

1,25-Dihydroxyvitamin D₃ Maintains Adherence of Human Monocytes and Protects Them from Thermal Injury

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Abstract

Adherence to a substratum is a characteristic feature of monocyte-macrophages which may be required for several effector functions. Human peripheral blood monocytes selected by adherence were found to readhere preferentially at 1 h to fibronectin or to a biological matrix. There was then a progressive decrease in the number of adherent cells, and by 48 h only 8–20% of monocytes remained adherent. This loss of adherence occurred while monocytes remained viable by criteria such as exclusion of trypan blue or release of lactate dehydrogenase.

1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) maintained the adherence of cultured monocytes to tissue culture plastic as well as to the biological matrix. This effect was concentration- and time-dependent, and suppressed by inhibitors of protein synthesis. Cellular proteins were labeled after incubation with [³⁵S]methionine. Analysis by two-dimensional gel electrophoresis revealed increased labeling of several distinct proteins in 1,25-(OH)₂D₃-treated monocytes compared with control monocytes. The increased loss of adherence and decreased overall protein synthesis observed in monocytes incubated at 45°C was partially prevented by preincubation of the cells with 1,25-(OH)₂D₃. We further evaluated the effects of thermal stress and 1,25-(OH)₂D₃ on protein synthesis by monocytes, and found that 1,25-(OH)₂D₃ increased the synthesis of heat shock proteins, protected normal protein synthesis, and increased the rate of recovery of normal protein synthesis after the thermal stress.

These observations suggest that 1,25-(OH)₂D₃ influences monocytes by preserving the synthesis of proteins, including those critical for the maintenance of cell adherence.

Introduction

The hormone 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃)¹ primarily affects cells in intestine, bone, and kidney where it binds to specific cytosolic receptors (1–2). More recently, significant

numbers of receptors for 1,25-(OH)₂D₃ have been measured in other normal or neoplastic cells, particularly monocytes and activated, but not resting, T lymphocytes (3–5). Further evidence has been presented in support of a possible role of 1,25-(OH)₂D₃ in modulating functions of these mononuclear cells in inflammation and immune cell reactions (6–8).

Our observations on the adherence of the human monocyte-related cell line, U937 (9), prompted us to investigate possible effects of 1,25-(OH)₂D₃ on adherence and other functions of mature human monocytes isolated from peripheral blood. Adherence of cells to a substratum is a prerequisite for the growth, proliferation, and differentiation of many normal eukaryotic cells (10–12). Although peripheral blood monocytes in culture adhere transiently to tissue culture plastic or glass surfaces alone, different components of biological matrices influence the adhesion, morphology, and function of these cells in vitro (13–15).

We report here that cultured human monocytes, once adherent, do not remain attached to their substratum but remain viable, and that incubation with 1,25-(OH)₂D₃ maintains their adherence to tissue culture plastic as well as to a biological matrix. Modulation of the synthesis of proteins involved in cell adhesion or protection from nonspecific injury are among several possible mechanisms by which 1,25-(OH)₂D₃ could maintain monocyte adherence. We therefore analyzed the cellular proteins synthesized by these cells by two-dimensional gel electrophoresis (16, 17) and found an increase in the synthesis of several proteins in 1,25-(OH)₂D₃-treated monocytes. Moreover, inhibitors of protein synthesis abolished the effects of 1,25-(OH)₂D₃ on maintenance of monocyte adherence. Exposure of the cultured cells to elevated temperatures was found not only to decrease protein synthesis but also to increase detachment of these cells. The decrease in protein synthesis induced by these elevated temperatures was partially prevented by preincubation of monocytes with 1,25-(OH)₂D₃. It had been previously observed that cells exposed to thermal or other stresses decrease overall protein synthesis yet increase the synthesis of specific proteins (termed heat shock proteins) (18–20). After exposure of monocytes to 45°C for 20 min we found an increased labeling of proteins consistent with heat shock proteins (major species 70,000 and 83,000 mol wt). Incubation with 1,25-(OH)₂D₃, however, led to an increased synthesis of the heat shock proteins accompanied by a relative preservation of total protein synthesis and an increase in the rate of recovery of normal protein synthesis.

Methods

Cells. Peripheral blood mononuclear cells from normal volunteers (provided by the Blood Bank, Massachusetts General Hospital) were recovered from Ficoll-diatrizoate gradients and plated at 3×10^6 cells/ml in 15-cm dishes (3025; Falcon Labware, Becton Dickinson & Co., Oxnard, CA) in Iscove's modification of Dulbecco's medium (IMD) (Gibco Laboratories, Grand Island, NY) containing 10% fetal calf serum (FCS) for 1 h. Adherent cells were then washed 3 times with Hanks' balanced salt

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1. *Abbreviations used in this paper:* 1,25-(OH)₂D₃, 1,25-dihydroxyvitamin D₃; FCS, fetal calf serum; IMD, Iscove's modification of Dulbecco's medium.

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solution without Ca^{++} or Mg^{++} (Whittaker M. A. Bioproducts, Walkersville, MD) and incubated overnight in IMD medium with 10% FCS. The next morning, the floating cells were removed, the adherent cells washed 3 times with Hanks' balanced salt solution, scraped, counted, centrifuged, and resuspended in IMD medium with 10% FCS at 0.5×10^6 cells/ml before use in the adherence assay. More than 97% of these cells were monocytes by morphology, using May-Grunwald-Giemsa staining.

The porcine renal tubular epithelial cell line LLC-PK₁ (21) was maintained in Dulbecco's modified Eagle's medium (Gibco Laboratories) with 10% FCS. All cell cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂.

Substrates. Laminin was a gift of Dr. H. Kleinman (Laboratory of Developmental Biology and Anomalies, National Institutes of Health, Bethesda, MD) (22). Fibronectin was purified from plasma by urea extraction (23). Types I and III collagens were pepsinized preparations prepared from human bone or human leiomyoma as previously described (24). These purified matrix components were coated onto 35-mm diameter dishes (3001; Falcon Labware) by adding 10 µg/dish in 1.5 ml distilled water or 0.05% M acetic acid, and by allowing the dish to remain uncovered overnight in a laminar flow hood. Control plates were coated with distilled water or 0.05 M acetic acid alone. In some experiments, bacteriological plastic was used as well as tissue culture plastic. The extracellular matrix of LLC-PK₁ cells was prepared by plating the cells at 0.25×10^6 cells/dish in 35-mm diameter dishes (3001; Falcon Labware) and maintaining the cultures for 4 d in Dulbecco's modified Eagle's medium with 10% FCS. At that time, cell density reached $\sim 2 \times 10^6$ cells/dish. The cells were removed by 30-min exposure to 0.5% Triton X-100 (25) at room temperature and five subsequent vigorous washes with phosphate-buffered saline (Gibco Laboratories). We have shown that this matrix contains fibronectin, laminin, and type IV collagen along with additional unidentified components (Healy, A. M., J.-M. Dayer, M. L. Stephenson, S. M. Krane, unpublished data).

Adherence assay. The assay for adherence was adapted from that of Kleinman et al. (26). After preparation of the substrate and the monocyte-enriched cultures (which we refer to as monocytes), the cells were incubated at 37°C on the substrate at 1×10^6 cells/dish in 2 ml IMD medium with 10% FCS. After incubation for the indicated time, floating cells were decanted, adherent cells released with trypsin EDTA and both cell populations counted electronically (Coulter Counter; Coulter Electronics Inc., Hialeah, FL). Percent adherence was calculated as adherent cells/total cells $\times 100$. All experiments were performed in triplicate.

Reagents. 1,25-(OH)₂D₃ (kindly provided by Dr. M. Uskokovic, Hoffman-La Roche Inc., Nutley, NJ) was dissolved in ethanol as a stock solution of 1.6 mg/ml and diluted with medium before addition to monocytes at time of plating. The ethanol concentration did not exceed 0.05% and appropriate controls were performed to exclude an effect of the ethanol on cell adherence. Human recombinant interferon gamma ($\sim 2 \times 10^6$ U/mg) was kindly provided by Genentech Inc., South San Francisco, CA. Cycloheximide (Aldrich Chemical Co. Inc., Milwaukee, WI) was used at 1 µg/ml after preliminary studies showed that toxic effects on the cells resulted from incubation with concentrations higher than 1 µg/ml.

Cell viability. Cell viability was assessed using trypan blue exclusion by estimating the number of stained cells among 200 cells in two individual fields. In some experiments, lactate dehydrogenase release was measured (27) in the supernatants at the end of cell culture.

Synthesis of heat shock proteins. Monocytes separated by adherence were plated at 3.5×10^6 cells/ml in 10 ml DME medium without L-methionine (78-0022; Gibco Laboratories) with 2% FCS, 25 mM Hepes, pH 7.4, with or without 10 ng/ml 1,25-(OH)₂D₃ for 48 h. The culture dishes were then heated at 45°C for 20 min in a waterbath and allowed to recover in the 37°C incubator for 2–30 h. The cells were then labeled with 150 µCi [³⁵S]methionine (Amersham/Searle Corp., Arlington Heights, IL) for 1 h. Floating and adherent cells were washed twice in phosphate-buffered saline (Gibco Laboratories), lysed together in 1.5 ml 70 mM sodium dodecyl sulfate, 60 mM Tris, pH 7.5, 20% glycerol (sample buffer), and the proteins resolved using sodium dodecyl sulfate poly-

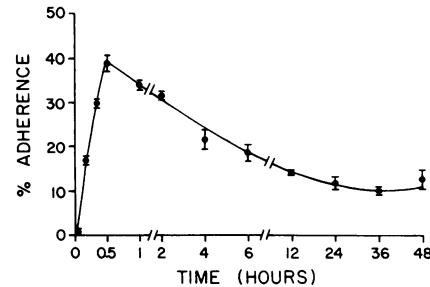


Figure 1. Time course of monocyte adherence to tissue culture plastic. Peripheral blood monocytes separated by adherence as described were plated at 0.5×10^6 cells/ml in 2 ml of IMD medium with 10% FCS on tissue culture plastic. At indicated times, floating and adherent cells were harvested and counted. Each point represents triplicate plates and bars indicate the SEM.

acrylamide gel electrophoresis in slab gels with polyacrylamide concentrations of 5 or 10% (28). Apparent molecular weight of synthesized labeled proteins was determined by comparison with standard marker proteins.

Two-dimensional gel electrophoresis. Monocytes separated by adherence were plated at $1-2 \times 10^6$ cells/ml in 10 ml DME medium without L-methionine but with 2% FCS, 25 mM Hepes, pH 7.4, with or without 10 ng/ml 1,25-(OH)₂D₃ for 48 h. The cells were then labeled with 200 µCi [³⁵S]methionine for 1 h. Floating and adherent cells were recovered, counted, washed twice in phosphate-buffered saline, and lysed together in lysis buffer (16, 17) at 10^6 cells/100 µl. Isoelectric focusing was carried out in glass tubes in the first dimension (16, 17). Ampholines were purchased from Bio-Rad Laboratories, Richmond, CA. Before the second dimension, the tube gels were placed in a freezer at -80°C , and then equilibrated in sodium dodecyl sulfate sample buffer for 2 h.

Statistics. The paired or unpaired Student's *t* test was used.

Results

Monocyte adherence to various substrates. Monocytes adhered rapidly at 37°C to tissue culture plastic. By 30 min $\sim 40\%$ were adherent, as in the experiment illustrated in Fig. 1. Although monocytes adhered to all surfaces, there was preferential adherence either to tissue culture plastic, LLC-PK₁ extracellular matrix, or fibronectin (Table I). It was not always possible, in

Table I. Adherence of Monocytes to Various Substrates

Substrate	No. of experiments	Percent adherence at 1 h	
		Mean	Range
Tissue culture plastic	11	41±1*	31–47
Bacteriological plastic	3	15±2	9–18
Fibronectin	9	46±3	32–61
Laminin	6	28±3	20–39
Collagen type I	8	34±4	18–52
Collagen type III	13	20±3	6–41
LLC-PK ₁ extracellular matrix	4	54±4	42–63

The purified matrix components were coated at 10 µg/ml in distilled water onto 35-mm dishes. Peripheral blood monocytes recovered from Ficoll-diatrizoate gradients and separated by adherence were plated at 2×10^6 cells/ml in 2 ml of IMD medium with 10% FCS. Floating and adherent cells were counted at 1 h. Experiments were performed in triplicate. * SEM.

view of the limited number of cells obtainable from an individual donor, to test each of the substrates with the monocytes from each donor, which could account for the broad range of adherence observed. There was a significantly greater adherence to type I collagen compared with type III collagen ($P < 0.05$). Although the adherence to type I collagen was not different from that to tissue culture plastic, the adherence to type III collagen was significantly less ($P < 0.05$). The adherence to laminin was also significantly less than that to tissue culture plastic or LLC-PK₁ matrix ($P < 0.01$). Addition of 1,25-(OH)₂D₃ did not alter the extent of adherence measured at 60 min as illustrated by the results of the experiment described in Fig. 2.

Effects of 1,25-(OH)₂D₃ and interferon gamma on monocyte adherence. When monocyte-enriched cells were maintained in culture after the initial adherence there was a progressive decrease in the number of adherent cells, from ~40% at 1 h to ~16% at 48 h as shown in the representative experiment in Fig. 1. A similar decrease was observed in the adherence of monocytes to the LLC-PK₁ extracellular matrix (Fig. 2). When 1,25-(OH)₂D₃ was added to the cultures at the time of plating, however, the adherence of monocytes measured at 48 or 72 h was maintained (Fig. 2). This effect of 1,25-(OH)₂D₃ was significant at 48 h ($P < 0.001$), was observed through 72 h, and could be reproduced with cells plated on tissue culture plastic as well as on fibronectin, collagen, or the LLC-PK₁ extracellular matrix.

This action of 1,25-(OH)₂D₃ on maintaining monocyte adherence was concentration-dependent (Table II). Although the concentrations at which 1,25-(OH)₂D₃ affected monocyte adherence varied from experiment to experiment, it should be emphasized that the same cells were not used in each experiment. Concentrations as low as 0.005 ng/ml (13 pM) were effective in some experiments, whether the monocytes were plated on tissue culture plastic (Table II) or on fibronectin (data not shown).

The maintenance of monocyte adherence by 1,25-(OH)₂D₃ was not associated with any detectable change in cell viability as assessed by exclusion of trypan blue (Table II) or release of lactate dehydrogenase. Lactate dehydrogenase activity was not detected in medium alone. There was no significant difference

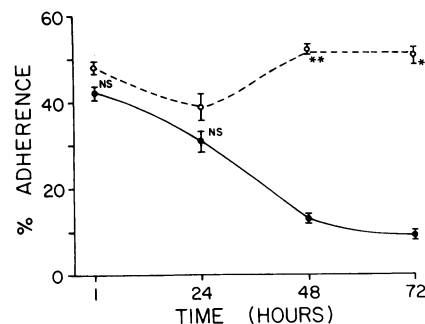


Figure 2. Monocyte adherence to the LLC-PK₁ extracellular matrix in presence or absence of 1,25-(OH)₂D₃. The extracellular matrix was prepared as described in 35-mm diameter dishes, and monocytes plated at 0.5×10^6 cells/ml in 2 ml IMD with 10% FCS, in the absence (—) or presence (---) of 1,25-(OH)₂D₃ (10 ng/ml). At indicated times, floating and adherent cells were counted, and percent adherence calculated from the ratio of adherent/total cells. Each point represents triplicate plates and bars indicate the SEM. Statistical differences are between 1,25-(OH)₂D₃-treated and untreated cells and were calculated from data at the same time point. NS, not significant. * $P < 0.01$. ** $P < 0.001$.

Table II. Effects of 1,25-(OH)₂D₃ on Maintaining Monocyte Adherence

1,25-(OH) ₂ D ₃ ng/ml	Adherence %
0	20±0‡
0.005	32±1*
0.01	36±0.5*
0.05	41±1*
1	42±1*
5	44±2*
10	43±0.5*
50	42±0*

Monocytes were incubated at 0.5×10^6 cells/ml in 2 ml IMD medium with 10% FCS and indicated concentrations of 1,25-(OH)₂D₃. At 48 h, floating and adherent cells were counted, and cell viability determined by trypan blue exclusion. Viability for adherent cells was ~100% and ranged for floating cells from 97 to 99%. Experiments were performed in triplicate.

* $P < 0.01$ as compared with control.

‡ SEM.

in enzyme release by monocytes cultured in the absence or presence of 1,25-(OH)₂D₃ at concentrations of 0.001–100 ng/ml (range in triplicate plates was from 25 to 37 U of lactate dehydrogenase/ml).

In order to determine whether 1,25-(OH)₂D₃ could also maintain the adherence of activated as well as resting monocytes, we incubated the peripheral blood adherent cells with interferon gamma, which had previously been shown to promote activation of monocytes (29). Cells were preincubated with interferon gamma, 100 U/ml, for 24 h, washed, and plated on tissue culture plastic for 48 h, with or without 1,25-(OH)₂D₃. As shown in Table III, preincubation with interferon gamma alone increased the percentage of monocytes which remained adherent at 48 h from 10 to 25% ($P < 0.01$). 1,25-(OH)₂D₃ further increased adherence of both control monocytes and monocytes preincubated

Table III. Effects of 1,25-(OH)₂D₃ and Interferon Gamma on Monocyte Adherence

1,25-(OH) ₂ D ₃ ng/ml	Monocyte adherence	
	Without preincubation with interferon gamma	With preincubation with interferon gamma
	%	%
0	10±1§	25±1
0.1	24±1‡	39±2‡
1	29±3*	39±2‡

Peripheral blood monocytes were incubated with or without interferon gamma, 100 U/ml, for 24 h in IMD medium with 10% FCS, then washed and plated on tissue culture plastic with indicated concentrations of 1,25-(OH)₂D₃ in IMD medium with 10% FCS. Floating and adherent cells were counted at 48 h. Experiments were performed in triplicate.

* $P < 0.05$ when compared with control.

‡ $P < 0.01$ when compared with control.

§ SEM.

with interferon gamma. In other experiments interferon gamma increased the adherence of monocytes not only to tissue culture plastic, but also to fibronectin or laminin. After 18 h of incubation with interferon gamma (100 U/ml), the number of monocytes which adhered at 1 h (measured in triplicate plates) to tissue culture plates increased from 21 to 35% ($P < 0.1$), to fibronectin, from 38 to 51% ($P < 0.05$), and to laminin, from 5 to 19% ($P < 0.01$).

Effects of $1,25-(OH)_2D_3$ on protein synthesis in unheated monocytes. We considered several possible mechanisms by which $1,25-(OH)_2D_3$ might exert its influence on monocyte adherence, among which were potential effects on the synthesis of proteins involved in cell adhesion. We were unable to detect significant differences in the pattern of [^{35}S]methionine-labeled cellular or medium proteins resolved by one-dimensional polyacrylamide gel electrophoresis, whether the monocytes were incubated with or without $1,25-(OH)_2D_3$. After resolution of the proteins on two-dimensional gels, however, we observed an increase in the labeling of several distinct proteins in the $1,25-(OH)_2D_3$ -treated monocytes. Two examples of the proteins resolved on two dimensional gels from $1,25-(OH)_2D_3$ -treated and -untreated monocytes from two separate experiments are shown in Fig. 3. Identification of these proteins and their possible role in cell adhesion is currently being pursued in our laboratory. No la-

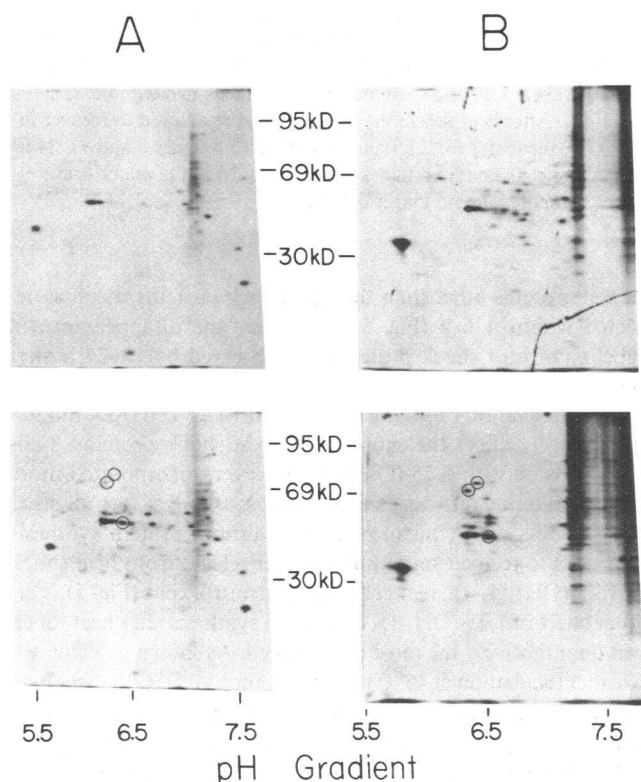


Figure 3. Protein synthesis of monocytes in presence or absence of $1,25-(OH)_2D_3$ analyzed by two-dimensional gel electrophoresis. Monocytes were prepared and cultured for 48 h with (*bottom*) or without (*top*) $1,25-(OH)_2D_3$ (10 ng/ml) as described. The cells were then labeled with 200 μ Ci (*A*) or 300 μ Ci (*B*) of [^{35}S] methionine for 1 h, recovered, counted, washed, and lysed in lysis buffer at 10^6 cells/100 μ l. Aliquot portions (20 μ l) were then loaded on each tube gel. Different cultures were used for the data shown in *A* and *B*. The major proteins whose labeling was consistently increased in monocytes cultured with $1,25-(OH)_2D_3$ are indicated by circles.

beling of proteins in the 220,000 mol wt range consistent with fibronectin was detected, either on two dimensional gels, or on 5% polyacrylamide gels under reducing conditions (Fig. 4, lanes 1 and 3). In Fig. 4, equal amounts of radioactivity from control- and $1,25-(OH)_2D_3$ -treated cultures were applied to each lane in order to detect qualitative differences.

Incubation of the cultured monocytes with cycloheximide (1 μ g/ml) for 48 h did not significantly alter the adherence of control cells (Table IV). The effect of $1,25-(OH)_2D_3$ on the maintenance of monocyte adherence was, however, completely abolished in the presence of cycloheximide.

Effects of $1,25-(OH)_2D_3$ on monocyte adherence and protein synthesis after heat shock. It had previously been shown that, in response to a thermal stress, cells exhibit a generalized decrease in protein synthesis while at the same time they synthesize distinct proteins, the heat shock proteins (18–20, 30). Experiments were therefore designed to determine if incubation of monocytes

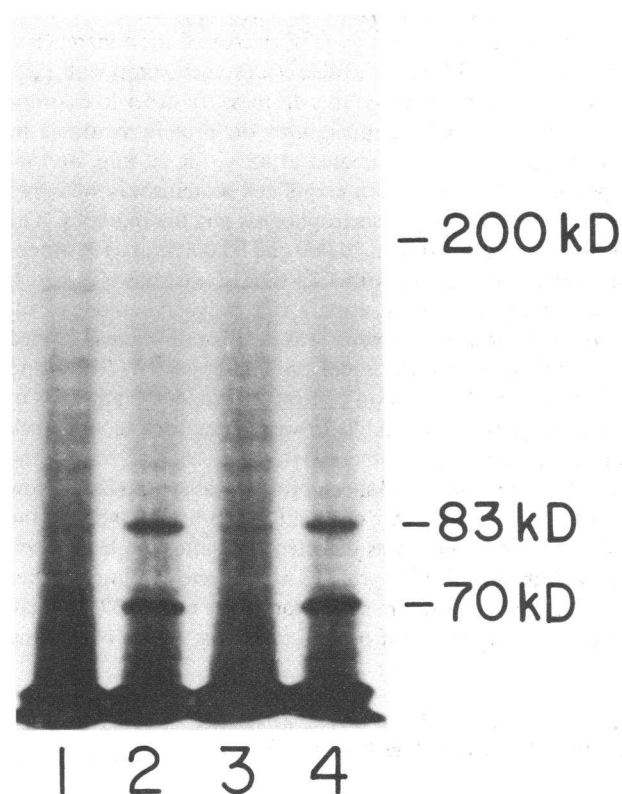


Figure 4. Production of heat shock proteins by monocytes. Monocytes were prepared, cultured, and heated to 45°C as described (lanes 2 and 4), allowed to recover in the 37°C incubator for 2 h, then labeled as described. Electrophoresis on sodium dodecyl sulfate polyacrylamide gels was performed using 5% polyacrylamide gels under reducing conditions. Equal quantities of radioactivity were applied to all lanes, in order to determine whether qualitative differences could be detected between control and $1,25-(OH)_2D_3$ -treated cells subjected to heat shock. Lanes 1 and 3, monocytes not exposed to 45°C. Lanes 2 and 4, monocytes were incubated with $1,25-(OH)_2D_3$ (10 ng/ml) for 48 h before experiments. Lanes 1 and 2, monocytes were incubated without $1,25-(OH)_2D_3$. The two major heat shock proteins were of 70,000 and 83,000 mol wt. The differences in the mobilities of proteins shown here and in Fig. 5 are explained by the different acrylamide concentrations. No protein band of molecular weight compatible with fibronectin (220,000 mol wt) was detected, even in monocytes incubated with $1,25-(OH)_2D_3$ (lanes 3 and 4).

Table IV. Effects of Inhibition of Protein Synthesis on Monocyte Adherence

	Monocyte adherence
	%
Control	17±1‡
1,25-(OH) ₂ D ₃	43±1.5*
Cycloheximide	12±0
1,25-(OH) ₂ D ₃ and cycloheximide	19±0.5

Peripheral blood monocytes were incubated for 48 h with or without 1,25-(OH)₂D₃ (10 ng/ml) and/or cycloheximide (1 µg/ml). Floating and adherent cells were counted at 48 h. Experiments were performed in triplicate.

* $P < 0.01$ when compared with control.

‡ SEM.

at elevated temperatures would alter their adherence. We found that incubation for 20 min at 45°C decreased the adherence of the cells measured 6 h later (Table V). Preincubation with 1,25-(OH)₂D₃ partially prevented this decrease. In order to examine the effects of elevated temperatures on protein synthesis by monocytes, they were incubated at 45°C for 20 min, and incorporation of [³⁵S]methionine into cell proteins was measured using polyacrylamide gel electrophoresis and fluorography. The appearance of new bands at 70,000 and 83,000 mol wt in monocytes incubated alone or with 1,25-(OH)₂D₃ is shown in Fig. 4. As seen in Fig. 5 A, when using a 10% polyacrylamide gel, incorporation into most proteins was decreased, whereas labeled bands consistent with heat shock proteins appeared (70,000 and 83,000 mol wt). In Fig. 5, in contrast to Fig. 4, the amounts of radioactivity applied to each lane were normalized to viable cell number. As described for other cells (30), the band corresponding to the 83,000-mol-wt heat shock protein was synthesized at low levels by monocytes under our culture conditions, whereas the 70,000-mol-wt protein was detected only after the heat shock (Fig. 5). Labeling of these proteins was detected within 2 h after exposure to 45°C. After reincubation of the cells at 37°C, there was a gradual recovery of protein synthesis; return of labeling

Table V. Effects of 1,25-(OH)₂D₃ on Monocyte Adherence Before and After Heat Shock

	Monocyte adherence	
	Control cells	1,25-(OH) ₂ D ₃ -treated cells
	%	%
Before heat shock	10±0.8*	35±3‡
After heat shock	4±0.7	14±1§

Peripheral blood monocytes were incubated for 48 h with or without 1,25-(OH)₂D₃ (10 ng/ml). Selected plates of monocytes were then heated to 45°C for 20 min (heat shock) and allowed to recover at 37°C for 6 h. Values are means of two different experiments, each performed in triplicate.

* SEM.

‡ $P < 0.01$ as compared with control cells.

§ $P < 0.001$ as compared with control cells after heat shock.

^{||} $P < 0.01$ as compared with unheated cells.

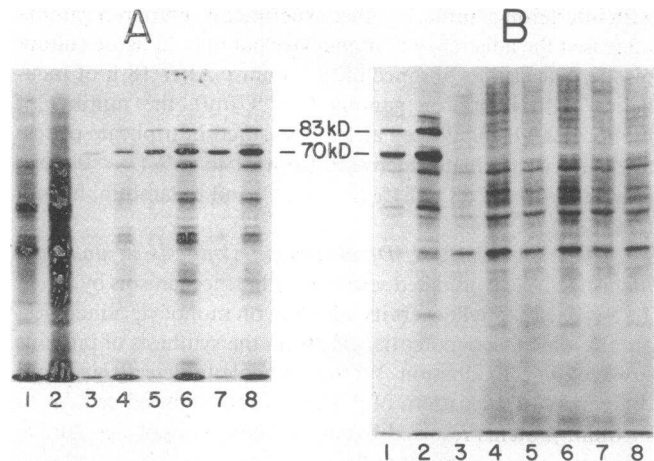


Figure 5. Effects of 1,25-(OH)₂D₃ on protein synthesis by monocytes after heat shock. (A) Monocytes were prepared, cultured, and heated to 45°C for 20 min (lanes 3–8) as described, allowed to recover in the 37°C incubator for 4 h (lanes 3 and 4), 6 h (lanes 5 and 6), and 8 h (lanes 7 and 8), and then labeled as described. Lanes 1 and 2, monocytes not exposed to 45°C. Electrophoresis was performed using 10% sodium dodecyl sulfate polyacrylamide gels under reducing conditions. The volume of sample applied to each lane was corrected for cell number. Lanes 2, 4, 6, and 8, monocytes were incubated with 1,25-(OH)₂D₃ (10 ng/ml) for 48 h before experiments. Lanes 1, 3, 5, and 7, monocytes were incubated without 1,25-(OH)₂D₃. The two major heat shock proteins were 70,000 mol wt and 83,000 mol wt. **(B)** Lanes 2, 4, 6, and 8, monocytes were incubated with 1,25-(OH)₂D₃ as in A. Lanes 1, 3, 5, and 7, monocytes were incubated without 1,25-(OH)₂D₃. After heat shock, the monocytes were allowed to recover in the 37°C incubator for 2 h (lanes 1 and 2), 10 h (lanes 3 and 4), 24 h (lanes 5 and 6), and 30 h (lanes 7 and 8). By 10 h, the major heat shock proteins were no longer detected.

of cell proteins other than the heat shock proteins was first detected within 4–6 h (Fig. 5 A) whereas the disappearance of labeling of heat shock proteins was observed by 10–24 h after heat shock (Fig. 5 B).

Preincubation of the monocytes with 1,25-(OH)₂D₃ did not qualitatively affect the synthesis of heat shock proteins. Cells preincubated with 1,25-(OH)₂D₃, however, incorporated more [³⁵S]methionine into heat shock proteins as well as into all other cell proteins than control cells. In addition, protein synthesis appeared to recover more rapidly after release from heat shock in 1,25-(OH)₂D₃-treated cells than in control cells (Fig. 5). This latter effect of 1,25-(OH)₂D₃ on protein synthesis after heat shock was quantitatively the most pronounced. As shown in Table VI, 6 h after incubation at 45°C and after return to 37°C the amounts of the 70,000-mol-wt and 83,000-mol-wt heat shock proteins as determined by densitometry were two and three times higher in 1,25-(OH)₂D₃-treated than in control cells, whereas the rate of overall protein synthesis in 1,25-(OH)₂D₃-treated cells was ~sixfold higher than that of controls.

Discussion

We have found in the present study that monocytes from peripheral blood, isolated by adherence, harvested, and allowed to readhere, do so more readily to a biological extracellular matrix, purified fibronectin, or tissue culture plastic compared with other single purified matrix components or plastic surfaces. Monocytes

Table VI. Effects of 1,25-(OH)₂D₃ on Protein Synthesis by Monocytes after Heat Shock

Time after heat shock	Proteins measured	Weight of corresponding areas on densitometer tracing	
		Control cells	1,25-(OH) ₂ D ₃ -treated cells
h		mg	mg
4	83,000 mol wt*	3	4
	70,000 mol wt*	4	8
	all other	31	76
6	83,000 mol wt	6	12
	70,000 mol wt	20	56
	all other	35	201
8	83,000 mol wt	9	16
	70,000 mol wt	22	54
	all other	47	122

After exposure of the fluorograms shown in Fig. 5 A for 6 h the relative amounts of proteins were determined by densitometry. The corresponding surface areas of the densitometer tracings were cut and weighed.

* Refers to the molecular weight of the two major heat shock proteins.

adhere to collagens, although the degree of adherence depends upon the type of collagen. Our observation that collagen type I is a better substrate for adherence than type III could account for previous, apparently contradictory, reports (11, 31, 32). Kleinman et al. (31) observed that monocytes adhered more readily to collagen compared with tissue culture plastic, whereas Bianco (32) noted that collagen coating prevented monocyte adherence and fibronectin was the major protein promoting adherence of monocytes to collagen. In our assays performed in the presence of 10% FCS, only type III collagen seemed to inhibit monocyte adherence.

It has been suggested that monocyte differentiation is influenced by the substrate that is used for culture, whether tissue culture plastic, collagens (15), or fibronectin (13). Collagens (15), fibronectin (33), or laminin (34) enhance phagocytosis by cultured human monocytes. Whereas fibronectin coating increases the number of adherent monocytes, laminin coating has been shown to decrease monocyte adherence (14, 34). In our studies, although the initial adherence rate appeared to depend on the substrate used, we observed that monocytes detached over time regardless of the substrate used to promote adherence, and by 48 h >80% of the monocytes were floating, as previously reported (35). Although monocytes float in the circulation, they do adhere once isolated and placed in culture. Adherence to the surface of vascular endothelium is the likely first step in the extrusion of leukocytes through the vessel wall and into the interstitial space during inflammation. The adherence of monocytes to an extravascular extracellular matrix may be involved in the function of these cells in inflammatory conditions. Adherence of monocytes has been related to their activation or differentiation state, function, and metabolism (36–38), and in particular to their production of active oxygen metabolites (37, 38).

We have shown here that 1,25-(OH)₂D₃ modulates the adherence of monocytes to tissue culture plastic as well as to biological matrices, and even at low concentrations maintains ad-

herence of these cells. The effects are consistent with those described in previous reports of reduced binding of macrophages from vitamin D-deficient rodents to bone in vitro (39). It seems likely that vitamin D, through its active metabolite 1,25-(OH)₂D₃, plays a role in monocyte adherence which can be distinguished from its role in monocyte differentiation, since we and others have shown that 1,25-(OH)₂D₃ modulates adherence of monocytes at several stages of differentiation, whether immature or transformed cells (9, 40), or resting or activated monocytes isolated from peripheral blood.

There are several possible mechanisms whereby 1,25-(OH)₂D₃ could exert its effects on cell adherence, which include maintaining the integrity of cell membranes, stimulating the production of adhesion glycoproteins, or altering contractile proteins involved in determination of cell shape. For example, 1,25-(OH)₂D₃ has been shown to increase fibronectin synthesis and the adhesiveness of human osteosarcoma cells (41). In our present studies of protein synthesis by monocytes we were unable to detect labeled proteins of molecular weight consistent with fibronectin in monocytes incubated for 48 h in the presence of 1,25-(OH)₂D₃. Fibronectin has been reported to be synthesized by monocytes under other conditions (42). We did observe, however, an increase in the synthesis of several distinct proteins by 1,25-(OH)₂D₃-treated monocytes, as analyzed by two-dimensional gel electrophoresis and fluorography. Further work is required in order to determine if these proteins are involved in cell adhesion. Inhibition of protein synthesis did not alter the adherence of monocytes which were not incubated with 1,25-(OH)₂D₃ but did abolish the effects of 1,25-(OH)₂D₃ on monocyte adherence. These observations suggest that protein synthesis is essential for the actions of 1,25-(OH)₂D₃ on maintenance of monocyte adherence. Tanaka et al. (43) and Abe et al. (44) have also shown that protein synthesis is required for the fusion of mouse alveolar macrophages induced by 1,25-(OH)₂D₃. This action of 1,25-(OH)₂D₃ may be mediated by polyamines (45). Although adherence of cells to a substrate and fusion to form multinucleated cells may be related events, we have not yet investigated the possible role of polyamines in the maintenance of monocyte adherence mediated by 1,25-(OH)₂D₃.

In order to further investigate the effects of 1,25-(OH)₂D₃ on cell adherence and protein synthesis we chose the situation of thermal stress. The decreased monocyte adherence after thermal injury could be related to the decrease in protein synthesis we observed in 1,25-(OH)₂D₃-treated and untreated monocytes. Thermal as well as other stresses induce the synthesis of heat shock proteins, which is probably a property of all species and all cells. We showed that monocytes produce at least two of the major heat shock proteins of 70,000 and 83,000 mol wt. The labeling (synthesis) of these proteins after exposure to elevated temperatures was associated with a decrease in normal protein synthesis. During the 10–24 h after recovery from thermal stress, the synthesis of heat shock proteins ceased, accompanied by restoration of normal protein synthesis. Preincubation of monocytes with 1,25-(OH)₂D₃ increased the labeling of the heat shock proteins, blunted the reduction in normal cellular protein synthesis, and increased the rate of recovery of normal cellular protein synthesis. The induction of heat shock protein synthesis, the decrease in normal protein synthesis, and the recovery of normal protein synthesis are regulated differently (18–20, 30). Restoration of normal protein synthesis takes place more gradually than the initial shift to heat shock protein synthesis (18). At least in *Drosophila*, during the decrease of normal protein

synthesis which occurs after heat shock, the messenger RNAs for the normal proteins are sequestered and protected from degradation, whereas during recovery, the messenger RNAs for the heat shock proteins are degraded (18, 19). How $1,25\text{-(OH)}_2\text{D}_3$ modulates these changes in protein synthesis remains to be elucidated. We have also not yet established that the effects of $1,25\text{-(OH)}_2\text{D}_3$ on protein synthesis in unheated and in heated monocytes are related events.

Heat shock proteins play a role in thermotolerance, in addition to protecting cells against other types of damage (46). It has recently been shown that bacteria (*Salmonella typhimurium*) become resistant to killing by hydrogen peroxide or heat if they are adapted by pretreatment with nonlethal levels of hydrogen peroxide (47). During adaptation to this oxidative stress, several proteins are induced, among which are heat shock proteins. Mutants of the bacteria which are more resistant than the wild-type parent to killing by heat and oxidizing agents also constitutively overexpress three heat shock proteins. By analogy, the adherent monocytes studied here produce active oxygen species such as H_2O_2 , and more H_2O_2 may actually be produced by monocytes incubated with $1,25\text{-(OH)}_2\text{D}_3$ (48). The greater induction of heat shock protein synthesis by $1,25\text{-(OH)}_2\text{D}_3$ may be related to the reported increased production of H_2O_2 in the presence of the hormone and to the protection of normal protein synthesis after the thermal stress. It is also possible that $1,25\text{-(OH)}_2\text{D}_3$ -treated cells could be protected from oxidative damage by enzymes such as superoxide dismutase or catalase (49) or other proteins such as metallothioneins (50).

Interferon gamma has several effects on monocytes, which include increasing H_2O_2 production (29), altering monocyte differentiation (51), and increasing interleukin 1 production by potentiating the actions of endotoxin (52, 53). We found here that interferon gamma alone also maintains monocyte adherence and that this effect is potentiated by $1,25\text{-(OH)}_2\text{D}_3$. Although mouse interferons (alpha and beta) have recently been reported to potentiate thermal injury in mouse 3T3 cells (54), we have not yet investigated the effects of interferon gamma on the synthesis of proteins after heat shock in human monocytes.

Recent reports from different laboratories suggest that $1,25\text{-(OH)}_2\text{D}_3$, in addition to its well known effects on calcium metabolism, might play a role in the regulation of the immune system (4-9). The results of the studies presented here provide further evidence that $1,25\text{-(OH)}_2\text{D}_3$ can modulate basic aspects of monocyte function such as maintenance of attachment to matrix components, and suggest additional approaches to examine possible roles of $1,25\text{-(OH)}_2\text{D}_3$ in cell-cell interactions as they pertain to a variety of inflammatory conditions. It is apparent that the effects of this hormone on monocyte function are profound and may be important in protecting the cells from different types of injury. The response to thermal injury which involves alteration of normal protein synthesis and induction of heat shock proteins is simply an example. Heat shock protein synthesis, and the recovery after heat shock, might prove a useful model for further study of some of the molecular mechanisms of action of $1,25\text{-(OH)}_2\text{D}_3$.

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