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SYNTHESIS OF 1,25-DIHYDROXYVITAMIN D_3 BY SPLEEN CELLS ISOLATED FROM TWO PATIENTS WITH MYELOFIBROSIS AND A NORMAL SUBJECT

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Summary—The ability to synthesise [^{3}H]1,25$(OH)_2D_3$ was studied in spleen cells incubated in short-term primary culture with [^{3}H]25$(OH)D_3$ which were isolated from two patients with idiopathic myelofibrosis and from one normal subject. Formation of a metabolite co-chromatographing with authentic 1,25$(OH)_2D_3$ on three different high-performance liquid chromatography systems was observed for cells from all three patients. [^{3}H]1,25$(OH)_2D_3$ synthesis was 0.37 and 1.6 fmol/10^6 cells/incubation for cells with a density below that of lymphocyte separating media (1.077 g/ml) for the two myelofibrotic patients respectively and 0.15 fmol/10^6 cells/incubation for the normal subject. The most likely cell type capable of this synthesis were those of the monocyte–macrophage lineage which would have been present in abnormally high numbers in the patients with myelofibrosis. However, the exact identity of the cell-type responsible could not be determined because of the heterogeneity of cell types present. The observation that spleen cells from two patients with myelfibrosis and from a normal accident victim could metabolise [^{3}H]25$(OH)D_3$ to its active form [^{3}H]1,25$(OH)_2D_3$ suggests a possible role for this metabolite in spleen haematopoiesis.

INTRODUCTION

Normally vitamin D_3 is sequentially hydroxylated to 25-hydroxyvitamin D_3 (25$(OH)D_3$) in the liver and then to either 24,25-dihydroxyvitamin D_3 (24,25$(OH)_2D_3$) or the active hormonal form 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) in the kidney. Synthesis of the latter is primarily stimulated by hypocalcaemia and parathyroid hormone and its calcium homeostatic actions are exerted in the intestine, bone and renal tubule after binding of 1,25$(OH)_2D_3$ to specific intracellular receptors [1]. More recently, studies have demonstrated that macrophages activated as a result of sarcoidosis [2] or peritonitis [3] or by treatment with gamma-interferon [4] or bacterial lipopolysacharides [5] *in vitro* can also synthesise 1,25$(OH)_2D_3$ from 25$(OH)D_3$. In the light of these observations we have examined whether cells isolated from the spleens of two patients with idiopathic myelofibrosis can also synthesise 1,25$(OH)_2D_3$. Myelofibrosis with myeloid metaplasia is a chronic proliferative disorder involving myeloid, megakaryocyte and erythroid series cells which is characterised by bone marrow fibrosis and splenomegaly with extramedullary hematopoiesis [6]. We have also examined a spleen from an accident victim to see if normal spleen cells can synthesise 1,25$(OH)_2D_3$.

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EXPERIMENTAL

Spleen biopsy samples were obtained following surgical removal of the spleens from two patients with idiopathic myelofibrosis both of whom had splenomegaly. Tissue from a third spleen was obtained immediately post-mortem from a healthy young accident victim. The spleen samples were finely chopped in phosphate buffered saline (PBS) to release the cells which were then separated into populations of low density white cells (< 1.077 g/ml) or high density white cells mixed with erythrocytes (> 1.077 g/ml) using Ficoll lymphocyte separating media (LSM) with a density of 1.077 g/ml (Flow Laboratories Ltd) centrifuged at 500 *g* for 20 min. These cells were then layered onto separate preformed 0–90% Percoll (Pharmacia) gradients (1.01–1.17 g/ml) [7] and centrifuged at 500 *g* for 20 min to further purify the low density white cells and to separate erythrocytes from the high density white cells.

Total cell numbers were estimated using a haemocytometer and Jenner–Giemsa stained cytocentrifuge preparations made with approximately 40,000 cells per slide were used to examine cell morphology.

To assess 25$(OH)D_3$ metabolism cells were resuspended in 10 ml of serum free RPMI-1640 medium containing 2 mmol/l glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 0.4 mmol/l HEPES buffer (Flow Laboratories Ltd) and incubated with 25 pg/ml [^{3}H]25$(OH)D_3$ (2.5×10^4 dpm/ml, 176 Ci/mmol, Amersham) at 37°C in an atmosphere of 95% air 5%

CO_2 for 16 h. The incubations were terminated by adding 10 ml of acetonitrile followed by preliminary purification on silica Sep-Pak cartridges (Waters Associates) [8] followed by high-performance liquid chromatography (HPLC) analysis. An aliquot of each sample was assessed initially on a normal phase HPLC column (Zorbax-SIL) eluted with a mobile phase of *n*-hexane:propan-2-ol:methanol, 110:6:4 or 110:4:4 by vol at 2 ml/min. Samples were also analysed on the normal phase column and on a reverse phase (Zorbax-ODS) column eluted with 5% propan-2-ol in dichlormethane (1.5 ml/min) and 15% water in methanol (1.5 ml/min) respectively to confirm the identity of the metabolic product. For each analysis eluant fractions were collected and the radioactivity associated with the substrate and $1,25(OH)_2D_3$ was estimated by liquid scintillation counting. Standard $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ (donated by Dr M. Uskokovic) were also chromatographed and the retention times of these markers were determined by u.v. absorption at 265 nm.

RESULTS

The formation of a tritiated metabolite of $[^3H]25(OH)D_3$, which co-chromatographed on the hexane-based normal phase HPLC with authentic $1,25(OH)_2D_3$, was demonstrated for spleen cells isolated from two myelofibrotic patients and by cells from a normal spleen (Fig. 1). Further chromatography of the $[^3H]1,25(OH)_2D_3$-like material on a second normal phase and on a reverse phase HPLC system also demonstrated co-chromatography with the authentic metabolite (Fig. 2), thus providing good evidence for its identity.

The density gradient separations yielded four major bands of spleen cells not all of which showed evidence of $[^3H]1,25(OH)_2D_3$ synthesis (Table 1). Unfortunately it was not possible to identify exactly which cells were responsible for $[^3H]25(OH)D_3$-1-hydroxylation because of the heterogeneity of cell types present and a tendency to aggregate. The lower denisty (< 1.077 g/ml) white cells isolated using LSM from both myelofibrotic spleens were separated into two further populations on 0–90% Percoll gradients, of which only one synthesised $[^3H]1,25(OH)_2D_3$. This band contained many myeloid series cells whereas the cells which did not metabolise $[^3H]25(OH)D_3$ were predominantly small lymphocytes. The higher density (> 1.077 g/ml) myelofibrotic spleen cells from the initial LSM preparation were separated into populations of erythrocytes or white cells on a 0–90% Percoll gradient. Of which, only the white cells formed $1,25(OH)_2D_3$ and although these were predominantly lymphocytes and polymorphs there were also other myeloid series cells present. In contrast only low density cells from the normal spleen synthesised $[^3H]1,25(OH)_2D_3$. This population of cells also contained monocyte macrophage series cells in addition to lymphocytes.

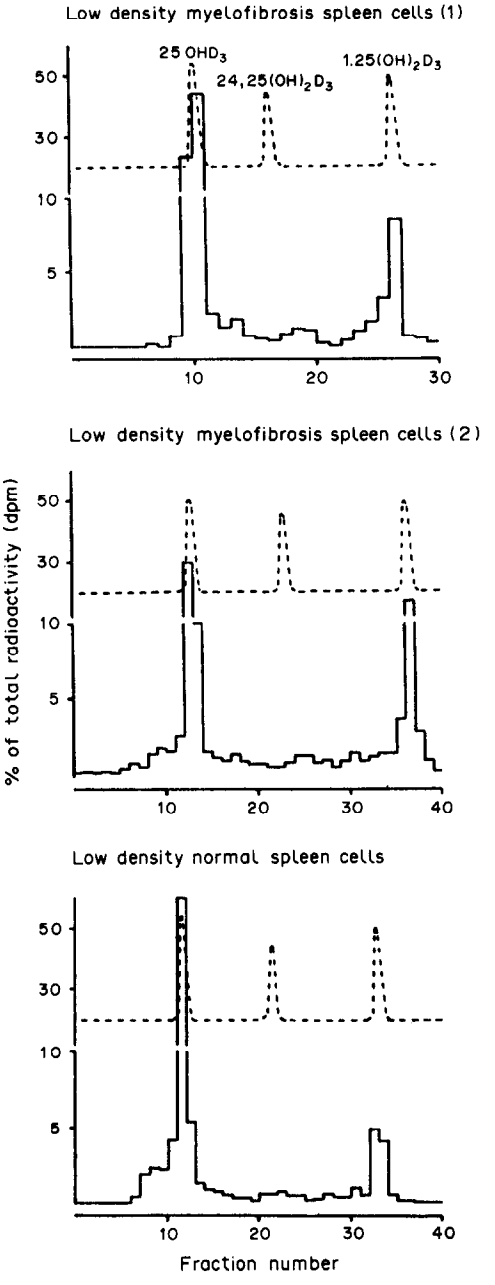

Fig. 1. Metabolism *in vitro* of $[^3H]25(OH)D_3$ by low density (< 1.077 g/ml,) spleen cells isolated on density gradients from myelofibrosis patients 1 (upper panel) and 2 (middle panel) and from a normal spleen (lower panel). Bars represent the amount of tritiated material in half minute fractions obtained by HPLC analysis of cell extracts on a Zorbax-SIL column developed with hexane, propan-2-ol, methanol (110:6:4, upper panel; 110:4:4, mid and lower panels) at 2 ml/min. Standard $25(OH)D_3$, $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ were used as markers and detected by u.v. absorption at 265 nm (dotted lines).

DISCUSSION

The results demonstrate that spleen cells from two myelofibrotic subjects and from an apparently healthy accident victim could metabolise $25(OH)D_3$

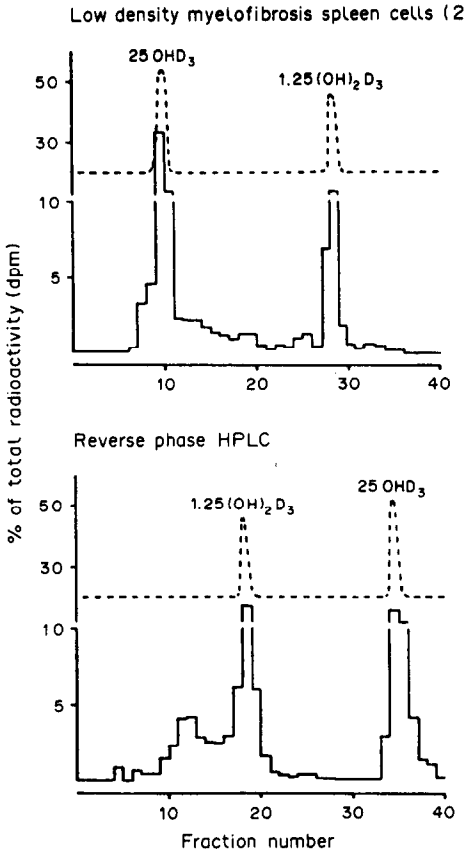

Fig. 2. Metabolism *in vitro* of [^{3}H]25(OH)D_3 by low density (< 1.077 g/ml) spleen cells isolated on density gradients from myelofibrosis patient 2. Bars represent the amount of tritiated material in half minute fractions obtained by HPLC analysis of cell extracts on a Zorbax-SIL column developed with 5% propan-2-ol in dichloromethane (1.5 ml/min, upper panel) and on a Zorbax-ODS reverse phase column developed with 15% water in methanol (1.5 ml/min, lower panel). Standard 25(OH)D_3, and $1,25(OH)_2D_3$ were used as markers and detected by u.v. absorption at 265 nm (dotted lines).

to its biologically active form, $1,25(OH)_2D_3$, after isolation on density gradients and incubation in short-term primary culture. Low density cells (< 1.077 g/ml) which formed $1,25(OH)_2D_3$ from the normal spleen were predominantly lymphocytes and myeloid series cells including many monocytes and macrophages. Similarly, the lower density cells which formed $1,25(OH)_2D_3$ from the myelofibrosis spleens were predominantly lymphocytes and myeloid series cells including monocytes, macrophages, polymorphs and myeloid series precursors. In contrast, the higher density (> 1.077 g/ml) myelofibrosis cells that formed $1,25(OH)_2D_3$ were predominantly lymphocytes and polymorphs with fewer myeloid series cells of other types. In all three patients the cells incapable of 25(OH)D_3 metabolism were predominantly small lymphocytes or erythrocytes.

The observations that sarcoidosis-activated alveolar macrophages and peritonitis-activated peritoneal macrophages synthesise $1,25(OH)_2D_3$ [2, 3] suggests that of the possible candidates spleen monocyte–macrophage series cells were the most likely cell-type to form this metabolite. Apparent differences in the numbers of myeloid series cells, particularly of monocytes and macrophages, may explain why the low density myelofibrosis cells appeared to form more $1,25(OH)_2D_3$ than cells isolated from the normal spleen. Similarly it may explain why the higher density myelofibrosis cells formed comparatively less $1,25(OH)_2D_3$ than the lower denisty cells. However, it is also possible that another cell type present in abnormally high numbers due to the increased haematopoiesis may have been responsible for the $1,25(OH)_2D_3$ synthesis by high density spleen cells from the myelofibrosis patients.

The formation of $1,25(OH)_2D_3$ by macrophages and the presence of specific $1,25(OH)_2D_3$ receptors in monocytes and activated lymphocytes [9] suggest that this metabolite has significant haematolymphopoietic functions in addition to regulating intestinal calcium absorption. *In vitro*, $1,25(OH)_2D_3$ has also been shown to promote the differentiation of monocytes into macrophages and the fusion of macrophages into multinucleated giant cells [10, 11]. Furthermore it enhances macrophage function in non-specific immune processes such as phagocytosis and inhibits interleukin-2 production by activated T-lymphocytes [9]. These observations also suggest that the actions of $1,25(OH)_2D_3$ on cells of the haematopoietic system could be regulated by locally synthesised rather than serum $1,25(OH)_2D_3$ formed in the kidney. These ideas are further supported by the observation in our study that cells from a normal spleen could synthesise $1,25(OH)_2D_3$. This study also suggests that there may be an increased capacity for the spleen to synthesise $1,25(OH)_2D_3$ in myelofibrosis as a result of the increased numbers of myeloid series cells and the increase in the size of the spleen.

Table 1. Summary of $1,25(OH)_2D_3$ synthesis by spleen cells isolated on density gradients from 2 myelofibrosis patients and a normal accident victim

	$1,25(OH)_2D_3$ Synthesis (fmol/10^6 cells/incubation)			
Cell density (g/ml)	< 1.077		> 1.077	
Myelofibrosis 1	0.38	0	0.13	0
Myelofibrosis 2	1.58	0	0.65	0
Normal	0.15	—	0	0
Main cell types	Lymphocytes and myeloid series	Small lymphocytes	Lymphocytes and polymorphs	Red blood cells

Recently it was proposed that $1,25(OH)_2D_3$ could modulate the activity of bone marrow cells in myelofibrosis by inhibiting the proliferation of megakaryocytes, which promote collagen synthesis and by increasing the number of activity of macrophages that mediate its degradation [12]. Attempts at treating myelofibrosis with $1,25(OH)_2D_3$ have had some success [13–15] as has treatment of a number of patients in which vitamin D deficiency appeared to be related to the development of bone marrow fibrosis and myeloid metaplasia in spleen [16]. Our study suggests that the development of myelofibrosis does not result from an inability to synthesise $1,25(OH)_2D_3$, although its synthesis would be linked to the availability of the substrate $25(OH)D_3$ and thus would be diminished by vitamin D deficiency.

Development of bone marrow fibrosis and extramedullary haematopoiesis in spleen could also be related to receptor abnormalities for $1,25(OH)_2D_3$. If this were the case then fibrosis could develop in an unregulated fashion ultimately leading to displacement of haematopoietic tissue to the spleen. If this tissue were itself responsible for $1,25(OH)_2D_3$ synthesis then its remote production would further facilitate bone marrow fibrosis because high enough concentrations of $1,25(OH)_2D_3$ may not be achieved within the bone marrow for local effects to be mediated.

In summary we have demonstrated the ability of spleen cells isolated from a normal subject and from two patients with idiopathic myelofibrosis to synthesise $1,25(OH)_2D_3$ from $25(OH)D_3$. Although we were unable to determine the exact cell-type responsible they were most likely to be monocyte-macrophage series cells.

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REFERENCES

1. Haussler M. R. and McCain T. A.: Basic and clinical concepts related to vitamin metabolism and action. *New Engl. J. Med.* **297** (1977) 974–983.
2. Adams J. S., Sharma O. P., Gacad M. A. and Singer F. R.: Metabolism of 25-hydroxyvitamin D_3 by cultured pulmonary alveolar macrophages in sarcoidosis. *J. Clin. Invest.* **72** (1983) 1856–1860.
3. Hayes M. E., O'Donoghue D. J. O., Ballardie F. W. and Mawer E. B.: Peritonitis induces the synthesis of 1α,25-dihydroxyvitamin D_3 in macrophages from CAPD patients. *FEBS Letts* **220** (1987) 307–310.
4. Koeffler H. P., Reichel H., Bishop J. E. and Norman A. W.: Gamma-interferon stimulates production of 1,25-dihydroxyvitamin D_3 by normal human macrophages. *Biochem. Biophys. Res. Commun.* **127** (1985) 596–603.
5. Reichel H., Koeffler P., Bishop J. E. and Norman A. W.: 25-Hydroxyvitamin D_3 metabolism by lipopolysaccharide-stimulated normal human macrophages. *J. Clin. Endocr. Metab.* **64** (1987) 1–9.
6. Kahn A., Bernard J. F., Cottreau D. and Boivin P.: A deficient G-6PD varient with hemizygous expression in blood cells of a woman with primary myelofibrosis. *Humangenetik* **30** (1975) 41–46.
7. Schumacher M., Schafer G., Holstein A. F. and Hilz H.: Rapid isolation of mouse leydig cells by centrifugation in Percoll density gradients with complete retention of morphological and biochemical integrity. *FEBS Letts* **91** (1978) 333–338.
8. Fraher L. J., Adami S., Clemens T. L., Jones G. and O'Riordan J. L. H.: Radioimmunoassay of 1,25-dihydroxyvitamin D_2: Studies on the metabolism of vitamin D_2 in man. *Clin. Endocr.* **19** (1983) 151–156.
9. Manolagas S. C., Provvedini D. M. and Tsoukas D. Interactions of 1,25-dihydroxyvitamin D_3 and the immune system. *Molec. Cell. Endocr.* **43** (1985) 113–122.
10. Abe E., Miyaura C., Tanaka H., Shaina Y., Kuribayashi T., Nishii Y., DeLuca H. F. and Suda T.: 1α,25-dihydroxyvitamin D_3 promotes fusion of mouse alveolar macrophages both by direct mechanisms and by spleen cell-mediated indirect mechanisms. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 5583–5587.
11. Bar-Shavit Z., Teitelbaum S. L., Reitsam P., Hall E., Trial J. and Kahn A.: Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natn. Acad. Sci. U.S.A.* **80** (1983) 5907–5911.
12. McCarthy D. M., Hibbin J. A. and Goldman J. M.: A role for 1,25-dihydroxyvitamin D_3 in control of bone marrow collagen deposition. *Lancet* **i** (1984) 78–80.
13. Arlet P. H., Nicodene R., Adoue D., Larregain-Fournier D., Delsol G. and Le Tallec Y.: Clinical evidence for 1,25-dihydroxycholecalciferol action in myelofibrosis. *Lancet* **i** (1984) 1013–1014.
14. Petrini M., Cecconi N., Azzara A., Ambrogi F. and Grassi B. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) in the treatment of idiopathic myelofibrosis. *Br. J. Haemat.* **64** (1986) 624–625.
15. McKinley R., Kwan Y. L., Ford D., Lam-Po-Tong P. R., Mason R. S. and Manoharan A.: Clinical and laboratory studies of 1,25-dihydroxy-cholecalciferol in myelofibrosis. *Br. J. Haemat.* **65** (1987) 624–625.
16. Yetgin S. and Ozsoylu S.: Myeloid metaplasia in vitamin D deficient rickets. *Heamatology* **28** (1982) 180–185.