1α,25-Dihydroxyvitamin D_3 directly induces fusion of alveolar macrophages by a mechanism involving RNA and protein synthesis, but not DNA synthesis

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The results of our present study indicate that 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] directly induces fusion of mouse alveolar macrophages without any participation of T-lymphocytes by a mechanism involving RNA and protein synthesis but not DNA synthesis. We have reported that 1α,25$(OH)_2D_3$ induces fusion of alveolar macrophages by a direct mechanism and by a spleen cell-mediated indirect mechanism [(1983) Proc. Natl. Acad. Sci. USA 80, 5583–5587]. Alveolar macrophages pretreated with or without anti-Thy 1.2 antibody and complement fused similarly when they were incubated with 1α,25$(OH)_2D_3$. The vitamin suppressed DNA synthesis, but it significantly enhanced RNA and protein synthesis. The 1α,25$(OH)_2D_3$-induced fusion was blocked by adding actinomycin D or cycloheximide, but not by hydroxyurea.

1α,25-Dihydroxyvitamin D_3 *Cell fusion* *Alveolar macrophage* *DNA synthesis* *RNA synthesis* *Protein synthesis*

1. INTRODUCTION

The metabolically active form of vitamin D_3, 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], accomplishes its biological activity in mineral metabolism through binding to its specific cytosol receptor in bone and intestine [1,2]. Recently, similar cytosol receptors for 1α,25$(OH)_2D_3$ have been found almost ubiquitously in several tissues and cells, suggesting that the role of this vitamin in biology extends far beyond its classical role in mineral metabolism [3].

We reported that 1α,25$(OH)_2D_3$ induces murine myeloid leukemia cells (M1) to differentiate into monocyte-macrophages [4]. Since then, it has been demonstrated that several mouse and human myeloid cells are similarly induced to differentiate into monocyte-macrophages by 1α,25$(OH)_2D_3$ [5–9]. Subsequently, it was reported that 1α,25$(OH)_2D_3$ promoted fusion of mouse alveolar macrophages directly and also by a spleen cell-mediated indirect mechanism [10]. The indirect effect of 1α,25$(OH)_2D_3$ was always higher than the direct one [10]. These results led us to examine the possibility that the trace lymphocytes which might have contaminated the preparation of alveolar macrophages might have been involved in the direct mechanism of the vitamin. We show here that 1α,25$(OH)_2D_3$ induces fusion of alveolar macrophages without any participation of T-lymphocytes by a mechanism involving RNA and protein synthesis, but not DNA synthesis.

2. MATERIALS AND METHODS

2.1. *Preparation and culture of alveolar macrophages*

Alveolar macrophages were obtained from 6–8-week-old male mice, ddy strain (Shizuoka Laboratory Animal Centre, Shizuoka, Japan), by the tracheobronchial lavage method and purified as in [10]. The culture medium was Eagle's

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minimal essential medium supplemented with 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 2-fold concentrated vitamins, and 5% heat-inactivated human serum obtained from healthy volunteers. For the fusion assay, 3 μl of the cell suspension of alveolar macrophages (1.2×10^4 cells) was placed in the centre of a multi-well (diam. 7 mm, Corning Glass Works, NY) with a Hamilton microsyringe. After incubation for 30 min, adherent cells were cultured with or without 12 nM 1α,25(OH)$_2$D$_3$ (a gift from Dr I. Matsunaga, Chugai Pharmaceutical, Tokyo) for up to 72 h. In some experiments, cells were cultured with 1α,25(OH)$_2$D$_3$ for 6–72 h, washed thoroughly, transferred to a fresh medium, and cultured without the vitamin for the remaining part of the 72 h culture period. At the end of culture, the adherent cell layers were washed, fixed and stained with May-Grünwald-Giemsa. The fusion rate was expressed as: (number of nuclei within giant cells consisting of 3 or more nuclei in a cell/total number of nuclei counted) $\times$ 100.

2.2. *Treatment with anti-Thy 1.2 antibody and complement*

To eliminate the participation of T-lymphocytes, if any, purified alveolar macrophages were incubated for 60 min at 4°C with 500-fold diluted anti-Thy 1.2 antibody (Cedarlane, Ontario), followed by incubation for 60 min at 37°C with 20-fold diluted rabbit complement (Cedarlane), and cultured as described above. Spleen cells obtained from mice of the same strain and those pretreated with the antibody and/or complement were cultured for 72 h with or without 2.5 μg/ml of concanavalin A (Con A, jack beans, grade IV, Sigma). DNA synthesis was examined by incubating spleen cells with 1 μCi/ml of [*methyl*-^{3}H]thymidine (Amersham) for the last 24 h of incubation.

2.3. *Measurement of the synthesis of DNA, RNA and proteins*

Alveolar macrophages (10^5 cells/well) were cultured for 48 h with or without 12 nM 1α,25(OH)$_2$D$_3$ and with either 1 mM hydroxyurea (Sigma), 0.48 nM actinomycin D (P-L Biochemicals), or 210 nM cycloheximide (Boehringer Mannheim). To examine DNA, RNA or protein synthesis, macrophages were incubated for the last 24 h of incubation with 10 μCi/ml of either deoxy[G-^{3}H]adenosine, [5,6-^{3}H]uridine, or ^{3}H-labelled amino acid mixture (Amersham), respectively, and incorporation of each radioisotope into trichloroacetic acid-insoluble materials was determined. Data are expressed as means ± SE of at least 3 independent sets of experiments. In each experiment, the triplicate assay was carried out. The statistical significance of difference between the controls and the experimental groups was analyzed by Student's *t*-test.

3. RESULTS AND DISCUSSION

Alveolar macrophages treated with 12 nM 1α,25(OH)$_2$D$_3$ began to fuse after incubation for 36 h and the fusion rate increased linearly for up to 60 h (fig.1A). The maximal fusion rate in this study (~80%) was much higher than that reported previously (~40%) [10]. This is due to a higher cell density of the macrophages placed in the centre of the well achieved by using a Hamilton microsyringe. When macrophages were treated with 12 nM 1α,25(OH)$_2$D$_3$ for 6–72 h and cultured in fresh medium without the vitamin for the remaining part of the 72 h, at least 18–24 h of the incubation time with 1α,25(OH)$_2$D$_3$ was necessary to make macrophages fuse (fig.1B). These results suggest that some slowly occurring cellular events are required for the induction of fusion of alveolar macrophages by the vitamin.

It has been reported that conditioned media of mouse splenic T cells treated with 1α,25(OH)$_2$D$_3$ contain a lymphokine which promotes fusion of mouse alveolar macrophages [10]. More than 99% of the cell preparation used here consisted of nonspecific esterase-positive macrophages, but the possibility could not be denied that trace lymphocytes contaminating the macrophage preparation might be involved in the fusion. However, alveolar macrophages pretreated with anti-Thy 1.2 antibody and complement to eliminate lymphocytes fused when 12 nM 1α,25(OH)$_2$D$_3$ was added just as the untreated control macrophages did (fig.2A). When spleen cells were treated with the same concentrations of anti-Thy 1.2 antibody and complement, the splenic DNA synthesis induced by Con A was suppressed completely (fig.2B), indicating that all T cells were inactivated by this treatment. These results indicate that

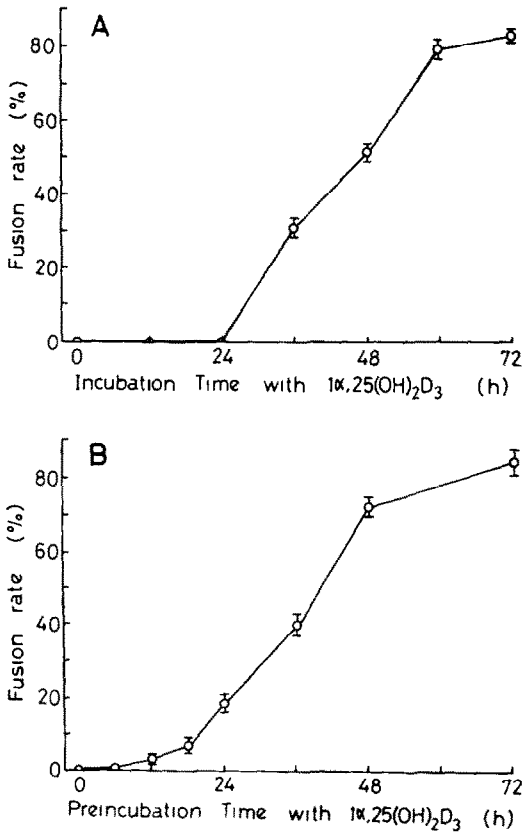

Fig.1. Time course of change in fusion of alveolar macrophages induced by $1\alpha,25(OH)_2D_3$. (A) Macrophages were incubated for 6–72 h with 12 nM $1\alpha,25(OH)_2D_3$, and fusion rate was examined at indicated times. (B) Macrophages were incubated for 6–72 h with 12 nM $1\alpha,25(OH)_2D_3$, then transferred to fresh medium, and incubated without the vitamin for the remainder of the 72 h culture period. The fusion rate was determined at 72 h in this experiment.

$1\alpha,25(OH)_2D_3$ directly promotes fusion of alveolar macrophages without any participation of T-lymphocytes. It has also been reported that the vitamin directly induces fusion of HL-60 cells, a human promyelocytic leukemia cell line, to form multinucleated giant cells with bone-resorbing activity [6].

Fig.3 shows the synthesis of DNA, RNA and proteins by alveolar macrophages. When macrophages were treated with 12 nM $1\alpha,25(OH)_2D_3$ for 48 h, DNA synthesis decreased to 60% of the control, whereas RNA and protein synthesis increased to about 150% of the control. $1\alpha,25(OH)_2D_3$ did not increase the total number of nuclei per well

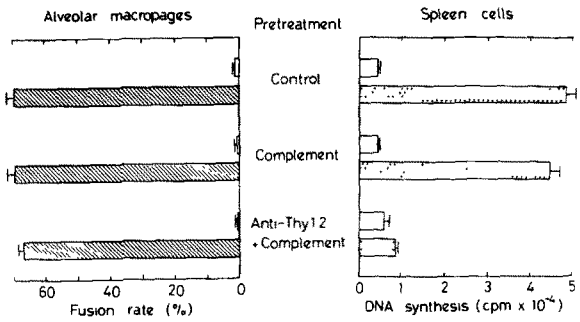

Fig.2. Effect of the treatment with anti-Thy 1.2 antibody and complement on fusion of alveolar macrophages and DNA synthesis of spleen cells. (Right) Alveolar macrophages pretreated with or without 500-fold diluted anti-Thy 1.2 antibody and/or 20-fold diluted complement were incubated for 72 h with (hatched bars) or without (unfilled bars) 12 nM $1\alpha,25(OH)_2D_3$, and the fusion rate was determined. (Left) Spleen cells pretreated with or without the same concentrations of anti-Thy 1.2 antibody and/or complement were cultured for 72 h with (dotted bars) or without (unfilled bars) 2.5 μg/ml of Con A. DNA synthesis was determined by incorporating 1 μCi/ml of [^{3}H]thymidine into trichloroacetic acid-insoluble materials during the last 24 h of culture.

(not shown). Thus, it is apparent that the formation of multinucleated giant cells is not due to nuclear replication.

Fig.4 shows the effect of inhibitors of DNA, RNA and protein synthesis on fusion of alveolar macrophages. Hydroxyurea, an inhibitor of nucleotide reductases and consequently an inhibitor of DNA synthesis, suppressed the $1\alpha,25(OH)_2D_3$-induced fusion dose dependently. In the presence of 1 mM hydroxyurea and 12 nM $1\alpha,25(OH)_2D_3$, DNA synthesis was inhibited to 10% of the control (fig.3A), whereas more than 50% of the macrophages fused to form multinucleated giant cells (fig.4A). These results suggest that DNA synthesis is not necessary in inducing fusion of alveolar macrophages.

In contrast, both actinomycin D and cycloheximide inhibited the $1\alpha,25(OH)_2D_3$-induced fusion of alveolar macrophages dose dependently. When macrophages were treated with either 0.48 nM actinomycin D or 210 nM cycloheximide, the fusion rate enhanced by the vitamin was suppressed almost completely (fig.4B,C), but RNA and protein synthesis was only reduced to 40–50% of the

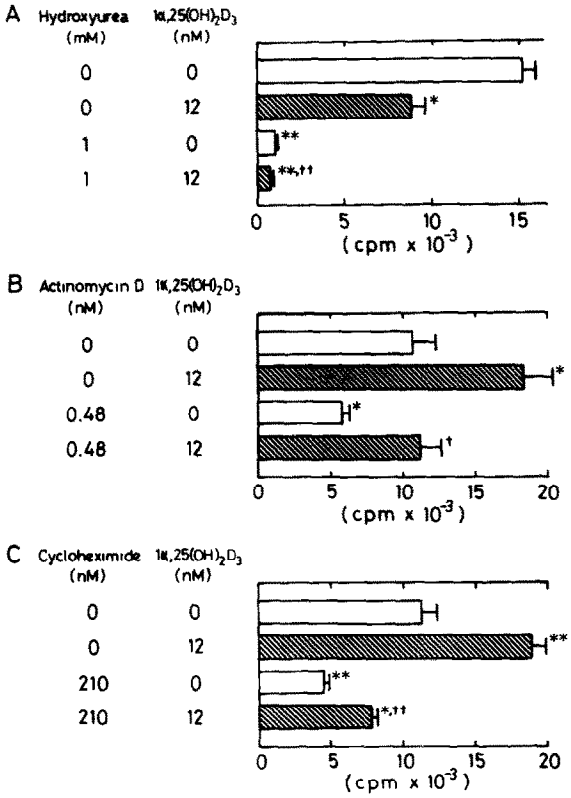

Fig.3. Effect of $1\alpha,25(OH)_2D_3$ and inhibitors on the synthesis of DNA, RNA and proteins. Alveolar macrophages were incubated for 48 h with or without 12 nM $1\alpha,25(OH)_2D_3$. To measure the synthesis of DNA (A), RNA (B) and proteins (C), 10 μCi/ml of deoxy[^{3}H]adenosine, [^{3}H]uridine or ^{3}H-labelled amino acid mixture, respectively, was added for the last 24 h of incubation and incorporation of each radioisotope into trichloroacetic acid-insoluble materials was determined. DNA synthesis was determined with or without 1 mM hydroxyurea, RNA synthesis with or without 0.48 nM actinomycin D, and protein synthesis with or without 210 nM cycloheximide. Significance of difference from each control culture without the vitamin: * $p < 0.05$; ** $p < 0.005$, and that from each control culture with the vitamin: $^+$ $p < 0.05$; $^{++}$ $p < 0.005$.

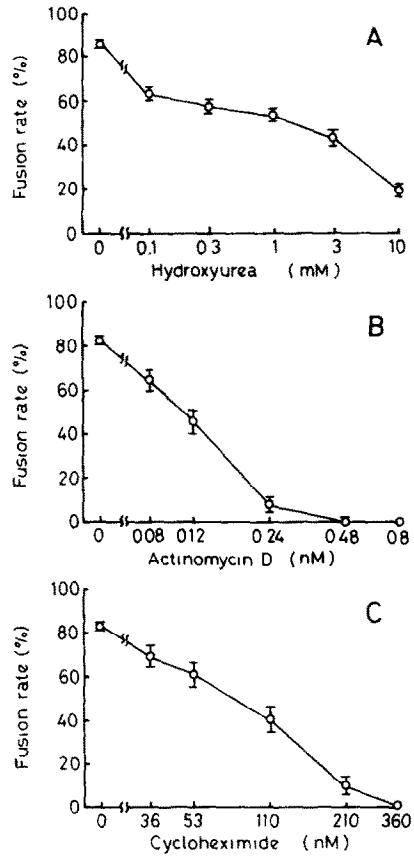

Fig.4. Dose response in the inhibitory effects of hydroxyurea, actinomycin D and cycloheximide on the fusion of alveolar macrophages induced by $1\alpha,25(OH)_2D_3$. After alveolar macrophages were cultured for 72 h with 12 nM $1\alpha,25(OH)_2D_3$ and with either hydroxyurea (A), actinomycin D (B), or cycloheximide (C), the fusion rate was determined.

enhanced levels (fig.3B,C). The concentrations of the inhibitors used here have been proved to be not cytotoxic. These results clearly indicate that $1\alpha,25(OH)_2D_3$-induced fusion of alveolar macrophages requires RNA and protein synthesis, but not DNA synthesis. The partial suppression of the fusion rate by hydroxyurea might have resulted from the side effect on RNA and protein synthesis.

It is known that $1\alpha,25(OH)_2D_3$ binds to specific cytosol receptors in target cells, translocates to the nucleus, and leads to the synthesis of new protein(s) before exerting its biological activity in mineral metabolism [1–3]. At present we have not succeeded in identifying the specific cytosol receptor for $1\alpha,25(OH)_2D_3$ in mouse alveolar macrophages, because more than 1×10^8 cells are required to examine $1\alpha,25(OH)_2D_3$ receptors. We can obtain only $4–6 \times 10^5$ cells from each mouse. However, the presence of $1\alpha,25(OH)_2D_3$ receptors in human peripheral monocytes [11] and in monocyte-macrophage lineage leukemia cells [7,12] suggests the possibility that a similar receptor exists in mouse alveolar macrophages.

We therefore suggest that a similar mechanism effects the direct action of $1\alpha,25(OH)_2D_3$ in inducing fusion of alveolar macrophages. First, the order of the potency of vitamin D_3 derivatives in inducing fusion of alveolar macrophages is closely related to that in binding to $1\alpha,25(OH)_2D_3$ receptors in several other tissues [10]. Second, $1\alpha,25(OH)_2D_3$ enhanced the synthesis of RNA and proteins (fig.3). Third, fusion was completely suppressed when either RNA or protein synthesis is inhibited (fig.4). The nature of protein(s) synthesized by alveolar macrophages treated with the vitamin is of considerable interest and is being explored in our laboratory.

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