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Apolipoprotein D transcription occurs specifically in nonproliferating quiescent and senescent fibroblast cultures

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We studied apolipoprotein D (apoD) mRNA in primary cultures of human diploid fibroblasts (HDF). In early-passage HDF no apoD mRNA was detected in replicating cells in sparse culture, but the gene was expressed in quiescent cells in confluent and in serum-starved cultures. In contrast, late-passage HDF expressed apoD mRNA in sparse culture, but the level increased after attainment of confluence. Thus fibroblasts, the common cell-type expressing apoD mRNA in vivo, express this characteristic following growth-arrest. The same pattern of activation was found in another fibroblast cell line deficient in apoB/E (LDL) receptors, excluding a role for cellular cholesterol delivery by the LDL-receptor pathway controlling apoD expression

Lipocalin (α2u-globulin) family; Growth arrest

1. INTRODUCTION

Apoliprotein D (apoD) is a glycoprotein of M_r 29 000 found mainly on the high density plasma lipoproteins (HDL) of man [1–6], baboon [7], rabbit and other species [8]. ApoD is an atypical apolipoprotein, nevertheless. The cloning and sequencing of the human cDNA revealed that it belongs to the Lipocalin (α2u-globulin) family [3,9]. Proteins in this family transport specific hydrophobic ligands (lipids) in a one-to-one molar ratio in a ligand-binding pocket composed of antiparallel β-strands [10–16]. The apoD gene is expressed in all 16 rabbit organs that we analysed [8] and many peripheral organs in man [3]. Being expressed mainly in peripheral organs such as spleen, lung, adrenal gland, central nervous system and male genital tissues, rather than liver and small intestine,it is unlike all other apolipoproteins. ApoD gene expression was found mainly in interstitial and connective tissue fibroblasts, with higher levels often near blood vessels, using in situ hybridization techniques with rhesus [17] and rabbit organs [29].

ApoD expression appears to be modulated in vivo: the protein increased over 500-fold in regenerating rat sciatic nerves [18–20]. Addition of androgens to human breast cancer cells in culture both stimulated apoD (GCDFP-24) secretion 3- to 4-fold and decreased cell proliferation to 50%, whereas estrogens had opposite effects on both parameters [21].

However, given that the cells that express apoD most often in vivo appear to be fibroblasts, we studied apoD mRNA levels in normal human diploid fibroblasts (HDF) in primary culture. We show here that apoD mRNA was detectable only in nonproliferating quiescent and senescent cultures, and that apoD expression was independent of exogenous cholesterol in this cell model.

Abbreviations: HDF, human diploid fibroblast; PDLs, population doubling levels; GCDFP, gross-cystic-disease-fluid protein.

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2. MATERIALS AND METHODS

2.1. *Cell cultures*

A human diploid fibroblast strain (HDF-1) (gift from Dr S. Lussier-Cacan, Institut de Recherches Cliniques de Montréal) was cultured from a skin biopsy from a 26-year-old man. We found that its in vitro life span before phase-out was about 56 population doubling levels (PDLs) [22]. Early- and late-passage cultures, at about 21 and 42 PDLs, had growth rates of 34 or 80 h per PDL, respectively. At least 30% of cells in the late-passage but none in the early passage culture had signs of senescence – large cytoplasm and nucleus [23]. A homozygous B/E receptor (LDL-receptor) deficient fibroblast strain (HFH) was obtained from the NIGMS Human Genetic Mutant Cell Repository (Camden, N.J., repository number GM1915). The PDLs of this culture were unknown but no cells had senescent morphology.

All cells were maintained at 37°C in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum and 1% nonessential amino acids. The levels of apoD gene expression seemed to vary from one serum batch to another but the pattern of gene expression remained the same.

Sparse cultures were plated at 8.5×10^3 cells/cm^2, cultured for about 48 h, then trypsinized and frozen in liquid N_2. Confluent cultures were initially plated at 2.5×10^4 cells/cm^2 and medium was renewed every 48 h until they attained confluence (estimated using the microscope) designated 'day 0 post-confluence' at that time. Generally medium was not renewed for these cells, except where indicated, until trypsinization

and freezing 0, 2, 7, or 14 days post-confluence. Serum-starved fibroblasts were initially plated as a sparse culture in 10% fetal calf-serum supplemented medium, which was changed to 0.5% fetal bovine serum about 24 h after plating. This 0.5% serum medium was replaced with fresh medium 48 h later, and the starved cells maintained in culture for 3 and 7 days. The growth state of some of the cultures was estimated by Northern blot analysis with a ^{32}P-labelled c-*fos* DNA probe.

2.2. *mRNA analysis*

Total RNA was extracted by the acid/guanidium thiocyanate/phenol/chloroform method [24] from 2–3 × 10^6 frozen cells. RNA concentration was measured by spectrophotometry [25]. Glyoxal-denatured RNA was electrophoresed in 1.1% agarose gel and transferred to Nytran membranes which were hybridized at 60°C [26]. The DNA probes were prepared by the random-primed oligonucleotide method [27] using the 800-bp *Eco*RI fragment purified from the pAPOD6 clone provided by Dr D. Drayna, Genentech Inc. [3]. After hybridization the membranes were washed in 2 × SET for 1 min at room temperature, 2 × SET containing 0.2% SDS at 60°C for 30 min, 1 × SET containing 0.2% SDS at 60°C for 30 min, and finally 1 × SET for 1 min at room temperature. 1 × SET contained 30 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA. After exposure, the probe was removed by washing with 18 mM NaCl, 0.1 mM EDTA, 1 mM sodium phosphate pH 7, 0.1% SDS, at 80°C for 10 min, and then rehybridized with ^{32}P-labelled actin cDNA probe or ^{32}P-labelled c-*fos* cDNA probe as indicated.

3. RESULTS AND DISCUSSION

The analysis of early-passage normal fibroblasts (21 PDLs) revealed detectable mRNA only after attaining confluence (Fig. 1). No message was seen in replicating sparse culture nor in the extract isolated on the same day when confluence of the culture was attained. Growth arrest at confluence was confirmed by a second hybridization of the same filter with the ^{32}P-labelled c-*fos* cDNA probe, and finding reduced expression of this oncogene [28] when compared to the replicating sparse culture (data not shown). ApoD mRNA was found in sparse culture when we used serum-starvation to induce growth-arrest (Fig. 1), but not in the replicating sparse cells, showing that establishment of confluence itself is not essential for activation of the apoD gene.

The same experiment was performed in parallel with late-passage normal fibroblasts (42 PDLs) of the same strain, that unlike the early-passage cells showed signs of senescence. In contrast to the early-passage cells the late-passage sparse cultures expressed apoD mRNA even with 10% serum supplementation (Fig. 1), but the level nevertheless increased after attainment of confluence, reaching a peak 2 days later. The subsequent decrease in late-passage cells 7 and 14 days post-confluence, and the low level of apoD mRNA in the 7-day serum-starved sparse culture, could be due to cell death. A decrease of c-*fos* mRNA levels is characteristic of senescent cultures [28]. The hybridization of the c-*fos*-cDNA probe was lower in the late- than in the early-passage sparse cells (not shown). In our experiments, c-*fos* expression decreased further after confluence in late-passage cultures (data not shown). Thus, apoD

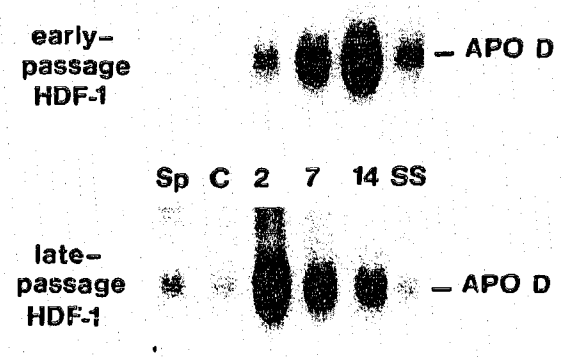

Fig. 1. Northern blot analysis of apoD mRNA levels in normal human diploid fibroblasts in early- and late-passage cultures. Total RNA (15 μg) extracted from various samples of the HDF-1 cell strain were analysed by Northern blot. The filter was hybridized with ^{32}P-labelled APOD6 cDNA, washed, and autoradiographed (16 h exposure). The early- and late-passage cultures had PDLs of 21 and 42 respectively. Sp, non-serum starved sparse cultures; C, cell cultures harvested by the day of confluence establishment; 2, 7, and 14, day-2, day-7 and day-14 post-confluence; SS, serum starved sparse cultures analysed after 7 days of starvation.

gene expression in normal human diploid fibroblasts is most likely specific to growth arrest and senescence in culture.

The medium was not renewed following confluence in the confluent cultures assayed in Fig. 1. Therefore, expression of the apoD mRNA could have occurred following the degradation of a potential inhibitor in the medium. However, the specific activation of the apoD gene in confluent cultures was observed in a subsequent experiment when the medium was changed every 2 days (data not shown). Thus, it appeared that neither the starvation of a particular component, nor incubation in conditioned medium was likely to have induced apoD mRNA expression. The expression in the sparse culture of late-passage cells (Fig. 1) was clearly not linked to degradation of a compound in the medium because this medium had been renewed every 2 days until cell harvest.

We could not exclude that the expression seen in Fig. 1 was not induced by the accumulation of a gene activator coming from the degradation of dead cells in the culture and so we tested that hypothesis with the following experiment. A replicating sparse culture (3 × 10^6 cells/350 cm^2) of early-passage HDF was incubated in complete medium to which we added cellular debris prepared by freezing, thawing and vortexing an identical culture. In a second experiment we added cholesterol (20 μg/ml) to a culture. No apoD mRNA was detected in either experiment after 2 days culture, before confluence was reached (data not shown), although 2 days sufficed for induction of apoD in confluent cells (Fig. 1). Therefore, we do not believe that either free cholesterol or cell debris was an inducer of apoD in our fibroblast cultures. It is more difficult to exclude the idea that the apoD gene was activated in cells undergoing a pro-

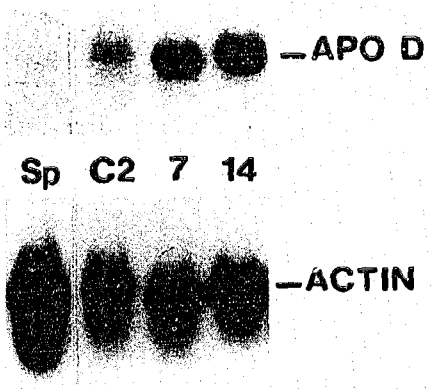

Fig. 2. Northern blot analysis of apoD mRNA levels in cells of the apoB/E (LDL)-receptor deficient fibroblastic strain (HDF). Total RNA (15 μg) extracted from various samples of the HFH cell strain were analysed by Northern blot. The filter was hybridized subsequently with APOD6 and actin cDNA-probes as indicated. Both autoradiographs were exposed for 16 h. Sp, non-serum starved sparse cultures; C2, 7, and 14, day-2, day-7 and day-14 post-confluence.

cess leading to cell death. But against this idea we did not detect any apoD mRNA in replicating sparse cultures of early-passage cells, despite there being 2–5% dead cells staining with Trypan blue, in these cultures. Thus apoD expression in these cultures was probably not linked to cell death.

The specific expression of apoD mRNA in nonproliferating quiescent fibroblast cultures was observed with two other strains of human fibroblasts, both having homozygous mutations affecting the B/E (LDL) receptor gene (Fig. 2 and further data not shown). Therefore, control of apoD mRNA expression does not seem to be related to the activity of the LDL-receptor pathway of cholesterol metabolism.

Soon after reaching confluence early-passage cells had no detectable apoD mRNA (sample C, Fig. 1), whereas the c-*fos* mRNA level was significantly lower in this sample than in the replacing sparse culture. These results together with the observation of a continued rise in apoD mRNA until 14 days post-confluence in this culture indicate that the induction of apoD transcription was probably a consequence of growth arrest. The biochemical stimulus, mechanism and function of this induction are still unknown. We could postulate that apoD plays a role in cellular homeostasis, required only under some metabolic conditions that occur following growth arrest, or that expression of apoD is a marker of the induction of fibroblasts into a particular functional state. The finding of high levels of apoD mRNA and protein in endoneurial fibroblasts during nerve regeneration [19,20] favours the hypothesis that exogenous factors may induce the expression of apoD and that fibroblasts that express it have developed a new function or activity.

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