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Regulation of TNF-α by 1α,25-dihydroxyvitamin D_3 in human macrophages from CAPD patients

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Background. We have previously reported that 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] accumulates in the dialysis fluid of uremic patients treated by continuous ambulatory peritoneal dialysis (CAPD). It has been reported that this metabolite regulates the production of cytokines by monocytes/macrophages. Since tumor necrosis factor-α (TNF-α) initiates an inflammatory cascade during peritonitis, the aim of the present study was to investigate the effect of 1α,25$(OH)_2D_3$ on the production of TNF-α by human peritoneal macrophages (HPMs).

Methods. HPMs were obtained from patients on CAPD. Cells were incubated with various concentrations of 1α,25$(OH)_2D_3$, 1α,24(S) dihydroxyvitamin D_2 [1α,24(S)$(OH)_2D_2$] or 25-hydroxyvitamin D_3 (25-OH-D_3) for 16 hours. This was followed by lipopolysaccharide (LPS; 1 μg/mL) incubation for 2.5 to 6 hours. TNF-α protein production was determined by enzyme-linked immunosorbent assay. TNF-α mRNA was assayed by the reverse transcriptase-polymerase chain reaction procedure, using internal synthetic mRNA standards for quantitative results.

Results. Incubation of HPMs with 1α,25$(OH)_2D_3$ prior to stimulation with LPS dose dependently inhibited the expression of TNF-α on both mRNA and protein levels. Similar results were obtained with the less calcemic vitamin D_2 analogue 1α,24(S)$(OH)_2D_2$. Incubation of HPMs with 25-OH-D_3 also revealed a down-regulation of TNF-α expression. Since this down-regulatory effect was blocked by ketoconazole, it is likely that this effect was caused by the conversion of 25-OH-D_3 into 1α,25$(OH)_2D_3$ by HPMs.

Conclusions. 1α,25$(OH)_2D_3$ has a potent inhibitory effect on the production of TNF-α by LPS-activated HPMs. We hypothesize that 1α,25$(OH)_2D_3$ may constitute a regulatory mechanism that, by controlling the intensity of the inflammatory response of the peritoneum, will moderate tissue damage during peritonitis.

It is well established that the physiological role of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$], the active metabolite of vitamin D_3, is not limited to mineral and skeletal homeostasis [1, 2]. In recent years, there has been increasing evidence pointing to the role of 1α,25$(OH)_2D_3$ and its analogues in the regulation of cell growth. This includes the antiproliferative effect on cancer cells and the induction of differentiation of leukemia cells into normal monocytes/macrophages [3]. Additionally, convincing evidence has been gathered indicating that 1α,25$(OH)_2D_3$ is an important immune response regulator at inflammatory sites. Such a property of 1α,25$(OH)_2D_3$ may find expression in the special microenvironment of the peritoneal cavity characteristic of the continuous ambulatory peritoneal dialysis (CAPD) technique. Thus, we have previously demonstrated that 1α,25$(OH)_2D_3$ accumulates in the peritoneal fluid of CAPD patients through two mechanisms: (*1*) 1α,25$(OH)_2D_3$ and its substrate 25$(OH)D_3$, combined with their binding protein leak from the blood stream through the peritoneal capillaries into the dialysate, and (*2*) 1α,25$(OH)_2D_3$ synthesized by the peritoneal macrophages, where the substrate is converted into the active metabolite of vitamin D [4–6].

We have demonstrated that 1α,25$(OH)_2D_3$ significantly increases superoxide generation and bactericidal activity of peritoneal macrophages [6]. This is particularly relevant in view of the importance of peritoneal macrophages in the host defense mechanism of the peritoneal cavity in CAPD patients. It is noteworthy, however, that in such patients, the capacity of macrophages to phagocyte microorganisms is severely limited because of the diluting effect of the infused dialysis solution on the peritoneal macrophage population. Consequently, within this context, the contribution of the peritoneal macrophages in combating bacterial invasion relies mainly on their ability to secrete inflammatory cytokine effectors, such as interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α).

It is interesting to note that in recent years, conflicting results have appeared in a number of studies on the regulation of cytokine expression by 1α,25$(OH)_2D_3$ [7–9]. These discrepancies may be caused by differences

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in laboratory techniques and conditions, cell type, etc. [10–14]. For example, using the human myelomonocytic cell line U-937, Fagan et al [12] and Prehn et al [10] showed that 1,25α$(OH)_2D_3$ causes a dose-dependent increase in TNF-α and IL-1β production in lipopolysaccharide (LPS)-stimulated cells. On the other hand, Riancho et al recently demonstrated a down-regulation of TNF-α expression [15] and Panichi et al showed a down-regulation of IL-1β and TNF-α expression in LPS-stimulated human peripheral blood monocytes [16]. To date, no data are available regarding the role of 1α,25$(OH)_2D_3$ in cytokine production by human peritoneal macrophages (HPMs). The present study was designed to investigate the regulatory effect of 1α,25$(OH)_2D_3$ on the generation of TNF-α by peritoneal macrophages of CAPD patients. We believe such an investigation could be of particular clinical value since in the peritoneal environment, there is a simultaneous up-regulation of TNF-α production and 1α,25$(OH)_2D_3$ synthesis in response to bacterial invasion.

METHODS

Materials

1α,25$(OH)_2D_3$ and 25-hydroxyvitamin D_3 (25-OH-D_3) were kindly provided by Hoffman La-Roche (Basel, Switzerland) and 1α,24(S)-dihydroxyvitamin D_2 by Bone Care International Co. (Madison, WI, USA). Each compound was dissolved in ethanol and stored in a concentrated solution at −20°C that was protected from light. Vitamin D metabolites were freshly diluted in the appropriate culture medium before each experiment. The final ethanol concentration did not exceed 0.1%.

Cell preparation

Human peritoneal macrophages were obtained from effluent dialysates of patients in end-stage renal disease undergoing CAPD treatment. All patients were treated with 1α-hydroxyvitamin D_3 (0.25 to 1 μg/day). This treatment was discontinued two weeks before the experiment took place. Isolation of macrophages from the dialysate fluids was performed as described elsewhere [4, 17, 18] with a few modifications. Complete dialysate effluent volume was centrifuged at 2000 r.p.m. × 15 minutes (Hettich, Rotanta/RP, Tuttlingen, Germany). Cells were washed with Hank's solution and centrifuged again at 1500 r.p.m. × 10 minutes. Then the cells were washed twice in RPMI-1640 medium (Bet-Haemek, Israel) and resuspended in RPMI-1640 medium, which contained 10% fetal calf serum (FCS), 2 mmol/L L-glutamin, 100 U/mL penicillin, 10 μg/mL streptomycin, and 12.5 U/mL nistatine (Biological Industries, Bet-Haemek, Israel). Cells were plated into a plastic flask and incubated for 90 minutes at 37°C in humidified atmosphere of 5% CO_2 and 95% air to allow the attachment of macrophages. Nonadherent cells were removed by discarding the medium and replacing it with fresh medium. The adherent cells were released by addition of phosphate-buffered saline and rolling glass sticks in the flask. Following isolation of macrophages, the cells were counted by a hemocytometer and adjusted to 1×10^6 cells/mL. More than 99% HPMs were viable as determined by trypan blue exclusion.

Incubation of HPMs

Primary cultures of HPMs (1×10^6 cells/tube) were seeded in plastic tissue culture tubes (12.4 × 75 mm; Falcon Plastics, Rohrchen, Greiner, Frickenhausen, Germany) by placing 1 mL cell suspension in 10% FCS containing RPMI-1640 medium into each tube and incubated as monolayers overnight. Treatments and activation were performed in triplicate as follows. Overnight supernatants were removed and the cells were incubated in RPMI medium containing 2% FCS with various concentrations of 1α,25$(OH)_2D_3$ (10^{-10} to 10^{-7} mol/L) for 16 hours. The cells were then exposed to 1 μg/mL of lipopolysaccharide (LPS; *Escherichia coli* 055:B5 LPS; Sigma, St. Louis, MO, USA) for six hours for protein expression or for 2.5 hours for mRNA expression. Cells were also incubated under the same conditions with various concentrations of the synthetic analogue 1α,24(S)$(OH)_2D_2$ (10^{-10} to 10^{-7} mol/L) or with 25-OH-D_3 (10^{-10} to 10^{-6} mol/L). Following the incubation period, tubes were placed on ice, and supernatants were collected and stored at −20°C for TNF-α protein determination. Cells were subjected to mRNA extraction procedure. A viability of more than 99% of cells was determined by Trypan blue exclusion in the HPM primary cultures. Similar incubations of the mice monocyte-macrophage-like cell line P388D1 with 1α,25$(OH)_2D_3$ (10^{-10} and 10^{-7} mol/L) were carried out using the same experimental protocol.

TNF-α mRNA analysis

Tumor necrosis factor-α mRNA was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) of total RNA extracted from HPMs. HPMs were incubated as described previously in this article. At the end of each experiment, the supernatants were removed, and cells were lyzed with 0.75 mL Tri Reagent (Molecular Research Center, Inc., Cincinnati, OH, USA). For TNF-α mRNA determination, we employed the methods of Bouaboula et al [19] and Legoux et al [20], using a quantitative RT-PCR procedure. This method uses internal standard RNA obtained from synthetic DNA construct containing TNF-α primer sites identical to the primer sites of native TNF-α mRNA.

cDNA preparation

For cDNA generation, a synthetic RNA mixture (0.0325 ng) and a reverse transcriptase reaction mixture

were added to each RNA sample. This mixture contained 1 μL of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT, 200 U/μL; GIBCO BRL, Gaithersburg, MD, USA), 4 μL of 5 × reverse transcriptase buffer (GIBCO BRL), 0.25 μL dithiothreitol (DTT; 0.1 mol/L; GIBCO BRL), 0.5 μL RNase inhibitor (40 U/μL; Boehringer Mannheim, Mannheim, Germany), 1 μL oligo-d(T) 12-18 mer (40 pmol/μL; Boehringer Mannheim), and 1 μL dNTP (2.5 nmol/μL of each nucleotide; Boehringer Mannheim). The tubes were incubated for one hour at 37°C. Then the volume of each sample was adjusted to 50 μL, and the enzyme was inactivated by incubation for 10 minutes at 65°C.

Polymerase chain reaction

Co-amplification of the standard RNA preparations together with native mRNA obtained from HPMs followed by quantitation of PCR products were carried out. Native TNF-α (427 bp), synthetic TNF-α (370 bp), and β-actin (263 bp) cDNA were then amplified by PCR using specific primers. A portion of 5 μL of reverse transcription product was added to 45 μL of PCR reaction mixture containing 30 μL H_2O, 2.5 μL 5′-primer (20 μmol/L), 2.5 μL 3′-primer (20 μmol/L), 2 μL dNTP (2.5 nmol/μL of each nucleotide; Boehringer Mannheim), 5 μL of reaction buffer, and 0.2 μL Red Hot DNA polymerase (5 U/μL; Advanced Biotechnologies, Surrey, UK). A negative control consisting of the reaction mixture without cDNA was included in each run. PCR for β-actin was performed at 25 cycles. The PCR was done with β-actin primers under the following conditions: 90 seconds at 94°C, then 10 cycles of 45 seconds each at 94°C, 1.5 minutes at 60°C, and 1 minute at 72°C. The last 15 cycles were run under the same conditions, but the 72°C step was prolonged by five seconds in each cycle. PCR with native and synthetic TNF-α primers was performed using the same protocol. Every experiment was amplified with at least two different cycle numbers to assure that amplification was in the exponential phase of PCR. We found that 30 cycles for TNF-α and 25 cycles for β-actin were in the exponential phase of amplification in our experiments, thus permitting comparison of mRNA levels in different samples. Under these conditions, we also found a linear dose–response of the PCR products to increasing doses of original cDNA. Eight microliters of each sample containing amplified cDNA of the two kinds of TNF-α, together with 8 μL of the corresponding sample containing amplified cDNA of β-actin, were loaded on an agarose gel (2%) containing ethidium bromide (0.5 μg/mL). A mixture of DNA size marker was run on the same gel (123 bp ladder; GIBCO BRL). PCR products were quantitated by a video densitometry of agarose gel with the UV GDS 5000 system (UVP Inc., San Gabriel, CA, USA). The TNF-α results were calculated for each treatment by the ratios of native TNF-α/synthetic TNF-α to compensate differences in RT and PCR efficiency, and results were then normalized according to β-actin levels to compensate differences in mRNA between the various samples. Results were compared with those of LPS-stimulated HPMs.

TNF-α protein determination

Tumor necrosis factor-α protein levels were measured in the supernatants of HPM cultures in triplicate by an enzyme-linked immunosorbent assay (ELISA) for human TNF-α using a commercially available paired antibodies assay (R&D Systems, Minneapolis, MN, USA), as per the protocol of the manufacturer. Supernatants of the P388D1 cell line were tested for TNF-α protein levels using an ELISA procedure specific for mice TNF-α (R&D Systems).

Determination of 1α,25(OH)$_2$D$_3$ production by HPM

Human peritoneal macrophages (1×10^6 cells/tube) in RPMI-1640 medium were incubated with 25-OH-D_3 (10^{-6} mol/L) as a substrate for 16 hours with or without the cytochrome P450 inhibitor, ketoconazole. The reaction was terminated by addition of 1 mL acetonitrile [high-performance liquid chromatography (HPLC) grade; Biolab, Jerusalem, Israel]. A tracer of 1000 CPM [^{3}H] 1α,25(OH)$_2$D$_3$ (Hamersham, Little Chalfont, Buckinghamshire, UK) was added to each sample for recovery estimation. Assessment of the 1α,25(OH)$_2$D$_3$ produced by HPM was carried out following lipid extraction and 1α,25(OH)$_2$D$_3$ isolation by a Sep-pak separation procedure. The isolated 1α,25(OH)$_2$D$_3$ fraction was subjected to a vitamin D receptor (VDR) radioligand competitive binding assay [21] using calf thymus VDR [22]. The results (pg/mL) were adjusted with respect to recovery values.

Statistical analysis

Results are presented as mean ± SEM. Student *t* test was used to compare TNF-α levels between treatments. *P* values below 0.05 were considered significant.

RESULTS

Effect of 1α,25(OH)$_2$D$_3$ on TNF-α mRNA levels

Reverse transcriptase-PCR of total RNA from HPMs yielded a band of mRNA for TNF-α at the expected size of 427 bp (Fig. 1A). The RT-PCR products of the synthetic RNA of TNF-α yielded cDNA at the expected size of 370 bp. The synthetic RNA of TNF-α accompanied each treatment in order to normalize the differences in loading amounts. As shown in Figure 1A and in its densitometric evaluation expressed in Figure 1B, TNF-α mRNA was detected in untreated primary HPM cultures only at minimal levels. However, TNF-α expression was found to be inducible by LPS. It is clearly shown in this

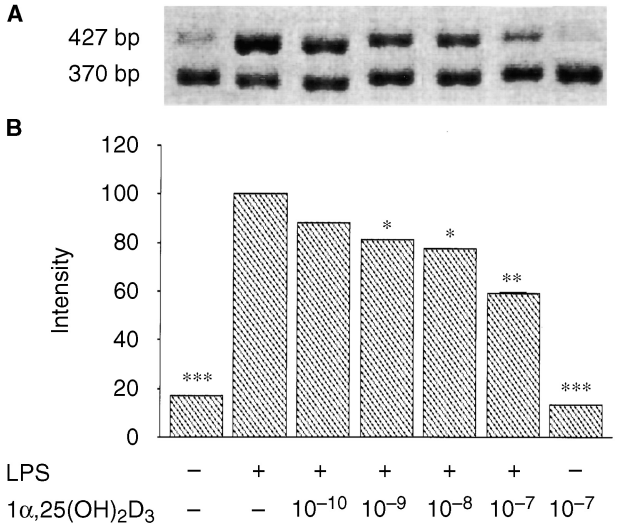

Fig. 1. Dose response of increased concentrations of 1α,25(OH)$_2$D$_3$ on tumor necrosis factor-α (TNF-α) mRNA production by stimulated human peritoneal macrophages (HPM). HPM (1×10^6 cells/tube) were incubated for 16 hours with increased concentrations of 1α,25(OH)$_2$D$_3$ (10^{-10} to 10^{-7} mol/L) followed by an additional 2.5 hours of incubation with lipopolysaccharide (LPS; 1 μg/mL). RT-PCR was performed on total HPM RNA and on synthetic RNA (RNA standards for TNF-α) using specific primers for TNF-α PCR products (at 30 cycles) were separated on 2% agarose gel containing ethidium bromide (*A*). Quantitative evaluations of the PCR products were performed by video densitometry, and the ratios between native and standard TNF-α bands were calculated. The results are expressed as percentage (mean ± SE) of TNF-α mRNA levels obtained by LPS induced HPM (*B*). The results represent three sets of experiments performed in triplicate on HPM prepared from different patients. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$.

study that incubation of HPMs (1×10^6 cells/tube) for 2.5 hours with LPS at a concentration of 1 μg/mL induced a very significant increase in TNF-α mRNA levels. On the other hand, preincubation for 16 hours with increased concentrations of 1α,25(OH)$_2$D$_3$ (10^{-10} to 10^{-7} mol/L) before the addition of LPS, induced a dose-dependent decrement in TNF-α mRNA. These effects were found to be at statistically significant levels. As shown in Figure 1B, a 20% decrease in TNF-α mRNA levels was observed in the presence of 10^{-9} mol/L 1α,25(OH)$_2$D$_3$ ($P < 0.05$) and a further decrease of 40% in TNF-α mRNA expression was obtained by incubating HPM with 10^{-7} mol/L 1α,25(OH)$_2$D$_3$ ($P < 0.01$).

Effect of vitamin D metabolites and analogue on TNF-α protein levels

Tumor necrosis factor-α expression on the protein levels also was examined in HPM media using a specific ELISA. Preincubation of HPM for 16 hours with increasing concentrations of vitamin D metabolites [1α,25(OH)$_2$D$_2$, 25-OH-D$_3$] or of the vitamin D analogue [1α,24(S)(OH)$_2$D$_2$], followed by activation with LPS (1 μg/mL) for an additional six hours, induced a dose-related decrement in TNF-α protein concentrations (Fig. 2). TNF-α production was down-regulated significantly ($P < 0.01$) even in the presence of physiological concentrations of 1α,25(OH)$_2$D$_3$ (10^{-10} mol/L) and reached a maximal effect at 10^{-8} mol/L (Fig. 2A). Similar incubation of the P388D1 monocyte-macrophage-like mice cell line with 1α,25(OH)$_2$D$_3$ (10^{-10} mol/L) revealed an inhibitory effect of 22% in TNF-α protein expression reaching 61.1% when higher concentrations (10^{-7} mol/L) of 1α,25(OH)$_2$D$_3$ were added (detailed results not shown).

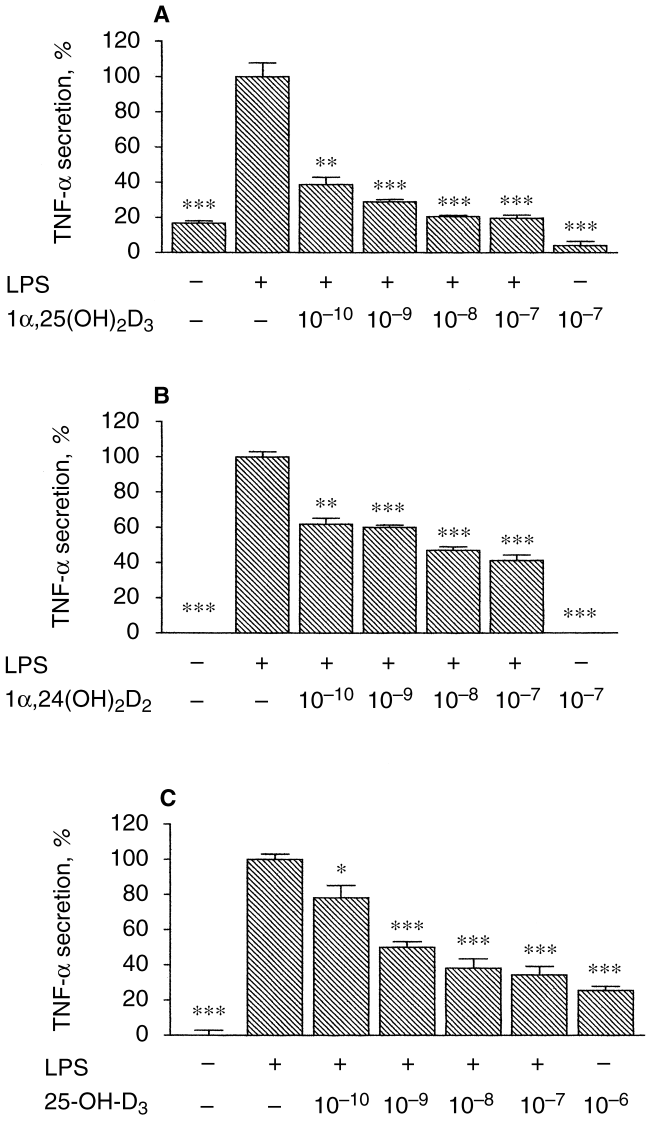

Fig. 2. Dose response of increased concentrations of 1α,25(OH)$_2$D$_3$ on TNF-α protein secretion from stimulated HPM. HPM (1×10^6 cells/tube) were incubated for 16 hours with increased concentrations (10^{-10} to 10^{-7} mol/L) of 1α,25(OH)$_2$D$_3$ (*A*), 1α,24(OH)$_2$D$_2$ (*B*), or 25-OH-D$_3$ (*C*) in concentrations of (10^{-10} to 10^{-6} mol/L). This was followed by additional six hours of incubation with LPS (1 μg/mL). Supernatants were collected and subsequently assayed for TNF-α protein levels by ELISA. The results are expressed as percentage (mean ± SE) of TNF-α protein levels obtained by LPS-induced HPM. The results represent three experiments performed in triplicate on cells obtained from different patients. ***$P < 0.001$; **$P < 0.01$.

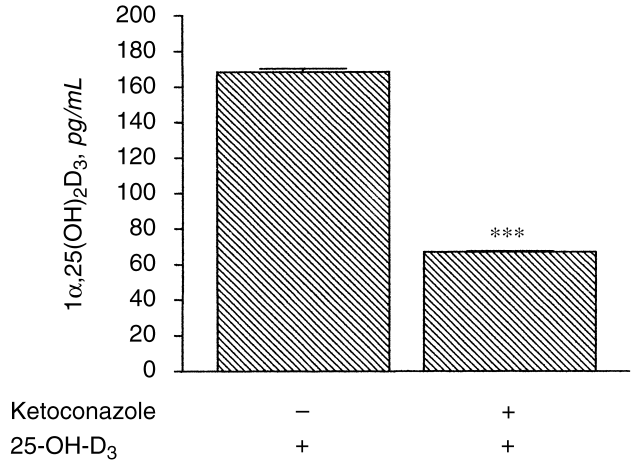

Fig. 3. Synthesis of 1α,25(OH)$_2$D$_3$ by HPM. HPM (1×10^6 cells/tube) were incubated for 16 hours with 25-OH-D$_3$ (10^{-6} mol/L) with or without ketoconazole. The reaction was stopped by addition of acetonitrile. Following extraction and chromatographic procedure, 1α,25(OH)$_2$D$_3$ concentrations were determined by a specific receptor binding assay. The results (mean ± SE) represent three experiments performed in triplicate on cells obtained from different patients. ***$P < 0.001$.

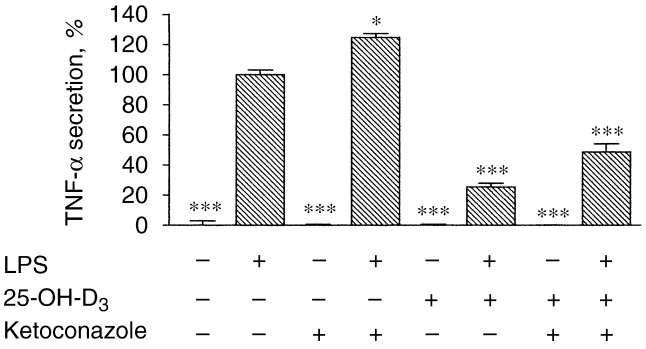

Fig. 4. Effect of the 25-OH-D$_3$-1α-hydroxylase inhibitor (ketoconazole) on TNF-α protein secretion by HPM. HPMs (1×10^6 cells/tube) were incubated for 16 hours in the presence of 25-OH-D$_3$ (10^{-6} mol/L) with or without ketoconazole (20 μmol/L). This was followed by additional six hours of incubation with LPS (1 μg/mL). Supernatants were collected and subsequently assayed for TNF-α protein levels by ELISA. The results are expressed as percentage (mean ± SE) of TNF-α protein levels obtained by LPS-induced HPM. The results represent three experiments performed in triplicate on cells obtained from different patients. ***$P < 0.001$; **$P < 0.01$.

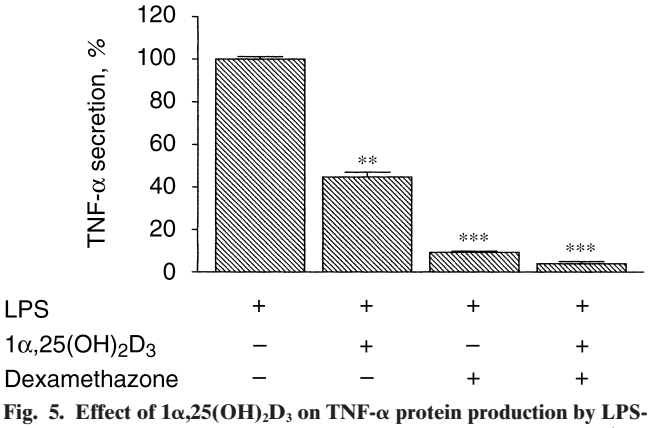

Fig. 5. Effect of 1α,25(OH)$_2$D$_3$ on TNF-α protein production by LPS-stimulated HPM as compared with that of dexamethasone. HPMs (1×10^6 cells/tube) were incubated for 16 hours with 1α,25(OH)$_2$D$_3$ (10^{-7} mol/L) or dexamethasone (10^{-8} mol/L) or both. This was followed by additional six hours of incubation with LPS (1 μg/mL). Supernatants were collected and subsequently assayed for TNF-α protein levels by ELISA. The results are expressed as percentage (mean ± SE) of TNF-α protein levels obtained by LPS-induced HPM. The results represent three experiments performed in triplicate on cells obtained from different patients. ***$P < 0.001$; **$P < 0.01$.

The results in Figure 2B summarize the effect of the synthetic vitamin D analogue 1α,24(S)-(OH)$_2$D$_2$ on TNF-α production. The effect of 1α,24(S)-(OH)$_2$D$_2$ on TNF-α expression was found to be dose dependent similarly to the effect of 1α,25(OH)$_2$D$_3$. A similar trend was observed also by incubating HPMs with 25-OH-D$_3$ (Fig. 2C). However, this effect of 25-OH-D$_3$ was found to be moderate, and higher concentrations of 25-OH-D$_3$ were required in order to exhibit the same down-regulation effect on TNF-α production. This down-regulatory effect caused by these vitamin D metabolites is specific for TNF-α. No change in IL-1β protein levels could be detected in simultaneous measurements (data not shown).

Conversion of 25-OH-D$_3$ to 1α,25(OH)$_2$D$_3$ and its effect on TNF-α expression

Human peritoneal macrophage obtained from patients on CAPD were able to produce a significant amount of 1α,25(OH)$_2$D$_3$ (168.5 ± 2 pg/mL) when incubated with 10^{-6} mol/L 25-OH-D$_3$ for 16 hours (Fig. 3). The addition of 20 μmol/L ketoconazole, the cytochrome P$_{450}$ inhibitor, significantly reduced ($P < 0.001$) the synthesis of 1α,25(OH)$_2$D$_3$ (Fig. 3), thus suggesting the presence of 25-hydroxy-1α-hydroxylase in these macrophages. This occurrence raises the question of whether the effect of 25-OH-D$_3$ in regulating the expression of TNF-α is a direct action of the metabolite per se or whether it relies on the macrophages' ability to convert 25-OH-D$_3$ to 1α,25(OH)$_2$D$_3$. To address this question, TNF-α was measured in supernatants of HPM incubated with 25-OH-D$_3$ (10^{-6} mol/L) with and without 20 μmol/L ketoconazole followed by activation with LPS. While incubation of HPM with 25-OH-D$_3$ followed by LPS (Fig. 4, lane 6) induced a decrement in TNF-α protein expression ($P < 0.001$), the addition of ketoconazole (Fig. 4, lane 8) blunted the down-regulation caused by 25-OH-D$_3$. Incubation of HPM with ketoconazole and various concentrations (10^{-10} to 10^{-7} mol/L) of 25-OH-D$_3$ (data not shown) revealed a similar trend. These results suggest that 25-OH-D$_3$ must be converted to 1α,25(OH)$_2$D$_3$ in order to express its down-regulatory effect on TNF-α generation.

The anti-inflammatory effect of 1α,25(OH)$_2$D$_3$ (10^{-7} mol/L) on HPMs, as expressed by the reduction in TNF-α production (Fig. 5, lane 2), was compared with that of dexamethasone (10^{-8} mol/L). Although dexamethasone

had a more potent inhibitory effect on LPS-induced TNF-α secretion (Fig. 5, lane 3) than $1\alpha,25(OH)_2D_3$, a combination of both of these anti-inflammatory agents produced a significantly greater inhibitory effect than dexamethasone alone (Fig. 5, lane 4, $P < 0.05$).

DISCUSSION

In recent years, there has been increasing evidence showing that beyond its role in divalent ion homeostasis, $1\alpha,25(OH)_2D_3$ may also affect immune cells and thus function as an important regulatory agent at sites of inflammation [8, 23]. The present data showing a regulatory role of $1\alpha,25(OH)_2D_3$ on TNF-α production by HPMs further emphasize this contention. We found that $1\alpha,25(OH)_2D_3$ induced a marked suppressor effect on TNF-α production by LPS-stimulated peritoneal macrophages. Incubation of HPM with $1\alpha,25(OH)_2D_3$ prior to exposure to LPS caused a significant inhibition of TNF-α expression by these cells. This inhibition of TNF-α expression by $1\alpha,25(OH)_2D_3$ was observed on both mRNA and protein levels and was dose-dependent. These results are strongly supported by the inhibition of TNF-α protein expression by $1\alpha,25(OH)_2D_3$, observed in the present study in a mouse monocyte-macrophage-like cell line (P388D1).

During peritonitis, upon contact with bacterial products, peritoneal macrophages initially secrete TNF-α. By activating other peritoneal resident cells (mainly the mesothelium), TNF-α creates a powerful proinflammatory cascade, which promotes massive recruitment of leukocytes into the peritoneal cavity. We have demonstrated in the past that $1\alpha25(OH)_2D_3$ is able to up-regulate the host defense mechanism of the peritoneal cavity of CAPD patients through a stimulatory effect of macrophages-generated superoxide, thus reinforcing the bactericidal capacity of these cells. This phenomenon may be particularly significant during peritonitis, since we have shown that under these conditions, peritoneal macrophages will up-regulate $1\alpha25(OH)_2D_3$ synthesis. From these findings, it would seem that during bacterial peritonitis, two opposing messages are relayed by $1\alpha25(OH)_2D_3$ to the macrophages: (*1*) up-regulation of superoxide production and (*2*) a suppressor effect on TNF synthesis. We can hypothesize that paradoxical as it may seem, these effects of $1\alpha25(OH)_2D_3$ have an important role in moderating the magnitude and the extent of the inflammatory response of the peritoneum to invading microorganisms. This contradictory pattern of behavior is known to occur during contact of macrophages with bacteria and can be illustrated by the production of the anti-inflammatory molecules: interleukin-1 receptor antagonist (IL-1RA) and soluble TNF receptor (sTNFR) concomitantly with the synthesis of potent proinflammatory cytokines IL-1 and TNF-α. The outcome of the disease and extent of tissue damage will depend on the fine balance between these two proinflammatory and anti-inflammatory systems. Adhering to this hypothesis, it is reasonable to assume that the concomitant but opposing effects of $1\alpha25(OH)_2D_3$, as described previously in this article, may have clinical relevance in CAPD since they moderate the damage to the peritoneum, which would otherwise occur because of disregulated local accumulation of proinflammatory molecules during peritonitis This concept is further supported by an in vitro experimental model [24] in which reactive oxygen molecules released by activated polymorphonuclear leukocytes caused severe damage to mesothelial cells.

The down-regulatory effect of $1\alpha,25(OH)_2D_3$ is not unique to TNF-α expression. $1\alpha,25(OH)_2D_3$ was found to decrease the synthesis of IL-2 and interferon-γ in activated lymphocytes, and also inhibited the expression of IL-1, IL-6, IL-12, and TNF-α in monocytes/macrophages [9, 11, 14]. In the case of IL-12, inhibition of the transcription factor nuclear factor-κB (NF-κB) by $1\alpha,25(OH)_2D_3$ has been suggested as the mechanism of inhibition of this cytokine in myelomonocytic cells [14]. Since NF-κB is also a major TNF-α transcription factor [25], the suppression of TNF-α by $1\alpha,25(OH)_2D_3$ could be explained by the same mechanism.

We have already demonstrated that there are two sources from which $1\alpha,25(OH)_2D_3$ accumulates in the peritoneal effluent of patients on CAPD [26]. The first, $1\alpha,25(OH)_2D_3$, together with its binding protein, leaks from the blood stream through the peritoneal capillaries, and second, this metabolite is synthesized by the peritoneal macrophages where the substrate $25(OH)D_3$ is converted into $1\alpha,25(OH)_2D_3$ [4, 5]. In the present study, we showed that the synthesis of $1\alpha,25(OH)_2D_3$ was suppressed by ketoconazole, an inhibitor of the enzyme 1α-hydroxylase. This finding strongly supports the notion that 25-OH-D_3-1α-hydroxylase mediates the conversion of $25(OH)D_3$ to $1\alpha,25(OH)_2D_3$ by the peritoneal macrophages.

It is well recognized that TNF-α plays a central role in the pathogenesis of several inflammatory conditions such as rheumatoid arthritis, inflammatory bowel diseases, and LPS-induced septic shock [27–31]. Therefore, the means of suppressing the biosynthesis or activity of TNF-α may have important clinical relevance. Indeed, patients suffering from rheumatoid arthritis and Crohn's disease were treated on an experimental basis with TNF-α–soluble receptors or with antibodies against TNF-α in order to decrease their rate of inflammation [32, 33]. In many cases, the inflammation is suppressed by dexamethasone, often at the cost of side effects. Although the present results may suggest the therapeutic advantage of $1\alpha,25(OH)_2D_3$ as an anti-inflammatory agent, practically its use is restricted because of its hypercalcemic activity [34]. In a previous study, we showed the antiproliferative

activity of the less calcemic and synthetic vitamin D_2 analogue, 1α,24(S)-$(OH)_2D_2$ [35]. This activity was found to be equipotential to that of 1α,25$(OH)_2D_3$ [34]. In the present study, it was found that incubation of HPM with 1α,24(S)$(OH)_2D_2$ revealed a significant down-regulation of TNF-α expression, although less potent than that observed with the native active metabolite of vitamin D_3. Since 1α,24(S)-$(OH)_2D_2$ has a lower calcemic effect than that of 1α,25$(OH)_2D_3$ [36], its pharmaceutical application as an anti-inflammatory agent in vivo is much safer and appears to be superior to that of 1α,25$(OH)_2D_3$.

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