Variations in 5'-Nucleotidase Activity in Established Mesenchymal Cell Lines from Syngeneic A/J Mice

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ABSTRACT. 5'-Nucleotidase activity was analyzed in four different mesenchymal cell lines (F, m, e and SP) established from syngeneic A/J mice. The 5'-nucleotidase activity of fibroblasts was lower in transformed cells (F and m) than in nontransformed cells (e). An increase in cell contact during confluence or during high cell density increased 5'-nucleotidase activity, and a decrease in cell contact caused a decrease in 5'-nucleotidase activity in both fibroblastic (F, m and e) and reticulum (SP) cell lines. These results are evidence that 5'-nucleotidase activity in mesenchymal cells is influenced by intercellular contact as well as transformation.

5'-Nucleotidase is a marker enzyme for cytoplasmic membranes, whose activities have been reported changed by malignant transformation, cellular aging and contact inhibition (2, 6, 9), but there have been conflicting reports regarding the changes in 5'-nucleotidase activity in neoplastic and transformed cultured cells. Some researchers have noted a decrease in 5'-nucleotidase activity in transformed fibroblasts (3T3/SV40) and in human leukemic lymphocytes (3, 7); whereas, others have reported an increase in 5'-nucleotidase activity in squamous cell carcinoma (C3H/N) (10). The confusion seems to stem from differences in the source of the cells used. Therefore, we have used cell lines derived only from A/J syngeneic mice and have compared their 5'-nucleotidase activity under critical experimental conditions.

MATERIALS AND METHODS

Cells: The cell lines used were all from syngeneic A/J mice. F cells were from spontaneous fibrosarcoma; m and SP cells from the spleen were spontaneously transformed *in vitro* and e cells from embryonal skin fibroblasts were considered nontransformed. Transplantable F and m cells in nu/nu mice have the histological appearance of fibrosarcoma, and SP cells have characteristics of the so-called reticulum cell sarcoma.

Determination of Tumorigenicity: Cells (2×10^6) were inoculated subcutaneously into 3-month old nu/nu or syngeneic A/J adult mice. When distinct tumors appeared at the inoculation site after one month, tumorigenicity was considered positive (+), otherwise it

Abbreviations used: MEM, minimum essential medium; 5'-AMP, 5'-adenosine monophosphate; Pi, inorganic phosphate.

was recorded as negative (—).

Cell Culture: 5×10^5 to 4×10^7 cells were cultured at 37°C in Type-40 flasks (45 cm², Ikemoto, Tokyo) for stationary culture, or in Erlenmeyer flasks (20 ml, Tokiwa, Tokyo) for suspension culture, containing Eagle's MEM (Nissui, Tokyo) with 12% calf serum (Flow laboratories, U.S.A.) and 0.5% lactalbumin hydrolysate.

Two types of suspension cultures were made; one was a shaking culture (46 cycle/min) as usual, the other was a stirred culture agitated with a magnetic stirrer at about 200 rpm to decrease cell-to-cell contact more than was possible in the shaking culture. Cells were counted in Neubaur hemocytometer. The viability of the cultured cells was determined by the dye exclusion test with 0.5% trypan blue. All the cells were checked for possible contamination by mycoplasma. There was no mycoplasma growth after two months of culture in mycoplasma agar base (1).

5'-Nucleotidase Activity Assay: Cells were harvested from the flasks with a rubber policeman and collected by centrifugation at 1,000 rpm for 10 min. The pellets formed were washed twice with 5 ml of 0.15 M NaCl to remove phosphate. The washed cells were lysed with 0.5 ml of distilled water then stored at -20°C until use. An addition of 0.5 ml of 0.1% Triton X-100, was made to the thawed burst cells, then they were sonicated with a sonifier (Branson, New York) for 30 sec. The resulting crude homogenates were used for the 5'-nucleotidase activity assay (11). A 0.6 ml portion of the homogenate was incubated in 0.6 ml of assay mixture composed of 0.1 M Tris-HCl (pH 8.5), 10 mM MgCl₂, 10 mM β -glycerophosphate and 10 mM adenosine 5'-monophosphoric acid at 37°C for 30 min. The reaction was terminated by the addition of cold trichloroacetic acid to a final concentration of 5%. After centrifugation, the inorganic phosphate (Pi) in the supernatant was measured by the method of Lowry and Lopez (4), and the protein content by the method of Lowry *et al.* (5). Enzyme activity was expressed as the amount of nmol Pi released from 5'AMP per min per mg of cell protein.

All assays were done in duplicate and their mean values recorded. Statistical significance was checked by Student's t test.

RESULTS

The phase contrast microscopy appearance of each cell line in the growth phase is shown in Fig. 1. A morphological distinction between fibroblastic cells and reticulum cells is clear, fibroblastic cells (F, m and e) generally having spindle shapes and reticulum cells (SP) being polygonal.

The tumorigenicity and doubling time of each cell line are shown in Table 1. The tumorigenicity of F cells was always positive in both nu/nu and adult A/J mice. The tumorigenicity of m and SP cells was positive in nu/nu mice, but negative in adult A/J mice. Embryonal skin fibroblasts (e) most probably were nontransformed, as their tumorigenicity was negative in both nu/nu and adult A/J mice. The doubling time was shortest in m cells and longest in e cells. The doubling time for F cells, which were always transplantable to both nu/nu and adult A/J mice, was longer than that

Fig. 1. Phase contrast microscopy appearance of established mesenchymal cell lines in the growth phase that were derived from A/J syngeneic mice. a: F cells from spontaneous fibrosarcoma; b: m cells from the spleen that are *in vitro* transformed fibrosarcoma; c: e cells from embryonal skin fibroblasts that are not transformed; d: SP cells from the spleen that are transformed *in vitro* and have characteristics of reticulum cell sarcoma. $\times 100$.

TABLE 1. CELL LINES FROM SYNGENEIC A/J MICE

Cell line	Origin	Doubling Time (h)	Tumorigenicity*	
			nu/nu	A/J
F	Spontaneous fibrosarcoma	24	+	—
m	Splenic fibroblasts transformed <i>in vitro</i>	18	+	—
e	Embryonal skin fibroblasts not transformed	36	—	—
SP	Splenic reticulum cells transformed <i>in vitro</i>	30	+	—

*Tumorigenicity (+): distinct tumors appeared within one month of a subcutaneous inoculation of 2×10^6 cells. Tumorigenicity (—): distinct tumors did not appear within one month of an inoculation of 2×10^6 – 2×10^7 cells.

of m cells which only showed positive tumorigenicity in nu/nu mice.

5'-Nucleotidase activity was measured by subtracting the effect of nonspecific phosphatases or alkaline phosphatase when β -glycerophosphate was added to the assay mixture (9). Changes in 5'-nucleotidase activity and the growth curve for each cell line are shown in Fig. 2. 5'-Nucleotidase activity increased with the period of culture, reaching a plateau at the confluent phase in all the cell lines. 5'-Nucleotidase activity in transformed cells (F and m) was significantly lower than that in non-transformed cells (e). The 5'-nucleotidase activity in the reticulum cells (SP), however, was much higher than in the three fibroblastic cell lines.

5'-Nucleotidase activities in high and low density culture systems were measured in each cell line (Figs. 3 and 4). Cells in denser cultures always showed higher 5'-nucleotidase activity than in stationary (Fig. 3) or shaking (Fig. 4) cultures.

Fig. 2. 5'-Nucleotidase activity (●—●) and the growth curve of each cell type (○---○) *in vitro*.

Fig. 3. 5'-Nucleotidase activity in stationary cultured cells. 5'-Nucleotidase activity (○—○) and the cell count (○---○) in high density cultures. 5'-Nucleotidase activity (●—●) and cell count (●---●) in low density cultures. High density culture vs low density culture for each cell line: $p < 0.02$

An increase in cell density makes the culture medium more acidic or induces the accumulation of lactate. The effect of acidity in the culture medium was investigated by incubating SP cells (4×10^6) with medium containing 8 to 32 mM (pH 6.6–7.2) lactic acid or sodium lactate for 24 h. 5'-Nucleotidase activity was not affected by acid or the accumulation of lactate (Fig. 5).

The effect of cell-to-cell contact on 5'-nucleotidase activity in SP cells also was tested. SP cells (4×10^6) were incubated with the medium in Erlenmeyer flasks; some in a regular shaking culture, others in a stirred culture. 5'-Nucleotidase activity was lower in the stirred culture cells than in the shaking culture cells (Fig. 6). The viability of the cells was about the same in both culture systems.

DISCUSSION

5'-Nucleotidase is known to be present in the plasma membrane and to catalyze the degradation of 5'-mononucleotides to nucleosides.

In our study, the 5'-nucleotidase activity in fibroblasts was lower (Fig. 2) in the transformed cell lines (m and F), irrespective of their high and low tumorigenicity (Table 1), than in nontransformed cells (e). This indicates that there is a decrease in one of the phenotypic characteristics during cell transformation.

Our results are in good agreement with those of previous reports (6, 9) that show there is lower 5'-nucleotidase activity in transformed fibroblasts (3T3/SV40) than

Fig. 4. 5'-Nucleotidase activity in shaking cultured cells. 5'-Nucleotidase activity (○—○) and the cell count (○---○) in high density cultures. 5'-Nucleotidase activity (●—●) and the cell count (●---●) in low density cultures. High density culture vs. low density culture for each cell line: $p < 0.02$

Fig. 5. Effect of added lactic acid (●—●) or sodium lactate (○---○) on 5'-nucleotidase activity in SP cells.

Fig. 6. 5'-Nucleotidase activity in SP cells in shaking (●—●) and in stirred (○—○) cultures. Shaking culture vs. stirred culture: $p < 0.02$

in normal fibroblasts (3T3). But, markedly high 5'-nucleotidase activity has been reported in squamous cell carcinoma (C3H/N) (10). This apparent contradiction may be due to differences in the 5'-nucleotidase activity of mesenchymal and epithelial cells. Tumors of mesenchymal origin have been reported as having characteristically smaller amounts of surface transport enzymes (such as alkaline phosphatase and/or 5'-nucleotidase) than do tumors of epithelial origin (12).

5'-Nucleotidase activity was changed by intercellular contact (Fig. 6), but the reason for the change is not clear. This same phenomenon has been reported for a plasma membrane enzyme, galactosyl transferase, by Roth and White (8). They showed that the galactosyl transferase activity of 3T3 cells was lower in a stirred culture than in a shaking one.

5'-Nucleotidase activity in SP cells, a so-called reticulum cell line, was exceedingly high. But, at this stage it is difficult to determine whether 5'-nucleotidase activity is lower in transformed reticulum cells in comparison to the activity in normal reticulum cells. So far, it has not been possible to establish a culture of a normal (nontransformed) reticulum cell line that corresponds to the nontransformed fibroblastic 3T3 cell line. We are now working on this problem in our laboratory, and hope to determine 5'-nucleotidase activity in normal reticulum cells in the near future.

Acknowledgements. We thank Drs. S. Sawada and S. Okada for their helpful suggestions about the reported research. This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

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(Received for publication, March 16, 1983)