

# An in vitro model for essential fatty acid deficiency: HepG2 cells permanently maintained in lipid-free medium

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**Abstract** A stable essential fatty acid-deficient cell type, known as HepG2-EFD, was derived from the lipoprotein-producing human hepatoma cell line HepG2. These cells are particularly useful for quantitative studies involving essential fatty acids (n-6 and n-3 fatty acids) in secreted lipoproteins. Radiolabeled essential fatty acids can be delivered to these cells without altering the specific activity of the fatty acids, since the deficient cells contain no endogenous essential fatty acids. Using these cells, radioactivity data (dpm) from metabolic studies can be converted directly to mass, and masses as low as a few pmoles can be accurately measured. HepG2-EFD cell cultures were established by growing HepG2 cells in medium containing delipidated serum. After 10 days of growth in delipidated medium, HepG2 cells were completely depleted of all essential fatty acids. Compensatory increases in nonessential fatty acids (n-9 and n-7 fatty acids) including 20:3n-9 (the Mead acid), which is the hallmark fatty acid of essential fatty acid deficiency, were also observed in HepG2-EFD cells. Despite the lack of exogenous fatty acids in the medium and the lack of essential fatty acids in the cells, export of very low density lipoprotein (VLDL)-associated apolipoprotein B by HepG2-EFD was the same as observed for parent HepG2 cells. However, the activity of beta-oxidation of fatty acids in HepG2-EFD cells was much lower than in the parent cell line. The doubling time was the same for both cell types, but depletion of essential fatty acids resulted in a phenotypic change, whereby HepG2 cells proliferated in large clusters of cells, but HepG2-EFD cells proliferated as homogeneously dispersed cell monolayers. Thus, cultures of HepG2-EFD cells provide an in vitro model for essential fatty acid deficiency, and the model has fatty acid changes consistent with those found in vivo in essential fatty acid deficiency. —Furth, E. E., H. Sprecher, E. A. Fisher, H D. Fleishman, and M. Laposata. An in vitro model for essential fatty acid deficiency: HepG2 cells permanently maintained in lipid-free medium. *J. Lipid Res.* 1992. 33: 1719-1726.

**Supplementary key words** essential fatty acids • arachidonate • Mead acid

In 1982, a stable essential fatty acid-deficient cell line was developed, named EFD-1, from the prostaglandin E<sub>2</sub>-

producing mouse fibrosarcoma line HSDM<sub>1</sub>C<sub>1</sub> (1). This line was established in an effort to study the regulation of eicosanoid precursor fatty acid metabolism, particularly arachidonate, the principal eicosanoid precursor, and other essential fatty acids (defined as all n-6 and n-3 fatty acids). EFD-1 has been found to possess the enzymatic machinery for eicosanoid production, but contains no fatty acid substrate from which to synthesize eicosanoids. Because there is no endogenous pool of arachidonate in EFD-1 cells, the specific activity of radioactive arachidonate does not change upon incorporation into these cells. Therefore, by using this arachidonate-free cell line, very small masses of arachidonate and arachidonate metabolites (as low as a few pmole) have been accurately quantitated by simple determination of radioactivity in several studies that would otherwise have been impossible (2-4). Use of the EFD-1 line was instrumental in studies involving the regulatory effect of intracellular pools of essential fatty acids on arachidonate uptake and release (3) and on arachidonate conversion to eicosanoids (2), and in studies involving the conversion of dihomogamma linolenic acid to one-series prostaglandins (4).

The packaging of eicosanoid precursor fatty acids, principally arachidonate, into newly synthesized lipoproteins could be studied in a highly quantitative fashion if a cultured cell were developed that synthesizes lipoproteins but contains no arachidonate. In this report, the development of an essential fatty acid-deficient culture line derived from the lipoprotein producing human hepatoma

Abbreviations: PBS, phosphate-buffered saline; VLDL, very low density lipoproteins; IDL, intermediate density lipoproteins; LDL, low density lipoproteins.

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cell line HepG2 is described. By maintaining HepG2 cells in medium containing delipidated serum for 10 days or more, a stable essential fatty acid-deficient culture from parental HepG2 cells was developed, designated HepG2-EFD, that is completely depleted of arachidonate, linoleate, and other essential fatty acids.

## MATERIALS AND METHODS

### Development and maintenance of HepG2-EFD

HepG2 cells were maintained and passaged in control medium (10% fetal calf serum in Dulbecco's minimal essential medium with low glucose and penicillin (170 U/ml), streptomycin (170  $\mu$ g/ml), and supplemental glutamine (2 mM)) (all from GIBCO). Cells were passed from one 100-mm diameter Petri dish to four dishes of the same size every 7 days. To establish the new HepG2-EFD cultures, parental HepG2 cells were plated into 100-mm diameter Petri dishes (Corning) and were maintained with daily medium change in delipidated medium (4.2% delipidated fetal calf serum in DMEM low glucose with penicillin, streptomycin, and glutamine as in control medium). Delipidated fetal calf serum was prepared from dialyzed fetal calf serum according to Capriotti and Laposata (5). More than 98% of total lipid phosphate and fatty acid is removed by this delipidation procedure. As HepG2-EFD cells became progressively depleted of essential fatty acids, the cultures were split 1 to 4 every 7 days as were the parent cultures. The HepG2-EFD cells grown in delipidated medium were stable for more than 6 months and presumably can be maintained permanently in lipid-free medium.

### Lipid extraction and gas liquid chromatography

Monolayers of cells were rinsed with ice cold phosphate-buffered saline (PBS), and the cells were then scraped into a final volume of 1 ml of cold PBS. This method of cell harvesting has been shown to preserve cellular arachidonate (6). [5,6,8,9,11,12,14,15-<sup>3</sup>H]arachidonate (0.2  $\mu$ Ci, 1 pmol) (DuPont-New England Nuclear), was added to each sample to allow determination of percent recovery of the fatty acids in the extracted samples. Total cell lipid was extracted according to the method of Cohen et al. (7) for total fatty acid analysis. Fatty acid methyl esters were prepared for gas chromatography as described by Turk et al. (8). Gas chromatography was performed on a WCOT Supelcowax 10 capillary column (Supelco) using a temperature program from 150° to 250°C increasing 10°C/min (split 6.3:1) in a Varian 3700 instrument equipped with a flame ionization detector. Peaks were identified by comparison with retention times of known standards and quantitated using a Hewlett-Packard 3392A integrator with methyl 17:0 (10 nmol) as an internal standard for quantitation.

### Determination of doubling time

To determine the rate of proliferation of HepG2-EFD cells and compare this rate with the parent HepG2 cell doubling time, 10<sup>5</sup> cells were plated in 35-mm diameter Petri dishes and grown with daily medium change for 10 days. On each day, two plates were harvested by trypsinization and the cells were diluted in cetrimide solution (82.3 mM hexadecyltrimethylammonium bromide/1.0 mM Na<sub>2</sub> EDTA/141.5 mM NaCl), and dispersed with a 16-gauge syringe. The cetrimide solution lyses the cells, but the nuclei remain intact. The nuclei were counted in a model F Coulter counter (Coulter Electronics).

### Lipoprotein production by HepG2 and HepG2-EFD cells

Apolipoprotein B was isolated by the isopropanol precipitation procedure (9). Briefly, logarithmically growing cells were incubated at 37°C for 4 h with 100  $\mu$ Ci [<sup>35</sup>S]methionine (1134 Ci/mmol) (DuPont-New England Nuclear) in methionine-free medium. After this incubation, the medium was collected and centrifuged to eliminate any cellular debris. The adherent cells were washed and scraped from the dish with a rubber policeman. An aliquot of medium was used for isolation of total labeled trichloroacetic acid-precipitable protein. From the remainder of the medium, 2 ml was removed. Human low density lipoprotein (200  $\mu$ g) was added as a carrier and recovery marker, and the sample was adjusted to 1.21 g/ml with KBr and placed in a centrifuge tube. A solution of saline (10 ml) at d 1.006 g/ml was carefully overlaid onto the sample. Centrifugation at 208,000 g and 4°C in a 50 Ti rotor was performed for 24 h, and the VLDL at the top of the tube was carefully aspirated. ApoB was then isolated from this material by an isopropanol precipitation method previously used to quantitatively isolate the apoB secreted by HepG2 cells in two previous studies (10, 11). The small amount of apoB in fetal bovine serum in the medium was not labeled and therefore did not interfere with the accurate determination of labeled material. Approximately 92% of apoB is secreted into the VLDL and IDL-LDL fractions by HepG2 cells, and of this the VLDL fraction contains 38% (12). It is likely that this is true for both HepG2 and HepG2-EFD cells, as the distribution of HepG2-derived apoB in the different density classes remains the same when large amounts of exogenous fatty acids are added to the culture medium (12), suggesting that the amount of fatty acid in the medium has no effect on apoB secretion. Total protein concentration was determined after dissolving the protein in NaOH using a Bio-Rad protein assay kit (Bio-Rad).

### Fatty acid oxidation studies

HepG2 and HepG2-EFD cells were grown to confluence in 100-mm diameter Petri dishes. The medium was removed from each dish and the cell monolayers were

washed three times with PBS warmed to 37°C. At this time, 8 ml of DMEM containing 1 mg/ml of fatty acid-free bovine serum albumin and either [1-<sup>14</sup>C]octanoate (1.87 mM, 53.5 mCi/mmol) or [1-<sup>14</sup>C]palmitate (1.75 mM, 57 mCi/mmol) was added. As a control, plates containing no cells were also incubated with the radioactive medium. After an 18-h incubation, the medium was removed from each plate and centrifuged to remove any cellular debris. The medium was then placed in a sealed container with a reservoir containing 0.5 ml Hyamine solution, and 0.5 ml of concentrated HCl was injected into the media. The <sup>14</sup>CO<sub>2</sub> was liberated and counted by recovering the Hyamine solution (13). The cell monolayers were recovered to determine the protein concentration.

## RESULTS

### Fatty acid composition of HepG2 and HepG2-EFD cell types

In order to establish a stable essential fatty acid-deficient cell line derived from HepG2 cells, HepG2 cells were maintained in medium containing delipidated fetal calf serum rather than lipid containing serum. At various times after switching to delipidated medium, cells were harvested, fatty acid methyl esters were prepared, and fatty acid analysis was performed by gas chromatography. The fatty acid compositions of HepG2 cells and HepG2-EFD cells are shown in Table 1.

All essential fatty acids (n-6 and n-3) were undetectable in HepG2-EFD cells. In addition, these cells showed compensatory increases in nonessential n-7 and n-9 fatty acids, including 20:3 n-9 (the Mead acid). In parent HepG2 cells, there was a large amount of cellular arachidonate (0.199 nmol arachidonate per nmol 16:0). In HepG2-EFD cells, a much smaller amount of fatty acid

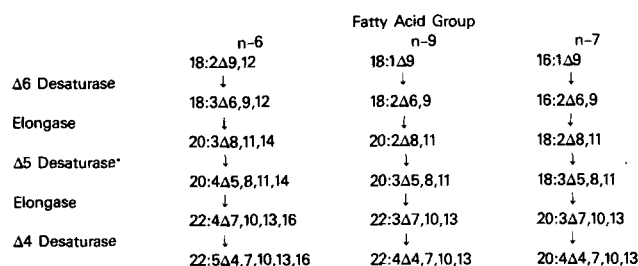


Fig. 1. Fatty acid metabolism of n-6, n-9, and n-7 fatty acids.

(0.049 nmol per nmol 16:0) was detected with a retention time very shortly before, but nearly identical to, that of arachidonate. Several experiments were performed to directly assess whether this represented an incomplete essential fatty acid-deficient state with residual arachidonate in the HepG2-EFD cells. It was suspected that 20:4Δ4,7,10,13, (the n-7 analog of arachidonate (20:4Δ5,8,11,14)) might be present in HepG2-EFD cells and have a retention time in the gas chromatograph very similar to that of arachidonate. Fig. 1 illustrates the metabolic pathways leading to the generation of n-6 and n-7 20:4 isomers and other related fatty acids. Using authentic methyl 20:4Δ4,7,10,13, it was demonstrated that 20:4Δ4,7,10,13 eluted very slightly before arachidonate. This elution sequence was expected, since for a given fatty acid, the sequential order of elution of isomers is n-9, n-7, n-6, and then n-3. To further confirm the conclusion that the residual peak in HepG2-EFD cells was 20:4 n-7 and not residual arachidonate, fatty acid methyl esters derived from the HepG2-EFD cells were spiked with authentic methyl arachidonate or methyl 20:4Δ4,7,10,13, and the fatty acid analyses were performed using different temperature programs in the gas chromatograph. In all variations of the temperature program, the peak in the HepG2-EFD cells suspected to be 20:4Δ4,7,10,13 more precisely co-chromatographed with 20:4Δ4,7,10,13 than with arachidonate. As a final proof that the peak eluting just before arachidonate was a distinct nonessential fatty acid, HepG2 cells were incubated in medium that contained twice-delipidated fetal calf serum instead of once-delipidated serum. It was previously shown that in the first delipidation, 98% of total fatty acid is removed and arachidonate is not detectable in this serum (5). The use of doubly delipidated serum further excluded the possibility that a small amount of residual arachidonate derived from the delipidated serum was present in the HepG2-EFD cells. The peak just before arachidonate persisted in cells maintained for 1 month in doubly delipidated serum (10% serum in medium), and, therefore, very likely represented a nonessential fatty acid. It is speculated that the fatty acid precursor of 20:4Δ4,7,10,13 in HepG2-EFD cells was 16:1Δ9.

A second issue with regard to fatty acid composition of the HepG2-EFD cells was the change in the pattern of

TABLE 1. Fatty acid composition of cells

Fatty Acid	HepG2 (n = 5)	HepG2-EFD (n = 5)
	<i>nmol relative to 16:0 (mean ± SEM)</i>	
16:0	1.000 ± 0.000	1.000 ± 0.000
16:1Δ9 (n-7)	0.926 ± 0.020	1.834 ± 0.035
18:0	0.304 ± 0.012	0.288 ± 0.013
18:1Δ9 (n-9)	1.497 ± 0.051	1.682 ± 0.090
18:2Δ8,11 (n-7)	0.139 ± 0.019	0.522 ± 0.051
<b>18:2Δ9,12 (n-6)</b>	0.074 ± 0.009	0.000 ± 0.000
<b>20:2Δ11,14 (n-6)</b>	0.025 ± 0.007	0.000 ± 0.000
20:3Δ5,8,11 (n-9)	0.142 ± 0.018	0.356 ± 0.014
20:3Δ7,10,13 (n-7)	0.028 ± 0.009	0.179 ± 0.028
<b>20:3Δ8,11,14 (n-6)</b>	0.038 ± 0.007	0.000 ± 0.000
<b>20:4Δ5,8,11,14 (n-6)</b>	0.199 ± 0.014	0.000 ± 0.000
20:4Δ4,7,10,13 (n-7)	0.000 ± 0.000	0.049 ± 0.009
<b>20:5Δ5,8,11,14,17 (n-3)</b>	0.010 ± 0.004	0.000 ± 0.000
<b>22:5Δ7,10,13,16,19 (n-3)</b>	0.017 ± 0.003	0.000 ± 0.000
<b>22:6Δ4,7,10,13,16,19 (n-3)</b>	0.078 ± 0.005	0.000 ± 0.000

Boldface print indicates essential fatty acids.

octadecadienoic acids. Linoleate is not a major fatty acid in fetal calf serum that was used in the maintenance of parental HepG2 cells. Linoleate is only 6.4% of total fatty acid in fetal calf serum (14). Thus, parental HepG2 cells were maintained in medium that contained linoleate, but in smaller amounts than in other sera such as horse serum. Linoleate (18:2 $\Delta$ 9,12) was completely absent in the HepG2-EFD cells, but 18:2 $\Delta$ 8,11 was detected. This n-7 fatty acid, like 20:4 $\Delta$ 4,7,10,13, is derived from the n-7 fatty acid 16:1 $\Delta$ 9 (Fig. 1). Having identified this fatty acid in HepG2-EFD cells, we subsequently established that 18:2 $\Delta$ 8,11 was also present in the HepG2 parent cell line though in smaller amounts (Table 1). It is unusual, although not unprecedented (15), that n-9 and n-7 polyunsaturated fatty acids will accumulate in measurable quantities in cell types not deprived of essential fatty acids.

Also noteworthy in these experiments were changes in the cellular content of the eicosatrienoic fatty acids (20:3). As expected, because they were essential fatty acid-deficient, HepG2-EFD cells contained the hallmark fatty acid of essential fatty acid deficiency, the Mead acid, 20:3 $\Delta$ 5,8,11, which is derived from oleate (18:1 $\Delta$ 9). As with 18:2 $\Delta$ 8,11 discussed above, the Mead acid was present in the HepG2 parent cell line as well as the HepG2-EFD line, though in much smaller amounts. Hyman, Stoll, and Spector (15) noted the presence of 20:3 $\Delta$ 5,8,11 in non-essential fatty acid-deficient 3T3 L1 cells before their differentiation into adipocytes. It is not clear why in their report, as in the present experiments, oleate was desaturated and elongated to 20:3 $\Delta$ 5,8,11 by cells not deprived of essential fatty acids, as the concentration of lipids in HepG2 cell medium containing 10% fetal calf serum should have been sufficient to prevent the development of essential fatty acid deficiency. The HepG2-EFD cells also contained the unusual fatty acid 20:3 $\Delta$ 7,10,13 (also present in smaller quantities in the parent cell line). This fatty acid, as seen in the metabolic diagram of Fig. 1, is derived from desaturation and elongation of 16:1 $\Delta$ 9 and is a nonessential n-7 fatty acid.

The final point with regard to the fatty acid composition of the two cell lines is that the n-3 fatty acids, 22:6, 22:5, and 20:5, were present in the parent cell lines but completely absent in the HepG2-EFD cells. This result is consistent with the facts that these fatty acids are not supplied in the delipidated serum and cannot be synthesized from the n-7 and n-9 fatty acids made *de novo* by the cells.

#### Time course for the development of essential fatty acid deficiency

The changes in cellular fatty acid composition as the HepG2 cells were deprived of essential fatty acids are shown in Fig. 2. After 10 days of growth in delipidated medium, and possibly after as little as 5-6 days based

upon our earlier experience with EFD-1 cells (1), the essential fatty acids, notably 18:2 n-6, 20:3 n-6, 20:4 n-6, 22:5 n-3, and 22:6 n-3, were completely lost from the cells. The n-9 and n-7 fatty acids, on the other hand, which are nonessential, showed compensatory increases, although the increase in oleate (18:1 $\Delta$ 9) was minimal. One day after changing to delipidated medium, increases were observed in the cellular concentrations of 20:3 $\Delta$ 8,11,14 and 20:4 $\Delta$ 5,8,11,14, which are n-6 fatty acid metabolites of 18:2 $\Delta$ 9,12 (linoleate). Linoleate did not show a similar increase and, in fact, showed a rapid decline in concentration. This is consistent with earlier observations using EFD-1 cells (1) that restriction of exogenous fatty acids induces  $\Delta$ 6 desaturase activity and increased conversion of linoleate to more elongated and desaturated n-6 fatty acids until the cellular linoleate is depleted. The observed increases in the cellular concentration of 22:5 $\Delta$ 7,10,13,16,19 and 22:6 $\Delta$ 4,7,10,13,16,19 one day after the cells were switched to delipidated medium were most likely a result of increased conversion of 20:5 $\Delta$ 5,8,11,14,17 to more elongated and desaturated n-3 metabolites. This would suggest that restriction of exogenous fatty acids also induces elongase and  $\Delta$ 4 desaturase activity. It has previously been reported that  $\Delta$ 4 desaturase activity is increased in essential fatty acid deficiency (16).

#### Functional and morphologic studies

Growth curves for HepG2 and HepG2-EFD cells are shown in Fig. 3. Both the HepG2 cells and the HepG2-EFD cells proliferated with a doubling time of 24-30 h. There was no significant difference in the growth rate of the two different cell lines, despite the fact that the HepG2-EFD cells were not supplied with any source of lipid in the tissue culture medium. This was not due to recycling of n-6 and n-3 fatty acids lost from and then reincorporated by the HepG2-EFD cells, as the essential fatty acid-deficient cells used in these studies had been maintained in lipid-free medium for 3-4 months, and by this time there were no essential fatty acids available for recycling.

Fig. 4 shows a comparison of HepG2 cells and HepG2-EFD cells by phase microscopy. The parent line grew in cell clusters, but deprivation of exogenous lipid changed the morphology of the cultures to a homogeneous distribution of cells. The two cell types were also examined by electron microscopy, and despite light microscopic changes in growing cell cultures, at the ultrastructural level no difference was found between the two cell lines (data not shown). Presumably, changes in surface molecules or other moieties responsible for the change from cell clustering to homogeneous cell distribution were not detectable by standard electron microscopy.

HepG2 cells are known to produce lipoproteins and secrete them into the tissue culture medium. We determined whether essential fatty acid deficiency resulted in

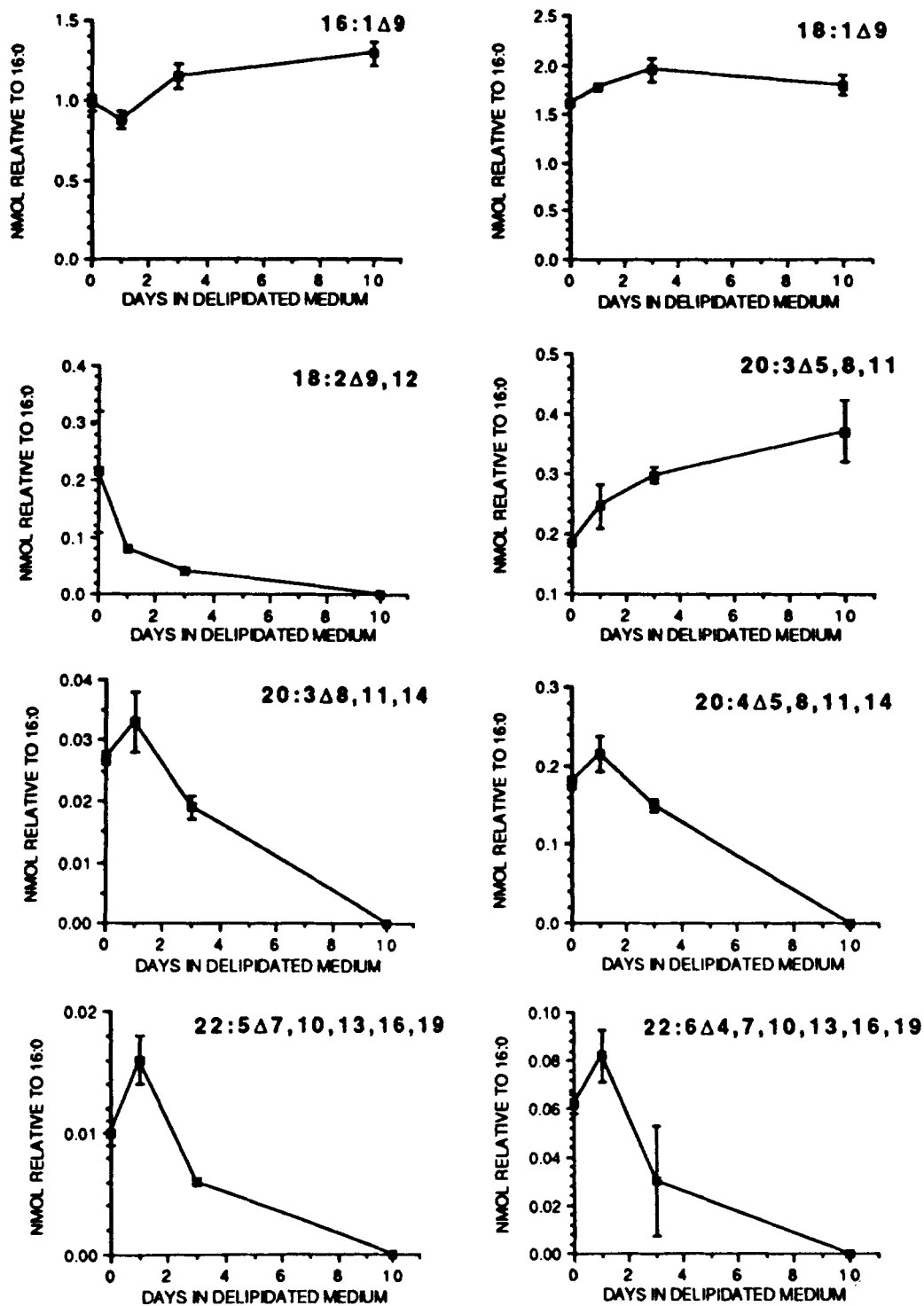
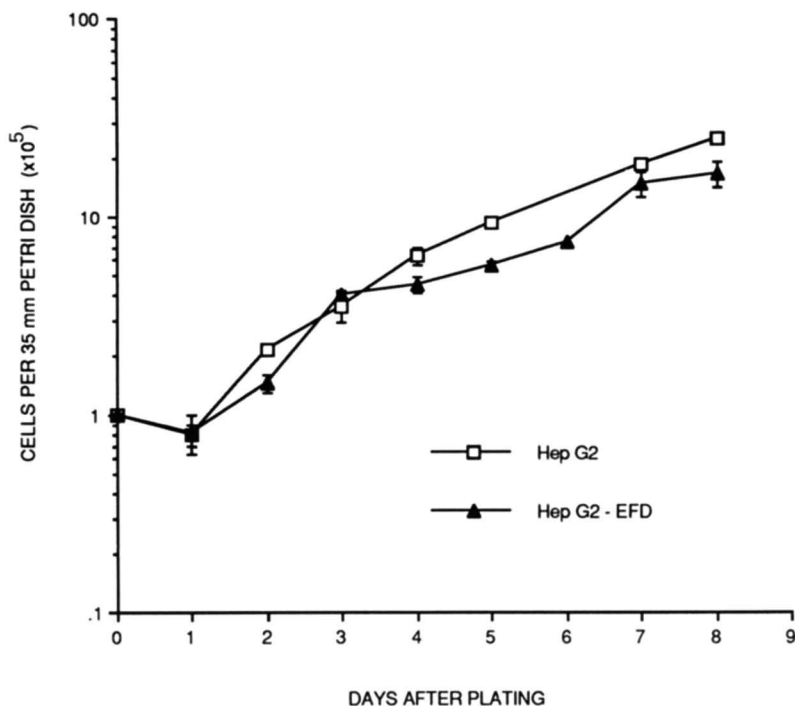


Fig. 2. HepG2 cells ( $4 \times 10^5$ ) were plated in 60-mm diameter Petri dishes and maintained in delipidated medium for up to 10 days. Duplicate plates were harvested for fatty acid analysis as described in Methods. Values represent mean  $\pm$  SEM of duplicate plates from one of two representative experiments.



**Fig. 3.** HepG2 and HepG2-EFD cells ( $10^5$  cells) were plated in 35-mm diameter Petri dishes with daily medium change. Cells were harvested and counted as described in methods. Values represent mean  $\pm$  SEM of duplicate plates from one of two representative experiments.

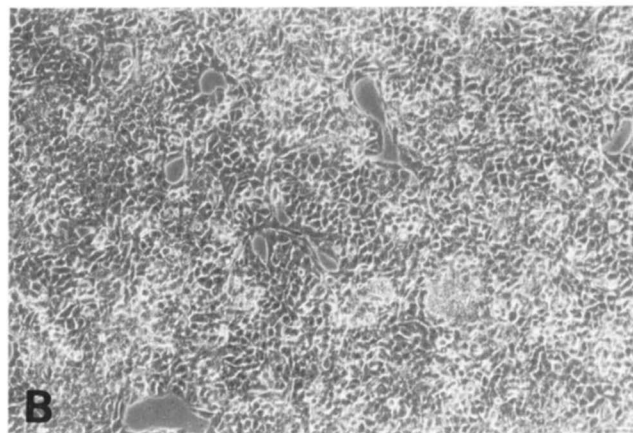
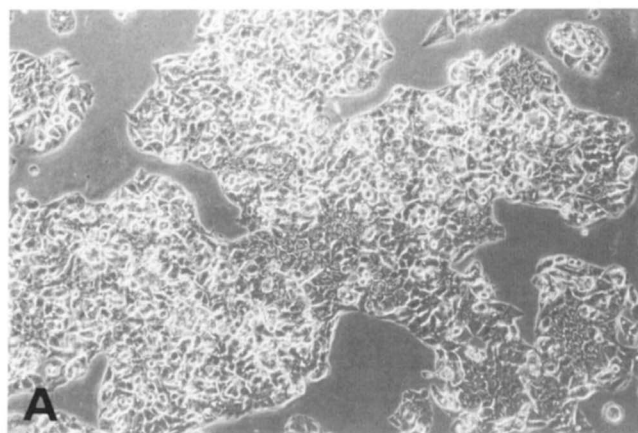
a change in the production and secretion of apoB. There was no difference in the apoB content of medium from HepG2 versus HepG2-EFD cells (dpm  $\times 10^5$  in 1.5 ml medium, mean  $\pm$  SEM; HepG2 cells =  $1.18 \pm 0.09$ ; HepG2-EFD =  $0.96 \pm 0.06$ ,  $n = 4$  for each,  $P = 0.08$  (NS), one of three representative experiments all of which showed no statistical difference).

The HepG2-EFD cells were found to have a decreased ability to oxidize both long chain and short chain fatty acids when compared to the parent HepG2 cells. The decrease relative to the HepG2 cell line was different for the

two fatty acid substrates. Relative to the parent cells, the oxidation of palmitate was reduced in the HepG2-EFD cells nearly 300-fold, while the decrease for octanoate was approximately 10-fold (Table 2).

## DISCUSSION

HepG2-EFD cells were derived from the HepG2 hepatoma cell line to develop a stable cell culture with unique advantages for mechanistic studies on essential fatty acids



**Fig. 4.** Phase contrast photomicrographs of HepG2 cells (A) and HepG2-EFD cells (B). Magnification 30 $\times$ .

TABLE 2. Fatty acid oxidation of cells

Cell Type	Palmitate (n = 4)	Octanoate (n = 4)
<i>cpm fatty acid oxidized/μg cell protein</i>		
HepG2	751 ± 64	2267 ± 145
HepG2-EFD	2 ± 1	207 ± 57

Values given as mean ± SEM.

in lipoprotein synthesis, particularly for studies on arachidonate packaging into newly synthesized lipoproteins. This new cell type, which has fatty acid changes consistent with those found *in vivo* in essential fatty acid deficiency, grows rapidly and produces apoB even in the absence of exogenously supplied lipids.

A number of tissue culture cell lines have been developed which display some but not all of the changes in fatty acid composition that occur in essential fatty acid deficiency *in vivo*. In some of these cell lines, arachidonate is depleted without accumulation of 20:3Δ<sup>5,8,11</sup> (17–19). Bailey et al. (20) described a tissue culture cell line that is deficient in arachidonate and accumulates 20:3Δ<sup>5,8,11</sup>, but does not accumulate oleate. Hyman et al. (15) showed that 3T3 L1 cells, before their differentiation into adipocytes, contained both 20:3Δ<sup>5,8,11</sup> and arachidonate. The EFD-1 cell line derived from the HSDM<sub>1</sub>C<sub>1</sub> mouse fibrosarcoma cell line has all the fatty acid changes seen in essential fatty acid deficiency, including decreased essential fatty acids and increased oleate and 20:3Δ<sup>5,8,11</sup> (1). The cell line we have characterized in this report derived from HepG2 cells shows fatty acid changes similar to those seen in EFD-1 mouse fibrosarcoma cells which closely reflect the fatty acid changes in essential fatty acid deficiency.

The absence of arachidonate in essential fatty acid-deficient cells permits the quantitation of very small masses of arachidonate and its metabolites (as little as a few pmole) by simple determination of radioactivity. This tremendous advantage exists because exogenous arachidonate, upon incorporation into cells, is not diluted by an endogenous arachidonate pool. The known specific activity of the exogenous arachidonate, therefore, does not change upon incorporation into the cell, and the relationship between radioactivity and mass remains known. Using the essential fatty acid-deficient mouse fibrosarcoma line EFD-1, several observations have been made that would have been impossible in cells with endogenous pools of fatty acid. It has been shown that the amount of cellular arachidonate regulates the percent of released arachidonate that is converted to eicosanoids versus the percent released from the cells as free arachidonate (2). EFD-1 cells have also been used to show that pre-existing pools of essential fatty acids (preloaded into EFD-1 cells)

regulate the amount of arachidonate that can be incorporated into the cell (3).

In the present studies it was surprising to find that the HepG2-EFD cells proliferated with the same doubling time as the HepG2 parent cell line. It was expected that the presence of lipid in the tissue culture medium would be a stimulus for a faster doubling time in the parent cell line. However, there was no apparent difference in the rate of growth, indicating that the cells were not metabolically dependent on exogenous fatty acids for growth.

The morphological changes seen in our previous studies when HSDM<sub>1</sub>C<sub>1</sub> mouse fibrosarcoma cells were converted to EFD-1 cells were obvious both in growing cell cultures and in electron micrographs at the ultrastructural level. By comparison, however, there were no obvious ultrastructural differences between the HepG2-EFD cells and the parent line. Unexpectedly, despite the lack of exogenously supplied lipids, the HepG2-EFD cells even retained their lipid droplets. This indicates that the cells synthesize enough lipid *de novo* to maintain a cytoplasmic storage pool of lipid droplets.

In addition to the marked difference in fatty composition between the essential fatty acid-deficient cells and the parent HepG2 cells, the HepG2-EFD cells were much less active in the beta-oxidation fatty acids. This may be a result of down-regulation of mitochondrial oxidation due to decreased supply of exogenous lipids. However, further studies will be needed to assess this possibility.

In summary, an essential fatty acid-deficient cell culture from HepG2 cells was developed which has been named HepG2-EFD. This stable cell type, with a fatty acid composition reflecting *in vivo* essential fatty acid deficiency, synthesizes apoB, proliferates with the same doubling time as its parent line, has markedly decreased beta-oxidation activity, and retains intracellular lipid droplets despite the complete absence of exogenously supplied lipids. ■■

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