosphorylation activity in the absence of an extracellular phosphoryl donor. This activity may be a mechanism for producing elevated extracellular ATP, particularly in the setting of apoptosis of tumor cells and angiogenesis required for tumor growth.

The human tumors MDA-MB-435s, Calu-1, WiDr, and PC-3 all express secreted NDPK-B activity (Figure 2). As we have shown, the structurally similar gallate compounds inhibit NDPK-B activity. These compounds are known to suppress cancer cell proliferation, inhibit invasion into Matrigel, and inhibit angiogenesis [14-18]. The anti-NDP kinase property reported here suggests an additional novel mechanism by which these compounds may be anti-tumorigenic. The gallate-related compounds inhibit NDPK-B transphosphorylation, and this may be related to angiogenesis inhibition.

Taken together, these findings suggest the hypothesis that inhibition of Nm23-H2 activity is mechanistically associated with inhibition of metastasis by multiple human tumors. Together with their action as Nm23-H2 inhibitors, the action of green tea catechin may suppress a critical step in metastasis, angiogenesis, by blocking extracellular ATP generation.

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