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Morphology and proliferation of B16 melanoma cells in the presence of lanthanoid and Al^{3+} ions

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The effects of trivalent metal ions such as lanthanoid (La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Er^{3+}, Yb^{3+}, Lu^{3+}) and Al^{3+} ions on the morphological change and proliferation of B16 melanoma cells in culture are discussed. These metal ions induced morphological transformations and decreased growth rates at doses of 1 mM. B16 melanoma cells treated with La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, and Gd^{3+} showed polyhedrical spreading. Elongation of axones was dependent on the metal ions. B16 melanoma cells treated with Er^{3+}, Yb^{3+}, Lu^{3+}, and Al^{3+} showed a long slender shape. Growth rates of melanoma cells in the presence of 1 mM of metal ions (La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Yb^{3+}, Al^{3+}) were significantly lower than that of control cells. Measurements of cell cycle indicated that the metal ions arrested the transitions from G_0/G_1 to S state.

Keywords: aluminium ion, B16 melanoma cell, lanthanoid, morphology, proliferation

Introduction

Lanthanoid ions (Ln^{3+}) are trace metal ions, and their ionic radii are near to that of calcium ion. It has been known that Ln^{3+} binds to biomembranes, proteins, nucleic acids, carbohydrates and cells (see review article, Evans 1990). The bioactivity of Ln^{3+} has been investigated for muscle cells (Weiss 1970, Weiss 1973, Triggle & Triggle 1976) and nerve tissue (Takata *et al*. 1966). The influences of Ln^{3+} on the cell proliferations have been investigated for several cell lines. For example, Ln^{3+} stimulated DNA synthesis of cultured fibroblast (Smith & Smith 1984). However, Ln^{3+} inhibited the lymphocyte proliferation (Yamage & Evans 1989). The therapeutic use of lanthanides as an anticancer agent has been proposed. Daily i.p. injection of 2.5 mg $LaCl_3$ retarded the growth of sarcoma in rats (Anghileri 1979). Administration of Sm-EDTMP (ethylenediaminetetra(methylenephosphonic) acid) to dogs bearing bone carcinoma has been found to result in regression of tumour growth (Ketring 1987). Gd-diethylenetriaminepentaacetic acid complex is accumulated in tumours, and clinically used as a NMR imaging agent (Bydder *et al*. 1985, Claussen *et al*. 1984, Heywang *et al*. 1986). Furthermore, it has been reported that Ln^{3+} potentially accumulated in several tissues of healthy humans and patients (Sabioni *et al*. 1982, Eramesta and Sihvonen 1971). Nevertheless, influence of Ln^{3+} on the cell function has not been sufficiently investigated. Investigation of the influences of lanthanides on cell function is considered to be very important to understand their role in the body and the correlation with disease.

In this study, we investigated the morphological changes and proliferative effects induced on cultured animal cells by different trivalent cations such as lanthanoid (La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Er^{3+}, Yb^{3+}, Lu^{3+}) and Al^{3+} ions. The main finding of this report is that these trivalent ions induced large morphological changes on B16 melanoma cells, but not on Hela cells, Neuro2-M and HepG2. Furthermore, growth rates of all the cells employed were suppressed in the presence of these trivalent ions.

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Materials and methods

Materials

$LaCl_3$, $CeCl_3$, $NdCl_3$, $SmCl_3$, $GdCl_3$, $ErCl_3$, $YbCl_3$, and $LuCl_3$ were obtained from Soekawa Rikagaku (Tokyo, Japan). $AlCl_3$ was obtained from Kanto Chemicals (Tokyo, Japan).

Cell culture

Mouse B16 melanoma cells, Hela cells, and neuroblastoma Neuro2-M were supplied by Japanese Cancer Research Resources Bank. Human hepatome HepG2 was obtained from Riken Cell Bank. The tumour cells were grown as monolayer in polystyrene tissue culture flasks at 37 °C under 5% CO_2 – 95% air in a water-jacketed incubator. Culture medium was Eagle's MEM (Nissui Pharmaceutical Co., Ltd., Japan) supplemented with L-glutamine (0.292 g L^{-1}, Nissui Pharmaceutical Co., Ltd., Japan) and 10% fetal bovine serum (FBS, Bocknek, Canada). Cells were subcultured by the treatment with 0.05% trypsin (Difco) and 0.02% ethylenediaminetetraacetic acid (EDTA, Kanto Chemicals, Japan) when they became confluent. Viability was determined by trypan blue dye exclusion test, and was more than 98%.

Morphology

Cells were seeded into a 96 well multiplate at a density of 5×10^2 cells/well. Cells were incubated in 5% CO_2–95% air at 37 °C overnight. Thereafter, cells were washed in serum culture medium, and incubated in fresh medium supplemented with a metal ion (1 mM) for 5 days. The shapes of cells were examined with a phase-contrast microscope (Nikon Diaphoto, Nikon Co., Ltd. Japan).

Cell growth assay

Cell growth was evaluated by counting cell numbers. Cells were seeded into a 24 well multiplate at a density of 1×10^5 cells/well. Cells were incubated in 5% CO_2–95% air at 37 °C overnight. Thereafter, cells were washed in serum culture medium, and incubated in fresh medium supplemented with different metal ions (1 mM) for 3, 5, and 7 days. The medium was replaced at 2 day intervals. The cells were collected by treating with phosphate buffer salt solution (PBS, pH 7.4) containing 0.05% trypsin and 0.02% EDTA. Cell number was counted by trypan blue exclusion method.

Cell growth was also evaluated by MTT assay. Cells were seeded into a 96 well multiplate at a density of 5×10^2 cells/well. Cells were incubated in 5% CO_2–95% air at 37 °C overnight. Thereafter, cells were washed in serum medium and incubated in fresh medium supplemented with a metal ion (1 mM) for 3, 5, and 7 days. The medium was replaced at 2 day intervals. After the medium containing the metal ion was removed, fresh serum medium (100 μl/well) and PBS (10 μl/well) containing MTT reagent (2 mg ml^{-1}) was added. Cells were incubated in 5% CO_2–95% air at 37 °C for 4 h. The supernatant was removed, and 100 μl dimethyl sulfoxide was added to each well. Absorbance at 550 nm due to formazane was measured by an ELISA reader.

Cell cycle was measured by the propidium iodide (PI) method. Cells suspended in 0.5 ml of serum medium were seeded into petri-dishes (6 cm dia.) at a density of 2×10^5 cells/well. Thereafter, serum medium (1.5 ml) supplemented with 1 mM of Gd^{3+}, Yb^{3+}, or Al^{3+} was added to the petri dishes. The cells were incubated in 5% CO_2–95% air at 37 °C for 24 and 48 h. The cells were treated with PBS containing trypsin–EDTA, and washed in PBS by centrifugation (700 r.p.m., 5 min) twice. The cells were fixed with cold 70% ethanol for 30 min, and washed in PBS by centrifugation (1000 r.p.m., 5 min) twice. The cell pellets were treated with RNaseA (2 mg) in 1 ml of PBS for 45 min, and the supernatants were removed by centrifugation (1000 r.p.m., 5 min). The cell pellets were treated with RNaseA (2 mg) in 1 ml of PBS for 45 min, and the supernatants were removed by centrifugation (1000 r.p.m., 5 min). The cell pellets were resuspended in 1 ml of PBS containing PI (0.05 mg ml^{-1}). The stained cells were analysed by flow cytofluorometer (Beckton-Dickinson).

Results and discussion

Morphological transformation

Several animal cells such as B16 melanoma, Hela, Neuro2-M, and HepG2 were cultured in the presence of 1 mM metal ion. Though no morphological changes of Hela cells, Neuro2-M, and HepG2 were observed (data not shown), B16 melanoma cells showed drastic morphological changes. Figure 1 shows morphologies of B16 melanoma cells cultured in serum medium containing 1 mM metal ions for 5 days. Morphologies of B16 melanoma cells were dependent on the variety of metal ions. The cells cultured in the presence of 1 mM Mg^{2+} and Fe^{3+} showed no morphological changes.

Shapes of control cells were mostly round. B16 melanoma cells treated with La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, and Gd^{3+} showed polyhedrical spreading. The outlines of nuclei in the cells were clearly observed. Elongation of axones was dependent on the kind of metal ions. The larger atomic number metal ions showed more significant elongation of axones. Especially, Gd^{3+}-treated cells showed apparent dendrites. The axons of the Gd^{3+}-treated cells appeared to be joined to neighbour cells; areas per cell of those cells were two or three times larger than that of the control cell.

B16 melanoma cells treated with Er^{3+}, Yb^{3+}, Lu^{3+}, and Al^{3+} showed long and slender shapes. Areas of those cells were slightly larger than that of control cells, and ellipticities (ratios of long axis and short axis; 4–10) of those cells were 2–5 times larger than that (about 2) of control cell.

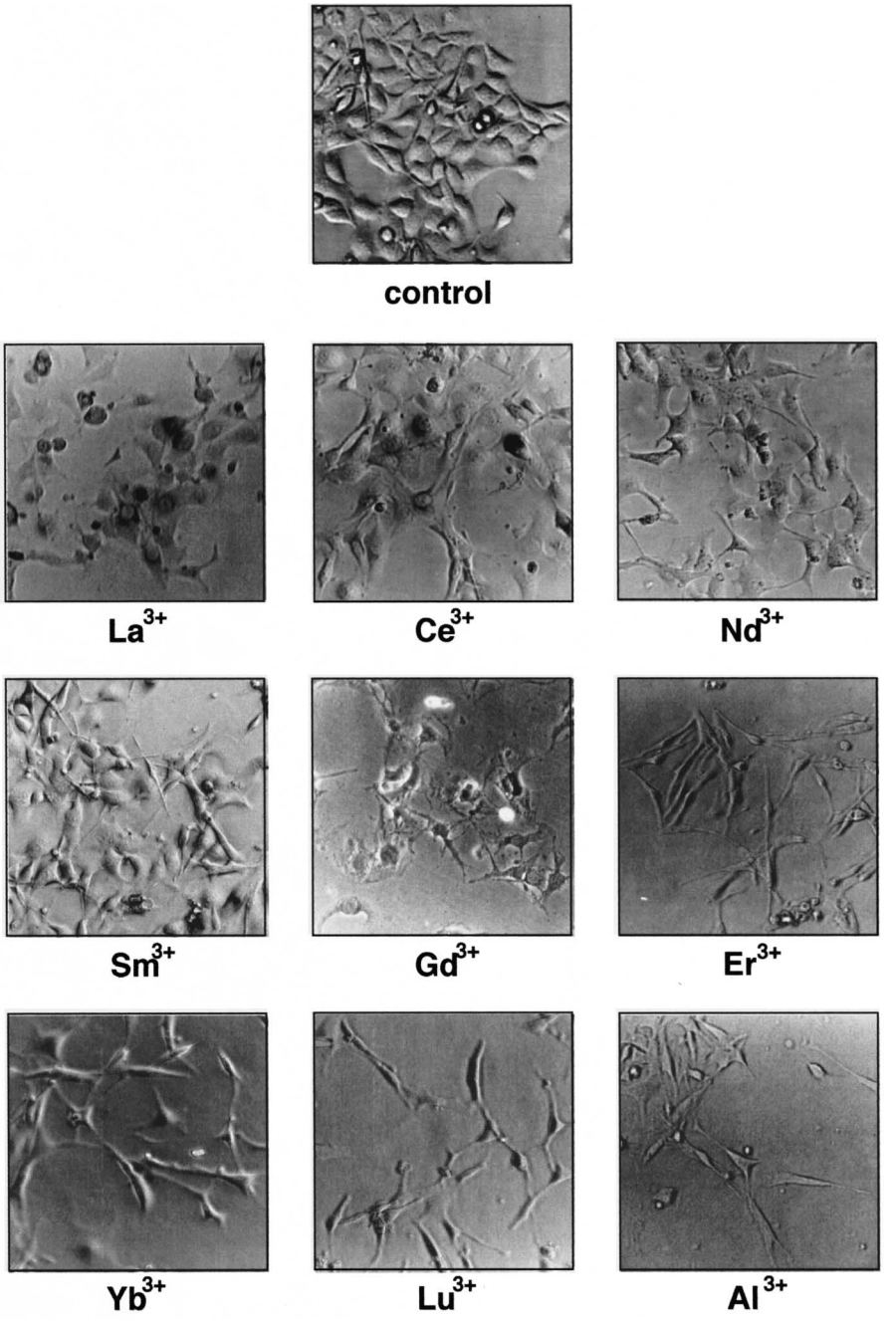

Figure 1. Morphologies of B16 melanoma cells cultured in serum medium supplemented with La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Er^{3+}, Lu^{3+}, and Al^{3+} ions for 5 days. Concentrations of the metal ion were 1 mM.

Experiments were carried out at 0.01, 0.1, 0.5, and 1 mM of metal ions. The morphologies of the cells cultured in the presence of 0.01 and 0.1 mM of metal ions were not different from that of control. Though the cells cultured in the presence of 0.5 mM of metal ions showed morphological changes similar to Figure 1 ([metal ion] = 1 mM), the morphological changes were small (data not shown).

Morphological changes as shown in Figure 1 were observed at 2–3 days after the addition of metal ions except Yb^{3+}. In the case of Yb^{3+}, morphological changes were observed after about 1 day. The cells did not show further morphological changes after prolonged cultivation. When the culture medium was replaced with fresh culture medium without metal ions and the cells were cultured for 2–3 days,

the cell morphologies were recovered in round form same as control. Morphological changes of B16 melanoma cells induced by the metal ions were reproducible on 5–10 separated runs.

Cell growth

The effects of Ln^{3+} and Al^{3+} ions on the proliferation of B16 melanoma cells were investigated by trypan blue exclusion method. Figure 2 shows the number of B16 melanoma cells cultured in 1 mM metal ions (La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Yb^{3+}, Al^{3+}) for 5 days. Cell proliferations were significantly inhibited in the presence of the metal ions. Yb^{3+} ion showed stronger inhibition than other metal ions. Figure 2 shows both the number of living cells and total cells. The ratios of living cells and total cells are defined as cell viabilities, and were more than 95%. These results suggest that the inhibition effect of metal ions on cell growth were not induced by the cytotoxicity.

Inhibition effects of the trivalent metal ions on the cell proliferations were also confirmed by MTT assay. Figure 3 shows time courses of cell growth. Growth rates of melanoma cells in the presence of 1 mM of metal ions (La^{3+}, Ce^{3+}, Nd^{3+}, Gd^{3+}, Yb^{3+}, Al^{3+}) were significantly lower than that of control cells. Especially, Yb^{3+} showed strong inhibition effect on cell growth. Er^{3+} and Lu^{3+} ions were also found to inhibit the cell proliferation as well as La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+},Gd^{3+}, and Al^{3+} ions in the separated run (data not shown). The results obtained by MTT assay (Figure 3) were well correlated with those by trypan blue exclusion method (Figure 2).

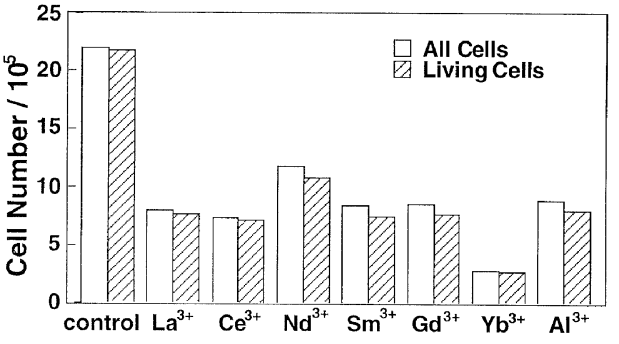

Figure 2. Cell numbers of B16 melanoma determined by a trypan-blue dye exclusion method. Cells were cultured in serum supplemented with La^{3+}, Ce^{3+}, Nd^{3+}, Sm^{3+}, Gd^{3+}, Yb^{3+}, and Al^{3+} ions for 5 days. Open columns represent the number of all cells, and shaded columns represent the number of living cells.

When melanoma cells were cultured in the presence of 0.01 and 0.1 mM of metal ion, MTT values were 1.3 times higher than that of control cells. However, increases of cell number and bromodeoxyuridine incorporation were not observed (data not shown). When the cells were cultured in the presence of 2.5 mM of metal ions, strong cytotoxicity was observed. At that concentration, aggregates of metal ions were detected in culture medium. The strong cytotoxicity was considered to be induced by the aggregation of metal ions. In serum-free medium, the metal ions easily form aggregates at 1 mM and showed strong cytotoxicity. Serum components suppressed the formation of metal ion aggregates.

To study the mechanisms for the inhibition of cell growth, cell cycles of B16 melanoma cells in the presence of 1 mM Gd^{3+}, Yb^{3+}, and Al^{3+} were measured by PI method. The results are summarized in Table 1. The results clearly indicate that transitions from G_0/G_1 to S were arrested by Gd^{3+}, Yb^{3+}, and Al^{3+}. Decrease of S state was reproducible in 3–4 separate runs. Decreases of cell growth are considered to be correlated with G_0/G_1 arrest.

Growth of other cells (Hela, Neuro2-M, and HepG2) are also inhibited in the presence of 1 mM trivalent metal ions. Though morphological changes were observed only for B16 melanoma cells, cell growth inhibitions were observed for all the cell lines employed in this study. Cell growth seems not to directly correlate with morphological changes.

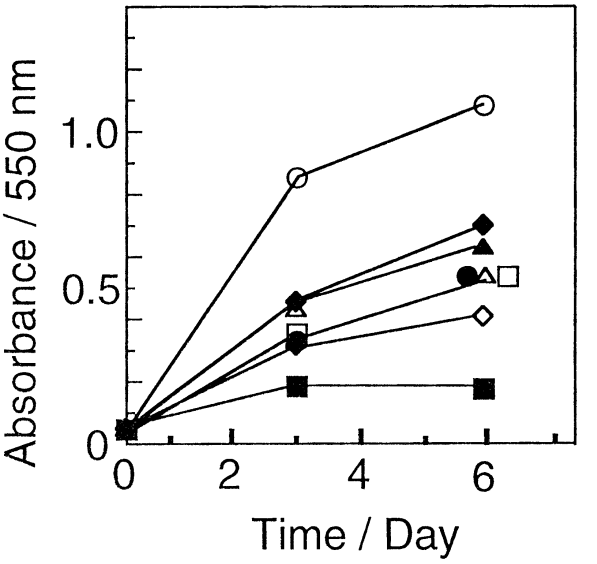

Figure 3. Time courses of cell growth determined by MTT assay. B16 melanoma cells were cultured in serum medium supplemented with (□) La^{3+}, (◆) Ce^{3+}, (△) Nd^{3+}, (◇) Sm^{3+}, (●) Gd^{3+}, (■) Yb^{3+}, and (▲) Al^{3+} ions for 3 and 6 days.

Table 1. Cell cycle of B16 melanoma cells cultured in serum medium supplemented with 1 mM Gd^{3+}, Yb^{3+}, and Al^{3+} for 24 h and 48 h

	24 h			48 h		
	G_0/G_1	S	G_2/M	G_0/G_1	S	G_2/M
control	50.4	31.3	18.3	60.6	23.7	15.7
Gd^{3+}	70.8	15.5	13.7	75.7	13.9	10.4
Yb^{3+}	67.3	11.5	21.2	66.0	15.4	18.6
Al^{3+}	70.0	17.0	13.0	69.0	11.4	19.6

Discussion

Lanthanoid and Al^{3+} ions induced drastic morphological changes in B16 melanoma cells. In general, cell morphology is known to correlate with the two- or three-dimensional structure of actin filaments. Interactions of Ln^{3+} with muscle actins in solution have been studied by Dos Demedios *et al.* When Gd^{3+} was present in excess of eight equivalents per mole of actins, polymerization of actins was inhibited (Dos Demedios & Barden 1977). Electromicroscopic examination showed that actin formed microcrystals and tubes in the presence of Gd^{3+} (Dos Demedios and Dickens 1978). Lanthanoids from Ce^{3+} to Ho^{3+} were able to promote the formation of actin tubes, and lanthanoids from Er^{3+} to Lu^{3+} produced amorphous precipitates (Dos Demedios *et al.* 1980). They suggested that this is connected with the stoichiometries of metal ion binding to actin. Furthermore, CD spectroscopy showed that Tb^{3+} reduced the amount of α-helix and β-sheet structure in actin (McCubbin *et al.* 1981). These results of Ln^{3+}–actin interaction suggest that Ln^{3+} internalized into cells have a potential to alter the polymerization and conformation of actin. Changes in synthesis and structures of actin will induce the changes of cell morphology.

If the metal ions directly regulate with the actin filament formation, they have to be internalized into cells. A possible process for the cell uptake of metal ions is transmembrane permeation through the plasma membrane. However, the lipid membrane is impermeable for metal ions (Bystrov *et al.* 1971, Fernandez *et al.* 1973). Furthermore, a transporter protein for Ln^{3+} and Al^{3+} has not been reported so far. Another possible process is endocytosis. Binding of Ln^{3+} to the cell surface has been shown previously. For example, addition of 1 mM La^{3+} increased the resting membrane potential of Ehrlich ascites tumour cells (Smith *et al.* 1972). Ln^{3+} can bind to sialic acid (Boyd *et al.* 1976) or proteins (Levinson *et al.* 1972) on the cell surface. The metal ions adsorbed on the cell surface would be internalized into cells by endocytosis or pinocytosis. Internalization of the Ln^{3+} aggregates into cells by endocytosis at prolonged incubation time has been evidenced by electron microscopy (Squir & Rooney 1976, Strum 1977).

We found two interesting phenomena about cell morphology induced by Ln^{3+}. One is that morphologies of B16 melanoma cells depend on the kind of metal ions. Another is that morphological changes induced by metal ions occurred only on B16 melanoma cells, but not on other cell lines tested (Hela cells, Neuro2-M or HepG2). B16 melanoma cells were very sensitive to lanthanoids and Al^{3+}. However, the reason is unknown. These results may suggest that B16 melanoma cells have a novel ion transporter system or produce second messenger responding to the addition of metal ions.

It has been known that cell morphology of melanoma cells is correlated with motility and metastasis. Therefore, the function of trivalent metal ions observed in this study will open new methodology for analysing the mechanism of metastasis and exploration of antitumour reagents.

The effect of metal ions on cell growth *in vitro* has been described in several papers. For example, Ln^{3+} inhibited the lymphocyte proliferation (Yamage & Evans 1989). In 3T3 fibroblast, vanadium compounds stimulated proliferation at 0.01 mM, and inhibited at 0.1 mM (Cortizo *et al.* 1997). In the present study, several trivalent ions involving Ln^{3+} and Al^{3+} ions showed the cell growth inhibition at 1 mM. The cell growth inhibition was induced by lengthened turn time of cell cycle, but not by the simple cytotoxicity. The reason for the decrease in cell growth rates should be complicated. However, recently the correlation of the intracellular Ca^{2+} concentration with gene expression and proliferation has been revealed (Dolmetsch *et al.* 1997, Ghosh & Greenberg 1997). If Ln^{3+} can bind Ca^{2+} transporter and inhibit Ca^{2+} influx (Katzung *et al.* 1973, Gill *et al.* 1981, Hagiwara & Byerly 1981), Ln^{3+} will be able to inhibit the cell proliferation.

We have shown here that the trivalent metal ions such as Ln^{3+} and Al^{3+} ions induced significant morphological changes and inhibited the cell growth. The trivalent metal ions are considered to have an important role as a modulator of the cell function. The present results suggest that the trivalent ions can modulate the biosynthesis and the signal transduction correlated with the formation of cytoskeleton and cell cycle.

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