# Human Melanocytes as a Target Tissue for Hormones: In Vitro Studies with 1α-25, Dihydroxyvitamin $D_3$, α-melanocyte Stimulating Hormone, and Beta-estradiol

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Clinical evidence exists which suggests that normal pigment cell (melanocyte) function is subject to hormonal influences, but the nature of these interactions at a cellular level is poorly understood. We have investigated the effects of the vitamin D-derived secosteroid hormone 1α-25,dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$), the pituitary-derived peptide alpha-melanocyte stimulating hormone (α-MSH), and the sex steroid beta-estradiol on melanocytes cultured from normal human foreskin.

Human melanocytes specifically internalized $1,25(OH)_2$ $D_3$ with high affinity ($K_d$ $0.5 - 0.8$nM). Incubation with $1,25(OH)_2D_3$ ($10^{-9}$M) for 48 h resulted in a 100% increase in 25-hydroxyvitamin $D_3$-24-hydroxylase activity and a 50% increase in tyrosinase activity. There was no significant effect of $1,25(OH)_2D_3$ on intracellular cyclic adenosine monophosphate (cAMP). In contrast to $1,25(OH)_2D_3$, α-MSH at a concentration of $5\times10^{-7}$M caused a sevenfold increase in intracellular cAMP after 12 min but only a modest increase (<20%) in melanocyte tyrosinase activity after 48 h. Incubation with beta-estradiol for 24 h caused a dose-dependent increase in tyrosinase activity. The maximal response varied from 145%–213% of basal activity depending on the donor source.

These results indicate that melanocytes from normal human foreskin in culture have the capacity to respond directly to several hormones. They also suggest that these cells form a useful model to study the effect of various hormones on pigment cell function. *J Invest Dermatol 91:593–598, 1988*

Several hormones are thought to influence skin pigmentation in vivo. These include sex steroids [1] and pituitary derived pro-opiomelanocortin related peptides [2]. It has been suggested that the hormonal form of vitamin D [1α-25,dihydroxyvitamin $D_3$ ($1,25(OH)_2D_3$)] may also influence pigment cell function. This metabolite, which is produced in relatively large concentrations in the kidney [3], is also synthesized by epidermal keratinocytes [4] and by some melanoma cell lines [5]. $1,25(OH)_2D_3$ binds to specific receptors in some melanoma cell lines [6,7] and exerts profound effects on their proliferative and melanogenic activities in vitro [6,8,9] and in vivo [10]. Pavlovitch et al [11] showed that the tyrosinase [E.C.1.14.18.1] response of rat skin to a dose of ultraviolet irradiation was dependent on the vitamin D status of the animal.

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Abbreviations:
α—MSH-alpha-melanocyte stimulating hormone
cAMP—cyclic adenosine monophosphate
CT—cholera toxin
EMEM—Eagles minimal essential medium with Earle's salts
FCS—fetal calf serum
IBMX—isobutylmethylxanthine
mEMEM—modified EMEM
PMA—phorbol-12-myristate-13-acetate
$1,25(OH)_2D_3$—1α-25, dihydroxyvitamin $D_3$
$25(OH)D_3$—25-hydroxyvitamin $D_3$
24-hydroxylase—25-hydroxyvitamin $D_3$-24-hydroxylase

The mechanisms involved in the actions of these hormones on pigmented cells are currently unclear. Some information is available from studies involving melanoma cells, but the hormonal responses of these cells are variable, even in qualitative terms [9,12–14]. The recent development of methods for the culture of melanocytes from normal skin [15,16] has facilitated the study of the effects of $1,25(OH)_2D_3$ and other hormones on the function of these cells.

## MATERIALS AND METHODS

**Materials** Eagle's minimal essential medium with Earle's salt (EMEM) was purchased from Gibco Laboratories (Grand Island, NY). Iscove's medium (containing bovine serum albumin), fetal calf serum (FCS), charcoal-stripped FCS, and trypsin were all purchased from Flow Laboratories Inc. (Sydney, Australia). Plastic tissue culture dishes and flasks were obtained from Nunc Inter Med (Roskilde, Denmark) and Corning Ltd., Laboratory Division (Staffordshire, UK). Phorbol-12-myristate-13-acetate (PMA), cholera toxin (CT), isobutylmethylxanthine (IBMX), bovine serum albumin, ethylene diamine tetra-acetic acid disodium salt (EDTA), geneticin, DNA standard, soy bean trypsin inhibitor, phenylthiourea, beta-estradiol, and synthetic human α-MSH were all purchased from Sigma Chemical Co. (St. Louis, MO). 25-Hydroxyvitamin $D_3$ and $1,25(OH)_2D_3$ were generously provided by Roche Pty Ltd (Dee Why, NSW, Australia). Dihydroxyphenylalanine (DOPA) and reagents used in buffers were obtained from Calbiochem Behring Co. (Kingsgrove, NSW, Australia). Bio-Gel HTP was purchased from Biorad, Australia. 1α,25-hydroxy-[26,27-methyl-$^3$H] cholecalciferol (180 Ci/mmol), 25-hydroxy-[26,27-methyl-$^3$H] cholecalciferol (20 Ci/mmol), and L-[3,5-$^3$H]-tyrosine (50–60Ci/mmol) were obtained from Amersham Australia Pty. Ltd. (Sydney, Australia). Soluene-350, Dimilume-30, Hionic-Fluor, and Instagel were purchased from Packard Instruments (Downers Grove, IL). The

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sonifier cell disrupter was purchased from Branson Sonic Power Company (Connecticut).

**Culture Conditions** Human melanocyte cultures were derived from neonatal foreskins as described by Eisinger and Marko [15] with minor modifications. The cultures were routinely grown in 80 $cm^2$ tissue culture flasks. The growth medium consisted of EMEM containing 2 mM glutamine, 2.25 g/l sodium bicarbonate, 16 nM PMA, 10 nM cholera toxin, and 5% v/v heat-inactivated FCS, pH 7.2. All cultures were treated for at least 3 d with 100 $\mu$g/ml geneticin [17].

**Experimental Conditions** Melanocytes near confluence were detached from the culture flasks with trypsin (0.05% w/v) and EDTA (0.02% w/v) in phosphate buffered saline. These cells were pelleted, resuspended in growth medium, and seeded at a density of $1-4 \times 10^4$ cells/$cm^2$ on to uncoated plastic 24 well plates or flasks except where otherwise indicated [18]. After incubation for 48 h the growth medium was replaced with EMEM devoid of PMA and CT (mEMEM) but containing up to 5% FCS which had been charcoal treated (charcoal-stripped FCS) in an attempt to remove endogenous steroids [7]. After preincubation with mEMEM for the stated times, the cells were either analyzed for $1,25(OH)_2D_3$ uptake or cyclic adenosine monophosphate (cAMP) production, or incubated for a further 24-48 h with $1,25(OH)_2D_3$, $\alpha$-MSH or with beta-estradiol. 1,25 $(OH)_2D_3$ and beta-estradiol were added in absolute ethanol (0.1% final concentration), while $\alpha$-MSH was added in phosphate buffered saline (1% final concentration). Control wells received the appropriate vehicle. At the end of the third incubation period (up to 48 h) tyrosinase and 24-hydroxylase were measured as described below.

**Uptake of $[^3H]1,25(OH)_2D_3$ by Intact Cells** These studies were performed in order to determine whether the observed effects of $1,25(OH)_2D_3$ were receptor-mediated. After preincubation in mEMEM for 24 h the melanocytes were detached and collected in phosphate buffered saline containing 1 mg/ml soy bean trypsin inhibitor. The cells were pelleted, washed once in binding buffer (EMEM containing 2 mM glutamine, 20 mM Hepes, 0.05% bovine serum albumin, pH 7.4), and resuspended in fresh binding buffer. An aliquot of the cell suspension was counted in a hemo-cytometer. The volume was adjusted to give a final cell concentration of $0.2 - 1.0 \times 10^6$ cells/ml. Uptake of $[^3H]$ $1,25(OH)_2D_3$ by intact cells was measured by the method of Manolagas and Deftos [19] modified as previously described [9]. Briefly, 500 $\mu$l aliquots of the cell suspension were added to polycarbonate tubes containing either $[^3H]1,25(OH)_2D_3$ alone (total binding) or $[^3H]$-$1,25(OH)_2D_3$ and a 500-fold excess of unlabeled $1,25(OH)_2D_3$ (non-specific binding). The tubes were incubated at 37°C with gentle shaking for 3 h when maximum binding occurred (data not shown). The cell suspension was pelleted and washed 3 times in 1 ml ice cold isotonic wash buffer (0.25 M sucrose, 0.01 M Tris-HCl, 0.025 M KCl, 0.001 M $MgCl_2$, 0.001 M EDTA, 0.012 M thioglycerol, 0.5% w/v bovine serum albumin, pH 7.4). The cells were resuspended in 500 $\mu$l isotonic wash buffer and an aliquot was taken for assessment of cell viability with trypan blue. This was found to be greater than 90% in all cases. Another aliquot was pipetted into a liquid scintillation vial containing an equal volume of Soluene-350. The mixture was heated to 50°C for 4 h, cooled and counted in 15 ml Dimilune-30 scintillant in a Packard 360 C liquid scintillation counter (Downers Grove, IL).

**Hydroxylapatite Assay of Nuclear Bound $1,25(OH)_2D_3$** Intact cells were incubated at 37°C for 3 h with $[^3H]$ $1,25(OH)_2D_3$ in the presence or absence of excess unlabeled ligand as described above. All subsequent procedures were performed at 0°C. The cells were washed with ice cold isotonic wash buffer and lysed by incubation with 1 ml hypertonic buffer (0.3 M KCl, 0.01 M Tris-HCl, 0.0015 M EDTA, and 0.005 M dithiothreitol, pH 7.4 containing 0.5% v/v Triton X-100) for 30 min with vigorous vortexing every 15 min. The internalized, receptor bound $1,25(OH)_2D_3$ was measured by a modification of the method of Wecksler et al [20]. One milliliter of a hydroxylapatite slurry (50% v/v in 0.01 M Tris-HCl/0.1 M KCl, pH 7.5) was added to the lysed cells and the mixture was incubated for 15 min with vortexing every 5 min. The mixture was centrifuged at 1000 g for 5 min and the hydroxylapatite pellet washed twice with buffer (0.01 M Tris-HCl, 0.001 M EDTA, pH 7.5). Radioactivity was extracted from the pellets by the addition of 1 ml ethanol and 0.5 ml dichloromethane. The mixture was kept at 30°C for 30 min, vortexed several times during this period, and centrifuged at 1000 g for 5 min. The supernatant was decanted into liquid scintillation vials. This procedure was repeated once and the supernatants for each tube pooled. After drying in air overnight the radioactivity was counted in 10 ml Instagel.

**24-Hydroxylase** Cells (approximately $3 \times 10^4$ cells/$cm^2$) were incubated for 48 h in mEMEM and then for a further 48 h in mEMEM containing various concentrations of $1,25(OH)_2D_3$. The cell monolayers were washed twice with phosphate buffered saline and 24-hydroxylase was measured using high performance liquid chromatography as previously described [5].

**Tyrosinase Activity** Tyrosinase activity, which is believed to be closely correlated with melanogenesis in melanocytes [18,21], was measured in cells (approximately $1.5 \times 10^4$ cells/$cm^2$) that had been preincubated for up to 48 h in mEMEM then incubated for a further 24–48 h in mEMEM or Iscove's medium containing the hormone under study. Although EMEM is a standard culture medium for melanocytes [15], the Iscove's medium used in these experiments contains bovine serum albumin and phospholipids that enhance the solubility of steroids and steroid-like compounds such as $1,25(OH)_2D_3$ (Mason et al, unpublished observations). Qualitatively similar results were obtained in the presence of either medium. After this incubation period, cell extracts were prepared by lysing in a phosphate buffer containing 0.1% Triton X-100 [9] and tyrosinase activity was measured by the method of Pomerantz [22]. This method measures the amount of $[^3H]H_2O$ released from $[^3H]$ tyrosine by tyrosinase in the presence of a catalytic amount of DOPA cofactor. All values were corrected for non-enzymic hydroxylation of $[^3H]$ tyrosine by subtracting the amount of $[^3H]H_2O$ formed by cell extracts containing 1mM phenylthiourea [18].

**cAMP Measurements** After preincubation with mEMEM for 48–72 h the cell monolayers (containing approximately $1 \times 10^4$ cells/$cm^2$) were washed twice with phosphate buffered saline and incubated for 10 min at room temperature in mEMEM containing 2% charcoal stripped FCS and 10 mM IBMX [23]. $1,25(OH)_2D_3$ or $\alpha$-MSH were then added and the cells were incubated for a further 12 min at room temperature. The medium was aspirated, the cells extracted with absolute ethanol and an aliquot assayed for cAMP by a radioimmuno-assay technique using $[^{125}I]$cAMP and a laboratory generated antibody [24]. Another aliquot of the cell extract was diluted in phosphate buffered saline, sonicated, and assayed for DNA content by the method of Kapuscinski and Skoczylas [25] using denatured, highly polymerized DNA from calf thymus as a standard. Although the concentration of IBMX used in these experiments is relatively high, previous studies in this laboratory have shown that under these conditions, hormonal effects on cAMP production are demonstrable in a wide variety of cell types, including pigment cells [9,23,24].

**Statistical Analysis** Groups were compared using Student's t-test for unpaired data.

## RESULTS

**Specific Uptake of $[^3H]$ $1,25(OH)_2D_3$ by Melanocytes** Intact melanocytes accumulated $[^3H]$ $1,25(OH)_2D_3$ in a specific, saturable manner (Fig 1). In order to obtain a quantitative measure of the translocation process [19], this data was analyzed by the method of Scatchard [26]. A straight line was obtained with an apparent dissociation constant (Kd) for the $1,25(OH)_2D_3$ intracellular binding site of 0.8nM and a total concentration (N max) of 7 fmol/$10^6$ cells (Fig 1, *inset*).

Scatchard analysis of the data generated with nuclear bound

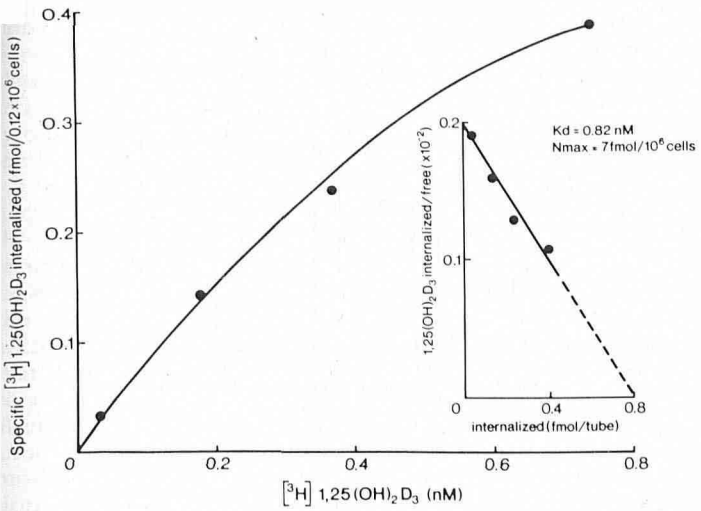


**Figure 1.** Specific [$^3$H]1,25(OH)$_2$D$_3$ uptake by intact human melanocytes. Cell suspensions containing $0.12 \times 10^6$ cells/500 $\mu$l were incubated with increasing concentrations of [$^3$H] 1,25(OH)$_2$D$_3$ in the absence or presence of excess unlabeled 1,25(OH)$_2$D$_3$. Specific uptake was calculated by subtracting uptake in the presence of unlabeled material from uptake in the absence of unlabeled material. *Inset:* Scatchard analysis of data.

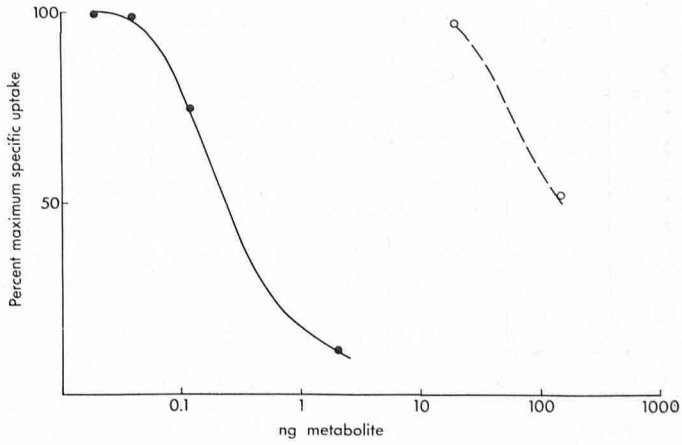


**Figure 2.** Competition of two vitamin $D_3$ metabolites for the 1,25(OH)$_2$D$_3$ binding site in intact human melanocytes. Cell suspensions containing $0.5 \times 10^6$ cells/500 $\mu$l were incubated with 20 pg [$^3$H]1,25(OH)$_2$D$_3$ and with increasing concentrations of unlabeled 1,25(OH)$_2$D$_3$ (●—●) or 25(OH)D$_3$ (○—○). Each *point* represents the mean of duplicate determinations. The values on the *abcissa* refer to the amounts of the two metabolites added to each tube (per 500 $\mu$l).

1,25(OH)$_2$D$_3$ gave very similar results. The apparent dissociation constant was 0.51 nM, while the total concentration was 10 fmol/$10^6$ cells (data not shown).

In the presence of 2 nmol unlabeled 1,25(OH)$_2$D$_3$ the specific uptake of [$^3$H] 1,25(OH)$_2$D$_3$ was reduced by approximately 50%, whereas at least 1000-fold molar excess of 25-hydroxyvitamin $D_3$ was required to effect a similar reduction in uptake (Fig 2).

**Effects of 1,25(OH)$_2$D$_3$ on 24-Hydroxylase Activity, Tyrosinase Activity and Melanocyte Numbers** Incubation of melanocytes with $10^{-10}$ or $10^{-9}$ M 1,25(OH)$_2$D$_3$ for 48 h resulted in a twofold increase in 24-hydroxylase activity (Fig 3). This response was biphasic with a maximal increase at $10^{-9}$ M 1,25(OH)$_2$D$_3$ (Fig 3).

Incubation with 1,25(OH)$_2$D$_3$ ($10^{-10} - 10^{-8}$ M) for 48 h in mEMEM resulted in a dose-dependent increase of tyrosinase activity (Fig 4*a*). Under these conditions the maximal tyrosinase response in melanocytes from three donors varied between 136% and 160% of control values. If melanocytes were examined after only 24 h incubation with 1,25(OH)$_2$D$_3$ in mEMEM the increases in tyrosinase activity were smaller and varied between 105% and 136% of control values, depending on the donor source (data not shown). Qualitatively similar results were obtained when the melanocytes were incubated with 1,25(OH)$_2$D$_3$ in Iscove's medium, except that increases of 147%–170% (at $10^{-9}$ M) of the control values were seen after only 24 h ($p < 0.01$).

Incubation with similar concentrations of 1,25(OH)$_2$D$_3$ also resulted in a decrease in cell numbers (Fig 4*b*). In three experiments, the magnitude of the decrease in cell numbers induced by 1,25(OH)$_2$D$_3$ at various concentrations was negatively related to the increase in tyrosinase activity ($n = 9$, $r = 0.744$, $p < 0.025$).

1,25(OH)$_2$D$_3$ had no significant effect on intracellular cAMP levels at any of the concentrations examined (data not shown).

**Effects of $\alpha$-MSH on cAMP Production and Tyrosinase Activity** Incubation of melanocytes with $\alpha$-MSH ($5 \times 10^{-7}$ M) for 12 min resulted in a sevenfold increase in intracellular cAMP concentrations (Fig 5).

In contrast, incubation of melanocytes with the same concentration of $\alpha$-MSH ($5 \times 10^{-7}$ M) for 48 h increased tyrosinase activity by only 12 ± 3% above control values ($n = 3$; $p < 0.05$). $\alpha$-MSH at this concentration and over this time period had no effect on cell numbers.

**Effect of Beta-estradiol on Tyrosinase Activity and Melanocyte Numbers** Incubation of melanocytes with beta-estradiol ($10^{-12} - 10^{-9}$ M) for 24 h resulted in a dose-dependent stimulation of tyrosinase activity and a 50% reduction in cell numbers with $10^{-10} - 10^{-9}$ M beta-estradiol (Fig 6). As with 1,25(OH)$_2$D$_3$ there was a negative correlation between the decrease in cell numbers and the increase in tyrosinase activity ($n = 15$, $r = -0.7257$, $p < 0.01$). The results in Table I show that the magnitude of the beta-estradiol-induced tyrosinase response was similar in cells from the same donor at different passages (cell line 0109, passages 5 and 6) but may differ between donors.

## DISCUSSION

On the basis of studies in intact animals 1,25(OH)$_2$D$_3$, $\alpha$-MSH, and sex steroids are regarded as probable regulators of pigment cell function [2,11,27]. The evidence that these hormones affect pigment cell function in vitro has been limited largely to studies with melanoma cell lines [8,9,12].

In some respects the results obtained with 1,25(OH)$_2$D$_3$ in human melanocytes are similar to those reported in human melanoma cells [5–7,9]. Human melanocytes bind 1,25(OH)$_2$D$_3$ with affinities similar to those reported for human and animal melanoma cells and for other types of skin cells [28]. They respond to this hormone with a large increase in 24-hydroxylase activity. This "classical" response has been shown in other cells to be a receptor-mediated event [29]. The lack of cAMP responses to 1,25(OH)$_2$D$_3$

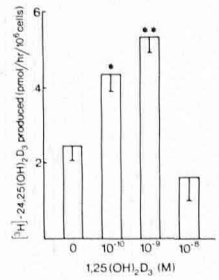


**Figure 3.** Effect of 48-h preincubation with 1,25(OH)$_2$D$_3$ on the 24-hydroxylase activities of human melanocytes. The cells were incubated with [$^3$H]25(OH)D$_3$ (0.4 $\mu$Ci/ml; specific activity 2 Ci/mmol) for 90 min. *Bar* heights indicate the mean ± 1SD of triplicate determinations. *Asterisks* refer to differences between treated and control values: *$p < 0.01$; **$p < 0.001$.

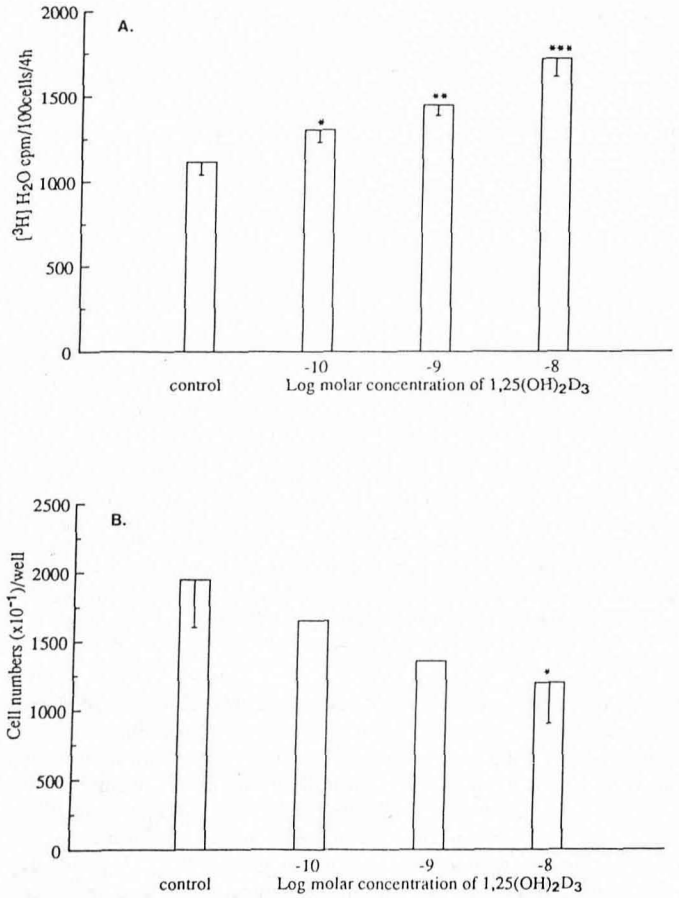


**Figure 4.** Effect of $1,25(OH)_2D_3$ on the tyrosinase activities and cell numbers of human melanocytes. Melanocytes were incubated for 48 h with $1,25(OH)_2D_3$ in mEMEM then assayed for tyrosinase activity (*A*) or counted (*B*). *Bar* heights indicate the mean ±1SD of triplicate determinations (*A*) and mean ± 1SD of triplicate determinations (0 and $10^{-8}$ M) (*B*) from one representative experiment. *Asterisks* refer to differences between treated and control values: *$p < 0.025$; **$p < 0.005$; ***$p < 0.001$.

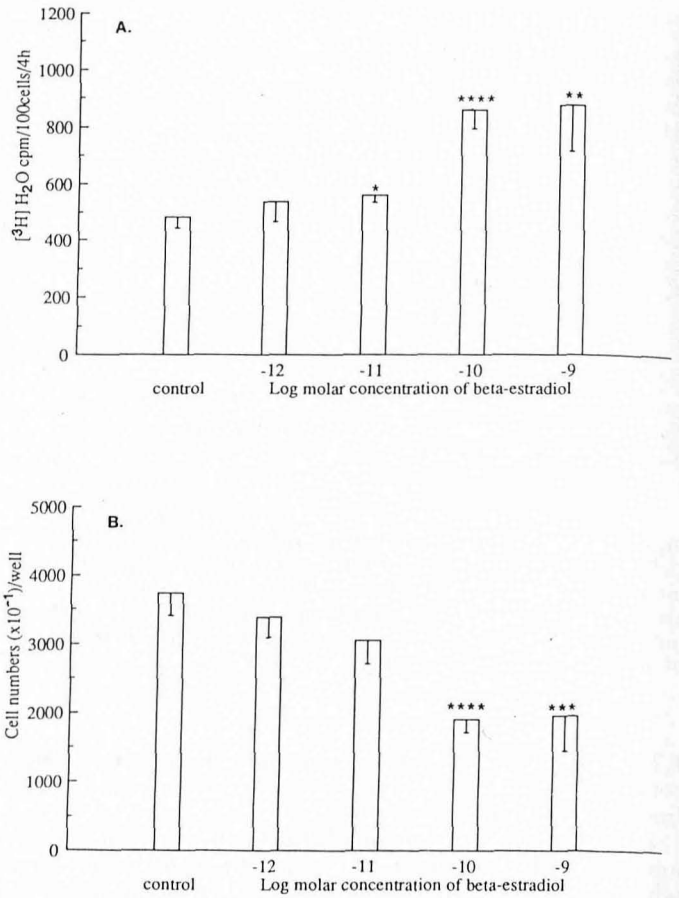


**Figure 6.** Effect of beta-estradiol on the tyrosinase activities and cell numbers of human melanocytes. Melanocytes, seeded on to extracellular matrix-coated wells [18], were incubated for 24 h with beta-estradiol then assayed for tyrosinase activity (*A*) or counted (*B*). *Bar* heights indicate the mean ±1SD for triplicate determinations from one representative experiment. *Asterisks* refer to differences between treated and control values: *$p < 0.05$; **$p < 0.02$; ***$p < 0.005$.

is in accord with the observation that the effects of steroid hormones are generally not mediated via the adenylate cyclase pathway [30].

It has previously been shown with melanoma cells [8,9] that the increase in tyrosinase activity induced by $1,25(OH)_2D_3$ is generally accompanied by a decrease in cell proliferation and it has been suggested that these effects reflect $1,25(OH)_2D_3$-mediated cell differentiation [31]. It is possible that culture in the presence of PMA may cause the melanocytes to become more "undifferentiated" when compared with normal melanocytes from intact skin and this may alter the expression of $1,25(OH)_2D_3$ receptors. However, the available evidence suggests that melanocytes propagated in vitro by the mitogens PMA and CT are phenotypically stable and relatively well differentiated [18]. The experiments reported here were performed after the removal of PMA and CT for 24–48 h. Under these condi-

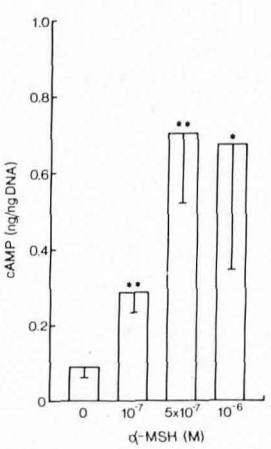


**Figure 5.** Effect of α-MSH on intracellular cAMP levels in human melanocytes. *Bar* heights indicate the mean ± 1SD for triplicate determinations from one representative experiment. *Asterisks* refer to differences between treated and control values: *$p < 0.05$; **$p < 0.02$.

**Table I.** Effect of Beta-estradiol on the Tyrosinase Activities of Human Melanocytes from Different Donors[a]

| Cell Line | Tyrosinase Activity cpm/$10^2$ cell/4 h | | |
|---|---|---|---|
| | Control | Beta-estradiol[b] | % Increase |
| 5/0109 | 418 ± 19 | 889 ± 29[c] | 213 |
| 6/0109 | 483 ± 39 | 872 ± 159[d] | 181 |
| 5/1108 | 1572 ± 108 | 2277 ± 141[e] | 145 |
| 5/1812 | 548 ± 21 | 1015 ± 60[f] | 185 |
| 4/2501 | 671 ± 12 | 965 ± 68[d] | 144 |

[a] Refer to legend to Fig 6 for experimental detail.
[b] The concentration of beta-estradiol used in these experiments was $10^{-9}$ M.
[c] $p < 0.001$, significantly different from control values.
[d] $p < 0.02$, significantly different from control values.
[e] $p < 0.01$, significantly different from control values.
[f] $p < 0.005$, significantly different from control values.

tions the subsequent state of differentiation in these cells, at least as assessed by tyrosinase activity, appears to be unaltered as only a small change (a decrease of approximately 10%–15%) in the tyrosinase activity per cell occurs (Ranson, unpublished data). Furthermore, tyrosinase responses to $1,25(OH)_2D_3$ are similar whether PMA/CT has been removed for 1, 2, or 4 d (Ranson, unpublished data).

Unlike melanoma cells, in the absence of PMA and CT the melanocytes cease to proliferate [18]. It is difficult, therefore, to attribute the effect of $1,25(OH)_2D_3$ (or beta-estradiol) on the numbers of attached cells to an inhibition of proliferation when, under the experimental conditions, no proliferation normally occurs. Thus the reason for the decrease in cell numbers in the presence of $1,25(OH)_2D_3$ or beta-estradiol is unclear. Although it is possible that either hormone is directly toxic to melanocytes, the negative correlation between tryosinase activity and cell numbers suggests an alternative explanation. Because the basal tyrosinase activity of melanocytes is generally much higher than that of melanoma cells even modest increases in tyrosinase activity may result in the accumulation of melanin precursors that are cytotoxic [32].

Our results are not in agreement with those of Tomita et al [33] who showed that a 6-d incubation with vitamin $D_3$ but not $1,25(OH)_2D_3$ caused an increase in the amount of tyrosinase in human melanocytes. However, the tyrosinase measurements in that study were expressed as units of relative staining intensity and were not corrected for cell numbers or protein concentrations. Furthermore, in the experiments of Tomita et al [33] melanocytes were cultured under PMA-free conditions for 9 d. Our studies using a similar system indicate a marked decrease in cell numbers under these conditions after even 5 d [18].

The production of vitamin $D_3$ [the precursor of $1,25(OH)_2D_3$] under the influence of ultraviolet light is diminished by the presence of melanin [34]. Colston et al [6] and Kuroki [28] have suggested that $1,25(OH)_2D_3$ may act in a negative feedback manner to reduce the photo-conversion of 7-dehydro-cholesterol to vitamin $D_3$. Our results, which indicate that $1,25(OH)_2D_3$ stimulates tyrosinase activity in melanocytes from normal skin, provide some evidence to substantiate this hypothesis and further support the theory that $1,25(OH)_2D_3$ is involved in the differentiation and development of skin [28].

Some murine and human melanoma cell lines respond to $\alpha$-MSH with large increases in cAMP followed by large increases in tyrosinase activity [14]. However, there are conflicting data concerning the presence or otherwise of direct effects of $\alpha$-MSH on normal melanocytes. Nielsen and Don [35], on the basis of unpublished data, reported that $\alpha$-MSH enhanced the tyrosinase activity of human melanocytes, whereas Halaban et al [21], who detected no effects, speculated that this phenomenon might be due to an inability of $\alpha$-MSH to increase intracellular cAMP concentrations in melanocytes. Our studies indicate that this postulated mechanism is incorrect. However, the large increases in intracellular cAMP concentrations were followed by only small increases in tyrosinase, a discrepancy that has also been described in relation to some melanoma cells [36]. The demonstrated effects of $\alpha$-MSH in vivo may be due to the presence of potentiating factors [37] that have yet to be identified. It is possible that other fragments of the pro-opiomelanocortin precursor also have direct effects on human melanocyte activity.

The presence of estrogen receptors was described in human melanoma cells many years ago [13,38,39] though the functional significance of these receptors has remained somewhat controversial [40–42]. To our knowledge, these are the first studies to show a direct biologic effect of an estrogen on normal melanocytes. The concentrations employed were in the physiologic range seen in pregnancy [43] and the estradiol-induced increase in tyrosinase may help to explain pregnancy-associated pigmentation.

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