**Calcium Dependency in the Growth of Adult T-Cell Leukemia Cells in Vitro**

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**ABSTRACT**

The effects of calcium, calcium antagonists, and calmodulin inhibitors on the growth of adult T-cell leukemia (ATL) cells were studied in vitro. Fresh ATL cells from patients and established ATL cell lines did not grow well in a low-calcium (<0.01 mm) medium. However, their growth was enhanced by the addition of calcium to the medium in a dose-dependent manner. The maximum response was induced at 4 mm calcium, which was higher than that of the normal serum calcium level, 2.5 mm. Other leukemia cells, except ATL, grew wel in the low-calcium medium, and their growth was not enhanced by the addition of calcium. Calcium antagonists and calmodulin inhibitors inhibited the growth of ATL cells at the concentration of $10^{-5}$-$10^{-7}$ m, while they did not inhibit the growth of other leukemia cells. Furthermore, the expression of interleukin 2 receptors (Tac antigens) on ATL cells was also enhanced by calcium and was inhibited by calcium antagonists and calmodulin inhibitors. In accordance with these results, the increase of the extracellular calcium concentration resulted in the increase of the intracellular calcium concentration in ATL cells, but not in other leukemia cells. These results suggest that calcium and calmodulin play a critical role in regulating the growth of ATL cells.

**INTRODUCTION**

(Atlas) found endemically in the southern part of Japan is one of the most severe cases of leukemias which are resistant to any kind of chemotherapy (1). Although it has become clear that ATL is caused by human T-cell leukemia virus I (2), the biological feature and the regulatory mechanism of the growth of ATL cells remain obscure. In clinical studies, we have often experienced that patients with ATL show hypercalcemia and that the number of ATL cells in the peripheral blood and the swelling of lymph nodes are often parallel with the increase in the serum calcium concentration (3). It has been reported that calcium plays an important role in the regulation of the growth and the functions of many kinds of cells (4, 5). These observations and reports suggest a possibility that calcium also regulates the growth of ATL cells in vivo. In order to understand this possibility, we have analyzed the effect of calcium, calcium antagonists, and calmodulin inhibitors on the growth and the expression of IL 2 receptors (Tac antigens) on ATL cells in vitro.

**MATERIALS AND METHODS**

Subjects. Seven patients with ATL admitted in our hospital were used for this study. The diagnosis for ATL was performed by using the following criteria: neoplastic cells have a highly convoluted nucleus; they have T-cell markers which form rosettes with sheep erythrocytes and are OKT 4 positive; and the patient has anti-ATL-associated antigen antibody in the serum.

Preparation of ATL Cells. Leukemia cells were collected from heparinized peripheral blood by a centrifugation over a lymphocyte separation medium (Litton Bionetics, Kensington, MD), washed with 10 mm PBS, and suspended in a calcium-free culture medium containing 1% FCS (Grand Island Biological Co., Grand Island, NY) which had been dialyzed against a calcium-free medium, penicillin (50 units/ml; Flow Laboratories, North Ryde, New South Wales, Australia) and streptomycin (50 μg/ml; Flow Laboratories).

Cell Lines. The established cell lines used in this experiment were ATL cells lines MT 1, MT 2, and HPB-CTRL; ATL cell line HPB-ALL; B-cell line NALM; monoblast cell line U 937; and myelocytic cell line K 562. These cell lines were kindly donated by Dr. S. Morikawa (Shimane Medical School, Izumo, Japan) and Dr. M. Yokoyama (Kurume University Medical School, Kurume, Japan). They were maintained in vitro by culturing in a RPMI 1640 medium (Grand Island Biological Co.) containing 10% FCS, penicillin, and streptomycin.

Preparation of Calcium-Free Culture Medium. For this study, we prepared a calcium-free culture medium based on the report by Corradin et al. (6). This contained 100 mg glucose, 800 mg sodium chloride, 40 mg potassium chloride, 4.8 mg sodium monohydrogen phosphate, 6 mg potassium dihydrogen phosphate, 4.9 mg magnesium sulfate, 4.7 mg magnesium chloride, 0.6 mg phenol red, 4 ml 50x concentrated essential amino acids, 5 ml 100x concentrated nonessential amino acids, 2.5 ml 100x concentrated nucleic acid precursors, 2 ml 100x concentrated vitamins, 2.5 ml 100x concentrated sodium pyruvate, 2 ml 100x concentrated glutamine, 0.45 ml 2 n sodium hydroxide, 0.5 ml N-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid, 1.3 ml 7.5% sodium bicarbonate, and 0.05 ml 0.1 M 2-mercaptoethanol in 10 ml water. All materials were purchased from Flow Laboratories and Sigma Chemical Co. (St. Louis, MO).

Assay of Leukemia Cell Growth. Leukemia cells (2 x 10⁶) were incubated in a 0.2-ml calcium-free culture medium containing 1% FCS and various concentrations of calcium chloride using microtiter culture plates (Falcon No. 3072; Falcon Plastics, Oxnard, CA) at 37°C for 48 h in 5% CO₂-95% air. The cells were pulsed for the last 24 h with 1 μCi [³²P]dThd (specific activity 5 Ci/mM; The Radiochemical Center, Amer- sham, England) and harvested by the aid of an automated cell harvester (Abekagaku Co., Funabashi, Chiba, Japan). The radioactivity incorporated into the cells was counted by a liquid scintillation spectrometer (7).

The results were expressed as the means of counts per minute of [³²P]dThd of triplicate cultures. The growth of cells was also detected by counting the number of recovered cells from the culture plates by a trypsin blue dye exclusion test.

Detection of IL 2 Receptors. Fresh ATL cells (1 x 10⁶) were cultured in a 1-ml calcium-free culture medium containing 1% FCS and various concentrations of calcium chloride using microtiter culture plates (Falcon No. 3003; Falcon Plastics) at 37°C for 48 h in 5% CO₂-95% air. The recovered cells (6 x 10⁶) were incubated with anti-Tac monoclonal antibody (kindly donated by Dr. T. Uchiyama, Kyoto University, School of Medicine, Kyoto, Japan) at 4°C for 3 h. After washing twice, the cells were incubated with [¹²⁵I]Protein A (The Radiochemical Center), about 50,000 cpm, at 4°C for a further 4 h. After washing 3 times, the radioactivity bound to ATL cells was counted by a gamma counter. The results were expressed...
as the mean ± standard error of counts per minute of triplicate cultures.

Measurement of Intracellular Calcium Concentration. MT 2 and K 562 cells (1 × 10⁶) were cultured in a 1-ml calcium-free culture medium containing 1% FCS and various concentrations of calcium chloride using culture tubes (Falcon No. 2058; Falcon Plastics) at 37°C for 24 h in 5% CO₂-95% air. The cells were washed with PBS, resuspended, and incubated with 50 μM quin 2/AM (Dojin Chemical Co., Kumamoto, Japan) at 37°C for 30 min. After washing twice with PBS, the cells were incubated at 37°C for a further 30 min. The fluorescence of quin 2-loaded cells was measured in a fluorescence spectrophotometer using an excitation wavelength at 339 nm and emission at 492 nm (9). The results were expressed as an arbitrary fluorescence intensity per 1 × 10⁷ cells.

RESULTS

Growth of ATL Cells Is Dependent on Calcium. At first, we studied the effect of the concentration of calcium in the culture medium on the in vitro growth of several kinds of cells. As shown in Fig. 1a, only some cases of ATL cells freshly prepared from patients proliferated in a low-calcium (<0.01 mM) medium. However, the addition of calcium chloride to this medium enhanced the proliferation of all ATL cells in a dose-dependent manner. The maximum response was induced at 4 mM calcium, which was higher than that of the normal serum calcium level, 2.5 mM. When established cell lines were used, ATL cell lines such as MT 1, MT 2, and HPB-CTL also showed a similar calcium dependency (Fig. 1b). On the other hand, other leukemia cell lines such as the myelocytic cell line K 562, T-cell line HPB-ALL, monoblast cell line U 937, and B-cell line NALM proliferated well in the low-calcium medium, and their proliferation was not enhanced by the addition of calcium. Fig. 2 shows the growth data of leukemia cells detected by the cell number. The result is the same as that expressed by [3H]dThd incorporation shown in Fig. 1. These results suggest that the growth of ATL cells is more dependent on calcium in the culture medium than other leukemia cells.

Calcium Antagonists and Calmodulin Inhibitors Inhibit the Growth of ATL Cells. Next we studied the effect of calcium antagonists and calmodulin inhibitors on the growth of several kinds of leukemia cells in vitro. This experiment was done in the presence of 4 mM calcium. As shown in Fig. 3, calcium antagonists such as diltiazem (Tanabe Seiyaku Co., Ltd., Osaka, Japan) and nifedipine (Bayer AG., Leverkusen, Federal Republic of Germany), which inhibit the influx of extracellular calcium into cells, inhibited the growth of ATL cells and ATL cell line MT 2 in a dose-dependent manner, while it did not inhibit the growth of other cell lines such as K 562 and HPB-ALL in a concentration of less than 10⁻⁴ M. These results correlate with the calcium dependency in the growth of ATL cells. The effect of calcium antagonists on the growth of ATL cells was not due to a nonspecific cytotoxic effect, because diltiazem and nifedipine are not cytotoxic in a concentration of less than 10⁻⁴ M. Furthermore, the inhibitory effect of calcium antagonists was overcome by the presence of a large amount of calcium (data not shown). Other calcium antagonists such as verapamil, nicaldipine, and nicorandil...
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had a similar effect (data not shown). Calmodulin inhibitors such as clomipramine (Ciba-Geigy, Ltd., Basel, Switzerland) and prenlimine (Hoechst AG., Frankfurt, Federal Republic of Germany) also had a similar effect on the growth of ATL cells in vitro (Fig. 4). These results further suggest that calcium and calmodulin play an important role in the growth of ATL cells.

Expression of IL 2 Receptors on ATL Cells Is Also Dependent on Calcium. On ATL cells, IL 2 receptors (Tac antigens) are constitutively expressed, and this is used as a marker of ATL cells, although their functional role for ATL cells is not known (10). Therefore we studied the effect of calcium on the expression of IL 2 receptors. As shown in Fig. 5, when ATL cells were cultured in a low-calcium medium, there was very little expression of IL 2 receptors on ATL cells. However, the addition of calcium chloride to the medium induced the expression of IL 2 receptors on ATL cells in a dose-dependent manner. The maximum response was obtained at 4 mM calcium. Furthermore, the calcium antagonist diltiazem and the calmodulin inhibitor clomipramine inhibited the expression of IL 2 receptors. These results suggest that the expression of IL 2 receptors on ATL cells is also regulated by calcium and calmodulin.

Intracellular Calcium Concentration of ATL Cells Was Changed by the Extracellular Calcium Concentration. In the above section, we demonstrated evidence that the growth of ATL cells was altered by changing the extracellular calcium concentration. We finally studied whether the change of the extracellular calcium concentration resulted in the change of the intracellular calcium concentration. MT 2 and K 562 cells were cultured at 37°C for 24 h in the medium containing various concentrations of calcium chloride, and the intracellular calcium concentration was measured using quin 2. As shown in Fig. 6, the intracellular calcium concentration of ATL cells increased with the increase in the extracellular calcium concentration and decreased due to the culture with diltiazem and clomipramine. On the other hand, the intracellular calcium concentration of K 562 cells was higher than that of MT 2 cells and was not changed by the extracellular calcium concentration. These results are consistent with the growth of leukemia cells. All these results suggest that the change of the extracellular calcium concentration results in the change of the intracellular calcium concentration and finally the change of the growth of ATL cells.

DISCUSSION

It is well known that calcium has a regulatory role in the growth and the functions of several kinds of cells including lymphocytes (4, 5). In a low-calcium medium, lymphocytes cannot be stimu-
lapsed to proliferate by mitogenic lectins (11). Calcium channel blocking agents (calcium antagonists) and calmodulin inhibitors suppress lymphocyte-proliferative response induced with mitogenic lectins (12, 13). Furthermore, the uptake of calcium by lymphocytes is induced shortly after the interaction with lectins (14). These results suggest that the calcium influx may be the initial intracellular mechanism that activates lymphocytes.

In this study, we found that the growth of ATL cells was limited in the low-calcium medium but was enhanced by the increase in the extracellular calcium concentration (Figs. 1 and 3). The intracellular calcium concentration of ATL cells was also increased by the increase in the extracellular calcium concentration (Fig. 6). Furthermore the growth and the increase in the intracellular calcium concentration of ATL cells were inhibited by calcium antagonists and calmodulin inhibitors (Figs. 3 and 4). The effects of calcium antagonists and calmodulin inhibitors on the growth of ATL cells were not due to the cytotoxic effect of these agents, because all agents were not toxic for cells at the doses used in this experiment, and the effect of calcium antagonists was overcome by the addition of a large amount of calcium chloride (data not shown). These results suggest that calcium plays an important role in the growth of ATL cells. On the other hand, other leukemia cells, except ATL, grew well in the low-calcium medium, and their growth was not enhanced by the presence of a high-calcium concentration (Figs. 1 and 2). Furthermore their growth was not inhibited by calcium antagonists and calmodulin inhibitors (Figs. 3 and 4). The intracellular calcium concentration of these cells was also not affected by the change of the extracellular calcium concentration. The difference of calcium dependency in the growth of ATL cells and other leukemia cells does not seem to be induced by the difference of times of passages, because all cell lines were passaged over a period of more than 1 yr by the same procedure in our laboratory. The expression of IL 2 receptors on ATL cells was also dependent on the extracellular calcium concentration (Fig. 5). This result suggests a possibility that calcium may primarily express its effect on IL 2-dependent regulation of the cell growth, because IL 2 receptors are expressed on ATL cells and established ATL cell lines but not on other leukemia cells. However, this possibility seems to be unlikely, because ATL cells grew well without IL 2, and their growth was not enhanced by the addition of IL 2 (data not shown). Furthermore we did not use IL 2 in this experiment and in maintaining all cell lines. The mechanism that causes the difference of calcium dependency in ATL cells and other leukemia cells is not clear at present. However, some enzymic systems required for the transport of calcium and for the synthesis of materials required for replication may be different in ATL cells and other leukemia cells.

Swierengo et al. found that neoplastic cells were less dependent for their growth on the calcium concentration in the culture medium, while normal cells were dependent on the extracellular calcium concentration. Thus they proposed that the calcium dependency served as an in vitro marker for neoplastic transformation (15). However, this is not applicable for leukemia cells as demonstrated here. Both ATL cells and other leukemia cells seem to be neoplastically transformed. The possibility for this difference may be that they primarily studied carcinoma cell lines derived from cervix, pharynx, kidney, mammary gland, and prostate, but they did not study leukemia cells. Thus, the calcium dependency may be different in cell types even if they are neoplastically transformed.

We often observed in clinical studies that the number of ATL cells in the peripheral blood parallels with the serum calcium level. We also reported that ATL cells produced bone resorption-stimulating factors which resulted in the release of calcium from bone marrow (3). Thus, the increase in the serum calcium level in ATL patients may result from the activation of the bone resorption systems. However, it is also possible that the high-calcium condition stimulates the growth of ATL cells in vivo. In fact, the maximum growth of ATL cells is induced at a higher concentration of calcium, 4 mm, than the standard medium containing 1.26 mm calcium and the normal serum calcium level, 2.5 mm. Thus the increased serum calcium level may be a better environment for the growth of ATL cells.

Although the molecular mechanism for calcium regulation in the growth of ATL cells is not clear yet, the data presented here give us a new aspect regarding both the biological feature of ATL cells and the possibility that the growth of ATL cells can be controlled by changing the extra- and the intracellular calcium concentration using calcium antagonists and/or calmodulin inhibitors not only in vitro but also in vivo.

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