Surface Membrane Nucleoside Triphosphatase Activity and Tumorigenicity of Cultured Liver Epithelial Cells

Shuichi Karasaki and Tohru Okigaki

Institut du Cancer de Montréal, Centre Hospitalier Notre-Dame, Montréal, H2L 4M1 and Département d’Anatomie, Université de Montréal, Canada (S. K.), and Pasadena Foundation of Medical Research, Pasadena, California 91101 (T. O.)

SUMMARY

A cell surface-located nucleoside triphosphatase activity can be assayed in liver epithelial cultures in situ with the incubation of intact cells in a medium containing [γ-32P]adenosine triphosphate and correlated with the tumorigenicity of these cells in neonatal Wistar rats. The ectoenzyme activity of normal diploid cell lines is minimal, whereas a considerably high activity has been found in all tumorigenic cell lines tested. The optimum condition for the adenosinetriphosphatase activity on the external surface. The iso-fixed cells of tumorigenic lines have Ca2+-stimulated adenosine triphosphatase activity along the entire surface membrane during the initial transformation. A significant increase in ATPase activity along the entire surface membrane during passages of rat liver epithelial cells in vitro can be demonstrated.

This cell surface alteration appears to begin coincidentally with the acquisition of tumorigenicity. Taking note of this, an analysis of tumorigenicity of cultured liver cells and the histochemical localization of surface ATPase activity was extended to several independently isolated cell lines from normal rat livers as well as some transformed liver cell lines. All of these cell lines were chosen from those that have shown evidence of specific functions on components characteristic of hepatocytes. In view of the problems inherent in the histochemical technique, it was desirable to add a biochemical procedure for the demonstration of an ATPase activity with intact cells in which viability was unimpaired during the enzyme assay. This cell surface alteration appears to begin coincidentally with the acquisition of tumorigenicity (18, 19). Taking note of this, an analysis of tumorigenicity of cultured liver cells and the histochemical localization of surface ATPase activity was extended to several independently isolated cell lines from normal rat livers as well as some transformed liver cell lines. All of these cell lines were chosen from those that have shown evidence of specific functions on components characteristic of hepatocytes. In view of the problems inherent in the histochemical technique, it was desirable to add a biochemical procedure for the demonstration of an ATPase activity with intact cells in which viability was unimpaired during the enzyme assay. At a later stage, Weinstein et al. (1) have demonstrated that incubating cultured cells with [γ-32P]ATP releases 32P into the medium in relation to membrane-bound ATPase. Applying this technique, the characteristics of membrane-bound ATPase of tumorigenic liver epithelial cells can be analyzed and compared with results of histochemical techniques. Finally, the possible functional significance of the phosphatase and its usefulness as a parameter for epithelial oncogenesis in vitro is considered.

MATERIALS AND METHODS

Cell Lines. Table 1 describes the origin of the cell lines established for this investigation. Normal diploid cell lines were isolated from the primary cultures of dissociated hepatocytes of Wistar rats (25). Other lines have been previously reported on (11, 18, 19, 26–28, 33, 35). Cell lines studied

DECEMBER 1976
have been in continuous culture for years (Table 2). As shown in Table 1, these cells may retain hepatocytic functions or components such as hydrocortisone-inducible tyrosine transaminidase activity (11, 33), peroxisomes (19), glycogen granules (11), and canaliculi (19).

**Cell Culture.** All cells were well grown in sealed flasks (Falcon Plastics, Oxnard, Calif.) at 37°C in Ham’s F12 medium (Grand Island Biological Co., Grand Island, N. Y.) with 10% fetal bovine serum (Grand Island Biological Co.) supplemented with streptomycin (100 μg/ml) and penicillin (100 units/ml). Cultures were subcultured twice weekly and kept at pH 6.8 to 7.1. For subculturing, weekly monolayers were dissociated with 0.25% trypsin in GIBCO Solution A (Grand Island Biological Co.) and 10% fetal bovine serum, containing 0.4% agarose (Grand Island Biological Co.). For growth in agar suspension, cells were transferred to separate scintillation vials containing 10 ml of Bray’s solution (New England Nuclear), and the radioactivity was determined in a Nuclear Chicago Mark II scintillation system.

Cell number was determined with a Coulter electronic counter following trypsinization of the cultures. The viability after ATPase assay was tested by trypan blue exclusion and was greater than 95%. Trypsinized cells were also assayed in suspension by the isotopic procedure.

For the colorimetric assay of ATPase activity, cells were suspended in 2.0 ml of the incubation medium. The reaction was carried out by adding 2 μmoles of ATP at 37°C and was terminated by chilling followed by the addition of 200 μl of 20% HClO₄. After a rapid centrifugation, a 2.0-ml aliquot of the supernatant was assayed for the presence of P by a modified Fiske and SubbaRow procedure (22, 30). Substrate specificity of the enzymatic hydrolysis of ATP was determined by substituting GTP, CTP, UTP, ADP, AMP, p-nitrophenyl phosphate or ß-glycerophosphate (all sodium salts, Sigma) for the ATP. The colorimetric method of analysis, however, required excessive amounts of ATP (more than 0.5 mM) and cells (more than 5 x 10⁶) and was not used routinely.

**Histochemistry and Electron Microscopy.** Cells were fixed in situ for 5 to 60 min in 2% glutaraldehyde (0.1 M sodium cacodylate-HCl buffer, pH 7.2, containing 4 mM CaCl₂) and washed in ice-cold 0.25 M sucrose for 3 hr. They were treated for 30 min by a modification of the method of Wachstein and Meisel (54) for histochemical demonstration of ATPase activity. The modified reaction medium contained 100 mM NaCl, 5 mM KCl, 4 mM CaCl₂, 1 mM MgCl₂, 30 mM Tris-acetate buffer (pH 7.2), and 10 μM ATP (disodium salt; Sigma Chemical Co., St. Louis, Mo.). Details on the conditions of modified assay systems are given in the legends to the charts and tables. For enzyme inhibition experiments, cultured cells were preincubated for appropriate periods in the culture medium containing inhibitors.

For the isotopic ATPase assays, the reaction was started at 37°C by adding [γ-³²P]ATP (tetraammonium salts; New England Nuclear, Boston, Mass.) and was usually terminated at 10 min. Following incubation, a 100-μl aliquot of the incubation medium and 2 ml of 1.5% molybdc acid in 0.5 mM H₂SO₄ were vigorously emulsified with 2 ml of benzene:isobutyl alcohol (1:1). Aliquots (100 μl) of the organic phase containing ³²P, and the aqueous phase ([γ-³²P]ATP) were transferred to separate scintillation vials containing 10 ml of Bray’s solution (New England Nuclear), and the radioactivity was determined in a Nuclear Chicago Mark II scintillation system.

The modified reaction medium contained 130 mM NaCl, 10 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 30 mM Tris-acetate buffer (pH 7.2), and 10 μM ATP (disodium salt; Sigma Chemical Co., St. Louis, Mo.). Details on the conditions of modified assay systems are given in the legends to the charts and tables. For enzyme inhibition experiments, cultured cells were preincubated for appropriate periods in the culture medium containing inhibitors.

For the isotopic ATPase assays, the reaction was started at 37°C by adding [γ-³²P]ATP (tetraammonium salts; New England Nuclear, Boston, Mass.) and was usually terminated at 10 min. Following incubation, a 100-μl aliquot of the incubation medium and 2 ml of 1.5% molybdc acid in 0.5 mM H₂SO₄ were vigorously emulsified with 2 ml of benzene:isobutyl alcohol (1:1). Aliquots (100 μl) of the organic phase containing ³²P, and the aqueous phase ([γ-³²P]ATP) were transferred to separate scintillation vials containing 10 ml of Bray’s solution (New England Nuclear), and the radioactivity was determined in a Nuclear Chicago Mark II scintillation system.

Cell number was determined with a Coulter electronic counter following trypsinization of the cultures. The viability after ATPase assay was tested by trypan blue exclusion and was greater than 95%. Trypsinized cells were also assayed in suspension by the isotopic procedure.

For the colorimetric assay of ATPase activity, cells were suspended in 2.0 ml of the incubation medium. The reaction was carried out by adding 2 μmoles of ATP at 37°C and was terminated by chilling followed by the addition of 200 μl of 20% HClO₄. After a rapid centrifugation, a 2.0-ml aliquot of the supernatant was assayed for the presence of P by a modified Fiske and SubbaRow procedure (22, 30). Substrate specificity of the enzymatic hydrolysis of ATP was determined by substituting GTP, CTP, UTP, ADP, AMP, ß-nitrophenyl phosphate or ß-glycerophosphate (all sodium salts, Sigma) for the ATP. The colorimetric method of analysis, however, required excessive amounts of ATP (more than 0.5 mM) and cells (more than 5 x 10⁶) and was not used routinely.

**Histochemistry and Electron Microscopy.** Cells were fixed in situ for 5 to 60 min in 2% glutaraldehyde (0.1 M sodium cacodylate-HCl buffer, pH 7.2, containing 4 mM CaCl₂) and washed in ice-cold 0.25 M sucrose for 3 hr. They were treated for 30 min by a modification of the method of Wachstein and Meisel (54) for histochemical demonstration of ATPase activity. The modified reaction medium contained 100 mM NaCl, 5 mM KCl, 4 mM CaCl₂, 1 mM MgCl₂, 40 mM Tris-maleate buffer (pH 7.2), 1 mM ATP (disodium salt; Sigma), and 0.3 mM Pb(NO₃)₂. Following incubation, the
samples were postfixed for 1 hr in s-collidine-HCl-buffered OsO₄, dehydrated through graded ethanol, and embedded in Epon for sectioning. Thick (1 μm) sections were treated with ammonium sulfide and examined with a phase-contrast microscope. Thin sections were either unstained or double stained with aqueous uranyl acetate and lead citrate according to their ability to grow in 0.4% agar suspension (31, 34, 39).

The results of tumorigenicity tests are listed in Table 2. All 4 normal cell lines and a transformed cell line, RL34-ESA, were nontumorigenic, when 10⁶ to 10⁷ cells were injected into newborn Wistar rats either s.c. or i.p. A positive result of tumorigenicity was obtained with s.c. transplantations of 3 x 10⁶ cells of RL34-HII. Injections s.c. of 10⁶ cells from RLC C-1, RLC-TVI, HNT, and HTC produced palpable tumors at the sites of injection within 10 days. The tumors grew continuously and, in many cases, revealed histological features resembling those of hepatocarcinomas (cf. Ref. 19).

Cytogenetic analyses of normal liver cell lines revealed that a majority of cells had a diploid number of chromosomes (Table 3). RL-1 demonstrated an epithelial appearance in early cultures but had shifted toward fusiform or bipolar appearance (cf. Refs. 11, 13, and 26) at the time of cytochemical studies and tumorigenicity tests. These serially subcultured cells were frozen and stored before entering a nondenuding stage in culture. RL-33, RL-34, and RL-36 were established as continuously growing lines with an epithelial appearance. Although the plating efficiency was often less than 1%, these epithelial cells revealed a relatively high growth rate during exponential phases (Table 3). Fig. 1 represents a phase-contrast micrograph of epithelial cells of the RL-34 line. Normal cells were extremely flat and adhesive to each other, as well as to the plastic substrate. As reported in other liver cell lines (3, 13, 15, 35, 37), they had little or no locomotion and continued to divide even when they were in close contact with each other. Their confluent cultures consisted of a monolayer of tightly packed but nonoverlapping cells. All transformed cell lines studied here consisted of aneuploid cells and had characteristics of epithelial- rather than fusiform-type cells. The plating efficiency was more than 10% in RL34-ESA and RL34-HII, while it reached 40% in RLC C-1, RLC-TVI, HTC, and HNT. Sparse cultures revealed irregularities in size and shape of cells. However, they often grew into a monolayer of tightly packed cells. There was a tendency for polygonal cells to pile up in multiple layers, but it was not a consistent feature of these transformed cell lines. No definite correlation could be established between the tumorigenicity and the cell generation time or saturation density of these cells (Table 3).

**RESULTS**

**Growth Properties of Cultured Liver Cells.** Those liver cell lines tested were defined as normal or transformed, according to their ability to grow in 0.4% agar suspension (Table 1). None of the normal liver epithelial cells grew in soft agar (31, 34, 39).

The results of tumorigenicity tests are listed in Table 2. All 4 normal cell lines and a transformed cell line, RL34-ESA,
reection products deposited on the extracellular side of entire plasma membranes. In Fig. 4, the free surfaces of epithelial cells showed numerous microvilli and were associated with the distinct reaction product. The intercellular boundaries of adjacent cells often ran undulating courses, and the patterns of lead precipitates followed exactly the courses of folded lateral membranes. In Fig. 5, the basal amount of reaction product varied from cell to cell or region to region, ranging from a near-complete absence to a dense band of lead precipitates on the plasma membranes. The cytochemical results given in Table 2 indicate that a correlation exists between membrane-bound nucleoside phosphatase activity of cultured liver cells and their tumorigenicity in newborn rats.

Biochemical Assays. Monolayer cultures of both normal and transformed cell lines were incubated in a physiologically balanced salt solution containing \( [\gamma-\text{P}]ATP \) in situ or following trypsinization. The viability of cells was not impaired during a 20-min period of incubation. The isotopic assay of ATP-splitting activity was found to be linear for at least 10 min and over the range of \( 10^4 \) to \( 3 \times 10^5 \) cells per assay vessel. Results of these experiments are presented in Table 3. The cells of transformed lines revealed a considerably high rate of ATPase activity: more than 40% of added ATP was hydrolyzed by \( 10^4 \) cells for 10 min. Exponentially growing cells exhibited an activity about 2 to 6 times that of confluent stationary cells. When dissociated by a trypsin treatment, the activity was unchanged in preconfluent cultures, whereas it was increased in confluent cultures roughly to the same degree as the untreated growing cells. Both growing and stationary monolayer cultures of the diploid cell lines exhibited a very low degree of ATPase activity, and trypsinization of those cultures did not increase it.

The release of \( \text{P}^32 \) from ATP correlated with an increase in \( \text{P}^32 \) in the reaction medium as measured by the colorimetric method. Virtually all (99.9% or more) of the \( \text{P}^32 \) added was recovered in the reaction medium after incubation with transformed or normal cells. Solubilization of the enzyme into the medium was negligible during the incubation period.

Some of the biochemical properties of surface-located ATPase were explored with the transformed RLC C-1 line and the normal cell line, RL-34, as a comparison. Substrate specificity was demonstrated utilizing the colorimetric procedures. Other nucleoside phosphates and organic phosphate compounds were substituted for ATP. The rates of hydrolysis of GTP, CTP, UTP, and ADP were, respectively, 80, 60, 30, and 5% of the rate of ATP hydrolysis. Neither RLC C-1 nor RL-34 hydrolyzed AMP, \( \beta \)-glycerophosphate, or \( p \)-nitrophenyl phosphate at pH 7.2. Chart 1 shows the dependency of the ATPase activity of RLC C-1 cells on cationic compositions. With Na\(^+\) and K\(^+\) in the isotonic incubation medium, the activity was very low. It required divalent cations, since the presence of 1 mM EDTA reduced the activity further by 50%. The addition of 1 mM Ca\(^{2+}\) to the monovalent cations increased the activity 5-fold. Mg\(^{2+}\) did not appreciably stimulate ATP hydrolysis and was inhibitory to the Ca\(^{2+}\) activation at the concentration of 1 mM. Thus, Ca\(^{2+}\) was the most effective cation for stimulating the hydrolysis of ATP. ATP hydrolysis was maximal at about 1 mM CaCl\(_2\), when NaCl and KCl were also present at physiological concentrations (Chart 2). One mM MgCl\(_2\) suppressed enzyme activation at Ca\(^{2+}\) concentrations greater than 1 mM. The effect of increasing concentrations of CaCl\(_2\) was dependent on NaCl and KCl, since ATP hydrolysis was significantly reduced when monovalent cations were replaced by isotonic sucrose (Chart 2). The pH optimum for the enzyme activity extended over a range of pH 6 to 7.5, with 2 maximal peaks at pH 6.0 and 7.2 (Chart 3).

At the optimal conditions of ionic composition and pH, ATP hydrolysis by \( 10^4 \) cells was maximal at about 1 mM ATP. The Lineweaver-Burk plots were obtained with RLC C-1 and 

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Chromosome no.</th>
<th>Cell generation time(^a) (hr)</th>
<th>Saturaion density(^b) (( \times 10^{-4} ))</th>
<th>ATPase activity (units(^c))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RL-33</td>
<td>42</td>
<td>22</td>
<td>79</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>RL-34</td>
<td>42</td>
<td>28</td>
<td>54</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>RL-36</td>
<td>42</td>
<td>32</td>
<td>60</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>Transformed(^d)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RL34-ESA</td>
<td>65-70</td>
<td>24</td>
<td>58</td>
<td>7.1 ± 2.1</td>
</tr>
<tr>
<td>RL34-HII</td>
<td>60-70</td>
<td>23</td>
<td>60</td>
<td>12.1 ± 4.3</td>
</tr>
<tr>
<td>RLC C-1</td>
<td>58-63</td>
<td>19</td>
<td>62</td>
<td>40.9 ± 14.9</td>
</tr>
<tr>
<td>RLC-TVI</td>
<td>58-63</td>
<td>23</td>
<td>72</td>
<td>14.6 ± 3.5</td>
</tr>
<tr>
<td>HNT</td>
<td>57-61</td>
<td>18</td>
<td>78</td>
<td>60.8 ± 14.3</td>
</tr>
<tr>
<td>HTC</td>
<td>60-70</td>
<td>19</td>
<td>65</td>
<td>42.6 ± 17.0</td>
</tr>
</tbody>
</table>

\(^a\) Cells in 5 ml F12-10% fetal cell serum were cultured in 25 sq-cm T flasks.

\(^b\) Cells were incubated in situ in the standard salt solution with 100 \( \mu \)M ATP; moles \( \text{P}^32 \), released from \( [\gamma-\text{P}]ATP \) per \( 10^4 \) cells per 10 min ± S.D.

\(^c\) \( 10^6 \) cells or less per flasks.

\(^d\) 3 \( \times 10^4 \) or more per flasks.

\(^e\) Cells positive for growth in soft agar.
Ca++ Membrane Activity and Tumorigenicity

Chart 1. Cation-dependent ATP hydrolysis by dissociated cells as a function of time. Cells (10⁶) of the RLC C-1 line were incubated at 37°C for varying times in 1 ml of an isotonic salt solution with Ca²⁺ (C), Ca²⁺ and Mg²⁺ (V), Mg²⁺ (V), or without divalent cations (C). The basic incubation medium contained 10 nmoles [γ-³²P]ATP, 30 mM Tris-acetic acid buffer (pH 7.2), 130 mM NaCl, and 10 mM KCl. The concentration of divalent cations, when present, was 1 mM. Pi release into incubation medium was expressed as nmoles Pi liberated per 10⁶ cells. RL-34 cells were incubated with Ca²⁺ and Mg²⁺ (C).

RLC-TVI when the reciprocals of the velocity of Ca²⁺-stimulated ATP hydrolysis were plotted against reciprocal substrate concentrations (Chart 4). The K₉, calculated for ATPase was 0.29 mM for RLC C-1 and 0.33 mM for RLC-TVI. The corresponding maximal velocities were 125 and 36 nmoles of Pi liberated per 10⁶ cells, respectively.

The direct effects of different agents on the ATPase activity were tested with RLC C-1 cells. La³⁺, which was known to be a specific antagonist of Ca²⁺ in a number of biological systems (14), was effective in suppressing 80% of ATP hydrolysis at the concentration of 0.2 mM. Ouabain, a cardiac glycoside which is a potent inhibitor of Na⁺-K⁺-ATPase (8, 22), did not inhibit it at the concentration of 10 μM. There is little reduction of enzyme activity in the incubation medium containing 10 μM Oncovin, 10 μM colchicine or 5 μM cytochalasin B. The addition of dithiothreitol up to 1 μM did not stimulate the activity.

RLC C-1 cells fixed for 5 min in 0.5% glutaraldehyde before assay showed 40% of the ATPase activity of unfixed cells. After routine glutaraldehyde fixation for electron microscopy (2%, 15 min to 1 hr), 30% of the activity was detectable. The Ca²⁺-stimulated ATP hydrolysis of the fixed cells was linear for a 20-min period and gradually reached a maximum of 30% hydrolysis of added ATP, within 1 hr (Chart 5). The reaction required divalent cations and was suppressed by La³⁺ but not by ouabain. The activity of fixed RL-34 cells was also low (Chart 5).
The primary purpose of this work is to investigate the surface alterations of cultured liver epithelial cells with reference to their tumorigenic potential. The morphological criteria often used for assessing neoplastic growth of fibroblast cultures such as piling up or criss-crossing of cells due to the loss of contact inhibition (4, 6, 31) cannot be reliably applied to epithelial cultures (20, 27, 31, 35, 38, 39). At the subcellular level, the increase of microvilli at the cell surface (19, 35, 39) and the loss of cytoplasmic microfilament bundles (15) have been observed in association with oncogenic conversion of liver epithelial cells, although these are hardly detectable as constant features. The present histochemical survey of an ATPase reaction of cultured liver cells has revealed a surface membrane alteration unique to tumorigenic cell lines and absent from diploid cell lines that were derived from normal rat livers. The nature of the nucleoside triphosphatase localized to plasma membranes has been characterized by a biochemical method. Since a close correlation exists between the activity of a surface-located ATPase and the tumorigenicity of cultured cells, the isotopic assay of $[^32P]ATP$ hydrolysis by intact cells may provide a rapid and quantitative method for screening oncogenic conversion in vitro of liver epithelial cells.

The hydrolysis of added ATP by intact cells has been described for other normal and neoplastic cell types including human glia-like astrocytes in culture (1), Ehrlich ascites cells (29), human granulocytes (5), and the nucleated erythrocytes of vertebrate species except mammals (36). In contrast to the present results with liver epithelial cell lines, Ågren et al. (1) have reported that human glia cell lines exhibit a decreased rate of surface-membrane ATPase activity following neoplastic transformation in vivo (glioma lines), as well as in vitro (glia cells exposed to SV40). Only a low or moderate activity of surface-membrane ATPase has been detected either in normal or transformed fibroblasts (1). The biochemical analyses with tumorigenic liver epithelial cells have characterized a $Ca^{2+}$-, $Mg^{2+}$-dependent ATPase on the basis of substrate specificities, activating cations, pH optima, sensitivities to inhibitors, and effects of fixation. The specific characteristic of the ATPase, especially with the pattern of $Mg^{2+}$-inhibition of $Ca^{2+}$-activation and sensitivity to $La^{3+}$, is somewhat different from the one described by Ronquist and Ågren (29) for the surface-located ATPase on intact Ehrlich ascites cells. The ectoenzyme activity demonstrated in transformed liver epithelial cells appears to be unique for tissue type or cell form rather than for a neoplastic state per se. In fact, morphology, behavior, and growth properties of cultured liver epithelial cells (4, 11, 13, 15, 19, 26, 35, 37–39) are quite different from those of fibroblasts (1, 4, 6), astrocytes (1), and asciites cells (9). Thus, the appearance of the ectoenzyme is a unique phenomenon of cytodifferentiation following a long-term cultivation of liver epithelial cells. The tumorigenic cell populations are clearly subject to selection processes in vitro. Cells expressing surface ATPase activity may have a growth advantage in vitro over other cells with lower activity that were suppressed and eventually eliminated from the culture. Whether a correlation of the enzyme activity to malignancy is applicable to other tumor cells that arose in vivo is not yet established. It should be noted, however, that the topographic pattern of surface enzyme reaction in the cultured liver cells is similar to those described in primary and transplantable hepatomas (9, 17, 18, 24).

It is well established that ATP cannot penetrate the plasma membrane barrier of intact cells (1, 5, 10, 22). Many physiological and biochemical studies with human erythrocytes and other cell types suggest that, except for the specialized surface regions of some differentiated cells (e.g., glia cells), the enzyme sites active in ATP hydrolysis are mostly located at the inner leaflet of the plasma membrane (2, 14, 22). Thus, the negative ATPase reaction with intact cells of normal liver cell lines reported in this study should not be interpreted as indicating an absence of this enzyme from the plasma membrane. Our histochemical studies of frozen-thawed and formaldehyde-fixed samples of cultured diploid liver cell lines have been in agreement with those of Williams et al. (37), who also showed positive reactions of ATPase activity at the plasma membranes as well as at the cytoplasmic sites. Since these preparative procedures could preserve cytoplasmic ATPase activity and permitted penetration of ATP into the cells (8, 10), the intracellular sites revealed enzymatic reactions.

As assayed biochemically, growing cells of all tumorigenic lines tested show a rapid hydrolysis of ATP added to a physiologically balanced salt solution. The ATP-splitting activity is due neither to leakage of enzyme from the cells into the medium nor to penetration of added substrate into the cells (1). The enzymatic activity of tumorigenic cell lines is probably different from that of the $Na^{+}$-$K^{+}$-dependent ATPase, since the latter was known to be completely suppressed by glutaraldehyde fixation (8, 16, 22). Only a $Ca^{2+}$-$Mg^{2+}$-dependent ATPase could survive in glutaraldehyde treatment with a long fixation time (16). The present histochemical study has demonstrated that enzymatic reaction products of glutaraldehyde-fixed cells are distributed randomly on the external side of entire plasma membranes. The reaction pattern revealed by electron microscopic histochemistry is presumably related either to the polarity of membrane enzyme constituents or the vectorial flow of enzymic end-products in the plasma membrane (2, 12, 22). The ATP-splitting activity of transformed liver cells could be ascribed to an exposure of enzyme proteins on the external surface that are cryptic in normal cells. Trypsinization of normal liver cells, however, was not enough to expose the cryptic sites. Some architectural changes of plasma membranes associated with neoplastic transformation may be responsible for the altered enzyme topography. The evidence that the ectoenzyme activity of growing cells is always many times higher than in stationary cells indicates that there may be a mechanism for generating the altered membrane conformation, which appears to be inherent in the process of membrane assembling or biogenesis following aberrant cell growth.

ACKNOWLEDGMENTS

We thank Luciano Borsato for valuable technical help and Bernard Szirth for assistance in photography. We also thank Shirley G. Quan and Marsha Davis who helped with preparations of cell cultures during the course of this work.

4496
REFERENCES


Fig. 1. Phase-contrast micrograph of a confluent monolayer of the normal rat liver cell line, RL-34 cells, indicating a flat and regular appearance of cellular morphology. x 400.

Fig. 2. Electron micrograph of RL-34 cells treated for the histochemical demonstration of ATPase activity. Thin section is parallel to the monolayer plane. No reaction is detectable. Microfilaments (F) and microtubules (T) are abundant in the cytoplasm just beneath the plasma membrane attached to the substratum. Organized bundles of microfilaments appear to extend from the cell surface toward the nuclei. The plasma membranes of adjacent cells are closely apposed with intercellular adhesion zones (A). x 10,000.

Fig. 3. Phase-contrast micrograph of RLC C-1 cells, treated for the cytochemical demonstration of ATPase activity. RLC C-1 cells are in a confluent region of culture. Arrows, positive reactions of ATPase activity along the intracellular boundaries and free surfaces. x 1,000.

Fig. 4. Electron micrograph of a thin section of RLC C-1 cells adjacent to that of Fig. 2. The entire surfaces of plasma membranes deposited opaque reaction products of ATPase activity (arrow). Many microvilli (MV) are protruding at the free surfaces. x 5,000.

Fig. 5. A thin section of RLC C-1 cells which is perpendicular to the plastic substratum (S). Opaque reaction products of ATPase activity are unevenly deposited on the external surface of plasma membranes as well as in the narrow intercellular space of lateral membranes (arrows). x 20,000.
Surface Membrane Nucleoside Triphosphatase Activity and Tumorigenicity of Cultured Liver Epithelial Cells

Shuichi Karasaki and Tohru Okigaki


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/36/12/4491

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.