25-Hydroxylation of vitamin D_3 in the human hepatoma cell lines Hep G2 and Hep 3B

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Abstract Two human hepatoma cell lines, Hep G2 and Hep 3B, were screened for vitamin D_3-25-hydroxylase enzyme activity by incubation with radioactive vitamin D_3. A compound co-chromatographing with 25-OH-D_3 was synthesized in both cell lines but its rate of synthesis was tenfold greater in Hep 3B than in Hep G2 cells. The identity of the compound was confirmed by comparing its chromatographic properties with authentic 25-OH-D_3 on three different high pressure liquid chromatography systems. Its production was suppressed by adding fetal calf serum (10%), lipoprotein-deficient fetal calf serum, or pure vitamin D-binding globulin to the medium. The mechanism of action of these plasma proteins appears to involve retardation of uptake of the substrate. These two cell lines offer considerable potential as defined in vitro models for studying the effects of physiological factors on the 25-hydroxylation of vitamin D_3. — **Tam, S-P., S. Strugnell, R.G. Deeley, and G. Jones.** 25-Hydroxylation of vitamin D_3 in the human hepatoma cell lines Hep G2 and Hep 3B. *J. Lipid Res.* 1988. **29:** 1637–1642.

Supplementary key words 25-hydroxyvitamin D_3 • vitamin D_3-25-hydroxylase • vitamin D-binding globulin

The enzyme vitamin D_3-25-hydroxylase is required for the first step in the activation of vitamin D_3 to the hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in all vertebrate species. Although it is generally believed that the second step, requiring the renal 25-OH-D_3-1-hydroxylase constitutes the main point of control of 1,25-$(OH)_2D_3$ synthesis (1,2), there is considerable evidence suggesting that hepatic vitamin D_3-25-hydroxylase can also be regulated by the concentration of plasma factors such as 25-OH-D_3 and possibly Ca^{2+}, PO_4^{3-}, and 1,25-$(OH)_2D_3$ (3–5).

The liver represents the major site for 25-hydroxylation of vitamin D_3 in the rat (6). 25-Hydroxylase activity has been demonstrated in vitro using rat liver homogenates (5), perfusion of intact liver (7), and isolated rat hepatocytes (8,9). It has also been demonstrated that vitamin D_3 can be taken up both by hepatocytes and nonparenchymal liver cells. However, 25-hydroxylation in vitro occurs only in hepatocytes (9). Recently, Oftebro and co-workers (10) have reported that a cytochrome P-450 enzyme system isolated from human liver mitochondria is able to convert vitamin D_3 into 25-OH-D_3. Despite the availability of a variety of in vitro systems, most of these models have drawbacks that relate to lack of an intact cell membrane or lack of extended viability. A defined established cell line would be advantageous for the study of the metabolism and regulation of vitamin D_3 and its metabolites. As yet, no liver cell lines have been utilized to examine this process.

In this article, we have investigated 25-hydroxylation of vitamin D_3 in two human hepatoma cell lines, Hep G2 and Hep 3B. These two cell lines have been shown to retain parenchymal cell morphology and to synthesize and secrete a variety of plasma proteins (11,12). Unlike Hep G2, the cell line Hep 3B also expresses the hepatitis B virus surface antigen and produces metastatic hepatocellular carcinomas when injected into nude mice (12). For these reasons, there has been a tendency to use Hep G2 cells as the preferred in vitro model for studying a variety of human hepatocyte functions. Hep G2 cells have been shown to express receptors for insulin and transferrin (13), estrogen (14), and low density lipoproteins (15), and to bind high density lipoproteins (16). It has been shown that Hep G2 cells also express the major enzymes of intra- and extracellular cholesterol metabolism (17). Although the spectra of proteins synthesized by both cell lines overlap extensively, they do differ in some important aspects. One of the striking differences between Hep G2 and Hep 3B cells is that the latter synthesize and secrete vitamin D-binding protein (DBP) while the former do not (18). This prompted us to investigate whether both cell lines express 25-hydroxylase activity and whether there is any relationship between the expression of DBP and vitamin

Abbreviations: 25-hydroxylase, vitamin D_3-25-hydroxylase; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OH-D_3, 25-hydroxyvitamin D_3; FCS, fetal calf serum; LPDS, lipoprotein-deficient fetal calf serum; DBP, vitamin D-binding globulin (Gc protein); HPLC, high pressure liquid chromatography.

D_3-25-hydroxylase. Some of our initial findings concerning the 25-hydroxylation of vitamin D_3 have been presented in abstract form (19).

EXPERIMENTAL METHODS

Cell culture

Experiments were carried out using two human hepatoma-derived cell lines, Hep G2 and Hep 3B, obtained from American Tissue Culture Collection. Cells were grown in T75 flasks containing 30 ml of Earle's minimal essential medium (MEM) supplemented with L-glutamine (4 mM), penicillin (100 I.U./ml), and streptomycin (100 μg/ml) containing 10% fetal calf serum (FCS), and incubated at 37°C in a humidified atmosphere of 95% air, 5% CO_2. Fresh medium was added every 2 days. For experiments, cells were plated in T25 flasks and grown to late log phase. Nearly confluent monolayers were washed three times with MEM and then incubated with [1,2-^{3}H]vitamin D_3 (10^5 cpm; sp act 20 Ci/mmol; Amersham, Oakville, Ontario, Canada) for 4 to 48 hr at 37°C in one of the five culture media described. Each flask contained 2 ml of medium. Cell viability was determined by the trypan blue dye exclusion method and cell number was determined by counting with a hemocytometer (14). In all experiments the number of dead cells never exceeded 5% of the total number of cells.

Culture media

Hep G2 and Hep 3B cells were incubated for 48 hr in one of the following five media: *1*) MEM; *2*) MEM + 10% FCS; *3*) MEM + 10% FCS + 1 μM estradiol; *4*) MEM + 10% lipoprotein-depleted serum (LPDS); *5*) MEM + DBP (25 μg/ml). LPDS was prepared by removing the lipoproteins by flotation at d < 1.25 g/ml as described (14). DBP was purchased from Sigma.

Extraction procedure and column chromatography

After incubation, the 2 ml of medium from each T25 flask was transferred to a capped glass test tube. Two ml trypsin/EDTA (0.25%; 0.02%) solution was added to each flask and incubated at 37°C for 10 min. The cell suspension thus obtained was either combined with the corresponding medium for extraction together or the cell suspension and incubation medium were kept apart during subsequent stages of analysis. Extractions were carried out by the modification of the method of Bligh and Dyer (20). Briefly, 2 ml of medium or cells was diluted with 5 ml of methanol and 2.5 ml of methylene chloride, vortexed, and allowed to stand for 30 min. A second 2.5-ml aliquot of methylene chloride was added, vortexed again, and then 5 ml of 25% saturated KCl solution was added. The tubes were centrifuged at 1,000 *g*, 0°C for 15 min in a Beckman J-6B centrifuge to give two layers with a pellet of precipitated proteins at the interface. The upper aqueous layer was removed by aspiration and the lower methylene chloride layer was transferred to a 5-ml Reactivial (Pierce, Rockford, IL) and evaporated under a stream of prepurified nitrogen. The extract was redissolved in hexane–isopropanol–methanol 96:3:1 (21) and an aliquot was subjected to HPLC on a Zorbax-SIL column (25 cm × 6.2 mm) using the same solvent mixture at a flow rate of 2.0 ml/min. Forty fractions (0.5 min/fraction) were collected. Identification of putative D_3 metabolites was by comigration of radioactive peaks with authentic 25-OH-D_3 detected using UV absorbance (UV max = 265 nm). The samples were counted in a Beckman liquid scintillation counter (Model 1800) at 45% efficiency. 25-Hydroxylase activity was calculated from the percentage of radioactivity recovered as 25-OH-D_3 and the specific activity of the substrate.

In some experiments, putative [^{3}H]25-OH-D_3 peaks were collected and rerun on further HPLC systems to confirm the identity of the peak. These HPLC systems are described in the legend to Fig. 1.

TABLE 1. Basal levels of 25-hydroxylation in the presence of fetal calf serum

Type of Incubation[a]	% Radioactivity Recovered as			
	Nonpolar cpm and esters	Vitamin D_3	25-OH-D_3	25-Hydroxylase
				fmol/10^7 cells per day
No cells	<0.01	97.00	0.32	0
Hep G2	0.06 ± 0.01	95.52 ± 0.20	0.88 ± 0.02	14 ± 1
Hep 3B	0.08 ± 0.01	91.90 ± 0.20	4.65 ± 0.20	107 ± 5

[a]Cells were incubated with [^{3}H]vitamin D_3 for 48 hr. Incubations were carried out in 2 ml MEM supplemented with 10% FCS. Control values represent the average of two experiments; experimental values represent the mean ± SEM of three experiments.

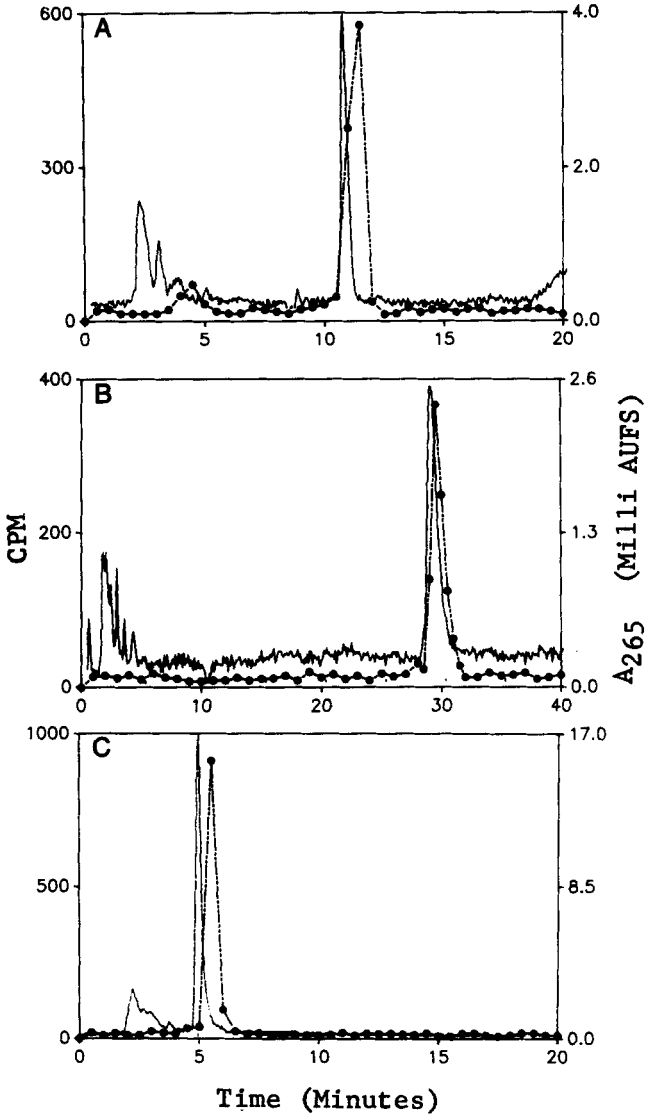

Fig. 1. Comigration of putative [^{3}H]25-OH-D_3 metabolite and authentic 25-OH-D_3 on three HPLC systems. The putative [^{3}H]25-OH-D_3, generated in Hep B3 cells, was purified on a Zorbax-SIL column. Authentic 25-OH-D_3 was added and the mixture was chromatographed on three different HPLC systems: *a*) Zorbax-SIL, hexane–isopropanol–methanol 96:3:1, flow rate 1 ml/min; *b*) Zorbax-CN, hexane–isopropanol 99:1, flow rate 2 ml/min; *c*) Zorbax-ODS, acetonitrile–water–methanol 90:5:5, flow rate 1 ml/min. Unbroken lines represent UV traces at 265 nm: (●-----●) represents radioactivity in 0.5-min fractions. The difference in elution times between the peaks of radioactivity and absorbance (265 nm) represents the deadspace (≈ 500 μl) separating the UV detector and the fraction collector. This difference appears smaller in panel B partly due to the flow rate being greater and partly due to the scale of the X-axis being more compressed.

RESULTS

Comparison of basal vitamin D_3- 25-hydroxylase activity in Hep G2 and Hep 3B cells

Initial experiments focused on whether we could demonstrate the formation of [^{3}H]25-OH-D_3 in hepatoma cell cultures. In the control experiments where [^{3}H]vitamin D_3 was incubated with 2 ml of MEM containing 10% FCS in the absence of cells for 2 days, little radioactivity (only 0.32% of total) was recovered in the fractions eluting in the position of authentic 25-OH-D_3 (**Table 1**). Small amounts of radioactivity migrating in this region of the chromatogram were not significantly above background and were attributable to minute amounts of impurities in the original substrate and to formation of nonenzymatic products during incubation. In contrast, when [^{3}H]vitamin D_3 was incubated with Hep G2 or Hep 3B cells and in the presence of 10% FCS for 2 days, small but significant amounts of radioactivity (0.88 ± 0.02% and 4.65 ± 0.2%, respectively) were found in the region of the HPLC profile corresponding to 25-OH-D_3. Further chromatography of the material produced by Hep 3B and Hep G2 cells revealed that it comigrated with authentic, standard 25-OH-D_3 on three different HPLC systems (**Fig. 1**). Over a 2-day period, net conversion of vitamin D_3 to 25-OH-D_3 by Hep G2 cells amounted to only 0.56% of total recovered radioactivity. Despite the relatively small amount converted, recovery of 25-OH-D_3 was still significantly higher than controls. In Hep 3B cells, the conversion rate was markedly higher, amounting to 4.33% of total recovered radioactivity. This suggested that the rate of hydroxylation was at least 8–10 times greater in Hep 3B than in Hep G2 cells (Table 1).

This suggestion was supported by subsequent experiments in which we studied the rate of formation of [^{3}H]25-OH-D_3 with time of incubation. It can be seen from examination of **Fig. 2** that Hep 3B cells synthesized [^{3}H]25-OH-D_3 at a linear rate over the full 2-day incubation period. The data presented in Fig. 2 also demonstrate the effect that FCS has on 25-hydroxylation.

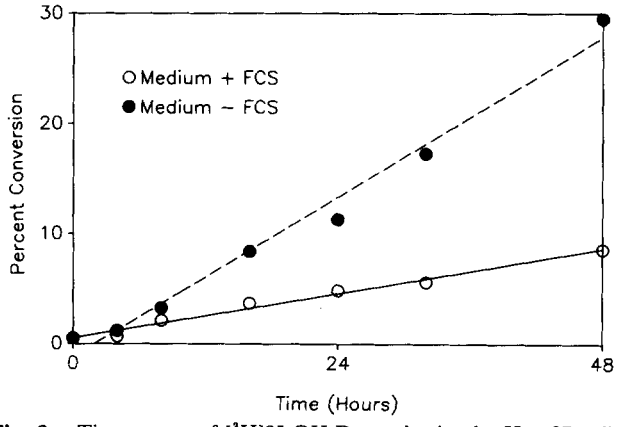

Fig. 2. Time course of [^{3}H]25-OH-D_3 production by Hep 3B cells. (●-----●) Cells incubated in medium without FCS; (○—○) cells incubated in medium with 10% FCS. Cells were grown to near confluence in MEM supplemented with 10% FCS. Cells incubated with 10% FCS had the medium changed at the beginning of the incubation. Cells incubated in the absence of FCS were washed three times with 5 ml PBS, and 2 ml MEM was added to each flask. Substrate was added in 10 μl EtOH at the start of the incubation. Results represent 25-OH-D_3 appearing in the medium. Values represent the mean of three experiments. Coefficient of variation did not exceed 5%.

TABLE 2. Effect of fetal calf serum on 25-hydroxylation

Type of Incubation[a]	% Radioactivity Recovered as: Nonpolar cpm and esters	Vitamin D_3	25-OH-D_3	25-Hydroxylase
				fmol/10^7 cells per day
Hep G2 + FCS	1.08 ± 0.05	93.70 ± 0.19	1.20 ± 0.05	22 ± 1
Hep G2 − FCS	3.71 ± 0.24	86.80 ± 0.26	2.66 ± 0.02	58 ± 1
Hep 3B + FCS	0.84 ± 0.02	84.22 ± 0.31	10.45 ± 0.32	252 ± 8
Hep 3B − FCS	1.50 ± 0.11	70.47 ± 0.25	22.71 ± 0.15	557 ± 4

[a]Cells were incubated with [^{3}H]vitamin D_3 for 48 hr. Incubations were carried out in 2 ml MEM in the presence or absence of fetal calf serum (FCS). Values represent the mean ± SEM of three experiments.

Factors affecting 25-hydroxylation in cell culture

Effect of fetal calf serum on vitamin D_3-25-hydroxylase activity. Since it was unclear whether vitamin D_3 delivery to the hepatocytes involved serum components such as lipoproteins or vitamin D-binding protein (DBP), we investigated the effect of omitting serum from the culture medium on the vitamin D_3-25-hydroxylase activity. Fig. 2 shows the effect of removal of FCS on the production of 25-OH-D_3 by Hep 3B cells. Both Hep 3B and Hep G2 cells showed a two- to threefold increase in 25-hydroxylase activity on removal of the FCS from the culture medium (**Table 2**). Examination of the cell pellet at various timepoints between 4 and 48 hr showed that the two- to threefold increase in hydroxylase activity resulting from the omission of FCS was also accompanied by a two- to threefold increase in total radioactivity associated with the Hep 3B cells (data not shown). The latter observation strongly suggests that one of the effects of FCS may be to slow the rate of uptake of vitamin D_3 by the cells.

Effects of vitamin D_3-25-hydroxylase activity after removal of lipoproteins or addition of DBP to culture media. We next investigated whether the addition or removal of isolated serum components such as lipoproteins or DBP would influence the 25-hydroxylase activity in these cell lines. The effect of removing lipoproteins was examined by culturing the cells in medium containing 10% lipoprotein-depleted serum (LPDS). The results obtained indicate that 25-hydroxylation of vitamin D_3 in Hep G2 and Hep 3B cells is not enhanced by removing exogenous lipoproteins (**Table 3**). The suppressive effect of lipoprotein-depleted FCS on 25-hydroxylase activity is equivalent to that of complete serum, suggesting that the possible sequestration of vitamin D_3 by these components does not have a significant effect on its availability for metabolism under the culture conditions we have used.

Experiments were also carried out to test whether the suppressive effect of serum could be attributed to DBP. Hep G2 and Hep 3B cells were incubated with [^{3}H]vitamin D_3 in the presence of DBP (25 μg/ml), but in the absence of FCS. Results from these experiments (Table 3) indicate that exogenous DBP alone can reduce the rate of 25-hydroxylation in both cell lines. The inhibition is approximately equivalent to that obtained by adding FCS.

Effect of 17β-estradiol on liver 25-hydroxylation. It has been shown in rodents that estrogens can influence the cytochrome P-450 system, including a variety of steroid hydroxylases (22). Consequently, we examined the possible effects of 17β-estradiol on 25-hydroxylase activity in Hep G2 and Hep 3B cells. Maintenance of Hep G2 and Hep 3B cells in medium adjusted to 1 μM in 17β-estradiol every 12 hr, over a period of 48 hr, did not alter the rate of conversion of vitamin D_3 to 25-OH-D_3 when compared to control cells. Estrogens at the concentrations

TABLE 3. Effect of lipoprotein-deficient serum and vitamin D-binding protein on 25-hydroxylation

Type of Incubation[a]	% Radioactivity Recovered as: Nonpolar cpm and esters	Vitamin D_3	25-OH-D_3	25-Hydroxylase
				fmol/10^7 cells per day
Hep 3B + FCS	1.60 ± 0.05	82.80 ± 0.68	8.28 ± 0.43	198 ± 11
Hep 3B − FCS	2.57 ± 0.10	71.86 ± 1.23	16.84 ± 1.20	410 ± 30
Hep 3B + LPDS	2.01 ± 0.08	81.98 ± 0.36	6.93 ± 0.14	164 ± 3
Hep 3B + DBP	2.07 ± 0.12	81.73 ± 0.27	8.01 ± 0.27	191 ± 7

[a]Cells were incubated with [^{3}H]vitamin D_3 for 48 hr. Incubations were carried out in 2 ml MEM supplemented with 10% fetal calf serum (FCS) or 10% lipoprotein-deficient fetal calf serum (LPDS) or purified DBP (5% physiological concentration; 25 μg/ml). Values represent the mean ± SEM of three experiments.

used appear to have no effect on the 25-hydroxylation in these two cell lines (**Table 4**).

DISCUSSION

In this study, we have screened two human hepatoma cell lines, Hep G2 and Hep 3B, for their ability to metabolize [^{3}H]-vitamin D_3 to [^{3}H]25-OH-D_3 during a 2-day culture period. By using different HPLC systems, we were able to demonstrate that the product comigrates with 25-OH-D_3 under a variety of column and solvent conditions. Despite the similarity of these two cell lines with respect to many parenchymal cell functions, we were able to show that Hep 3B cells have a ten-fold higher 25-hydroxylase activity than Hep G2 cells. At the moment, the rate of conversion of vitamin D_3 to 25-OH -D_3 in normal human liver is not known. Consequently, it is not possible at present to judge whether Hep G2 cells are defective in 25-hydroxylation or whether Hep 3B cells have an abnormally high 25-hydroxylase activity. This reservation notwithstanding, the availability of two highly differentiated hepatoma cell lines with very different basal rates of 25-hydroxylation provides an attractive in vitro model for studying the regulation of hydroxylase activity by such factors as 25-OH-D_3, 1,25-$(OH)_2D_3$, Ca^{2+} and PO_4^{3-}, the roles for which remain controversial.

One problem with the new models was the interassay variability between experiments. In our hands the basal 25-hydroxylase activity of Hep 3B averaged 260 ± 66 (SD) fmol/10^7 cells per day (n = 22) in the presence of fetal calf serum and 550 ± 98 (SD) fmol/10^7 cells per day in the absence of fetal calf serum (n = 32). Thus, in some experiments reported here (e.g., Table 1), the 25-hydroxylase activity is lower than normal, whereas in others (e.g., Table 4) activity is higher than normal. Some biological variation is thus to be expected and may be due to variations in cell density and/or minor differences in incubation conditions. In all experiments reported here we used an internal control to offset the variation in basal 25-hydroxylase activity. Nevertheless, all experiments were reproducible and gave the same qualitative change in 25-hydroxylase activity.

The removal of FCS from culture medium clearly increased the rate of vitamin D_3-25-hydroxylation in both cell lines. This raised the possibility that *1*) the influence of serum on cell growth resulted in an inhibition of 25-hydroxylation, or *2*) components in the serum were sequestering the substrate and thus slowing its rate of uptake and subsequent conversion to 25-OH-D_3. Results from experiments using LPDS and supplementation of the culture medium with DBP suggest that the latter possibility is more likely to be the case. The two prime candidates in the serum likely to sequester vitamin D_3 are lipoproteins and DBP. The fact that the suppressive effect of FCS is still present after removal of lipoproteins by ultracentrifugation indicates that they are probably not the major sequestering agent of vitamin D_3. Since purified DBP at a concentration far less than the circulating level resulted in a decrease in the rate of hydroxylation equivalent to that observed with 10% FCS, our results strongly suggest that this is the primary component in serum responsible for inhibiting vitamin D_3 uptake by the cells. With the increased rate of conversion of vitamin D_3 to 25-OH-D_3 in Hep 3B in the absence of FCS, it is likely that we could shorten the incubation time to hours rather than 2 days and still generate measurable amounts of product. This will offer advantages for examining the short-term regulation of 25-hydroxylation of vitamin D_3 by hormones or ions. It is also of interest to note that Hep 3B cells are capable of synthesizing DBP and displaying vitamin D_3-25-hydroxylase activity while in Hep G2 cells DBP is undetectable and 25-hydroxylase activity low. Thus, there exists a possibility that the regulation of these proteins may be linked.

It is perhaps also worth noting that Hep G2 cells have an estrogen 2α-hydroxylase activity five- to tenfold lower than Hep 3B cells (S-P. Tam and P. H. Jellinck, unpublished results) suggesting that Hep G2 cells might have a defective or suppressed cytochrome P-450 system. How-

TABLE 4. Effect of 1 μM estradiol of 25-hydroxylation

Type of Incubation[a]	% Radioactivity Recovered as			
	Nonpolar cpm and esters	Vitamin D_3	25-OH-D_3	25-Hydroxylase
				fmol/10^7 cells per day
Hep G2 − E_2	4.23 ± 0.47	90.80 ± 1.05	1.15 ± 0.15	21 ± 4
Hep G2 + E_2	5.90 ± 0.32	88.40 ± 0.26	1.53 ± 0.09	30 ± 2
Hep 3B − E_2	2.07 ± 0.07	80.20 ± 0.81	15.10 ± 0.43	367 ± 11
Hep 3B + E_2	2.70 ± 0.15	80.20 ± 0.40	14.40 ± 0.37	350 ± 9

[a]Cells were incubated with [^{3}H]vitamin D_3 for 48 hr. Incubations were carried out in 2 ml MEM supplemented with 10% FCS in the presence or absence of 1 μM estradiol (E_2). Values represent the mean ± SEM of three experiments.

ever, owing to the lack of information on the physiological levels of different hydroxylases in normal human liver, it is not possible to assess which of the two cell lines mimics the in vivo situation more accurately. The availability of specific cDNA probes for different human P-450 isoenzymes (23–25) should allow us to answer these questions in the future.

Note added in proof: Recent mass spectrometry of the putative 25-OH-D_3 produced by Hep 3B cells gave a spectrum identical to authentic 25-OH-D_3 with molecular ion at *m/z* 400 and major fragments at *m/z* 271, 253, 136, and 118.

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