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The specific production of the third component of complement by osteoblastic cells treated with 1α,25-dihydroxyvitamin D_3

Toshiyuki Sato[1,2], Mei Hua Hong[1], Cheng He Jin[1], Yoshiko Ishimi[1], Nobuyuki Udagawa[1], Toshimasa Shinki[1], Etsuko Abe[1] and Tatsuo Suda[1]

[1]*Department of Biochemistry, School of Dentistry, Showa University, Tokyo 142* and [2]*Exploratory Research Laboratories III, Daiichi Pharmaceutical Co., Tokyo 134, Japan*

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A 190 kDa protein was purified from conditioned media of mouse marrow-derived stromal cell (ST2) cultures treated with 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$) and identified as the third component of mouse complement (C3). Northern and Western blot analysis revealed that the production of C3 by ST2 and primary osteoblastic cells was strictly dependent on 1α,25$(OH)_2D_3$, but the production by hepatocytes was not. Adding 1α,25$(OH)_2D_3$ together with mouse C3 antibody to bone marrow cultures greatly inhibited the formation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclast-like multinucleated cells. Adding C3 alone induced no TRAP-positive cell formation. These results suggest that, in bone tissues, C3 is specifically produced by osteoblasts in response to 1α,25$(OH)_2D_3$ and somehow involved in inducing differentiation of bone marrow cells into osteoclasts in concert with other factors produced by osteoblasts in response to 1α,25$(OH)_2D_3$.

Complement C3; 1α,25-Dihydroxyvitamin D_3; Osteoblastic cell

1. INTRODUCTION

It is well established that 1α,25$(OH)_2D_3$, the active form of vitamin D_3, induces bone resorption in two distinct ways; one is the recruitment of new osteoclasts and the other is the activation of quiescent osteoclasts [1–4]. Osteoclasts are unique multinucleated cells present only in bone, with characteristics such as tartrate-resistant acid phosphatase (TRAP) activity, abundant calcitonin receptors and bone-resorbing activity [5–9]. We previously reported that osteoclast-like cells which satisfied the above criteria were formed in co-cultures of primary osteoblastic cells isolated from mouse calvaria and mouse spleen cells in the presence of 1α,25$(OH)_2D_3$ [10]. Separate cultures of either osteoblastic cells or spleen cells induced no osteoclast-like cell formation [10]. The primary osteoblastic cells could be substituted by mouse bone marrow-derived stromal cells (ST2) in inducing osteoclast-like cell formation in co-cultures with spleen cells [11]. This indicates that osteoblastic cells or marrow-derived stromal cells are somehow involved in osteoclast differentiation. In this study, we found a 190 kDa protein produced by ST2 cells and primary osteoblastic cells treated with 1α,25$(OH)_2D_3$. The 190 kDa protein was unequivocally identified as the third component of mouse complement (C3). The possible biological significance of the specific production of C3 by osteoblasts was also examined.

Correspondence address: T. Suda, Department of Biochemistry, School of Dentistry, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan. Fax: (813) 3784 5555

2. MATERIALS AND METHODS

2.1. *Cells and culture*

ST2 cells (purchased from Riken Cell Bank, Tsukuba, Japan) and primary osteoblastic cells prepared from mouse calvaria were cultured overnight in α-minimal essential medium (α-MEM) containing 10% fetal bovine serum (FBS) in 24-multiwell dishes, and further cultured for 3 days in 0.5 ml of methionine-free α-MEM containing 0.1% bovine serum albumin (BSA) and 2 μCi of [^{35}S]methionine with and without 10^{-8} M 1α,25$(OH)_2D_3$. Conditioned media were collected, and 20 μl of the media was heat-denatured and applied to 7.5% SDS polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. The methods of Laemmli [12] were followed with a minor modification.

2.2. *Purification and identification of the 190 kDa protein*

For purification of a protein present in conditioned medium of ST2 cell cultures treated with 1α,25$(OH)_2D_3$, the cells were cultured for 3 days in the presence of 10^{-8} M 1α,25$(OH)_2D_3$ in α-MEM containing 0.1% BSA, and 2 liter of conditioned media was collected. Purification was performed sequentially by DEAE-sepharose, hydroxyapatite column chromatography and HPLC (fitted with DEAE-5PW), and the purified protein was finally applied to SDS-PAGE under a reducing or a non-reducing condition. The proteins on the gels were stained with Coomassie brilliant blue and transferred onto Immobilon (Millipore, Bedford, MA) by electro-blotting, and their N-terminal amino acid sequences were determined using a protein sequencer (Applied Biosystems, model 470A, Foster, CA).

2.3. *Northern blot analysis*

ST2 cells, primary osteoblastic cells, and primary hepatocytes were cultured for 2 days in the presence or absence of 10^{-8} M 1α,25$(OH)_2D_3$ in α-MEM containing 10% FBS and the total RNAs were extracted from the cells by the guanidine thiocyanate/CsCl

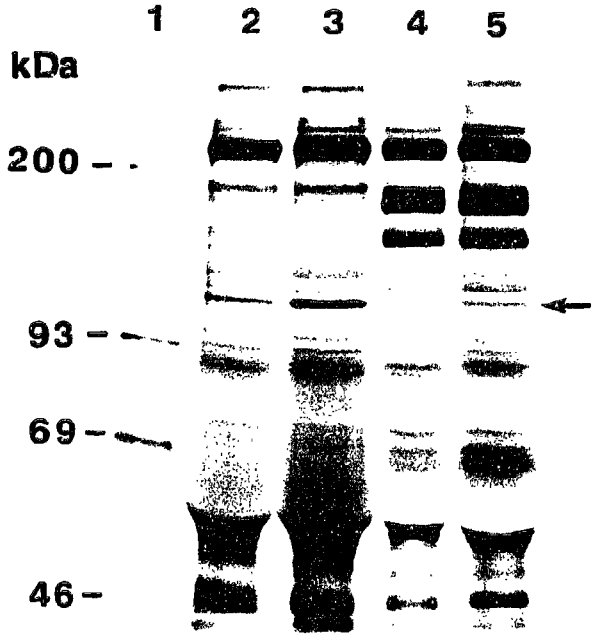

Fig. 1. The effect of $1\alpha,25(OH)_2D_3$ on the production of [^{35}S]methionine-labeled proteins by the mouse marrow-derived stromal cell line, ST2, and mouse primary osteoblastic cells. ST2 cells (lanes 2 and 3) and primary osteoblastic cells (lanes 4 and 5) were incubated for 3 days in the absence (lanes 2 and 4) or presence (lanes 3 and 5) of 10^{-8} M $1\alpha,25(OH)_2D_3$ and proteins in the conditioned media were analyzed under a reducing condition on SDS-PAGE. Lane 1 indicates standard protein markers. The arrow indicates the 116 kDa protein.

method [13]. The RNAs were separated by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde, transferred to a nylon membrane, and hybridized with ^{32}P-labeled cDNA probes encoding mouse C3 or tubulin.

2.4. *Mouse bone marrow cultures*

Mouse bone marrow mononuclear cells (1.5×10^6/ml) were cultured in 24-multiwell dishes with an antibody against mouse C3 (Cappel, West Chester, PA) at a final dilution of $1/10^4$ with or without 10^{-8} M $1\alpha,25(OH)_2D_3$ in 0.5 ml of α-MEM containing 10% FBS. On day 4, 0.4 ml of the old medium was replaced with fresh medium. After the culturing, the adherent cells were washed, fixed and stained for TRAP, non-specific esterase (NSE) and alkaline phosphatase (ALP) activities, as reported previously [4].

2.5. *Radioimmunoassay of mouse C3*

The contents of C3 in conditioned media of cell cultures and in mouse serum and calvaria were determined by radioimmunoassay using ^{125}I-labeled purified C3 and its polyclonal antibody. The sensitivity for the assay was 1 ng/tube. For in vitro experiments, conditioned media of ST2 and bone marrow cell cultures treated for 3 days with and without 10^{-8} M $1\alpha,25(OH)_2D_3$ were used. For in vivo experiments, vehicle (ethanol) or 500 ng of $1\alpha,25(OH)_2D_3$ was injected once subcutaneously into vitamin D-deficient mice prepared by the methods of Suda et al. [14] and the serum and calvaria were prepared 72 h after the injection. The calvaria was homogenized with 50 mM Tris-HCl buffer (pH 7.4) and centrifuged, then the resulting supernatant was used for the assay.

3. RESULTS AND DISCUSSION

Fig. 1 shows the SDS-PAGE of proteins produced by ST2 cells and primary osteoblastic cells. $1\alpha,25(OH)_2D_3$ greatly increased the production of a 116 kDa protein under a reducing condition by both ST2 cells and primary osteoblastic cells (Fig. 1). The 116 kDa protein was purified and applied to SDS-PAGE under a reducing or a non-reducing condition. The apparent molecular weight of the protein isolated was 190 kDa under non-reducing conditions, and 116 and 65 kDa under reducing conditions (Fig. 2A). The N-terminal amino acid sequence of the 116 kDa protein (number 1–10) was identical to that of the α-chain of mouse C3 except for number 8 which was not identified [15–18] (Fig. 2B). The sequence of the 65 kDa protein (number 1–7]

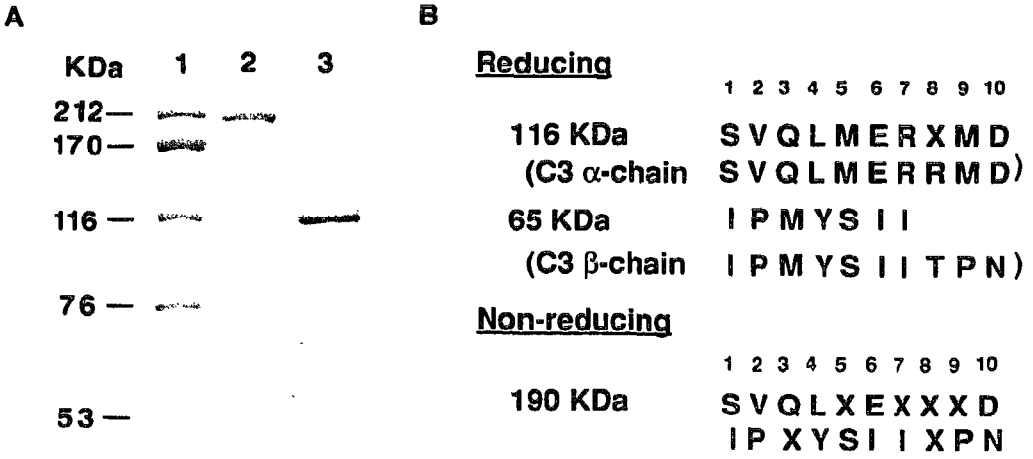

Fig. 2. SDS-PAGE (A) and the N-terminal amino acid sequences (B) of the purified protein. (A) The protein was applied to SDS-PAGE under non-reducing (lane 2) or reducing (lane 3) conditions with standard protein markers (lane 1), and stained with Coomasie brilliant blue. (B) Eluted proteins (190, 116 and 65 kDa) from lanes 2 and 3 were subjected to a protein sequencer. The amino acid sequence of hepatic C3 previously reported is shown in parenthesis (12–15). The symbol X indicates an amino acid residue which was not identified.

was identical to that of the β-chain of mouse C3 [15–18] (Fig. 2B). The 190 kDa protein obtained under non-reducing conditions gave two different N-terminal amino acid residues in each Edman degradation, which exactly coincided with those of the 116 and 65 kDa proteins (Fig. 2B). The 190 kDa protein immunoreacted with a polyclonal mouse C3 antibody (data not shown). The mRNA expression of C3 was greatly and specifically increased by $1\alpha,25(OH)_2D_3$ in both ST2 cells (Fig. 3, lanes 1 and 2) and primary osteoblastic cells (Fig. 3, lanes 3 and 4). This also confirms that the purified protein is identical to mouse C3.

It is known that more than 90% of the C3 present in the blood stream is supplied by the liver [19]. We examined the mRNA expression of C3 in hepatocytes and its regulation by vitamin D. The mRNA expression by hepatocytes similarly occurred even in the absence of $1\alpha,25(OH)_2D_3$ (Fig. 3, lanes 5 vs 6). Also, the [^{35}S]methionine-labeled 116 kDa band produced by hepatocytes appeared irrespective of the presence or absence of $1\alpha,25(OH)_2D_3$ (data not shown). These results indicate that the hepatic production of C3 is not regulated by $1\alpha,25(OH)_2D_3$. This may be explained in part by the absence of $1\alpha,25(OH)_2D_3$ receptors in the liver [20].

The biological significance of the C3 production by bone marrow-derived stromal cells and osteoblastic cells in response to $1\alpha,25(OH)_2D_3$ is most interesting. To address this issue, we examined the role of C3 in osteoclast-like cell formation using mouse marrow cultures. TRAP-positive multinucleated osteoclast-like cells were formed adjacent to the colonies of ALP-positive osteoblastic cells, when marrow cells were cultured with $1\alpha,25(OH)_2D_3$ (Fig. 4B). We have reported that these TRAP-positive multinucleated cells have calcitonin receptors and bone-resorbing activity [4]. When a polyclonal antibody against mouse C3 (goat IgG fraction) together with $1\alpha,25(OH)_2D_3$ was added, the formation of both ALP-positive osteoblastic cell colonies and TRAP-positive multinucleated cells was greatly inhibited (Fig. 4C). Instead, numerous NSE-positive mono-nuclear cells, probably monocyte-macrophages, appeared (Fig. 4C). TRAP-positive cell formation was not inhibited by adding non-immune goat IgG fraction (data not shown). Adding purified C3 alone induced no TRAP-positive cell formation. These results indicate the possibilities that C3 is one of the factors produced by osteoblasts in response to $1\alpha,25(OH)_2D_3$ and that C3 is somehow involved in determining the differentiation pathways of bone mar-

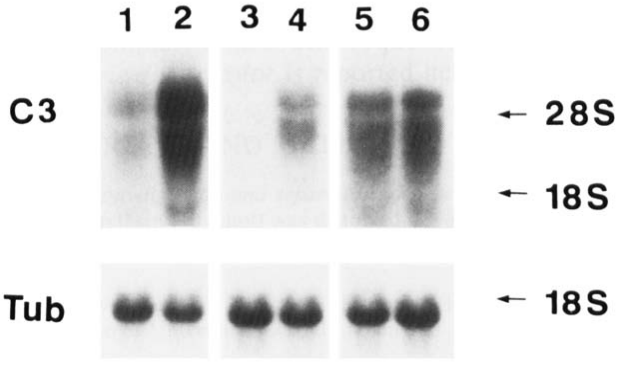

Fig. 3. The effect of $1\alpha,25(OH)_2D_3$ on the mRNA expression of C3 by ST2 cells (lanes 1 and 2), primary osteoblastic cells (lanes 3 and 4) and primary mouse hepatocytes (lanes 5 and 6). Cells were cultured for 2 days in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of 10^{-8} M $1\alpha,25(OH)_2D_3$. The positions of C3 and tubulin are indicated as C3 and Tub, respectively.

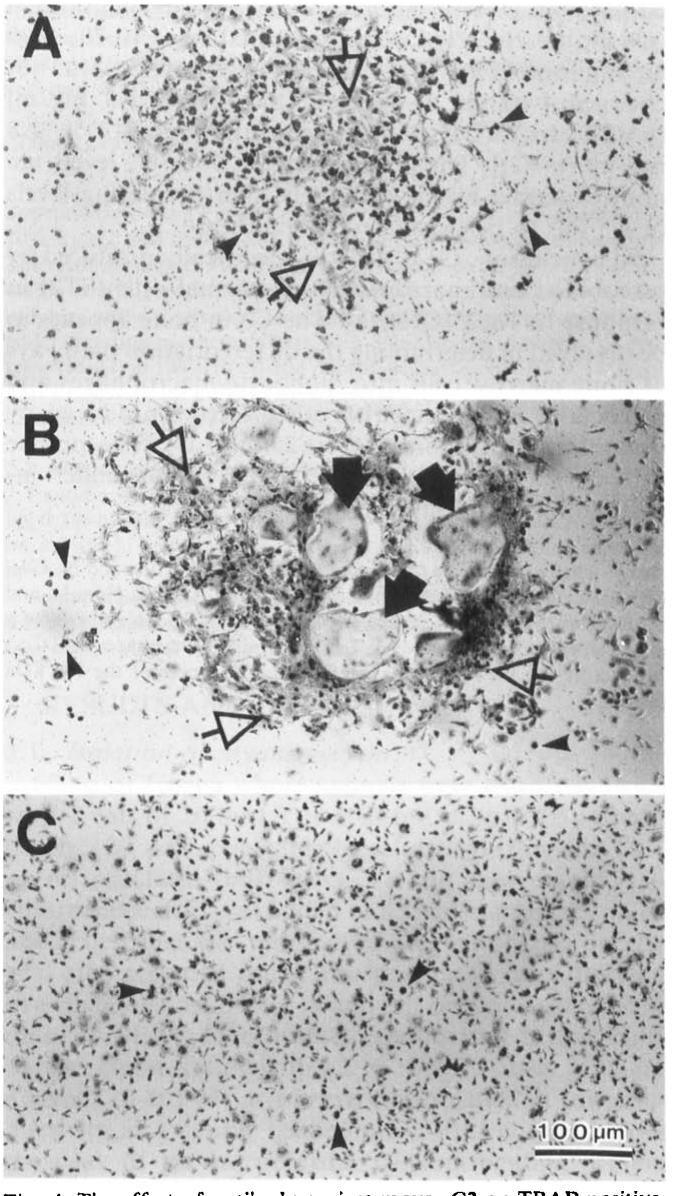

Fig. 4. The effect of antibody against mouse C3 on TRAP-positive osteoclast-like cell formation in mouse bone marrow cultures. Mouse marrow cells were cultured for 8 days without (A) or with 10^{-8} M $1\alpha,25(OH)_2D_3$ (B), or with an antibody against C3, together with 10^{-8} M $1\alpha,25(OH)_2D_3$ (C). The arrows indicate TRAP-positive mononuclear and multinucleated osteoclast-like cells (➡), typical NSE-positive monocyte-macrophages (►), and colonies of ALP-positive osteoblastic cells (⇢).

row cells into osteoclasts and monocyte-macrophages in concert with other $1\alpha,25(OH)_2D_3$-dependent factors.

It is important to determine whether the vitamin D-dependent production of C3 by osteoblastic cells and marrow-derived stromal cells is biologically relevant to bone metabolism. The circulating level of C3 in mice was about 1.5 mg/ml, which was at least 500- to 1000-fold higher than the C3 concentration in the conditioned media of ST2 cells and bone marrow cells treated with $1\alpha,25(OH)_2D_3$ for 3 days. However, when $1\alpha,25(OH)_2D_3$ was administered into vitamin D-deficient mice, the calvarial level of C3 greatly increased [vitamin D-deficient mice, 0.18, 0.24 ng/mg protein ($n=2$); $1\alpha,25(OH)_2D_3$-supplemented mice, 0.78, 0.90 ng/mg protein ($n=2$)], whereas the serum level was not changed [vitamin D-deficient mice, 1.4, 1.5 mg/ml ($n=2$); $1\alpha,25(OH)_2D_3$-supplemented mice, 1.5, 1.8 mg/ml ($n=2$)]. The reason why the bone C3 levels are not influenced by the extremely high circulating levels of C3 has to be elucidated in the future.

In conclusion, C3 is a factor specifically produced by osteoblasts and marrow-derived stromal cells (ST2) in response to $1\alpha,25(OH)_2D_3$. The C3 in bone appears to be involved in determining the differentiation pathways of bone marrow cells into monocyte-macrophages and osteoclasts in concert with other factors produced by osteoblasts in response to $1\alpha,25(OH)_2D_3$. The precise role of C3 in osteoclast differentiation is under investigation in our laboratory.

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