Effect of Anti-NaPi2b Monoclonal Antibody on Phosphate Transport in Renal Cancer Cell Line SK-RC-18

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ABSTRACT: The sodium-dependent phosphate transporter NaPi2b (*SLC34A2*, NaPi-IIb, NPT2b) is involved in regulation of inorganic phosphate metabolism. Its expression was detected in a set of normal and malignant tissues predominantly on mRNA level. Here we describe NaPi2b protein expression in tubules of human kidney by using immunohistochemical analysis with anti-NaPi2b mAb. Patch-clamp technique was used to study effect of anti-NaPi2b mAb on sodium-dependent phosphate current in renal cancer cells (SK-RC-18 cell line) expressing high level of NaPi2b protein. We demonstrate that incubation of SK-RC-18 cells with anti-NaPi2b mAb decreases phosphate current in 70% cells while unspecific antibody A33 does not produce any effect. Thus, anti-NaPi2b mAb can serves as a potential specific inhibitor of NaPi2b mediated phosphate transport. NaPi2b and anti-NaPi2b mAb have a great potential for diagnosis and treatment of different pathologies related to aberrant expression and function of NaPi2b phosphate transporter.

KEY WORDS: NaPi2b, *SLC34A2*, SK-RC-18, kidney, mAb

I. INTRODUCTION

The sodium-dependent phosphate transporter NaPi2b (*SLC34A2*, NaPi-IIb, NPT2b) belongs to SLC34 family of phosphate transporters and is involved in regulation of inorganic phosphate metabolism and the maintenance of phosphate homeostasis by inorganic phosphate (P*i*) absorption in small intestine via brush border membranes.[1] The level of NaPi2b is controlled by several hormones, kinases and metabolic factors according to the body's Pi needs.[2,3,4,5,6,7]

Besides small intestine NaPi2b protein expression was reported in a set of other normal tissues. Expression studies were performed for human, mouse, and rat NaPi2b on mRNA and protein levels. According to Northern blot analysis, highly abundant NaPi2b gene expression was shown in human lung, small intestine, kidney, testis, uterus, prostate, pancreas, thyroid and salivary glands etc.[8,9] On the protein level its

expression was shown at different sites including brush border membranes and/or apical (luminal) surface of epithelial cells of ducts of some organs such as epididymis, liver, lung, mammary and salivary glands.[10,11,12,13,14] In epididymis, NaPi2b phosphate transporter was shown to be involved in sperm maturation while in liver it might play an important role in the regulation of bilary Pi concentration.[10] In lungs, NaPi2b is expressed at the apical side of alveolar type II cells where it is involved in Pi uptake for the synthesis of surfactant products.[12] In secreting mammary glands, a role of NaPi2b could be envisaged in delivering Pi into milk during lactation.[13] Deregulation of the NaPi2b transporter function as a result of mutations in human *SLC34A2* gene may lead to the pulmonary alveolar microlithiasis, autosomal recessively inherited disease which is characterized by deposition of calcium-phosphate precipitates in lungs.[15,16] NaPi2b aberrant expression was described also in some malignancies such as ovarian, papillary thyroid, breast and lung cancers[17,18,19,20,21,22,23,24] where its function remains to be elucidated. In ovarian cancer, NaPi2b (known as MX35 antigen) is investigated as a potential target for therapeutic monoclonal antibody MX35 which showed significant clinical efficacy during treatment of ovarian cancer micro-metastatic disease.[25,26]

Playing an essential role in phosphate homeostasis in a human body NaPi2b could be considered as a target for inhibition of hyperphosphatemia in patients with this failure. There are some compounds that have been reported as inhibitors of sodium dependent phosphate transport including phosphonoformic acid, 2V-phosphophloretin, and JTP-59557.[27,28,29] However all mentioned inhibitors are not strictly specific for sodium-dependent phosphate transport mediated by NaPi2b. Correspondingly the search of alternative approaches is appreciated. Using cellular models expressing the wild type or mutant forms of recombinant NaPi2b transporter we have shown that MX35 monoclonal antibody significantly decreases phosphate uptake mediated by NaPi2b and thus can be considered as a specific inhibitor of NaPi2b transport function.[30] Recently, monoclonal antibody directed to the extracellular domain of NaPi2b protein (188-361aa) was generated in our laboratory.[31] Intriguingly, this antibody recognizes the same region of extracellular domain of NaPi2b protein (311-340 aa) where the epitope for MX35 monoclonal antibody is located.[31,32]

Based on these considerations it can be concluded that NaPi2b is a promising target for diagnosis and treatment of many different pathologies linked with altered function and expression of NaPi2b. The aim of this study was to analyze an effect of anti-NaPi2b mAb on the functioning of endogenous form of NaPi2b on the model of renal cancer cell line using patch-clamp technique.

II. MATERIALS AND METHODS

II.A. Cell Line and Antibodies

SK-RC-18 renal cancer cell line was obtained from Dr. G.Ritter (Memorial Sloan-Kettering Cancer Center, New-York, USA). SK-RC-18 cell line cells were cultured in minimal essential medium (DMEM) with fetal bovine serum (10%), L-glutamin

(2mM), penicillin (100 U/ml), and streptomycin (100 mkg/ml). MAbs against extracellular domain of NaPi2b were generated by hybridoma technology[31] and mAb of hybridoma clone L2 (20/1) was purified from ascites of mice at the concentration of 1 mg/ml. The unspecific A33 mAb against of colon cancer antigen A33 were generated previously in our laboratory.

II.B. Immunohistochemical Analysis

Immunohistochemistry was performed according to standard protocol. Representative sections of tumor samples were prepared from paraffin blocks and stained with hematoxylin – eosin as previously described.[31] Endogenous peroxidase was quenched with H_2O_2 (3%) in PBS. After blocking of nonspecific binding by avidin – biotin blocking solution (Vector Laboratories, Burlingame, CA, USA), tissue sections were incubated overnight with anti-NaPi2b mAb (10μg/ml) at 4°C. Then, sections were probed with biotinylated secondary antibodies for 2h at room temperature (goat anti-mouse biotinylated IgG, Sigma, 1:400), followed by incubation with avidin – biotin – peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The immune complexes were developed with diaminobenzidine solution. Hematoxylin was used for counterstaining. Prepared slides were examined with the use of Zeiss Universal microscope (Zeiss, Germany); images were captured using digital Axiocam software.

II.C. Patch-Clamp Technique

Electrophysiological recordings were made on the cells of SK-RC-18 renal cancer cell line. Electrophysiological experiments were carried out on SK-RC-18 cells incubated with anti-NaPi2b L2 (20/3) and unspecific A33 mAbs overnight. 30 μl of mAbs with the antibody concentration of 1 mg/ml were added to SK-RC-18 cells in 1 ml of DMEM medium in 1,5 cm plate with 80% confluence of cells.

II.D. Solutions

The extracellular solution (standard experimental solution) with (in mM) 100 NaCl, 2 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, 5 HEPES, pH 7.4 (adjusted using Tris) was used as proposed by Forster.[33] Solutions containing the required concentrations of P_i were prepared by adding 1M KH_2PO_4 (pH 7.4) to standard experimental solution. The concentration of Pi was 1 mM as described by Forster[33] and Murer[34]. The intracellular solution for the patch pipettes was120mM CsF and 20mM Tris-Cl, pH was adjusted to 7.2.

II.E. Electrophysiological Recordings

Patch-clamp protocol usually used for investigation of NaPi2b transport function in oocytes[35] was adopted to mammalian cells of SK-RC-18 cell line during this study. Electrical activity mediated by NaPi2b transporter was recorded in a whole cell configuration of conventional patch-clamp method.[36] All the experiments were conducted at room temperature. Patch pipettes had resistance of 3 to 4 MOhm when filled with intracellular solution. Just before the electrophysiological procedures DMEM medium was replaced with the extracellular solution. In order to record

NaPi2b-mediated ionic current, extracellular solution was replaced with the experimental solution containing Pi. Subsequently voltage step (of ramp type) was applied to the cell. The ramp type of stimulus providing a linear voltage change between two given potential values was applied to the cell.[35,36] During the ramp-protocol the experimental cell was clamped to -140 for 3 sec with subsequent linear ramp to +20 over 40 sec. The procedure was repeated with a period of 1 minute. Before the Pi-mediated currents measurements each experimental cell was subjected to at least 3 control ramps.

III. RESULTS AND DISCUSSION

Three types of anti-NaPi2b monoclonal antibodies (L2 (20/3), L3 (28/1) and L2 (2/1)) were generated and described earlier for analysis of NaPi2b expression in different cell lines.[31]

The highest level of NaPi2b expression was detected in the cells of SK-RC-18 cell line with anti-NaPi2b mAbs (clones L2 (20/3) and L3 (28/1)) by WB analysis.[31] NaPi2b protein was also recognized in ovarian cancer cell line OVCAR3 by these antibodies but to a smaller extent than in SK-RC-18 cells.

Strong NaPi2b protein expression which was observed in renal cancer cell line SK-RC-18 by Western-blot analysis[31] and NaPi2b mRNA transcripts abundance in human renal tissues which was showed by Northern-blot analysis[8,9] prompted us to investigate NaPi2b protein expression in normal human kidney with anti-NaPi2b monoclonal antibody (clone L2 (20/3).

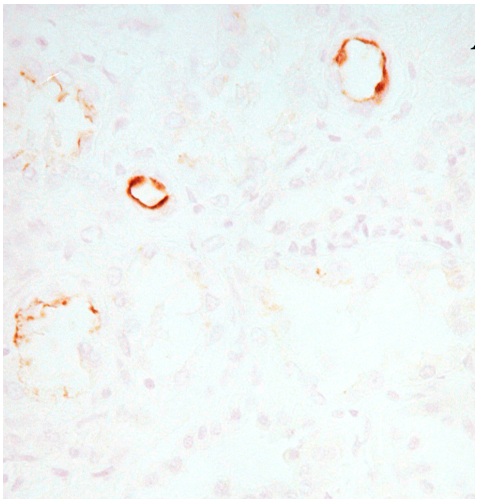

FIG 1. Immunohistochemical staining for anti-NaPi2b mAb in human normal kidney. Magnitude – x400

Figure 1 represents results of immunohistochemical staining of human normal kidney with anti-NaPi2b mAb, clone L2 (20/3). Positive anti-NaPi2b mAb staining is

observed on the apical surface of epithelial cells lining some renal tubules; however additional analysis should be performed for exact identification of tubules type.

For investigation of the influence of anti-NaPi2b mAb on NaPi2b function in further experiments we used renal cancer cell line SK-RC-18 which exhibits high level of expression of endogenous NaPi2b. NaPi2b transport function was monitored by patch-clamp technique.

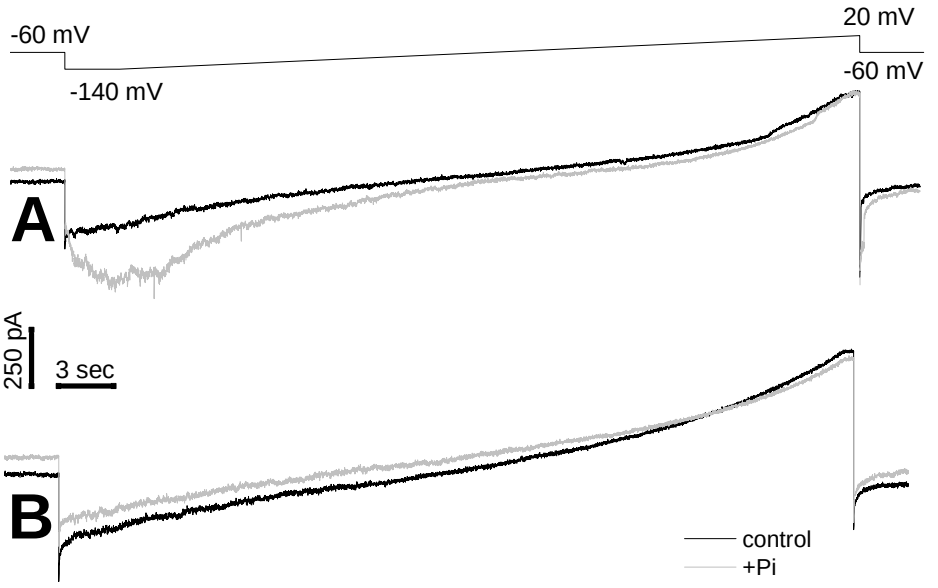

FIG 2. Anti-NaPi2b mAb L2 (20/3) blocks Pi mediated current in SK-RC-18 cells. The upper part of the figure depicts the voltage ramp protocol used for the registration of NaPi2b-mediated ionic current. **A.** Electrical activity of SK-RC-18 renal cancer cells. Application of ramp-type voltage stimulus to SK-RC-18 renal cancer cells with no Pi in the extracellular solution resulted in a "control ramp trace" (black line). Administration of Pi to the extracellular solution resulted in an additional current (grey line) in the negative voltage range (-140 – -40) as compared to the "control ramp" values. **B.** Electrical activity of SK-RC-18 renal cancer cell line that were incubated with anti-NaPi2b mAb L2 (20/3). Application of Pi (grey line) renders no effect when compared with the "control ramp" values (black line) suggesting that anti-NaPi2b mAb L2 (20/3) blocks Pi mediated current caused by NaPi2b transport activity

The SK-RC-18 cells, while being flowed by Pi (1 mM), revealed an increase in the current values in the negative voltage range in 88% cells (n = 7 of 8). Similar result (80%) was obtained with the SK-RC-18 cells that were incubated with unspecific A33 mAb. However, SK-RC-18 cells that were incubated with anti-NaPi2b mAb L2 (20/3)

showed an increase of current value only in 30% of cells (n = 5 of 16) in response to the application of 1 mM Pi. About 70% of these cells were insensitive to the application of 1 mM Pi (n = 11 of 16).

Figure 2 represents the current induced by the application of Pi in standard solution to control SK-RC-18 cells and SK-RC-18 cells that were incubated during overnight with anti-NaPi2b mAb L2 (20/3).

IV. DISCUSSION

In our previous study we have analyzed the effect of MX35 monoclonal antibody on NaPi2b mediated phosphate transport on HEK293 cells which stably express recombinant wild type and mutant forms of NaPi2b transporter using phosphate uptake assay[30]. Our data demonstrated 1,8-fold decrease of NaPi2b-mediated phosphate transport in HEK293 cells stably expressing wild type NaPi2b after MX35 antibody application, which was considered as a specific inhibitor of NaPi2b transport function.

To analyze anti-NaPi2b mAb impact on NaPi2b transporter activity we used cell line expressing endogenous form of NaPi2b and applied patch-clamp technique for monitoring of NaPi2b activity. It should be noted that the activity of phosphate transporters has been investigated so far predominantly by labeled phosphate uptake assay or by two-electrode-voltage clamp technique on *X.laevis* oocytes.[33,38,39,40] Under voltage clamp conditions, superfusion of oocytes expressing the type II sodium dependent phosphate transporters with 1mM P_i in the presence of Na^+ (100mm) elicits inward current at negative membrane potentials.[40] However, the expression system on X.laevis oocytes requires the cRNA synthesis of target gene and is not convenient for many reasons. Patch-clamp technique is more convenient tool which allows recording of inward current mediated by electrogenic molecules including phosphate transporter NaPi2b in cultivated mammalian cells.

In the course of this study we applied patch-clamp technique to measure sodium dependent phosphate current in NaPi2b positive SK-RC-18 cells (see Methods). It was shown that incubation of SK-RC-18 cells with anti-NaPi2b monoclonal antibody decreased current value in 70% of SK-RC-18 cells while unspecific antibody A33 did not have any effect on these cells.

During immunohistochemical analysis of human renal tissue with anti-NaPi2b mAb which specifically recognizes recombinant and endogenous forms of human NaPi2b we have revealed NaPi2b protein expression in some renal tubules. Additional analysis is needed to specify the type of these tubules. However, based on the data about MX35 antigen expression in collecting ducts in human kidney[41] we can conclude with a high probability that NaPi2b can be expressed in the same ducts of human kidney since the molecular identity of both proteins was convincingly proven recently by several techniques.[32] Moreover, a luminal location of NaPi2b protein has also been observed in the collecting ducts of the flounder and zebrafish kidney.[42,43]

Phosphate balance in the human body is tightly coordinated by a complex process involving various organs and tissues, including the small intestine, kidney,

parathyroid gland and bone. Members of SLC34 family are expressed in small intestine and in renal proximal tubules, two important sites that control the extracellular concentration of Pi.[1,44] NaPi2a (SLC34A1) and NaPi2c (SLC34A3) are commonly expressed in the apical brush border membrane of renal proximal tubule and are responsible for renal phosphate re-absorption. NaPi2b is mostly localized in the small intestine and takes part in phosphate absorption via apical brush border membranes. Recently, it has been reported that mice knockout for both NaPi-2a and NaPi-2c, while resulting in severe hypophosphatemia, still secured some renal phosphate re-absorption.[45] These results imply the involvement of other secondary active phosphate transporters along the nephron.[46]

Taking into account this assumption, the fact that NaPi2b transcripts are highly abundant in the human kidney[8,9] and our observation on NaPi2b protein expression in human renal tubules we supposed that NaPi2b is expressed in renal tubules and might be involved in phosphate re-absorption in kidney. Thus, NaPi2b can be considered as a target not only for inhibition of small intestine phosphate absorption but also for renal re-absorption as well in patients with hyperphosphatemia.

Patch-clamp technique which was applied during this study to measure phosphate mediated current on cells of SL-RC-18 renal cancer cell line allowed us to investigate the impact of anti-NaPi2b mAb on transport activity of phosphate transporter NaPi2b. It was shown that anti-NaPi2b monoclonal antibody could affect NaPi2b mediated phosphate transport in cells of renal cancer cell line SK-RC-18. We conclude that anti-NaPi2b monoclonal antibody can be considered as potential specific inhibitor for regulation of phosphate homeostasis in patients with hyperphosphatemia and, possibly, other pathologies including cancer. Further experiments using *in vivo* models have to be performed to assert this with confidence.

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