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THE ROLE OF N-6-ISOPENTENYL ADENINE IN TUMOR CELL GROWTH

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SUMMARY: When cell extracts from Ehrlich ascites tumor cells were assayed for isopentenyl adenine content and correlation with cell growth stage by radioimmunoassay, concentrations of low statistical significance were obtained. High performance liquid chromatographic analysis of cell extracts showed undetectable levels of isopentenyl adenine or 8-hydroxy-isopentenyl adenine, a known metabolite. Thus these substances do not seem to be required for cell division in Ehrlich ascites tumor cells.

Studies in several laboratories have provided evidence that cells require, in addition to cholesterol, a non-sterol metabolite of mevalonate metabolism to support growth. Kaneko, et al., using mouse L-cells, showed that treatment with compactin, a potent inhibitor of HMG-CoA reductase, made cell growth dependent on the presence of mevalonate in the culture medium (1). Habenicht, et al., using PDGF-stimulated smooth muscle and Swiss 3T3 cells, demonstrated that compactin arrested the growth of these cultured cells, even in the presence of lipoproteins or cholesterol (2). Quesney-Huneeus, et al., showed that baby hamster kidney cell growth was blocked by compactin at the beginning of S-phase (3).

The identity of the mevalonate metabolite required for cell growth is still uncertain. When the various end products of the

Abbreviations. HPLC, high performance liquid chromatography; i6A, N-6-isopentenyl adenine; i6Ado, N-6-isopentenyl adenosine; 8-OH-i6A, 8-hydroxy-N-6-isopentenyladenine; RPIA, R-(N-phenylisopropyl)adenosine; Mev, mevinolin; MVL, mevalonolactone.

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branching mevalonate pathway were tested in compactin-arrested BHK cell cultures, Quesney-Huneeus, et al. found that only isopentenyl adenine (i6A) and its hydroxylated metabolite zeatin were capable of restoring growth (4). Neither cholesterol, ubiquinone, nor dolichol were effective. However, Perkins, et al. were unable to demonstrate restoration of DNA synthesis in compactin treated, Con A-induced lymphocytes with i6A (5). In addition, Sinensky and Logel have reported that the growth of the CHO cell mutant Mev-1 (which are blocked in the synthesis of mevalonate) is not restored by the addition of i6A (6).

To explore the relationship of i6A to mammalian cell growth, we have employed Ehrlich ascites tumor cells and attempted to measure directly the levels of this substance and to correlate its level with cell growth.

MATERIALS AND METHODS

Cell culturing. Ehrlich ascites tumor cells were obtained by transplantation in the peritoneal cavity of ICR mice. Cells were harvested 2-7 days following inoculation.

Effect of mevinolin on cell growth. Ehrlich ascites tumor cells were incubated **in vitro** with 1mM mevinolin and injected intraperitoneally into ten mice. Mice were boosted daily with 1mM mevinolin. At day four the mice were killed and the tumor cells removed from the peritoneal cavity and counted. The effect of mevalonate and isopentenyl adenine on the growth of mevinolin-inhibited cells was assessed by coinoculating cells daily with 1 mM mevalonate or 0.05 mM isopentenyl adenine.

Preparation of antisera. Antisera to periodate oxidized isopentenyl adenosine conjugated to hemocyanin by the procedure of Eichler and Glitz (7) were raised in rabbits and fractionated by ammonium sulfate precipitation and DEAE-cellulose column chromatography. Specificity of the antiserum was similar to that from previous reports, showing equal cross-reactivity towards i6A and i6Ado, less reactivity towards 8-hydroxy-i6A, and little or no reactivity with adenosine, zeatin, or N-methyl adenosine (8).

Preparation of labeled i6A. [2-^{3}H]-i6A was prepared from [2-^{3}H]-adenosine (Schwartz-Mann) by alkylation with dimethylallyl bromide according to the procedure of Robins and Trip (9) to form [2-^{3}H]-isopentenyl adenosine. Deribosylation was achieved with 88% formic acid at 65°. The product was radiochemically pure as judged by TLC [chloroform-methanol (3:1)]. The product had a uv absorption maximum at 269 nm and an extinction coefficient of 19.4, in agreement with published values (10). The labeled i6A

was diluted with unlabeled i6A to a final specific activity of 865 cpm/pmole.

Isolation of N-6-isopentenyl adenine from tumor cells. Tumor cells were washed in normal saline and counted. Ice cold 10% TCA was then added to the washed, resuspended cell pellet and the mixture centrifuged. TCA solubl material was then neutralized with KOH. When samples were to be assayed by radioimmunoassay ^{3}H-isopentenyl adenine was added as a tracer while unlabeled R-(N-phenylisopropyl)adenosine (535 pmoles, a gift from Dr. R. A. Olsson) was added as an internal standard when HPLC analysis was performed. The mixture was partially purified using a C-18 Sep-Pak (Waters Associates). The i6A-containing fraction, obtained by washing the column with 20% methanol followed by elution with 100% methanol, was evaporated to dryness and then either redissolved in ethanol for radioimmunoassay or in 50% methanol for HPLC analysis.

Radioimmunoassay for N-6-isopentenyl adenine. Quantitation of i6A levels was made by RIA analyses of partially purified tumor cell extracts according to the procedure of MacDonald, et al. (11). All manipulations were carried out in 1.5 ml polypropylene tubes. Standard curves were determined using stock solutions of i6A in methanol which was removed by evaporation under nitrogen prior to the addition of the assay buffer. The assay was linear from 15 to 300 pmoles i6A in a total volume of 0.45 ml.

High performance liquid chromatography. Samples were applied to a 4x250 mm, 5 u Partisil C-18 column (Whatman) in 50% methanol and eluted isocratically with the same solvent (buffered with 3 mM triethylammonium acetate, pH 7) at a flow rate of 1.5 ml/min. The effluent was monitored at 269 nm. The overall yield of the internal standard was 55%.

Preparation of 8-hydroxy-N-6-isopentenyl adenine. i6A was converted to its 8-hydroxy derivative by treatment with xanthine oxidase (generously provided by Dr. Michael Barber) according to the procedure of Chen et al. (12).

RESULTS

Incubation *in vitro* of Ehrlich ascites tumor cells with 1 mM mevinolin led to inhibition of cell growth *in vivo* by 40% (Fig. 1). Higher doses, although more inhibitory, led to cytotoxic effects. Growth was restored to essentially normal rates by coinoculation with 1 mM mevalonate but not with 0.05 mM isopentenyl adenine.

Ehrlich ascites tumor cells at various stages of cell growth were examined for i6A content by radioimmunoassay as shown in Table I. The large scatter in the data points (caused by the fact that the assay was at its extreme limit) precluded any definitive

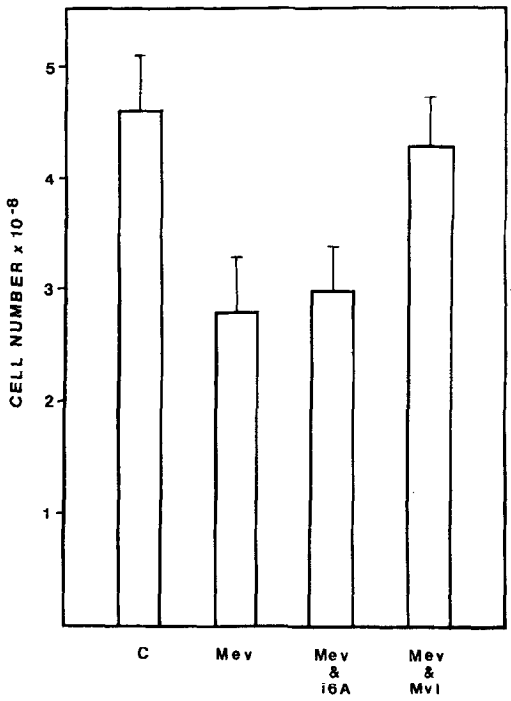

Fig. 1: Effect of mevinolin on tumor cell growth. Tumor cells pretreated with mevinolin (Mev) were inoculated into mice as described in Materials and Methods. Mice were coinoculated with either 1 mM mevalonolactone (Mvl) or 0.05 mM isopentenyl adenine.

conclusions correlating i6A levels and growth stage. The values for cross-reacting material at days 3 and 4, though of questionable significance, prompted us to attempt a more direct determination. High performance liquid chromatographic analysis (with a

TABLE I

Isopentenyl adenine levels in Ehrlich ascites tumor cells during growth

Days post-inoculation	Isopentenyl adenine levels (pmoles/10^6 cells)
2	0.516 ± 0.645 (7)
3	1.080 ± 2.000 (8)
4	1.373 ± 1.921 (5)
5	0.137 ± 0.315 (2)
6	0.229 ± 0.417 (2)
7	0.096 ± 0.121 (5)

Values in parentheses indicate numbers of independent determinations. 3 - 10 x 10^7 cells were used per radioimmunoassay point.

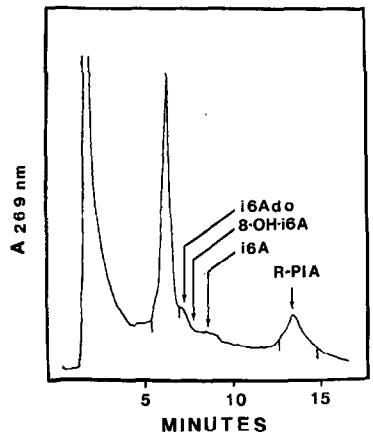

Fig 2: High performance liquid chromatography of extracts from Ehrlich ascites tumor cells. The TCA-soluble extract from 1.4 x 10^9 cells was supplemented with R-(N-phenylisopropyl)adenosine and partially purified prior to chromatography (see Materials and Methods). Full scale absorbance, 0.04 O.D. units.

detection limit of 10 pmoles) gave the results shown in Fig. 2.In the chromatographic profile of an extract from $7x10^7$ cells, there were no peaks comigrating with i6A or 8-hydroxy i6A. The shoulder on the main unidentified peak comigrated with i6Ado. Integration values for this peak, assuming the extinction coefficient of i6Ado, indicate a maximum concentration of about 0.18 pmoles per 10^6 cells (0.004 nmoles/g).

DISCUSSION

A non-sterol metabolite of the branching mevalonate pathway has been implicated in the control of mammalian cell growth. The known non-sterol end products of this pathway in mammalian cells include ubiquinone, dolichyl phosphate, isopentenyl adenine in tRNA, and heme a. Siperstein's group has published evidence implicating i6A in the growth of cultured BHK cells (4), while others have failed to show this effect with other cell types (5,6).

We were unable to detect isopentenyl adenine or its metabolite 8-hydroxy-isopentenyl adenine by either radioimmunoassay or

HPLC. A small peak of a uv absorbing material comigrating with i6Ado was detected, however its identity is not certain. The level of isopentenyl adenosine in yeast has been recently determined to be 2-2.3 nmole/g (13), while the levels of the cytokinin zeatin in growing plants has been reported as 0.025-0.05 nmole/g (11,14). The value we obtain by HPLC (0.004 nmole/g) is a maximum estimate and is substantially less than any reported values in cells where cytokinins are known growth effectors.

Confirmation that i6A is not required for cell growth is seen in the studies with mevinolin-treated cells in vivo. Mevalonate was able to restore cell growth while i6A was ineffective.

Recently Schmidt, et al. reported that cellular proteins in 3T3 cells incorporated labeled mevalonate post-translationally (15). In addition, Maltese and Aprille have shown that mevinolin administration to cultured neuroblastoma cells completely inhibited ubiquinone synthesis (16). The relationship of these findings to cell growth is as yet unknown.

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REFERENCES

1. Kaneko, I., Hazama-Shimada, Y., and Endo, A. (1978) Eur. J. Biochem. 87, 313-321.
2. Habenicht, A.J.R., Glomset, J.A., and Ross,R. (1980) J. Biol. Chem. 255, 5134-5140.
3. Quesney-Huneeus, V., Wiley, M.H., and Siperstein, M.D. (1979) Proc. Nat. Acad. Sci. 76, 5056-5060.
4. Quesney-Huneeus, V., Wiley, M.H., and Siperstein, M.D. (1980) Proc. Nat. Acad. Sci. 77, 5842-5846.
5. Perkins, S.L., Ledin, S.F., and Stubbs, J.D. (1982) Biochim. Biophys. Acta 711, 83-89.
6. Sinensky, M. and Logel, J. (1985) Proc. Nat. Acad. Sci. 82, 3257-3261.
7. Eichler, D.C. and Glitz, D.G. (1974) Biochim. Biophys. Acta 335, 303-317.
8. Milstone, D.S., Vold, B.S., Glitz, D.G., and Shutt, N. (1978) Nucl. Acids Res. 5, 3439-3455.

9. Robins, M.J. and Trip, E.M. (1973) Biochemistry 12, 2179-2187.
10. Robins, M.J., Hall, R.H., and Thedford, R. (1967) Biochemistry 6, 1837-1848.
11. MacDonald, E.M.S., Akiyoshi, D.E., and Morris, R.O. (1981) J. Chromatog. 214, 101-109.
12. Chen, C., Smith, O.C., and McChesney, J.D. (1975) Biochemistry 14, 3088-3093.
13. Laten, H.M. and Zahareas-Doktor, S. (1985) Proc. Nat. Acad. Sci. 82, 1113-1115.
14. Weiler, E.W. (1980) Planta 149, 155-162.
15. Schmidt, R.A., Schneider, C.J., and Glomset, J.A. (1984) J. Biol. Chem. 259, 10175-10180.
16. Maltese, W.A. and Aprille, J.R. (1985) J. Biol. Chem. 260, 11524-11529.