Volume 323, number 1,2, 89–92 FEBS 12477 May 1993

Vitamin D binding protein is produced by human monocytes

A. Sabbatini[a], M. Petrini[a], L. Mattii[a], P. Arnaud[b] and R.M. Galbraith[b]

[a]*U.O. Ematologia, Clinica Medica 1, University of Pisa, Via Roma 67, 56100 Pisa, Italy* and [b]*Department of Microbiology and Immunology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425-2230, USA*

Received 25 February 1993

The expression of the DBP (vitamin D binding protein) gene was investigated in monocytes and in peripheral blood lymphocytes. DBP message was amplified through 35 cycles of PCR amplification using specific oligonucleotide primers. PCR products of the expected size were further identified by Southern blotting using a specific DBP probe. No expression of the DBP gene could be detected in peripheral blood lymphocytes, nor in the monocyte-derived U 937 cell line. In contrast, message for DBP was identified in monocytes activated with lipopolysaccharide when analyzed between 6 and 10 h following stimulation. These results suggest that the temporal expression of the DBP gene could play a major role in the activation of monocytes by 1-25$(OH)_2D_3$.

Vitamin D binding protein; Monocyte; Gene expression; PCR

1. INTRODUCTION

Vitamin D binding protein (DBP), a 56,000 m.wt. α2-globulin, represents the major serum carrier for vitamin D metabolites [1,2]. In addition, it binds actin [3,4] and facilitates depolymerization of filamentous F actin to the globular (G) form [5]. DBP also binds unsaturated fatty acids [6] and, at the cellular level, it has been shown to enhance neutrophil chemotactic activity of C5a and C5a des Arg [7,8]. Preincubation of DBP with 1,25$(OH)_2D_3$ completely abolishes its cochemotactic activity [9], suggesting that binding of vitamin D may modify different functions of DBP.

In the early eighties it was shown, by indirect immunofluorescence, that DBP is present on intact lymphocytes [10]. The protein is expressed on B lymphocyte membranes and closely associated with surface immunoglobulins [11]. DBP is also expressed on monocytes [12]. Further studies suggested that DBP is selectively related, on B lymphocyte membranes, to surface IgM, although it was not found on lymphocyte from B chronic lymphocytic leukemia carrying different subclasses of immunoglobulins [13]. Resting T lymphocytes are mostly negative for surface DBP but the protein is expected to be present on activated T lymphocytes where it has been demonstrated to be related to the Fc γ receptor [14,15]. It has been demonstrated that the protein is hidden in part in the membrane of circulating monocytes [16]. This suggests that DBP could play several roles at the cell membrane level in addition to modulate the effects of vitamin D on lymphocytes [17]. This is confirmed by the finding that an antiserum against DBP inhibits human natural killer activity [18]. Thus DBP seems to be possessing a major potential role in the immune function. In this view, it will be quite surprising that any immunocompetent cell is not involved in DBP synthesis. It has been generally accepted that DBP is synthesized by liver [2]. In all rat tissues examined low levels of DBP RNA transcripts could be detected by polymerase chain reaction [19]. In humans extraliver synthesis of DBP has not been demonstrated. By Northern blot analysis, DBP gene expression was not detected in several cells, including B lymphocytes, resting and stimulated T lymphocytes, the B lymphoblastoid cell line GM 1500, the T-cell lymphoma Sup T1 cell line and placental cytotrophoblast cells [20]. The monoblastic human cell line U 937 was also checked for DBP expression both by Northern blot and by PCR but no specific signal was found [20]. As monocytes are involved in synthesis of 1,25$(OH)_2D_3$ [21] and show major reactivity to anti DBP antibodies [12] we decided to reevaluate the expression of DBP in resting and LPS activated human normal monocytes. Monocyte depleted human peripheral blood lymphocytes and U 937 cells were also examined.

Correspondence address: M. Petrini, U.O. Ematologia, Clinica Medica I, Via Roma 67, 56100 Pisa, Italy. Fax. (39) (50) 502617.

Abbreviations: DBP, vitamin D binding protein; PCR, polymerase chain reaction, LPS, lipopolysaccharides; dNTPs, triphosphate nucleotides; SDS, sodium dodecylsulphate; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; FCS, fetal calf serum.

2. MATERIALS AND METHODS

2.1. *Cells*

Hep 3B cells (human hepatoma cell line) were routinely cultured in Eagle's minimal essential medium, supplemented with L-glutamine (2

mM), penicillin (100,000 U/l), streptomycin (100 mg/l), fungizone (250 mg/l) and 10% fetal calf serum (FCS), and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air.

U 937 cells were continuously cultured at 37°C, 5% CO_2 in RPMI 1640 supplemented with 10% FCS, 1% glutamine 200 mM and gentamycin 100 mg/ml. Cells were sub-cultured twice a week and resuspended at a concentration of 5×10^5/ml. Cells were examined in the log growth phase

Human lymphocytes were recovered from peripheral blood obtained from healthy donors after Lymphoprep purification. Mononuclear cells were recovered and allowed to adhere to plastic dishes at the concentration of 10^7/dish in PBS with Ca^{2+} and Mg^{2+} plus 20% FSC at 37°C (5% CO_2) for 1 h. The supernatant was recovered and cells resuspended in fresh PBS supplemented with 20% FCS and allowed to adhere for an additional 60 min Monocytes in the recovered cell populations were <1% as resulted by cytofluorimetric analysis using CD 14 monoclonal antibody. Lymphocytes were then pulsed with LPS 10 μg/ml.

Monocytes were recovered from healthy donors from mononuclear cells partially purified by differential centrifugation with an apparatus for cyto-apheresis. Cells representing >90% mononuclear were stored in liquid nitrogen in 10% DMSO. The experiments were conducted with cells washed in PBS with Ca^{2+} and Mg^{2+} supplemented with 20% FCS and allowed to adhere in plastic dishes at a concentration of 15×10^6/ml. After 1 h at 37°C the supernatant was discarded and the dishes were washed three times with 37°C PBS. Adherent cells were >80% monocytes as shown by cytofluorimetric analysis with CD14 monoclonal antibody and cytochemical reaction for alpha naphthyl butyrate esterase. 10 ml of PBS supplemented with 20% FCS were then added to the dishes together with LPS at a final concentration of 10 μg/ml. Both lymphocyte and monocyte enriched populations were tested for specific mRNA after 0, 3, 6, 10, 24, 30 h following the LPS stimulation.

2.2. *RNA isolation and reverse transcription (RT)*

Total RNA from human monocytes, lymphocytes, Hep 3B and U 937 cell lines was isolated following the acid guanidinium thiocyanate–phenol/chloroform method [22]

Total RNA was then reverse-transcribed into cDNA as follows: 2 μl of each sample (200 ng) were mixed with a solution containing 5 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 1 mM each dNTP (Perkin-Elmer), 2.5 mM random hexamers (Perkin-Elmer-Cetus), 1 U/μl RNase inhibitor (Perkin-Elmer-Cetus), 2.5 U/μl reverse transcriptase (Perkin-Elmer-Cetus) in a final volume of 20 μl.

2.3. *Oligonucleotide synthesis*

The downstream (5′-GTGCACACAAAAACGTCC-3′) and upstream (5′-GTCCTGCTGTACCTCTGC-3′) primers for human DBP were designed to amplify a 375 bp fragment and span exon–intron boundaries in order to prevent contamination of the amplification product of mRNA by amplified genomic DNA.

Oligonucleotides were synthesized on an automated oligonucleotide synthesizer (Applied Biosystems, Model #380B, Foster City, CA) and desalted through a Sephadex G-25 column. Purity was assessed by electrophoresis on a 15% polyacrylamide gel containing 7 M urea

2.4. *PCR*

The PCR reaction was carried out in a final concentration of 2 mM $MgCl_2$, 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 0.2 mM dNTP, 2.5 U/100 ml AmpliTaq DNA polymerase (Perkin-Elmer Cetus), and 0.15 mM of each primer. The mixture was overlayed with mineral oil and amplified using a DNA thermal cycler (Perkin-Elmer Cetus). The amplification program was set to denature at 95°C for 1 min, anneal at 55°C for 1 min and extend at 70°C for 1 min for a total of 35 cycles.

The absence of contaminants was routinely checked by running control samples that contained no RNA, no reverse transcriptase or only the PCR reaction buffer.

2.5. *DBP probe*

A clone was isolated from a human liver cDNA expression library made in λgt11 (a gift from Frank Gonzales, Laboratory of Molecular Carcinogenesis, National Cancer Institute) using specific antiDBP antibodies. The insert was excised from the purified phage using *Eco*RI digestion, and the four fragments obtained (140, 310, 505, 776 bp, respectively) were subcloned individually in the phagemid p-Bluescript SKII (Stratagene) and sequenced using the dideoxy method of Sanger. Their sequence showed to be identical to that published in the literature [23,24].

The 505 bp fragment was used as a probe for the hybridization procedure. The H-DBP probe was biotinylated by nick translation and the reaction was performed according to the 'non-isotopic system for DNA probe labeling' (Oncor, Gaithersburg, MD).

2.6 *Southern blot analysis*

8 μl of each PCR product were electrophoresed in a 5% acrylamide gel (75 volts for 90 min), stained with ethidium bromide and photographed. After an incubation in a 0.25 N final concentration of sodium hydroxide (10 min at room temperature), samples were blotted onto a nylon membrane (Oncor, Gaithersburg, MD) which was baked for 1 h at 80°C. Hybridization was performed overnight at 42°C using 25 ng/ml final concentration of biotinylated HDBP probe. The filter was then washed twice in 0.16 × SSC and 0.1% SDS at room temperature for 5 min, once at 50°C for 30 min in 0.16 × SSC, 0.1% SDS and Wash Enhancer (80 μl/100 ml) (Oncor, Gaithersburg, MD) and two more times in 1 × SSC at room temperature for 5 min. The filter was then developed with the 'Oncor non-isotopic system for detection' based on the biotin/streptavidin/alkaline-phosphatase interaction.

2.7. *Immunofluorescence*

The presence of DBP on the membrane surface of cells was examined by immunofluorescence. Briefly, cells were washed in cold PBS containing 0.1% sodium azide and then incubated for 30 min at 4°C with 10% of rabbit IgG to human DBP (Dako) After appropriate washing, cells were incubated with a fluoresceinated swine anti rabbit Ig antiserum. After appropriate washes cells were examined under a fluorescence microscope and a cytofluorimetric apparatus. Appropriate negative controls were performed. Specificity of the rabbit anti human DBP antibody has been previously reported [11]. In particular, no cross reactivity between anti human DBP Ig and FCS was detected by Outcherlony double diffusion method. Results were expressed as percentage of positive cells.

Table I

Sequence of the oligonucleotides used as primers for PCR amplification

Oligonucleotides	Nucleotide position	Polarity	Sequences (5′ → 3′)	Ref.
1 H-DBP primer I	589–606	(+)	GTCCTGCTGTACCTCTGC	[25]
2 H-DBP primer II	963–946	(−)	GTGCACACAAAAACGTCC	[25]

3. RESULTS

DBP was detected by immunofluorescence on >80% of monocyte enriched human peripheral blood cells and in 20% monocyte depleted cells. DBP positivity appeared as minute bright granules on cell membranes. U 937 cells were negative for DBP.

Total RNA obtained from Hep 3B and U 937 cell lines and from human lymphocytes and monocytes (before and after LPS stimulation) was subjected to RT/PCR analysis to study the human DBP gene expression. Hep 3B cell line was selected as a positive control since it has been shown to express the DBP gene [24] and secrete the DBP protein [25].

As described in section 2, the oligonucleotides were designed so that the mRNA amplification product (375 bp) would not be confused with any genomic DNA contamination.

The PCR products were first analyzed by gel electrophoresis and ethidium bromide staining. At this stage monocytes after 6 and 10 h from LPS stimulation and Hep 3B were positive for the 375 bp fragment when all the other samples showed a number of faint, non specific bands but not that of the expected size (Fig. 1). Samples were then blotted onto a nylon membrane and hybridized with the biotinylated human DBP probe to determine the presence of a specific amplification product. This procedure demonstrated that the band found in monocytes after a 6 and 10 h stimulation was specific for DBP (Fig. 2). Resting and monocytes stimulated for 3, 24, 30 h, resting and stimulated lymphocytes and U 937 cells were completely negative.

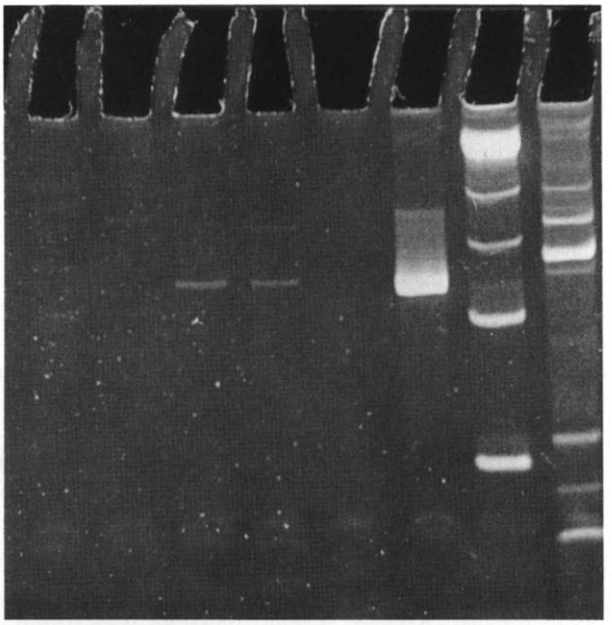

Fig. 1. Ethidium bromide-stained 5% acrylamide gel for DBP PCR products (expected size 375 bp). Lanes 1–5, LPS activated monocytes after, respectively, 0, 3, 6, 10, 24 h of incubation. Lane 6, Hep 3B cell line. Lane 7, molecular weight marker (*Eco*RI digested p-Bluescript SK II (2,961 bp)) containing the four subcloned fragments of human DBP cDNA (140, 310, 505, 776, respectively). Lane 8, U 937 cell line.

4. DISCUSSION

DBP is present on the surface of several human cells including native B lymphocytes and monocytes. At the present time, no definitive data showing the origin of

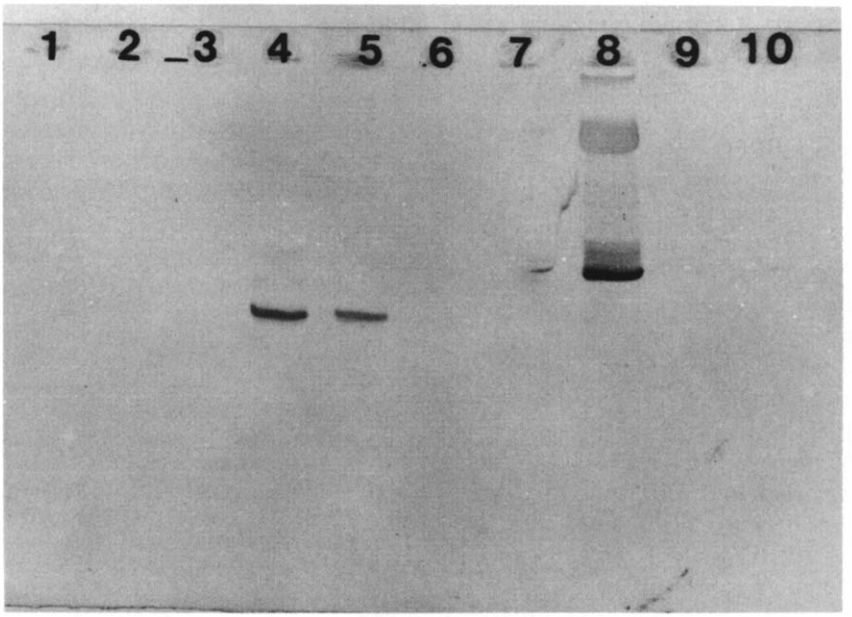

Fig. 2. Southern blot of PCR products for DBP. Products of PCR were run in a 5% acrylamide gel, electrophoretically transferred on a nylon membrane and hybridized with a biotinylated *Eco*RI fragment (505 bp) of DBP cDNA. Lane 1, empty. Lanes 2–7, monocytes activated with LPS for 0, 3, 6, 10, 24, 30 h, respectively. Lane 8, the four fragments obtained after *Eco*RI digestion of human DBP (140, 310, 505, 776) were run in this lane. As expected only the 505 bp fragment hybridized with the biotinylated specific probe. Lanes 9–10, empty.

membrane DBP have been reported. In a previous work we were not able to show any specific binding of DBP on native peripheral blood cells [11]. Since then, albumin, which is structurally and evolutionarily related to DBP, has been shown to bind with specificity, saturability and reversibility to a number of cells [26,27]. The origin of DBP bound on human peripheral cells is unknown. By contrast, it has been clearly demonstrated that blood peripheral cells as well as several cell lines including U 937 do not express any message for this protein [20]. Although the positivity of U 937 by immunofluorescence had been related to a cross reactivity of the anti human DBP with DBP present in fetal calf serum [20], in our experiments U 937 was completely negative suggesting that this cross reactivity was not present in our antiserum. In agreement with a previous report [20] we could not detect any message following PCR amplification in U 937 cells nor in monocyte depleted human peripheral blood lymphocytes or resting monocyte enriched cells.

In contrast, a clear specific message was found in human monocytes (but not in lymphocytes) after LPS stimulation. This positivity was present only at 6 and 10 h after LPS stimulation. Thus a possible synthesis of DBP may have happened during a very restricted period of time following monocyte activation. It is logical to postulate that DBP present on monocytes derives at least partially from this synthesis. DBP has been postulated to prevent several activities of vitamin D_3, and, in this respect, $1,25(OH)_2D_3$ is known to play a major role on monocyte/macrophage activities and especially during the process of differentiation [28]. It is therefore possible that synthesis of DBP during the early phase of monocyte activation could protect cells from vitamin D_3 activity. In this respect, γ interferon-induced production of $1,25(OH)_2D_3$ is detectable after 24 h of culture. Thus these phenomena appear temporally separated during cell activation. At the beginning of the activation production of DBP for a short period of time could prevent the vitamin D_3 effect resulting in monocyte differentiation; later, a suppression of DBP synthesis together with the synthesis of $1,25(OH)_2D_3$ in monocytes induced by γ interferon could contribute to control both monocyte and lymphocyte functions. The fact that DBP is synthesized by monocytes does not mean that the bound protein does not originate from plasma, although the deep location of DBP in the monocyte membranes [16] is not in favor of the latter. In contrast, this could be the case for lymphocytes where specific mRNA is not present even following PCR amplification. However, our results do not rule out the possibility that DBP synthesis could occur in lymphocytes activated by different mechanisms.

Acknowledgements: This research was supported in part by AIRC, Milan.

REFERENCES

[1] Daiger, S.P., Schanfield, M.S. and Cavalli Sforza, L.L. (1975) Proc. Natl. Acad. Sci. USA 72, 2076–2080.
[2] Putnam, F.W. (1977) The Plasma Proteins: Structure, Function and Genetic Control (Putnam, F.W. Ed.) vol. 3, pp. 333–357, Academic Press, New York.
[3] Van Baelen, H., Bouillon, R. and De Moor, P. (1980) J. Biol. Chem. 255, 2270–2272
[4] Haddad, J G. (1982) Arch Biochem. Biophys 213, 538–544.
[5] Korn, E.D. (1982) Physiol. Rev. 62, 672–737.
[6] Williams, M.H., Van Alstyne, G. and Galbraith, R.M. (1988) Biochem. Biophys. Res. Commun. 153, 1019–1024
[7] Kew, R.R and Webster, R O (1988) J. Clin. Invest. 82, 364–369.
[8] Perez, H.D., Kelly, E., Chenoweth, D. and Elfman, F (1988) J. Clin. Invest. 82, 360–363.
[9] Petrini, M., Azzara, A , Carulli, G , Grassi, B , Ambrogi, F and Galbraith, R.M. (1991) J. Endocrinol. Invest 14, 405–408
[10] Constans, J., Oksman, F. and Viau, M. (1981) Immunol. Lett. 3, 159.
[11] Petrini, M., Emerson, D.L. and Galbraith, R M. (1983) Nature 306, 73–74.
[12] Petrini, M., Galbraith, R M., Werner, P.A.M , Emerson, D.L. and Arnaud, P. (1984) Clin Immunol. Immunopathol. 31, 282–295.
[13] Petrini, M , Carulli, G., Ambrogi, F. and Galbraith, R.M. (1986) N. Engl J. Med. 314, 1514–1515
[14] Petrini, M., Galbraith, R.M., Emerson, D.L., Nel, A.E. and Arnaud, P. (1985) J. Biol Chem. 260, 1804–1810.
[15] Machii, T , Kimura, H., Ueda, E., Chujo, T., Morita, T., Katagiri, S., Tagawa, S. and Kitani, T (1986) Acta Haematol. 75, 26–29.
[16] McLeod, J.F., Kowalsky, M.A and Haddad, J.G. (1986) Endocrinology 119, 77–83.
[17] Vanham, G., Van Baelen, H., Tan, B.K. and Bouillon, R. (1988) J Steroid. Biochem 29, 381–386.
[18] Chujo, T., Machii, T., Tagawa, S., Kuratsune, H., Ueda, E , Kimura, H. and Kitani, T. (1989) Clin. Exp. Immunol. 76, 154–158
[19] McLeod, J F. and Cooke, N.E. (1989) J. Biol Chem. 264, 21760–21769.
[20] Gouth, M., Murgia, A , Smith, R.M., Prystowsky, M.B., Cooke, N.E. and Haddad, J.G. (1990) Endocrinology 127, 2313–2321.
[21] Koeffler, H.P., Reichel, H., Bishop, J E. and Norman, A.W. (1985) Biochem Biophys. Res. Commun 127, 596–603.
[22] Chomczynski, P. and Sacchi, N (1987) Anal. Biochem. 162, 156–159.
[23] Yang, F., Brune, J.L., Naylor, S.L., Cupples, R.L., Naberhaus, K H. and Bowman, B.H. (1985) Proc Natl Acad. Sci USA 82, 7994–7998
[24] Cooke, N.E and David, E.V. (1985) J. Clin. Invest 76, 2420–2424.
[25] Haddad, J.G., Aden, D.P. and Kowalski, M.A. (1983) J. Biol. Chem 258, 6850–6854.
[26] Ockner, R.K., Weisiger, R A. and Gollan, J.L (1983) Am. J Physiol. 245, G13–G18.
[27] Ghitescu, L.A., Fixman, M., Simionescu, M. and Simionescu, N. (1986) J. Cell. Biol 102, 1304–1311.
[28] Suda, T. (1989) Proc. Soc. Exp Biol. Med. 37, 214–220.