The anti-proliferative effect of lithium chloride on melanoma cells and its reversion by myo-inositol

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Summary The effect of LiCl on melanoma cell growth and differentiation was studied in mouse and human melanoma cell lines. LiCl markedly inhibited B16 and HT-144 melanoma cell growth in vitro. Clonogenicity in soft agar of the melanoma cells was also markedly inhibited by LiCl. Pretreatment of B16 mouse melanoma cells with LiCl delayed the appearance of melanoma tumors in syngeneic mice. Growth inhibition of cells was accompanied by morphological and biochemical alterations. LiCl induced cell enlargement and formation of dendrite-like structures. The activity of NADPH cytochrome c reductase, an enzymatic marker of endoplasmic reticulum was significantly (2-3 fold) increased. Addition of myo-inositol to cell cultures partially reversed the anti-proliferative and morphological effects of LiCl on melanoma cells. This finding may suggest that the anti-proliferative effect of LiCl is related to its effect on phosphatidylinositol metabolism.

Monovalent cations play a role in regulation of cell growth and differentiation. Lithium has been shown to stimulate growth of several normal and cancer cells. 3T3 mouse fibroblasts, mammary epithelial cells and mammary tumour cells were stimulated to proliferate by lithium chloride (LiCl) (Plushne et al., 1980; Horii & Oka, 1979; Rybak & Stockdale, 1981). This agent also potentiated the mitogenic response of B and T cells (Hart 1981, 1982; Bray et al., 1981).

LiCl was found to modulate haematopoiesis by influencing pluripotential and committed stem cell proliferation and differentiation toward granulocytes (Gallicchio & Chen, 1981; Morley & Galbraith, 1978; Levitt & Quensenberg, 1980). Axolotl-embryo’s ectodermal cells were induced to differentiate into mesenchyme, nerve cells and melanophores by LiCl (Lovtrop & Perris, 1983). LiCl has also been shown to potentiate the anti-melanoma effect of bleomycin in mice bearing melanoma tumours (Ballin et al., 1983).

The biochemical basis of lithium’s actions on cell growth and differentiation is unknown. However, in different tissues lithium has been shown to affect cyclic nucleotide levels (Dovsa & Mecher, 1970; Wang et al., 1974) glucose metabolism (Nordenberg et al., 1982; Dempsey et al., 1976) and phosphatidylinositol metabolism (Allison & Stewart, 1971; Allison et al., 1976; Berridge & Irvine, 1984).

In the present study the direct effect of LiCl on melanoma cell growth and differentiation is evaluated. We demonstrate that LiCl inhibits melanoma cell growth and clonogenicity and delays the appearance of tumours in syngeneic mice. The anti-proliferative effects of LiCl are accompanied by morphological and biochemical phenotypic alterations. Addition of myo-inositol to cell cultures partially reverses the effects of LiCl on melanoma cells. This finding may suggest that the anti-proliferative effect on LiCl is related to its effect on phosphatidylinositol metabolism.

Materials and methods

Cells, media and reagents

A melanotic clone of mouse B16 melanoma F10, kindly donated by Dr. Abraham Raz, the Weizmann Institute, was used in most experiments. In selected experiments a human amelanotic melanoma cell line HT-144 from the American Type Culture Collection, Maryland was used. The cells were cultured in RPMI 1640, supplemented with 10% foetal calf serum and antibiotics, at 37 °C in a humidified atmosphere of 5% CO2 and 95% air. B16 F10 cells were passaged three times weekly and HT-144 cells 1–2 times weekly. Media and supplements were obtained from Biol. Industries, Israel and lithium chloride from BDH chemicals, Ltd., Poole, UK.

Cell growth experiments

In most cases 103 cells were plated in 1.5 ml culture medium, in 3.5 cm culture dishes. When cells were cultured for more than 3 days, 3 × 104 cells were plated in 10 ml culture medium in 8.5 cm culture dishes. This was done to avoid cell crowding. Two to four hours later, after most cells were attached to the bottom of the petri dish, LiCl or myo-inositol were added as indicated in legend to Figure 1 and Table IV. The cultures were refed every 48 h with growth medium supplemented with LiCl. Cell growth was determined by counting the cell number after 1, 2 and 4 days in culture. The human line had a relatively lower proliferation rate, and therefore the effect of LiCl on cell number was determined after 3 to 5 days of treatment. The cells were detached with EDTA (1 mM) and counted in a Coulter counter. Viability of the cells was assessed by the trypan blue dye exclusion test.

Clonogenic assay

The effect of LiCl, in the presence and absence of myo-inositol, on the clonogenic potential of F-10 melanoma stem cells was investigated by modification (Ellason et al., 1984) of the soft agar method of Hamburger and Salmon (1977). Briefly, 2 × 103 single and viable cells in 1 ml RPMI medium containing 10% FCS and 0.3% agar were plated as a single layer in 30 mm bacterial dishes (Sterilin). The various concentrations of LiCl and myo-inositol were dispersed in the agar layer. The plates were incubated at 37 °C in 5% CO2, humidified atmosphere and the colonies were scored after 12 days.

Assessment of tumorigenicity

C57/B16J, 6–8 week old mice, bred and supplied by the laboratory animal unit, Beilinson Medical Center, Petah-Tikva, Israel, were used. To test the tumorigenic potential of untreated and LiCl treated melanoma cells, mice were inoculated s.c. on the dorsum with 5 × 104 viable treated or

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untreated cells, suspended in 50 µl PBS. Tumour growth was quantified and expressed as tumour volume as previously described (Nordenberg et al., 1985; 1986). Unpaired t-test was used for statistical analysis.

Cell morphology

Cells were plated at 5 × 10⁴ cells ml⁻¹ (5 ml) in petri dishes (6 cm diameter) in growth medium. Two to four hours later, after cells have been attached, LiCl at 10 mM was added for 48–72 h. Cell morphology was visualized by light microscopy following fixation and staining in situ as previously described (Nordenberg et al., 1986; Wasserman & Icekson-Kessler, 1984). For assessment of morphology by scanning electron microscopy (SEM) cultures were treated as previously described (Nordenberg et al., 1986). Cells were examined by a Jeol JSM 35 SEM (Cohen, 1974).

Determination of NADPH cytochrome c reductase

For determination of NADPH cytochrome c reductase 5-7 × 10⁴ cells were incubated in 10 ml culture medium. LiCl and myo-inositol were added as described in legend to Table II for 72 h. For extraction of NADPH cytochrome c reductase, ~3 × 10⁶ cells were washed with PBS, scraped with a rubber policeman and placed in glass tubes. Extracts were prepared by repeated (3 times) freezing and thawing of 1.5 × 10⁶ cells in 0.1 ml Tris-HCl buffer (100 mM pH = 7.4) containing MnCl₂ (1 mM) and CaCl₂ (1 mM).

Enzyme activity was determined spectrophotometrically at 30°C as described by Phillips and Landgon (1962) using 2,6-dichlorophenol-indophenol as a substrate. Enzyme activity was expressed as OD per cell number or per DNA content. DNA was measured in the cell lysates by the method of Burton (1956).

Results

Inhibition of cell growth by LiCl

The effect of LiCl on cell growth in vitro was examined by culturing melanoma cells in the absence and presence of LiCl for 1-5 days. LiCl at 5-20 mM induced a dose-dependent inhibition in cell growth (Figure 1A). Forty-eight hours of LiCl (5 mM) treatment resulted in a decrease in B16 F10 melanoma cell proliferation of about 50% (Figure 1A). Growth of a human amelanotic melanoma cell line (HT-144) was also inhibited by LiCl (Figure 1B). The results depicted in Table I show that LiCl inhibited the ability of the B16 melanoma cells to form colonies in soft agar. The effect of LiCl was dose dependent. LiCl at 5 mM decreased clonogenicity by 58%.

Table 1 Inhibition of clonogenicity by LiCl and its partial reversal by myo-inositol

| Addition                  | No. of colonies |
|---------------------------|-----------------|
| None                      | 132 ± 35        |
| LiCl (2.5 mM)             | 89 ± 34         |
| LiCl (5 mM)               | 55 ± 4          |
| LiCl (10 mM)              | 17 ± 4          |
| LiCl (20 mM)              | 1 ± 2           |
| Myo-inositol (1 mM)       | 68 ± 4          |
| LiCl (2.5 mM) + myo-inositol (1 mM) | 108 ± 39        |
| LiCl (5 mM) + myo-inositol (1 mM) | 91 ± 20         |
| LiCl (10 mM) + myo-inositol (1 mM) | 60 ± 18         |
| LiCl (20 mM) + myo-inositol (1 mM) | 3 ± 2           |

B16 mouse melanoma cells were plated on semi solid agar for 12 days. Values are means ± s.d. for 3-4 experiments.

Figure 1 The effect of LiCl on melanoma cell growth. B16 mouse melanoma cells (A) or HT-144 human melanoma cells (B) were incubated with LiCl at various concentrations for different time intervals (C1); initial cell density (C2); cell density of incubated cultures. Vertical lines express s.d. of 3-4 experiments.

Effect of LiCl on tumorigenicity of melanoma cells in syngeneic mice

Mice injected s.c. on their dorsum with 5 × 10⁴ viable, untreated melanoma cells, developed spherical tumours on their back within 10-12 days after inoculation. The tumours killed the animals 19-30 days after inoculation. Preincubation of the melanoma cells for 2-6 days with LiCl (5-10 mM) prior to inoculation of 5 × 10⁴ viable cells, resulted in a delay of tumour appearance (Figures 2, 3). The tumours formed by LiCl-treated cells were significantly smaller than those formed by untreated cells. Longer follow-up could not be conducted, since most control mice die between days 19-30 after inoculation of melanoma cells. Mice inoculated with LiCl-treated cells survived for ~10 days longer than control mice.

Effects of LiCl on cell morphology and NADPH cytochrome c reductase

Treatment of melanoma cells with LiCl altered morphology of the cells (Figure 4). The LiCl treated cells possess very
**Figure 2** Effect of LiCl on tumorigenicity of B16 melanoma cells. Mice were inoculated with $5 \times 10^4$ viable untreated cells ([ ]) or cells that have been pretreated with LiCl (10 mM) for 5 days ([ ]). Tumour appearance and size were followed as described in Materials and methods. The results represent 1 out of 4 independent experiments. Values are means of tumours from 8 mice in each group. Vertical lines represent s.e.

**Figure 3** The effect of myo-inositol on tumorigenicity of untreated and LiCl-pretreated B16 melanoma cells. Mice were inoculated with $5 \times 10^4$ viable untreated ([ ]) or with $5 \times 10^4$ cells that had been pretreated for 3 days with the following: LiCl (5 mM), ( ); Myo-inositol (1 mM), ( ); or LiCl (5 mM) + myo-inositol (1 mM), ( ). Values are means of tumours from 7 mice in each group. Vertical lines represent s.e.

**Figure 4** The effect of LiCl on the morphology of B16 melanoma cells. Cells were treated with LiCl (10 mM) for 2-3 days and prepared for light and scanning electron-microscopy as described in Materials and methods. Light micrographs of untreated (A) and LiCl-treated cells (B) (H&E, ×400). Scanning electron micrographs of untreated (C) and LiCl-treated cells (D) (×500).
long dendrite-like appendages. The cells also seem to be more flattened as compared to the untreated cells. Viability of these cells was not reduced compared to untreated cells. Removal of LiCl from the cultures and addition of fresh medium resulted in reversal of the morphological alterations within 5–7 days. Similar morphological changes were induced by differentiating agents, such as butyric acid, dimethylsulphoxide and retinoids in different cancer cells (Simmons et al., 1975; Huberman et al., 1979; Reese et al., 1985; Nordenberg et al., 1986; Mache et al., 1978). These changes are thought to reflect a more differentiated state.

Melanocytic differentiation is associated with an increase in the endoplasmic reticulum and development of Golgi complexes (Jimbo & Vesugi, 1982; Beiter & Wennersten, 1983). We measured the effect of LiCl on the activity of NADPH cytochrome c reductase, an enzymatic marker of the endoplasmic reticulum.

Treatment of B16 melanoma cells with LiCl resulted in a significant concentration dependent increase in the activity of NADPH cytochrome c reductase (Tables II, and IV).

Table II The effect of LiCl on NADPH cytochrome c reductase activity in B16 melanoma cells

| Additions      | NADPH cytochrome c reductase |
|----------------|-------------------------------|
|                | OD 10^{-4} cells min^{-1}    | OD mg^{-1} DNA min^{-1} |
| None           | 5.46 ± 0.51                  | 2.07 ± 0.12              |
| LiCl (10 mM)   | 7.42 ± 0.69b                 | 5.70 ± 0.74              |

*P<0.05; *P<0.02. Cells were treated with LiCl for 3 days. Extractions and determination were described in Materials and methods. Values are means ± s.e. for 4–8 independent experiments.

Table III The effect of myo-inositol on the induction of NADPH cytochrome c reductase activity by LiCl

| Addition          | Enzyme activity OD mg DNA h^{-1} |
|-------------------|---------------------------------|
| None              | 3.7 ± 0.4                       |
| Myo-inositol (5 mM)| 5.0 ± 0.7                       |
| LiCl (5 mM)       | 5.5 ± 0.6*                      |
| LiCl (1 mM) + myo-inositol (5 mM)| 4.7 ± 0.5  |

*LiCl vs. none, P<0.02. Enzyme was extracted and measured as described in Materials and methods. Number of experiments are given in parentheses.

Reversal of the effects of LiCl by myo-inositol

It has been shown that LiCl affects phosphatidylinositol metabolism in brain (Allison & Stewart, 1974; Allison et al., 1976). The enzyme myo-inositol phosphate phosphatase was shown to be inhibited by LiCl in vitro (Hallcher & Sherman, 1980; Naccarato et al., 1974). We considered the possibility that the action of LiCl on melanoma cells might be related to an effect on phosphatidylinositol metabolism. Addition of myo-inositol together with LiCl, or prior to LiCl to the culture medium partially reversed the inhibitory effect of LiCl on cell growth (Table IV) and restored the original cell morphology (Figure 5). The degree of reversion could not be altered by increasing myo-inositol concentrations. Myo-inositol also reversed the effects of LiCl on clonogenicity (Table I) and tumorigenicity (Figure 3). Interestingly, myo-inositol by itself elicited an inhibitory effect on melanoma cell clonogenicity and tumorigenicity. While studying the possible reversion of LiCl-induced activation of NADPH cytochrome c reductase by myo-inositol we observed that myo-inositol by itself induced this enzyme in melanoma cells. When LiCl and myo-inositol were added together, there was no additive or synergistic effect (Table III).

Discussion

Leukaemic cell lines can be induced to undergo terminal differentiation by a variety of chemical agents (Friend et al., 1971; Scher & Friend, 1973, Leder & Leder, 1975). In contrast, cancer cell lines derived from solid tumours have not been shown to undergo terminal differentiation by the chemical inducers of differentiation. However, these cancer cell lines can be induced to develop a variety of phenotypic features, some of which characterize the mature cell counterpart. Several studies, including our recent findings, have shown that chemical inducers of cell differentiation retard melanoma cell growth and tumorigenesis. Growth inhibition was accompanied by differential effects on melanin biosynthesis (Huberman et al., 1979; Nordenberg et al., 1985; 1986). Normal melanocytic maturation is characterized by melanosome formation and development of rough endoplasmic reticulum and Golgi complexes (Beiter & Wennersten, 1983; Jimbo & Vesugi, 1982).

LiCl is shown to exert anti-proliferative effects on melanoma cell lines. It inhibits melanoma cell growth in vitro, as assessed by cell counts and clonogenic assays. LiCl also delays the growth of inoculated melanoma tumours in syngeneic mice. Growth inhibition was accompanied by morphological alterations and by a significant increase in the activity of NADPH cytochrome c reductase, an enzymatic marker of endoplasmic reticulum. Melanin content, however, was only slightly elevated (20–70% in different experiments). Although LiCl does not cause complete differentiation of the melanoma cells, it does induce the appearance of phenotypic features that are associated with the mature melanocyte.

The anti-melanoma effects of LiCl are shown to be partially reversed by the addition of myo-inositol to cell cultures (Tables I and IV and Figures 3 and 5). The reversion of the effects of LiCl by addition of myo-inositol suggests that LiCl affects melanoma cells by interfering with phosphatidylinositol metabolism, most probably by its inhibitory effect on myo-inositol-1-phosphatase (Hallcher & Sherman, 1980; Naccarato et al., 1974). Interestingly, myo-inositol, per se, exerted some anti proliferative effects on melanoma cells. This might offer an explanation why reversal by myo-inositol is only partial. Other possibilities for a partial reversion are that the carrier systems for inositol are saturated at 1 mM, but cannot provide sufficient substrate for the cell, or may LiCl is acting at another site as well.

Table IV Reversion of the inhibitory effects of LiCl on cell growth by myo-inositol

| Additions                      | Cell number x 10^3 |
|--------------------------------|-------------------|
| None                           | 6.5 ± 0.9         |
| Myo-inositol (1 mM)             | 5.8 ± 0.6         |
| Myo-inositol (5 mM)             | 5.6 ± 0.2         |
| LiCl (5 mM)                    | 2.6 ± 0.3         |
| LiCl (5 mM) + myo-inositol (1 mM)| 4.2 ± 0.4         |
| LiCl (5 mM) + myo-inositol (5 mM)| 4.5 ± 0.5         |

*10^6 B16 F10 melanoma cells were incubated for 48 h in culture medium with and without LiCl, myo-inositol or a combination of both. Cells were counted as described in Materials and methods. Values are means ± s.e. for 5 independent experiments. LiCl vs. none P<0.01 and LiCl + myo-inositol vs. LiCl<0.02.
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Figure 5 The effect of myo-inositol on LiCl-induced morphological alterations: (A) untreated cells; (B) cells treated with LiCl (5 mm) for 48 h; (C) cells treated with LiCl (5 mm) and myo-inositol (1 mm) for 48 h; (D) cells treated with myo-inositol (1 mm) for 48 h (Papanicolaou staining, ×400).
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