NHE1 Expression and Drug Resistance in Tumor Cells

Ismail M. H[1]. and Shazib P[2].

Department of Biological Sciences, National University of Singapore,
10 Kent Ridge Crescent, Singapore 119260

ABSTRACT

It was recently shown that acidification of intracellular milieu is critical to induce efficient caspase activation and cell death following drug-induced apoptosis, with evidence linking the acidification to the inhibition of Na^+/H^+ exchanger in a variety of cell types. NHE1 has long been established as a major player in maintaining the cell pH in animal tissue. Thus in this project, we have tried to establish the NHE1 expression of some cell lines and made an effort to dissect a relationship with the intracellular pH, cell sensitivity, and their response to drug treatments. We optimized the western blotting protocol to detect the NHE1 and fluorescence assay to detect changes in the pHi. We established that NHE1 expressions in the different cell lines assayed are the same except in HL60, which shows negligible expression. Phosphorylated bands are present in all these tumor cell lines but not in the normal NIH3T3 cells. In addition hyper-phosphorylated NHE1 is present in the HCT116 cells. We also show that most of the tumor cell lines have slightly alkaline pHi and treatment with etoposite induces a drop in pHi. We concluded from our data that the activity of NHE1 is not regulated at the transcription level but could be due to the activity of the channels that are too subtle to be detected by western blotting.

INTRODUCTION

Programmed cell death (apoptosis) is a normal physiological process, which occurs during embryonic development as well as in maintenance of tissue homeostasis. Failure to undergo apoptosis in response to appropriate signals may results in cancer.

Apoptosis is a programmed process where a cell actively participates in its own destructive processes. It is characterized by certain morphological changes, which include changes in the plasma membrane such as loss of membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Finally, the dying cells become fragmented into apoptotic bodies, which are rapidly eliminated by phagocytic cells. This last step is required to fulfill the definition of apoptosis. On the other hand, necrosis occurs as a result of cell injury. It begins with swelling of the cell and mitochondrial contents, followed by rupture of the cell membrane. Unlike apoptosis, necrosis triggers as an inflammatory reaction, which is detrimental to the surrounding tissue.

It has been shown that acidification of the intracellular milieu is critical to induce efficient caspase activation and cell death following drug-induced apoptosis (Pervaiz *et al.*, 2001). Drug-

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[1] Student
[2] Supervisor, Associate Professor

induced intracellular acidification has been linked to the inhibition of Na^+/H^+ exchanger in a variety of cell types (Kaufman *et al.,* 1993).

NHE are considered important regulators of cytoplasmic pH, especially with fluctuations of more than 0.2 units. They facilitates the electroneutral uptake of extracellular Na^+ with concomitant extrusion of cytosolic H^+. Up to now six isoforms (NHE1-NHE6) have been identified (Maly *et al.,* 2002). The Na^+/H^+ exchanger isoform1 (NHE1) is ubiquitously expressed the mammalian species. It is an integral membrane phosphoglycoprotein of approximately 110KDa spanning the membrane 12 times with a sizeable hydrophilic tail. The transmembrane region is concentrated in the amino-terminal domain of the protein, while the carboxylic terminus constitutes the cytosolic tail. Transport activiy of NHE1 involves phosphorylation of the protein, believed to be in the C-terminus of the regulatory domain (Putney *et al.,* 2002). However, the rate of cations exchange is dictated primarily by the intracellular pH (pHi). The pHi sensitivity of NHE1 is attributed to the presence of an allosteric modifier site. When protonated, the modifier site activates cation exchange, protecting the cytosol against excessive acidification. As pHi approaches the cell's normal physiological set point, deprotonation of the allosteric site curtail the activity of the antiporter, preventing excessive alkalization (Aronson, 1985). Although the transport event does not require metabolic energy, in intact cells, ATP is required for the optimal functioning of the antiporter. ATP increases the sensitivity of the antiporter towards protons (Levine *et al.*, 1993).

MATERIALS AND METHODS

Cells were cultured with their standard culture medium specified by ATCC. Western blotting with 10% resolving gel was carried out to detect the NHE1 protein expression. Fluorescence assay with BCECF-AM dye was used to detect the intracellular pH of the cell.

RESULTS AND DISCUSSION

Figure 1: Western blot bands for Basal NHE1 Expression (Optimized Protocol)

We first establish the basal NHE1 expression in different tumor cell lines. However, to do so with great resolution and accuracy, we need to optimize the western blotting procedure for the detection of NHE1. As the NHE1 proteins have a high MW, we have used resolving gel of 10% polyacrylamide . Lowering the gel concentration increases the mobility of the protein in a given electric field, thus allowing the different forms of NHE1 band to separate distinctively. One of the few technical problems faced in our lab was efficient detection of NHE1 protein bands. We have deduced for the first time that loading higher concentrations of protein (250ug) and using the optimal current and time for transfer can lead to detection of added bands of NHE1. Prior to this, protocol used in other labs was perhaps not optimal to pick up details of NHE1 bands.

Results were intriguing to reveal (see figure 1), three different bands for NHE1 are detected as compared to only one type by the initial protocol (figure not shown). This attests towards the fact of optimizing western blot procedure has been described elsewhere in this report. To date, no protocol described was able to detect the three different forms of NHE1 bands.

We speculate that the three different forms of NHE1 bands at (110kD), are the basal, phosphorylated (>110kD) and hyper-phosphorylated (>>110KD) form of the same NHE1 protein (see figure 4). We conclude this from the fact that the NHE1 antibody used is monoclonal, and by nature react only to a single epitope that is highly specific. Cross-reactivity of the antibody with other isoforms of NHE is non-existence due to the absence of significant homology of the NHE1 peptide chain with the other (NHE2-NHE6) isoforms (Chemicon, 2002). The addition of phosphate groups would increase the overall effective mass of the peptide chain and thus would move slower in a given electric field and time, thus these bands would appear higher than the normal NHE1 band (110kD).

We looked at the NHE1 expression for the following tumor cell lines, Raji, Jurkat, CEM/Bcl2, HL60, CEM/Neo, HCT116/Bax+/-, HCT116/Bax-/-, HCT116 and we use the NIH3T3, normal mouse fibroblast as a negative control (see figure 1). Our objective was to take cell lines of different backgrounds to help elucidate basal NHE1 expression in different tumor cell lines. Our data repeatedly has shown and clearly establishes that the basal NHE1 expressions for the different tumor cell lines, NIH3T3, Raji, Jurkat, Cem/Bcl2, Cem/Neo, HCT116/Bax+/-, HCT116/Bax-/-, HCT116, are approximately the same and perhaps the changes in expression are subtle. This data is further confirmed with the use of PARP antibody, which inferred that not only have we load equal amount of protein but the amount of protein transferred to the PVDF membrane is also of equal amount (as the PARP bands are of equal intensity and size). It can be further inferred that the intensity of the phosphorylated band/s in these tumor cells is approximately equal.

However, a third band speculated to be the hyper-phosphorylated form of NHE1 was only present in HCT116 colon carcinoma cell lines. We hypothesize that these hyper-phosphorylated bands could hinder colon carcinoma cells to maintain powerful adhesions, as NHE-1 also functions as a membrane anchor. Whether this phosphorylation is a modification at C-terminal regulatory domain needs to be determined. (Putney *et al.,* 2002).

However, the other aspect of finding phosphorylation in all tumor cell lines points out to the fact that NHE1 channel helps regulate pHi homeostasis consequently leading to cell proliferation and ultimately survival. This was concomitant with the findings in the literature (Putney *et al.,* 2002). Interestingly in NIH3T3 cells (normal mouse fibroblasts), the phosphorylated form of NHE1 was absent. Thus we propose that tumor cell lines may confer resistance by NHE 1 activity that requires phosphorylation and normal human cell lines do not possess this characteristic.

Another important finding in our data was negligible expression of NHE1 in human leukemia cell line (HL60). We thus believe that this cell line might contain other isoforms of NHE (isoform2 - isoform6). Indeed, HL60 cells show sensitivity to undergo drug induced apoptosis.

With this data we proceeded with second set of experiments, measuring the basal intracellular pH (pHi) of the above mentioned cell lines with an attempt to correlate the basal pHi with the NHE1 expression (graph not shown). Interestingly, most of the tumors cell lines we checked had a pH slightly in the alkaline range that is beneficial for cell survival (7.2 to 7.6). A

strengthening correlation could not be established by looking at actual NHE1 activity. Therefore we concluded that the activity of NHE1 in regulating cell pH is not regulated at the transcription level (i.e. the expression of the proteins) but could be due to the activity of the channels that are too subtle to be detected by western blotting.

That brings us to our third set of experiments, where the cell lines are subjected to drug treatment to see whether drug sensitivity and drop in intracellular pH have any relationship with NHE1 expression or activity. The cell lines were subjected to 6hr treatment to etoposide at an apoptotic concentration of 20uM. Etoposide's apoptosis inducing activity has already been established and is also being studied in our lab. As expected etoposide dropped pHi in most of the tumor cell lines but relationship of this drop and NHE1 expression could not be established due to time constraint (graph not shown).

Future aims of this project involve studying changes in NHE1 expression or phosphorylation status of NHE1 after treating with known and unknown chemotherapeutic agents and co-relate these findings by measuring NHE1 channel activity. These experiments would establish concrete evidence that NHE1 channel activity is an important aspect then looking only at NHE1 expression. A tumor cell may have the same expression as another tumor but the functional state of the channels can be different. Whether this functional state is a parameter in determining the fate of the cell remains to be determined.

This project has paved way not only in our understanding of NHE1 protein expression but provided an insight to develop better chemo-therapeutic agents that can exploit NHE1 activity and intracellular pH for programmed execution of cancer cells.

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