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Growth Kinetic Studies of Methionine Dependence in Co-culture of Monolayer and Anchorage Independent Mouse Cell Lines

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Abstract. *Non-transformed C3H/10T1/2 Cl 8 mouse embryo fibroblasts and malignant R.1.1 mouse T-lymphoma cells were examined for their ability to utilize homocysteine thiolactone (Hcy-tl) instead of methionine (Met) for growth. The non-transformed fibroblasts showed only a slightly slower growth rate in Hcy-tl supplemented medium, while the T-lymphoma cells showed an absolute requirement for Met, defined as methionine dependence. A co-culture system was established where both monolayer growing fibroblasts and lymphoma cells in suspension were grown in the same culture vessel. In Met supplemented medium both cell types proliferated, but when Hcy-tl replaced Met only the fibroblasts were able to grow. Two major conclusions were drawn: 1) The inability of the lymphoma cells to utilize Hcy-tl was not due to formation and release of toxic agent(s) to the growth medium. 2) It was possible to exploit the metabolic defect of methionine dependence to select for the growth of non-transformed cells from malignant cells.*

Homocysteine, the metabolic precursor of methionine (Met), has been shown to support growth of cells in culture with varying efficiency (1, 2, 3). Initial experiments (4, 5) indicated that loss of ability to utilize homocysteine was linked to neoplastic transformation, and malignant cells were defined as "methionine dependent". However, several investigations have revealed a more complex nature of this phenomenon since some normal cells have been shown to require Met for growth, and different malignant cells have been shown to be Met independent (6, 7, 8, 9).

Abbreviations: Hcy-tl = homocysteine thiolactone; Met = methionine.

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The biochemical basis of Met dependence is not well understood. No enzyme defect has been demonstrated in such cells, but there are reports of low concentration of free Met and low ratio between S-adenosylmethionine and S-adenosylhomocysteine, suggesting decreased overall transmethylation rate (1).

In a detailed study of both non-transformed and several malignant lines of C3H/10T1/2 mouse embryo fibroblasts, we showed that the malignant cell lines were capable of utilizing homocysteine thilactone (Hcy-tl) for growth, but at a slower rate than their non-transformed counterpart (9). Homocysteine in its reduced form was highly toxic to these cells, while the disulfide or the thiolactone of homocysteine was non-toxic. Furthermore, one transformed cell line showed altered glutathione content when transferred to medium containing Hcy-tl instead of Met (9). These observations point to the possibility that cellular utilization of Hcy-tl results in formation of toxic agent(s), eliminated through a detoxification mechanism in non-transformed cells. In methionine dependent cells this mechanism may be defective and cause apparent lack of ability to utilize Hcy-tl for growth. If toxic agent(s) are not efficiently handled, they may be released into the culture medium and suppress growth of normal cells. This possibility is evaluated in the present report.

Since Met dependence is largely associated with malignant cells, an interesting idea is to exploit this metabolic defect to promote selectively the growth of normal cells from a mixture of normal and malignant cells.

To explore these possibilities, the present paper describes the establhishment of a co-culture system where anchorage-independent mouse leukemic cells and non-transformed monolayer-growing mouse embryo fibroblasts are grown in the same medium in the same culture vessel. This system enables us to determine the growth rates of both cell types in Hcy-tl supplemented medium, and compare this with data obtained from separately growing cultures. Growth kinetic data of this co-culture system are presented, and the absolute Met requirement of the leukemic cells is exploited in selecting for the non-transformed cells by growing both cell types in Met deficient medium supplemented with Hcy-tl.

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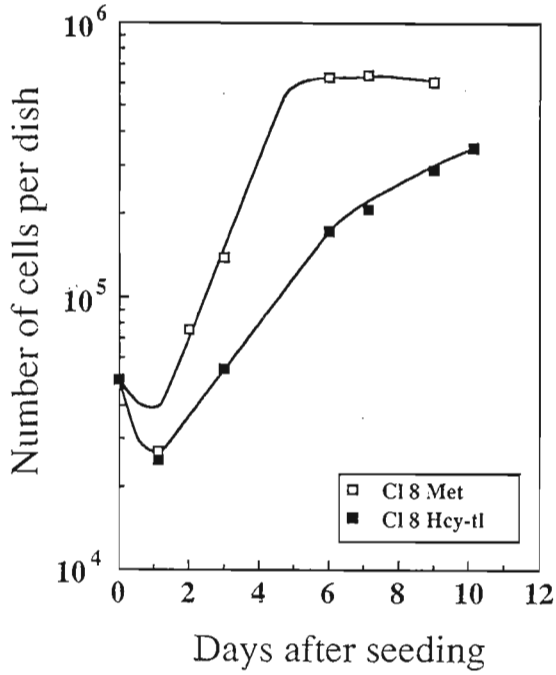

Figure 1. *Growth of C3H/10T1/2 Cl 8 mouse embryo fibroblast cells in Met deficient medium supplemented with either 0.1 mM Met or 0.1 mM Hcy-tl. 50000 cells were seeded per dish (60 mm) at day 0. At the times indicated two dishes from each group were trypsinized and the cells counted. The growth curves are representative examples of four different experiments.*

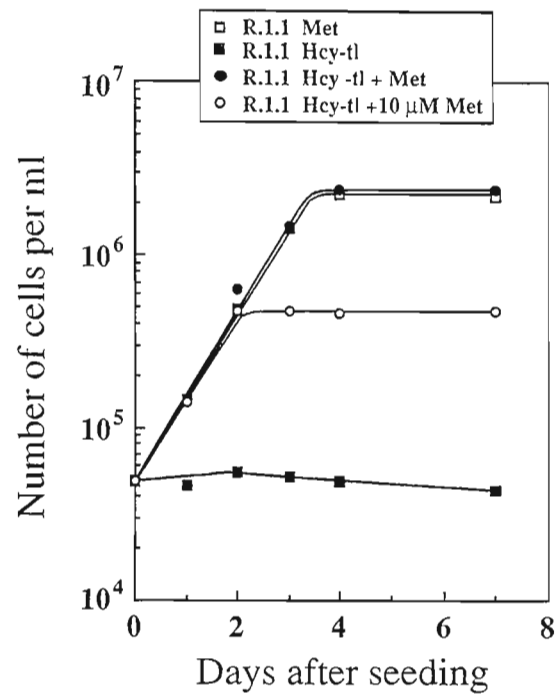

Figure 2. *Growth of R.1.1 mouse T-lymphoma cells in Met deficient medium supplemented with either 0.1 mM Met, 0.1 mM Hcy-tl, 0.1 mM Hcy-tl plus 0.1 mM Met or 0.1 mM Hcy-tl plus 10 µM Met. 50000 cells per ml were seeded in culture flasks (25 cm^2) at day 0. At the times indicated, aliquots of 0.5 ml each from two different vessels were removed from each group, and the cells counted. The growth curves are representative examples of four different experiments.*

Materials and Methods

Chemicals. L-Hcy-tl, L-Met, vitamin B_{12} (cyanocobalamin), folic acid and Met-free RPMI 1640 medium were from Sigma Chemical Co., St. Louis, MO. Complete RPMI 1640 medium was from Flow Laboratories and foetal calf serum was from Sera-Lab Ltd., Sussex, England.

Cell lines and culture conditions. Stock cultures of the non-transformed mouse embryo fibroblasts C3H/10T1/2 Cl 8 cells were grown as described elsewhere (9). The mouse T-lymphoma cell line R.1.1 (10) was obtained from Dr. Dennis A. Carson at Scripps Clinic and Research Foundation, La Jolla, CA. Stock cultures of R.1.1 were maintained in suspension culture in RPMI 1640 medium supplemented with 10% heat inactivated foetal calf serum and routinely diluted in fresh medium twice a week. Cells were grown in tissue culture plastic dishes (6 cm; Nunc, Denmark) at 37°C in an atmosphere of 5% CO_2 in air and a relative humidity of 95%.

Growth experiments with separate cell lines. C3H/10T1/2 Cl 8 and R.1.1 cells were grown in Met-free RPMI 1640 medium supplemented with 1.5 µM vitamin B_{12} (cyanocobalamin), 11.3 µM folic acid, 10% dialyzed, heat inactivated foetal calf serum and either 0.1 mM L-Met or 0.1 mM L-Hcy-tl unless otherwise indicated. Cl 8 cells were seeded at a density of 50000 cells per dish, and two parallel dishes from each group were removed at the times indicated, trypsinized and the cells counted in a Coulter Counter Model ZM (Coulter Electronics Ltd., England).

R.1.1 cells were seeded at a density of 10^5 cells/ml in a total volume of 5 ml/dish. Two parallel dishes from each group were removed at the times indicated and samples of the cell suspensions counted.

Co-culture of non-transformed and malignant cells. C3H/10T1/2 Cl 8 cells were seeded at a density of 50000 cells per dish in Met-free RPMI 1640 medium supplemented as above. Exponentially growing stock cultures of R.1.1 cells were harvested by centrifugation for 7 min at 500 X g, washed and resuspended (5 X 10^6 cells/ml) in Met-free RPMI 1640 medium supplemented as above, but without Met or Hcy-tl. 24 hours after seeding of the fibroblasts, the medium was removed and replaced with fresh medium as above (5 ml/dish) containing either 0.1 mM Met or 0.1 mM Hcy-tl, and 10^5 R.1.1 cells per ml were added from the Met-free cell suspension. At the times indicated two parallel dishes from each group were removed, and samples of the suspension cells were counted. In dishes with Met-containing medium, the lymphoma cells outnumbered the fibroblasts and attached to the fibroblasts, making it impossible to quantitate the latter cells. In dishes containing Hcy-tl, however, it was possible to remove the R.1.1 cells for counting, and thereafter the fibroblasts were trypsinized and counted.

Results

Growth support of Hcy-tl in separately growing cultures. The ability of the non-transformed C3H/10T1/2 Cl 8 cells to utilize Hcy-tl for growth is demonstrated in Figure 1. The cells thrived in Hcy-tl supplemented medium, but grew at a slightly slower rate than in medium supplemented with Met. The malignant R.1.1 cells, however, exhibited an absolute requirement for Met since the cells did not grow at all when Hcy-tl replaced Met in the growth medium (Figure 2). Co-addition of Met and Hcy-tl supported growth to the same

extent as Met alone, demonstrating that the lack of growth in Hcy-tl supplemented medium was not due to a direct toxic effect of Hcy-tl. Furthermore, co-addition of 0.1 mM Hcy-tl and 10 μM Met resulted in optimal growth until mid-log phase where growth ceased abruptly (Figure 2), probably due to depletion of Met.

Co-culture of non-transformed and malignant cells. The growth of Cl 8 and R.1.1 cells in co-culture is shown in Figure 3 and Figure 4. In Met-supplemented medium, the lymphoma cells grew at a rate similar to that observed when cultured alone (Figure 2) with a doubling time of 13.2 hours. In co-culture in Met supplemented medium, the lymphoma cells totally outnumbered the fibroblasts, probably due to the shower growth rate of the latter, which had a doubling time of 20.4 hours when cultured alone. A major portion (50-70%) of the fibroblasts were covered with lymphoma cells (up to 20 per fibroblast cell) within 24 hours after seeding. Shaking of the cultures or mild treatment with trypsin (0.01%, 5 min at 37°C) did not separate the cells. After 2-3 days in co-culture the lymphoma cells seemed closely and strongly associated with the fibroblasts. However, cells attached to the fibroblasts constituted a negligible part of the total number of lymphoma cells. Consequently, determination of the number of lymphoma cells was not affected.

In contrast, the attachment of lymphoma cells to the fibroblasts made it nearly impossible to make accurate determinations of the number of fibroblasts in co-culture in Met supplemented medium. But when Hcy-tl replaced Met in the medium, the lymphoma cells did not grow at all, and the number attached to the fibroblasts was too small to affect cell counting of the latter. In this case, it was therefore possible to determine the number of both cell types with equal accuracy.

Substituting Met with Hcy-tl in the medium resulted in the selective growth of the non-transformed cells, while the lymphoma cells did not proliferate at all (Figure 3). The non-transformed cells had a doubling time of 38.2 hours in the co-culture experiment. This equals the growth rate (doubling time of 40.1 hours) observed when the fibroblasts were cultured alone in Hcy-tl supplemented medium (Figure 1).

These results strongly indicate that the inability of the lymphoma cells to utilize Hcy-tl for growth was not due to production and release of toxic agent(s) in response to Hcy-tl.

Furthermore, these results show that in this cell culture system, it was possible to obtain selective growth of non-transformed cells in co-culture of non-transformed and malignant mouse cells.

The morphological appearance of the non-transformed C3H/10T1/2 Cl 8 cells and the R.1.1 T-lymphoma cells in co-culture is demonstrated in Figure 4. Without attachment of any lymphoma cells, the fibroblasts appeared normal in morphology, both in Met and in Hcy-tl supplemented medium. Covered with lymphoma cells, however, the fibroblasts had a round, swollen and rugged morphology. In Met supplemented medium, the lymphoma cells thrived with a typical round and smooth shape (Figure 4 A). In contrast, when Hcy-tl replaced Met in the medium, the lymphoma cells appeared shrunk and wrinkled as seen in Figure 4 B.

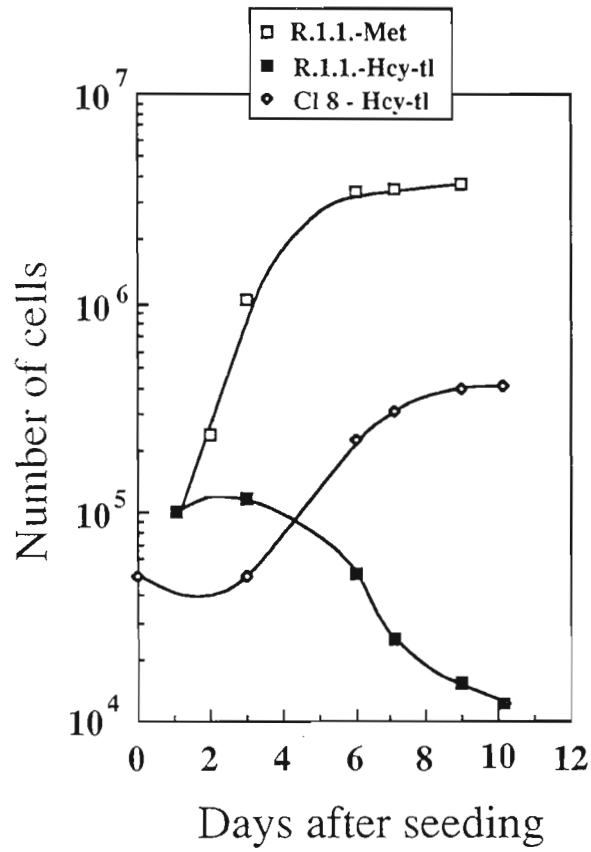

Figure 3. *Growth of C3H/10T1/2 Cl 8 mouse embryo fibroblast cells and R.1.1 mouse T-lymphoma cells in co-culture in Met deficient medium supplemented with either 0.1 mM Met or 0.1 mM Hcy-tl. 50000 Cl 8 cells were seeded per dish (60 mm) at day 0, and 10^5 R.1.1 cells per ml were added to each dish at day 1. At the times indicated, aliquots of 0.5 ml each were removed from two dishes in each group, and the R.1.1 cells counted. In dishes with Hcy-tl in the medium the R.1.1 cells were removed, and the remaining Cl 8 cells were washed, trypsinized and counted. The growth curves are representative examples of four different experiments.*

Discussion

The idea of exploiting therapeutically the metabolic defect of methionine dependence has been put forward previously (1, 11, 12). Recently, Stern and Hoffman (13) described the selective growth of normal human fibroblasts in co-culture with methionine dependent malignant cells in methionine deficient medium supplemented with homocysteine. Under these conditions they obtained selective killing of the cancer cells by use of cytostatic drugs.

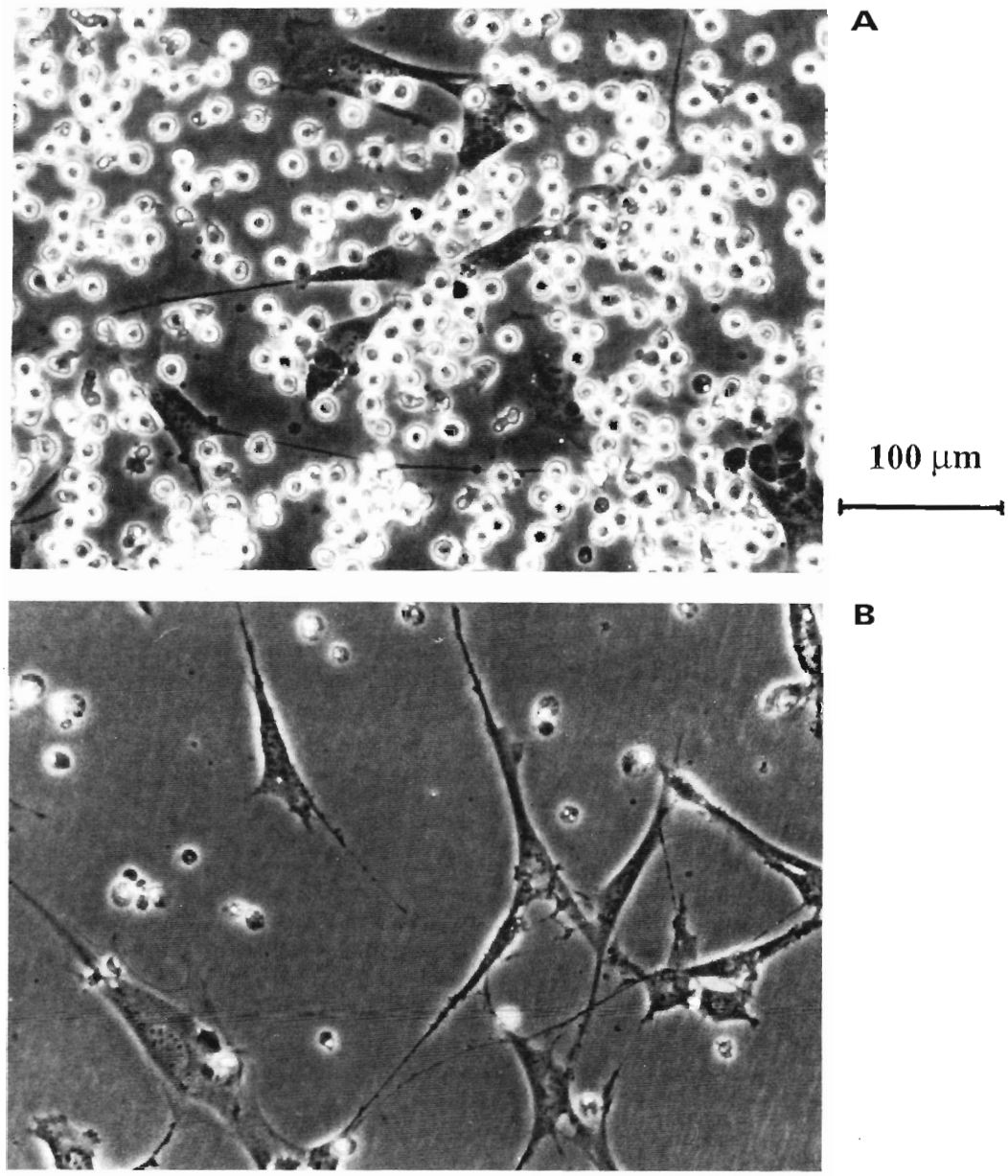

Figure 4. *C3H/10T1/2 Cl 8 mouse embryo fibroblast cells and R.1.1 mouse T-lymphoma cells in co-culture in Met deficient medium supplemented with either 0.1 mM Met or 0.1 mM Hcy-tl. Cells were grown at conditions identical to those described in the legend to Figure 3. Photomicrographs were taken at day 3 after seeding of Cl 8 cells. Bar = 100 μm.*

The present paper describes a co-culture system consisting of anchorage dependent non-transformed mouse fibroblasts and mouse leukemic cells growing in suspension. Grown separately, these two cell lines behaved according to the hypothesis of methionine dependence (1), since the non-transformed cells thrived in Hcy-tl supplemented medium, while the leukemic cells demonstrated complete inability to utilize Hcy-tl for growth (Figures 1,2).

The results obtained with these two cell lines in co-culture experiments confirmed the results of Stern and Hoffman (13) regarding the selective growth of non-transformed cells. In addition, this co-culture system allowed us to determine cell growth kinetics, demonstrating that the growth rate of the normal cells was not reduced by the presence of the leukemic cells in methionine deficient medium supplemented with Hcy-tl (Figure 3). In contrast, the latter cells died after some days in culture under these conditions (Figures 3, 4).

Obviously, comparing growth kinetics (Figures 1,3) may

reveal the presence of factors in the medium affecting growth, which would not be recognized by qualitative (13) or end-point analysis (12).

Some observations have pointed to the possibility of production of toxic agent(s) during cellular utilization of homocysteine. In its reduced form, homocysteine was toxic to both non-transformed and malignant fibroblasts (9), and toxicity against endothelial cells seemed to be mediated by production of hydrogen peroxide (14). The malignant fibroblast cell line Cl T422 (15) had markedly reduced growth rate when Hcy-tl replaced Met in the medium and showed altered content of the detoxifying agent glutathione under these conditions (9).

The demonstration of absolutely no reduction in growth rate of fibroblasts in co-culture with leukemic cells in Hcy-tl supplemented medium (Figures 1,3), is not consistent with production and release of toxic agent(s) into the culture medium. Therefore, the methionine dependence of the leukemic cells did not seem to be mediated by formation and release of toxic agent(s) during cellular utilization of Hcy-tl.

In conclusion, the methionine dependence of R.1.1 mouse T-lymphoma cells is probably related to a defect in the metabolism of homocysteine, and the inability to utilize Hcy-tl for growth was not caused by production and release of toxic agent(s). It was possible to exploit this metabolic defect to promote the selective growth of non-transformed fibroblasts in co-culture with totally Met dependent leukemic cells.

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