Impairment of Sodium Pump and Na\textsuperscript{+}/H\textsuperscript{+} Antiport in Erythrocytes Isolated from Cancer Patients

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ABSTRACT

We sought to determine whether the impairment of sodium pump activity and Na\textsuperscript{+}/H\textsuperscript{+} exchange reported in tumorigenic cells was specific to these cells or more general. Sodium pump activity and Na\textsuperscript{+}/H\textsuperscript{+} exchange were measured in erythrocytes from 49 cancer patients and 51 healthy subjects. Cancer patients with a newly detected cancer or in relapse and without associated pathologies known to modify these sodium transporters were included in this study.

Two sodium pump statuses reflecting its physiological modulation were evidenced for healthy subjects (103.2 ± 0.19 and 194 ± 0.68 mW/liter of cells). In cancer patients, only one basal status lower than those of controls was observed (83.2 ± 0.5 mW/liter of cells; P < 0.0001). Cooperativity of the Na\textsuperscript{+}/H\textsuperscript{+} antiporter is the same in cancer patients and controls (2.58 ± 0.27 versus 2.60 ± 0.15). The intracellular pH (pHi) dependence curve of the antiporter was shifted toward more acidic values, and optimal pHi was lower in cancer patients than in controls (5.80 ± 0.03 versus 6.08 ± 0.02; P < 0.0001). The mean maximal rate and the Keq of H\textsuperscript{+} for the Na\textsuperscript{+}/H\textsuperscript{+} antiporter were higher: 8.4 ± 1.2 versus 4.6 ± 0.4 mmol H\textsuperscript{+}/liter of cells/h (P < 0.01) and 514 ± 12 versus 322 ± 16 nm (P < 0.05), respectively.

Alterations of these Na\textsuperscript{+} transporters, therefore, were not restricted to cancerous cells. Among the alterations, the acidic shift in the pHi dependence of Na\textsuperscript{+}/H\textsuperscript{+} exchange appears associated with cancer because this behavior has never been reported in other pathologies.

INTRODUCTION

Changes in ion transport across cell membranes during control of cell proliferation and carcinogenesis are now well documented. In particular, it was shown that the malignancy of human cancers is correlated with the increase in the intracellular Na\textsuperscript{+}/K\textsuperscript{+} ratio due to a net increase in the sodium content (1). The increase in the Na\textsuperscript{+}/K\textsuperscript{+} ratio was also observed during rat liver carcinogenesis (2). Similarly, an increase in amiloride-sensitive sodium transport was described in a mouse model of colonic carcinogenesis (3). The increase in intracellular sodium is accompanied by an increase in pH\textsubscript{i2} and involves an activation of the Na\textsuperscript{+}/H\textsuperscript{+} antiport (4—6). In addition, the expression of a plasma membrane-associated oncoprotein of the ras family has been demonstrated to activate the antiport (7). Therefore, antiport stimulation during cellular proliferation and carcinogenesis seems well admitted, even if the exact significance of this stimulation is unclear (8, 9).

In addition to the Na\textsuperscript{+}/H\textsuperscript{+} exchange, the sodium pump is an important mechanism in regulating intracellular sodium. Na\textsuperscript{+},K\textsuperscript{+}-ATPase is apparently not less efficient in tumor cells than in normal tissues (10); nevertheless, its activity is altered in some cancerous cells and during induction of colon cancer (11, 12). Moreover the H-ras oncogene induces an impairment of Na\textsuperscript{+},K\textsuperscript{+}-ATPase in cells transfected with this oncogene (13).

These findings raised the question of whether these impairments of sodium pump and Na\textsuperscript{+}/H\textsuperscript{+} antiport were specific to tumorigenic cells, or whether, in a more generalized manner, they also affect other cells in patients suffering from various types of cancer. We therefore studied the sodium pump and Na\textsuperscript{+}/H\textsuperscript{+} antiport activities in erythrocytes from cancer patients and from healthy subjects for comparison.

Classically, Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity in erythrocytes is measured in vitro under maximal pump current conditions after lysis of the cells. We used micrcalorimetry because one can determine global sodium pump activity under experimental conditions that are similar to the physiological conditions, with fresh and intact cells suspended in their own plasma (14, 15). The activity of Na\textsuperscript{+}/H\textsuperscript{+} antiport was determined by a classical titrimetric method (16).

PATIENTS AND METHODS

Subjects. Forty-nine patients with newly detected cancer or in relapse were consecutively included in the study between October 1993 and January 1995. Eligibility criteria to include patients were histologically proven cancer and no radiation therapy or anticancerous chemotherapy within the previous 12 months. All patients were free of associated medication and intercurrent illness known to modify the activity of the sodium pump and Na\textsuperscript{+}/H\textsuperscript{+} antiport (for a review, see Refs. 17 and 18). Moreover, the clinical status of all patients eliminated the possibility of a protein-calorie malnutrition. Performance index according to WHO classification was ≤2. The studied population consisted of 29 males and 20 females, ranging in age from 23 to 78 years. The characteristics of the patients are reported in Table 1. As a control group, 51 students and staff members of the hospital units (18 males and 33 females, ranging in age from 23 to 50 years) were studied.

Reagents. All reagents were obtained from Sigma Chemical Co. (St. Quentin Fallavier, France). Ethyl isopropyl amiloride was graciously provided by Merck Sharp and Dohme, Inc. (Rahway, NY).

Erythrocyte Preparation. All venous blood samples were collected in the morning between 8 and 9 a.m. in tubes containing heparin-lithium and were used the same day. Venous blood (30 ml) was centrifuged for 10 min at 600 × g, then plasma was collected and recentrifuged for 10 min at 2000 × g. To separate erythrocytes from leucocytes and platelets, we suspended blood cells in a washing solution containing 149 mM choline chlorine, 1 mM MgCl\textsubscript{2}, and 10 mM Tris 4-morpholinepropanesulfonic acid at pH 7.4 and 4°C. Then the cell suspension (final Hct = 50%) was filtered through cellulose according to the method of Beutler et al. (19) and centrifuged for 10 min at 2000 × g. The erythrocyte pellet was then resuspended in appropriate medium for the different measurements (see below).

Sodium Pump Activity Measurements. For each subject, the erythrocyte pellet was resuspended in the cleared plasma of each subject (Hct = 10%). Sodium pump activity was determined as described previously using a LKB flow bioactivity monitor 2277 microcalorimeter (LKB-Produkter AB, Stockholm, Sweden) thermostated at 37°C (15). Briefly, thermograms were recorded by pumping an erythrocyte suspension through the microcalorimeter cell in closed circuit (volume = 3 ml). When the steady state in HP was reached, the 14 mM ouabain solution was added at a flow rate of 1 ml/h for 6 min (final concentration, 0.40 mM). Ouabain addition provoked a decrease in its value was expressed as mW/liter of cells.

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2 The abbreviations used are: pHi, intracellular pH; Hct, hematocrit; HP, heat production.
the antiport was determined by varying the intraerythrocytic pH from 5.6 to 6.5. Briefly, after separation, the erythrocytes were incubated in a hypertonic medium containing inhibitors of Na⁺ transport (ouabain and bumetanide) with pH adjusted to obtain adequate pHᵢ. Next, pHᵢ was clamped by addition of 4,4′-diisothiocyanato-stilbene-2,2′-disulfonic acid and methazolamide, which inhibited the anion exchanger and carbonic anhydrase, respectively. In these conditions, pHᵢ modification did not significantly change intracellular Na⁺,K⁺, and water content.

Na⁺/H⁺ antiport activity was determined by two steps as follows: (a) H⁺ efflux was measured in an efflux medium containing 0.1 mM ouabain, 0.15 mM MgCl₂, 3.5 mM glucose, 0.01 mM bumetanide, 40 mM sucrose, 0.5 mM methazolamide, 0.1 mM phloretin, and 150 mM NaCl (Na⁺ efflux medium); and (b) the same measurement was performed in an efflux medium in which NaCl was replaced by 150 mM KCl (K⁺ efflux medium).

For each step, the acid-loaded cells suspended in the efflux medium (Na⁺ or K⁺; Hct = 5%; 37°C) were maintained at a constant extracellular pH (8.00 ± 0.01) by adding 0.1 M KOH with a pH-stat apparatus (Radiometer, Copenhagen, Denmark), and the KOH volume added was recorded versus time. The volume values were transmitted in ASCII mode by the serial module of the pH-stat and acquired using a program elaborated in our laboratory (resolution = 0.1 μl). H⁺ efflux was calculated using the KOH adding rate measured during the linear variation of additions (between 100th and 400th seconds).

Hemolysis was determined at the end of each experiment. For this purpose, the reaction mixture was centrifuged for 10 min at 1850 × g, and the absorbance of the supernatants was measured at 540 nm. The relative hemolysis was determined by comparison with a sample showing 100% hemolysis. Hemolysis was determined by comparison with a sample showing 100% hemolysis.

**RESULTS**

**Sodium Pump Activity.** Distributions of the HP values corresponding to the energy expenditure by the sodium pump for healthy subjects and cancer patients are reported in Fig. 1. For healthy erythrocytes, the present results confirmed our previous findings (15). There are two different levels of sodium pump activity: one with a mean HP value of 10.3 ± 0.2 mW/liter of cells, the other with 19.4 ± 0.8 mW/liter of cells. In contrast, for erythrocytes from cancer patients, only one mean level of 8.3 ±0.5 mW/liter of cells was noted that the sodium pump activity in patients never reached the high level observed in healthy subjects. In addition, there is no correlation between the pump activity and the age of the patients (not shown).

**Na⁺/H⁺ Antiport Activity.** Fig. 2 illustrates the mean pHᵢ dependence of Na⁺/H⁺ exchange in healthy subjects (n = 10) and cancer patients (n = 15). As the pHᵢ decreases, Na⁺/H⁺ antiport activity first increases then decreases. As shown in Fig. 3, in all cancer patients, the optimal mean pHᵢ was lower than in healthy subjects: 5.80 ± 0.03 versus 6.08 ± 0.02, respectively (P < 0.0001). Because of this difference, the dependence of the Na⁺/H⁺ exchange on intracellular H⁺ concentration was analyzed according to previous reports (16, 22) by using the rearranged form of the classical Hill equation:

\[
\log \frac{v}{V_{\text{max}} - v} = n_{\text{app}} \log [H_1] - \log K' 
\]

where \(v = \text{measured Na}^+/{H}^+ \text{ antiport activity, } V_{\text{max}} = \text{measured maximum rate from the pH}_1 \text{ activation curve, } [H_1] = \text{intracellular H}^+ \text{ concentration, and } K' = ([H_1]_0)^{n_{\text{app}}}. \) A plot of \(\log[v/(V_{\text{max}} - v)]\) of H⁺ efflux versus \(\log[H_1]\) yielded a straight line (Fig. 4). The slope gives the Hill coefficient \(n_{\text{app}}\), which provides an index of the cooperative effect of Hᵢ on the Na⁺/H⁺ antiport regulatory sites. The
intercept at the X axis at Y = 0 gives log[H+]_{50}, which corresponds to
the H+ concentration (K_m) that yields 50% of the V_{max}.

The Hill coefficients (n_{Hill}) were similar in both groups
(2.58 ± 0.27 versus 2.60 ± 0.15). The K_m was higher in cancer
patients than in healthy subjects (514 ± 12 versus 322 ± 16 nM;
P < 0.05).

Results in Fig. 2 showed that the maximal activity of Na+/H+ antiport is higher in cancer patients. To confirm this difference, we
measured the Na+7H+ exchange in 30 additional cancer patients and
33 additional healthy subjects at pH 5.80 ± 0.05 and 6.10 ± 0.05,
respectively. Distributions of the values obtained are presented in Fig.
5. Because the V_{max} of Na+/H+ exchange was not normally distrib-
uted for the cancer group, the variable was analyzed with a logarithm
transformation. The V_{max} mean value was significantly higher in
cancer patients (n = 45) than in healthy subjects (n = 43): 8.4 ± 1.2
versus 4.6 ± 0.4 mmol H+/liter of cells/h; P < 0.01.

DISCUSSION

We have determined in the erythrocytes of cancer patients and
healthy subjects both the energy expenditure due to the sodium pump
and the Na+/H+ antiport activities. This study was designed to
support the hypothesis that alterations in the activity of the two ionic
transporters, reported in tumorigenic cells (1—13), might be more
generalized and related to cancer.

In the control group, there were two levels of sodium pump activity
(Fig. 1), which reflects its modulation. Indeed, we reported previously
that for the same healthy subject, the sodium pump activity can
permute from a low to a high value, corresponding to a basal and a
stimulated status, respectively (15). In contrast, there was only one
level of activity in cancer patients, and it was lower than the basal
status of the control group. Moreover, the activity of the sodium pump
in each patient never reached the values corresponding to a stimulated
status in controls. Taken together, these findings imply a decreased
activity and a lack of modulation of sodium pump in the erythrocytes
of cancer patients. The decrease in activity is related neither with the
age of the patients (see "Results") nor with a protein-calorie malnu-
trition (see "Subjects").

Using experimental conditions that were similar to the physiolog-
ical conditions, with fresh and intact cells suspended in autologous
plasma, we clearly evidenced an impairment of sodium pump in
erythrocytes from cancer patients. This finding shows that impairment
of sodium pump is not restricted to tumoral cells. However, even if
this defect is more widely related to cancer, it does not appear highly
specific because it has been also observed in other pathologies
(15, 17).

Our comparative study of the functional status of erythrocyte
Na+/H+ antiport has evidenced some original differences. Determi-
nation of the pH dependence of Na+/H+ exchange revealed a highly
significant acidic shift for cancer patients as compared with healthy
subjects. Indeed, the V_{max} of Na+/H+ exchange is reached at pH 5.80
and 6.08 for patients and controls, respectively (Fig. 2). In addition,
the optimal pH of each cancer patient was systematically found more

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Fig. 3. Optimal pH for activation of erythrocyte Na+/H+ exchange in control (n = 10)
and cancer (n = 15) groups. Columns, mean; bars, SE.

Fig. 4. Hill plots of activation of erythrocyte Na+/H+ exchange by cellular pH in
control subjects (○) and cancer patients (●). Each point results from treatment of mean
data issued from the pH dependence curves depicted in Fig. 2. Dashed line, 50% of the
V_{max}.

Fig. 5. V_{max} of erythrocyte Na+/H+ exchange measured at pH = 6.10 ± 0.05 for
control subjects (n = 43) and pH = 5.80 ± 0.05 for cancer patients (n = 45). Dashed
lines, mean V_{max} values.

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acidic than that of each control (Fig. 3). Thus, this modification seems linked to cancer status.

The Hill coefficient ($n_{app}$) mean values did not differ significantly between patients and controls (Fig. 4), which indicates an identical cooperative effect of intracellular H on the Na+/H+ antiport. The mean $K_m$ we obtained for healthy subjects agrees perfectly with a previous report (16). In contrast, the higher $K_m$ in cancer patients evidenced a lower affinity of Na+/H+ antiport for intracellular H+ in patients with regard to controls.

The mean $V_{max}$ of erythrocyte Na+/H+ exchange was about 83% higher in cancer patients than in controls (8.4 versus 4.6 mmol H+/liter of cells/h). The patients exhibited large interindividual differences in the $V_{max}$ of Na+/H+ exchange and a skewed distribution: 40% of them had activities higher than the maximum value reached by controls. (Fig. 5). The mean global Na+/H+ exchange value was not significantly different in patients with newly detected cancer or in relapse (not shown). It has been reported that the population of young erythrocytes is larger in some cancer patients than in healthy subjects (23). Therefore, the age of erythrocytes may account for a small part of our results on Na+/H+ activity (24).

As for the sodium pump, impairment of the Na+/H+ antiport is not strictly restricted to tumoral cells because it is observed in erythrocytes from cancer patients. However, an increased $V_{max}$ has also been described in hypertension (22). Thus, this increase cannot be considered as characteristic of cancer, all the more because 60% of patients exhibited values similar to those of controls (Fig. 5). Modulation of the Na+/H+ antiport by factors such as hormones and growth factors is well established and is generally associated with an increase in affinity for intracellular H ($K_m$ decrease), coupled with an alkaline shift of the pH dependence curve (25). We showed a decrease in affinity of the Na+/H+ antiport for intracellular H; such a decrease has been reported in an experimental model supplemented with fibroblast growth factor (26). However, we showed an acidic shift of optimal pH, which seems specific to erythrocytes from cancer patients. Indeed, each of the patients studied exhibited a systematically lower optimal pH than that of controls (Fig. 3). Such a finding has not yet been reported for other pathophysiological conditions. Moreover, we found no correlation between our data and the erythrocytic sedimentation rate of patients (not shown), which eliminates the possible involvement of an inflammatory status linked to cancer. It remains to be determined which factors (genetic or environmental) account for the impairment of the Na+/H+ antiport in erythrocytes, which have no apparent bearing on cancer tissues.

In conclusion, whatever the mechanism responsible for our findings, this study shows that impairment of Na+ transport is not encountered only in tumorigenic cells. Indeed, the sodium pump and Na+/H+ averaged activities were, respectively, decreased and increased in erythrocytes from cancer patients with regard to controls. Moreover, the optimal pH, corresponding to the $V_{max}$ of Na+/H+ exchanger in cancer erythrocytes, is shifted to acidic pH, which appears to be specifically associated with cancer pathology.

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