Correlation of Malignancy with the Intracellular Na\(^+\):K\(^+\) Ratio in Human Thyroid Tumors\(^1\)

Imre Zs.-Nagy,\(^2\) György Lustyik, Géza Lukács, Valéria Zs.-Nagy, and György Balázs

F. Verzár International Laboratory for Experimental Gerontology (VILEG), Hungarian Section [I. Zs.-N., Gy. L., V. Zs.-N.], and First Department of Surgery, University Medical School [G. L., G. B.], H-4012 Debrecen, Hungary

ABSTRACT

Energy-dispersive X-ray microanalysis was applied on human intraoperative biopsy materials of different thyroid tumors. To ensure suitability of these tissue pieces for quantitative microanalysis in freeze-fractured, freeze-dried bulk specimens, sampling was carried out with strictly defined criteria. Benign adenomas and differentiated and anaplastic carcinomas were selected for the studies on the basis of pathohistological investigations of the same specimen. The results of the tumor cells were compared to those obtained in apparently normal human epithelial cells. The number of normal cells analyzed was 349, whereas in the tumors 408, 423, and 891 cells were measured in the benign, differentiated, and anaplastic groups, respectively. Intracellular monovalent contents were calculated as percentage of cell dry mass; then, Na\(^+\):K\(^+\) molar ratios were calculated for each cell individually. Due mostly to the increase of Na\(^+\) content, the distribution histograms of the Na\(^+\):K\(^+\) molar ratio show an increase in the number of cells with a higher Na\(^+\):K\(^+\) ratio with increasing malignancy of the tumors studied. The differences proved to be statistically highly significant by the \(\chi^2\) test. Thus, in human thyroid, increasing malignancy is associated with increasing intracellular Na\(^+\):K\(^+\) ratio.

The results give further support to the theory of C. D. Cone (J. Theor. Biol., 30: 151–181, 1971) according to which the sustained depolarization of the cell membrane results in an increased rate of cell division.

INTRODUCTION

In 1971, Cone (5) formulated a "unified theory" on the basic mechanism of normal mitotic control and oncogenesis. This theory predicts that sustained depolarization of the cell membrane (i.e., an increase of the intracellular Na\(^+\):K\(^+\) in the resting cell) is involved in the regulation of cell divisions during both normal and malignant growth of tissues. Several studies published during the 1970s (2, 6, 7, 10, 18, 19, 23) demonstrated an intimate correlation between cell membrane functions and the regulation of mitotic activity. These studies have contributed indirect and circumstantial support to Cone's (5) hypothesis. Other investigators (15, 21, 22) doubt the theory of Cone (5).

The introduction of X-ray microanalytic methods in biology allowed direct measurements of intracellular and intranuclear elemental concentrations to be performed. A correlation of increased proliferation with increased intracellular Na\(^+\):K\(^+\) ratio has been described in various cell types: (a) normal animal cells; (b) transformed cells (hepatomas and mammary adenocarcinomas); (c) normal rapidly or slowly dividing cells (3, 4, 24); and (d) cancer cells of invasively growing human urogenital tumors (26). Although these data support the hypothesis of Cone (5), obviously, they still do not prove the causal role of high intranuclear sodium content in the regulation of mitogenesis. Nevertheless, evidence is accumulating nowadays for such a causal interrelationship. Namely, Koch and Leffert (11) have shown in vitro in hepatocyte cultures, and also in vivo in regenerating liver, that sodium influx is a necessary prerequisite for the initiation of cell proliferation, because a specific sodium influx inhibitor (amiloride) is able to block the cell growth in both systems. Moolenaar et al. (14) published the same observations on serum-stimulated N1E-115 neuroblastoma cells. Furthermore, Mummery et al. (16) have demonstrated that amiloride specifically inhibits the transient increase of Na\(^+\) influx at the G1-S-phase transition of neuroblastoma cells, whereas it has little effect on Na\(^+\) or K\(^+\) influx in other phases of the cell cycle.

In light of the above results, the necessity of collecting more data on human cancer cells of different origin and various levels of malignancy is obvious. By means of an energy-dispersive X-ray microanalytical method (27) applied by us formerly on human urogenital invasive cancers (26), we measured the intranuclear monovalent ion contents in samples of the human thyroid gland removed under circumstances allowing us to perform a safe quantitative analysis. The results described in this paper support the idea that the increase of malignancy of human cancers is correlated with the increase of the intranuclear Na\(^+\):K\(^+\) ratio due mostly to a net increase of the sodium content.

MATERIALS AND METHODS

Sampling Criteria. Human tumors can be used for X-ray microanalysis of the monovalent electrolytes (Na\(^+\), K\(^+\), Cl\(^-\)) if the following criteria are met: (a) sampling must be carried out before any cytostatic treatment has been started; (b) the exact diagnosis can be established by detailed pathohistological investigations (We always used parallel samples from which the pathologist gave us the diagnosis.); (c) the biopsy material must be mechanically and metabolically intact. Special care must be taken by the surgeon to assure that the shortest possible time (less than 1 min) elapses between the closure of blood circulation and the deep freezing of the sample; (d) sampling is allowed from humans only if the surgical intervention is absolutely necessary in the interest of the patient; (e) the removal of some normal tissue for control purposes is possible without any damage to the patient. In the case of thyroid tumors, one can obtain apparently intact thyroid tissue from those cases in which lobectomy or total thyroidectomy must be performed due to the presence of single or multiple nodules which are suspected to be malignant.

Materials. Sixteen patients suffering from thyroid tumors were involved in our studies (4 males, 12 females). The age of the patients was between 36 and 67 years except 2 cases (12 and 26 years, respectively). According to the pathohistological diagnoses, the patients could be classified into 3 main groups: (a) benign adenomas (5 cases); (b) differ-

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\(^2\) To whom requests for reprints should be addressed, at VILEG Hungarian Section, University Medical School, H-4012 Debrecen, Hungary.

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entiated carcinomas, including follicular, papillary, or mixed ones (5 cases); and (c) anaplastic carcinomas (6 cases). Sampling was carried out in more cases; however, we used the samples for microanalysis only if the pathologist established the above diagnoses. Cases of thyroiditis were excluded from the present material.

Control tissue was obtained from 7 patients with benign adenomas and differentiated carcinomas, in which we could find sufficiently large tissue pieces containing no nodules or other pathologically altered parts. The electron microscopic morphology of the tissue always documented the normal state of the cells analyzed in the control group; only the epithelial cells bordering the colloidal mass of the thyroid acini were measured. However, it is conceivable that the adjacent pathological process may have some influence on the nearby, apparently intact thyroid tissue; i.e., our control cells may not be really "normal." Nevertheless, we had to accept this compromise because intact normal human thyroid tissue is not available.

**X-Ray Microanalysis.** The removed tissue samples were prepared for energy-dispersive X-ray microanalysis by means of the freeze-fracture, freeze-drying technique described in detail elsewhere (27). Application of this method for human urogenital cancers has been described by Zs.-Nagy et al. (26). Identical preparative procedure and instrumentation were used for the present studies. The main steps of the procedure are: (a) deep freezing of the resected sample in isopentane cooled by liquid nitrogen to its freezing point; (b) fracturing of the tissue samples by cold scissors still in the deep-frozen state, in order to explore the intact cell compartments inside the tissue; (c) freeze-drying of the samples; (d) X-ray microanalysis of the nuclei at 10 kV accelerating voltage in a scanning electron microscope with no coating layer (The incident beam current was kept at 45 μA, whereas the effective beam current in the specimen was in the range of 1 to 2 pA, when obtaining a count rate of 450 cps. The distance between the specimen and the X-ray detector was 36 mm, and the takeoff angle was 28 degrees.); (e) computerized evaluation of the X-ray spectra, obtained by using the mass fraction method of Hall et al. (9), extended for bulk specimens (27); (f) calculation of the monovalent electrolyte concentrations of the nuclear water. For additional technical or methodological details, see Refs. 26 and 27.

**Statistical Evaluation of the Data.** For each cell nucleus analyzed, elemental concentrations of Na+, Cl−, and K+, as well as Na+:K+ molar ratios, were calculated. In the first step of statistical elaboration of the data, the individual averages of these parameters within the control and each tumor group were calculated. It was evident that the basic data displayed a fairly narrow Gaussian distribution in the normal thyroid cells and that their scatter was considerably broader in the tumor samples. For example, Chart 1 shows the Na+:K+ ratios found in the normal thyroid cell nuclei of each individual, as well as 2 kinds of possibilities for further handling of these data: (a) to calculate the average of the individual averages with the respective standard deviation; (b) to pool together the measured cells and give the average and standard deviation for the total pool. Chart 1 clearly shows that pooling of the data represents the real range in which the Na+:K+ ratios are scattered much better than the first method mentioned above. This statistical approach appears to be even more valid for the malignant tumors because, due to the much broader scatter of the basic data, the average of the individual means may be relatively more misleading. Pooling together the data is justified furthermore by the fact that Student’s t test did not reveal any significant difference between the individual averages within each experimental group. The pooled data of elemental concentrations were compared again between different groups by using Student’s t test (Table 1). However, due to the fact that the distribution of the increasing malignancy of the tumors, we applied the χ² test for the comparison of histograms (Chart 4).

**RESULTS**

**Morphology of the Normal and Cancerous Tissue Samples.** The freeze-fracture, freeze-drying method results in freeze-dried bulk specimens with broken surfaces allowing exploration of the internal compartments of cells which remained intact during the dissection procedure. This method has been elaborated for the purpose of X-ray microanalytical determination of intracellular and intranuclear monovalent elemental concentrations; therefore, the specimen quality is not ideal for morphological studies by scanning electron microscopy (27). Nevertheless, the quality of the obtainable image is sufficient for recognition of the main cell compartments, such as nucleus, cytoplasm, cell borders, etc. (Figs. 1 to 4). The normal thyroid epithelial cells forming the thyroid acini can be distinguished readily, and the colloidal substance demonstrated by immersion refractometry (1), results-

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**Table 1**

**Monovalent electrolytes in cell nuclei of normal thyroid and tumorous cells**

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Dry mass (%)</th>
<th>Intranuclear water (mEq/kg water)</th>
<th>Total monovalents (mEq/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal cells (349)</td>
<td>0.277 ± 0.000</td>
<td>0.389 ± 0.008</td>
<td>2.921 ± 0.002</td>
</tr>
<tr>
<td>Benign adenomas (408)</td>
<td>0.328 ± 0.014</td>
<td>0.486 ± 0.015</td>
<td>2.803 ± 0.025</td>
</tr>
<tr>
<td>Differentiated cancers (423)</td>
<td>0.429 ± 0.014</td>
<td>0.617 ± 0.015</td>
<td>3.034 ± 0.035</td>
</tr>
<tr>
<td>Anaplastic carcinomas (891)</td>
<td>0.592 ± 0.014</td>
<td>0.824 ± 0.013</td>
<td>2.889 ± 0.024</td>
</tr>
</tbody>
</table>

- Calculated assuming 75 weight % intranuclear water content. All the monovalent concentrations of the tumor cells differ significantly with p < 0.001 from the respective normal values except the Na+ content of benign adenomas, where p < 0.01, and the K+ content of the anaplastic carcinomas, where the difference from the normal cells is not significant.
- Numbers in parentheses, number of nuclei.
- Average ± S.E.
Intracellular Na*:K+ Ratio in Thyroid Tumors

...ing in a spongy structure of larger than average “hole size” than in the cytoplasm after freeze-drying. Also, one can recognize the characteristic morphology of the cells in benign adenomas and in differentiated and anaplastic carcinomas (Figs. 2 to 4).

**X-Ray Microanalysis.** The cell nuclei for microanalysis were selected randomly in each type of tumor. The only parameter that we considered was nuclear size. Obviously, the plane of breaking could pass at different heights through the nearly spherical nucleus, and if this plane was far from the equatorial one, the nucleus was broken into a smaller and a larger segment. Since in the bulk specimen we can see only the surface, it is impossible to decide whether at a given place the smaller or the larger segment of the nucleus will be found below the surface. Unfortunately, one cannot completely avoid the danger of overpenetration of the nuclei by selecting the largest ones, if the average nuclear diameter is below about 8 μm. Evidence has been published elsewhere (25, 27) regarding the penetration depth of the 10-kV electron beam into the freeze-dried biological mass. Since most of the nuclei of the normal thyroid epithelium are smaller than 8 μm (Fig. 1), it is certain that some part of the X-ray counts collected over the nucleus come from the cytoplasm below the broken nuclei. However, this does not represent any difficulty for determination of Na*:K+ ratios, since the nuclear membrane does not play a barrier role for the light elements such as Na+ and K+ (17). Therefore, the mean ratio of these elements must be equal in the nucleus and the cytoplasm. On the other hand, the phenomenon of the possible overpenetration of the nuclei represents a difficulty if our data were to be converted to absolute concentrations, since the nuclei contain somewhat more water than does the cytoplasm (1). As we shall point out later, absolute concentrations cannot be calculated without knowing the exact water content of the analyzed cell compartment.

A further possibility of error in measuring normal thyroid epithelial cells may be that, if due to special topographical circumstances of breaking the tissue, the colloidal substance lies very near the analyzed cell, resulting in analysis of colloid. Such cases, however, are easily recognized on the following basis. Chart 2 demonstrates a typical X-ray spectrum taken from a normal thyroid epithelial cell (Chart 2A) and one taken from the central part of the colloid substance of an acinus (Chart 2B). It is clear from these spectra that the colloid substance contains almost no phosphorus and a very low concentration of K+, whereas it is extremely rich in sulfur. At the same time, the sulfur content of the cells is rather low. If we obtained a considerably increased sulfur content accompanied by unusually low phosphorus and K+ peaks over an epithelial cell, the beam overpenetrated the cell into the colloid, and such spectra were discarded. The form of such spectra was so characteristic that it was not necessary to establish any numerical cutoff point for the selection of unsuitable spectra.

It is very important to perform analysis only on living cells. Although the tumor samples were always taken from regions of tissue where no apparent macroscopic signs of necrosis could be observed, we cannot exclude a priori the presence of some necrotic cells in our samples. However, on the basis of the criteria that we elaborated and described in detail elsewhere (26), one can safely distinguish and exclude from analysis the necrotic cells from the living ones. The main signs of necrosis apart from the morphological appearance are the absence of any ion gradient (as compared to the extracellular space) and the accumulation of Ca2+ ions.

Chart 3 shows a typical X-ray spectrum recorded from the nucleus of an anaplastic cancer cell. The high sodium peak is evident, accompanied by some increase in Cl− content and decrease of the K+ peak.

Table 1 summarizes the results obtained in the normal epithelial cells and 3 different classes of thyroid tumors. Since the method of quantitation used by us (26, 27) gives mass fraction values in the dry mass of the cells for the measured elements, we present here these values as percentages by weight. At least 40 to 50 cells were measured, usually from 2 to 3 different tissue pieces prepared from the biopsy material of each patient.
It is evident from the data of Table 1 that the Na\(^+\) and Cl\(^-\) contents of the nuclear dry mass increase parallel with the increased malignancy of the tumors studied. At the same time, the K\(^+\) content, although displaying some statistically significant changes, varies relatively little and does not show any clear tendency.

Table 1 reports also the concentrations of the monovalent ions calculated for the intranuclear water in mEq/kg units. In order to calculate these concentrations, the actual dry mass content (i.e., the water content) of the nuclei must be determined. Since this parameter was not measured in the present material, we assumed uniformly a value of 25% by weight for the dry mass (i.e., 75% by weight for water) for the values reported in Table 1. We should like to stress, however, that our calculated values cannot be taken as true absolute concentrations, since differences in the nuclear dry mass content may influence the figures obtained very strongly. For example, if the dry mass content of the nuclei is only 20% instead of the assumed 25%, all the monovalent concentrations calculated for the water will be lowered to 75% of the values shown (for details, see Equation 1 of Bertoni-Freddari et al. [1]). Differences of several percentage points in the total dry mass may well exist between the normal and tumor cells, bringing the absolute values of the monovalent concentrations back into the physiologically accepted range of approximately 300 mEq/kg water.

Chart 4 shows the distribution histograms of the Na\(^+\):K\(^+\) ratios calculated individually for each nucleus. It should be stressed that this parameter does not depend at all on the actual dry mass content of the cells; therefore, its increase from the normal value toward the anaplastic carcinomas may represent real differences between various states of the thyroid tissue. It is noteworthy that the \(\chi^2\) test of statistical comparison revealed very highly significant differences between any pair of the histograms \((p < 0.001)\). It is obvious from the histogram of Chart 4 that each type of tumor studied contains a population of cells having Na\(^+\):K\(^+\) ratios in the normal range. However, there is an additional cell population in the tumors with considerably increased Na\(^+\):K\(^+\) ratios, and the proportion of these cells increases parallel with the malignancy of the tumors. One might assume that cells with extremely high Na\(^+\):K\(^+\) ratios (e.g., 1.5 or above) may be injured or seriously disturbed, even if they are not dead cells according to our criteria mentioned above. However, even if we exclude these cells from consideration, the mean values may be somewhat lower, but neither the shape of the histograms nor the significance levels will be altered considerably.

The Na\(^+\):K\(^+\) ratios presented in the histograms were calculated for each nucleus individually, and the averages of these ratios are shown in Chart 4. For mathematical reasons, this average of the ratios is not equal to the ratio of the average molar concentrations presented in Table 1. However, the latter values show similar tendencies.

**DISCUSSION**

The applicability, reliability, and limitations of the bulk specimen
X-ray microanalysis for human unogenital tumor cell nuclei have been discussed in detail in our earlier paper (26). Therefore, we shall not discuss the validity of the approach further. We are of the opinion that this method is also valid for human thyroid tumors, since the sampling criteria were met.

The present results demonstrate that the malignancy of the thyroid tumors is correlated with their intracellular Na⁺:K⁺ ratio. As used here, malignancy is a clinical term which describes the proliferative capacity of the tumors, the invasive character of the tumor, and the tendency to form metastases.

The possible mechanisms of action by which an increased Na⁺:K⁺ ratio may affect regulation of cell division are still not quite clear. We listed some theoretical possibilities in our earlier paper (26). Even if the theory of Cone (5) is true, the absolute value of the Na⁺:K⁺ ratio cannot be regarded as the only factor regulating cell division, since even physiologically normal cells display a variation in this parameter. For example, normal thyroid cells have a Na⁺:K⁺ ratio twice as high as that of urinary bladder epithelial cells (26). The membrane potential of any cell type is determined by the actual Na⁺:K⁺ ratios in the extra- and intracellular spaces, as well as by the permeability ratio of the cell membrane for these ions. In normal resting cells, these parameters are regulated according to the state of differentiation and type of the cells, resulting in some variations between the different cell types. Nevertheless, the increase of the intracellular Na⁺:K⁺ ratio and the increase of the PmNa/PmK ratio lead to the depolarization of the cell membrane as described by the Goldman equation. Therefore, if we detect an increased Na⁺:K⁺ ratio, it will contribute toward relative depolarization of the cell membrane. It is also possible that the chromatin of differentiated cells binds less Na⁺.

The mechanism of the increase in intracellular Na⁺ content in the tumor cells is unknown. Theoretically, one can assume 2 basic phenomena, either of which (or their combination) may result in this increase: (a) it is possible that the Na⁺ permeability of the cell membrane remains normal, but that the membrane-bound Na⁺:K⁺-dependent ATPase (the "pump enzyme") is defective, thereby allowing the extra- and intracellular space to equilibrate more or less quickly the concentration gradient for Na⁺; (b) the Na⁺ permeability of the resting cell membrane may be higher (being much lower normally than that for K⁺). As a consequence of increased Na⁺ influx, the pump enzyme will be activated but may not be able to pump out the excess Na⁺, since the influx is too high. It is interesting to note that both of these mechanisms may be associated with cancerous growth. For instance, in chemically induced liver cancers, the disappearance of the pump enzyme was shown to be the very first and persisting detectable histochemical alteration (for details, see Ref. 8). On the other hand, our preliminary histochemical studies on the thyroid tumors revealed a marked increase of the pump enzyme activity in the anaplastic cancer cells as compared to the normal thyroid cells or to the less malignant thyroid tumor cells.

Although this fact still does not directly prove an increased sodium permeability of the cell membrane, it offers strong support for such an assumption. Similarly, Rozengurt and Mendoza (20) have demonstrated an increased sodium permeability of serum-stimulated fibroblasts compared to quiescent cells and of transformed compared to normal cells. The possibility that human carcinogenesis may result from an increased sodium permeability of the cell membrane is not merely of theoretical significance. It seems to be reasonable to assume that sodium channel-blocking drugs, such as amiloride, may inhibit the growth of cancer cells in humans as they inhibit cell proliferation in experimental systems (11, 14, 16).

In earlier observations (12), we showed that the DNA content of cell nuclei in thyroid cancers is very aneuploid, whereas benign adenomas are less aneuploid. Recently, we performed parallel measurements of DNA content and the intranuclear Na⁺:K⁺ ratios on the thyroid biopsy material presented in this report. The results of these studies revealed a strong correlation between increased aneuploidy and increased intracellular Na⁺:K⁺ ratio (13). These data give further support to the hypothesis of Cone (5) that sustained membrane depolarization increases DNA synthesis and cell division.

Some previously published reports deny a regulatory role for intracellular Na⁺ in mitogenesis (15, 22). The intracellular Na⁺ content in those experiments was determined by atomic absorption spectroscopy, the results of which are subject to extensive alterations due to the preparative steps applied to cultured fibroblasts. The methodological controversy, however, will be treated elsewhere. ⁴

Note Added in Proof

Since this manuscript was completed, Mizukami et al. (Lab. Invest., 48: 411-418, 1983) published data on the ouabain-sensitive, potassium-dependent p-Nitrophenylphosphatase activity in human thyroid carcinoma cells. It has been shown that this enzyme shows a marked change in its localization in the thyroid papillary carcinomas and the biochemically measured activity of it was 10 times higher, as expressed per mg of DNA, than that in the normal thyroid cells.

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