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ORGANELLE MEMBRANE-CELL FUSION: DESTRUCTION OF TRANSPLANTED MITOCHONDRIAL PROTEINS IN HEPATOCYTE MONOLAYERS

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Deliberate miscompartmentalization of liver outer mitochondrial membrane (OMM) proteins and liver mitochondrial proteins has been achieved by polyethylene-glycol mediated OMM vesicle-hepatocyte or mitochondrial-hepatocyte fusion. Reductively methylated OMM and mitochondrial proteins (^{3}H) are destroyed at rates remarkably similar to those for OMM ($t_{\frac{1}{2}}$, 60-70 h) or mitochondrial proteins ($t_{\frac{1}{2}}$, 84-104 h) in liver *in vivo* when studied over 4-5 days in hepatocyte monolayers cultured in conditions giving stabilized endogenous protein catabolic rates mimicking endogenous *in vivo* rates. Destruction of transplanted OMM proteins is partially sensitive to chloroquine, supporting some lysosomally mediated autophagic destruction of long-lived transplanted OMM proteins in hepatocyte monolayers.

Introduction

The mechanism(s) of intracellular protein degradation in animal cells is not understood, although ordered protein catabolism takes place in all cells and is essential for normal differentiative transitions and modulation of the differentiated phenotype [1]. The use of hepatocytes to study protein dedradation has been limited to experiments completed in a few hours [2]. However, most liver organelle proteins have long half-lives [3]. Therefore the study of the degradation of liver organelle proteins in hepatocytes requires measurements over an extended culture period. We report the establishment of non-proliferating hepatocyte monolayers which maintain a steady state of protein metabolism over 4-5 days.

Destruction rate of proteins may be determined by recognition domains (signals) on individual proteins or proteins may be destroyed in populations characterised by common recognition signals or

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cytomorphological location [4]. These hypotheses are not testable if all the proteins in cells are radiolabelled by amino acid precursors but can be tested if radiolabelled proteins are introduced into target cells. We have transplanted reductively methylated OMM vesicle and mitochondrial preparations [^{3}H] to hepatocytes by polyethylene glycol mediated fusion. The data suggest that liver OMM vesicles or vesicular proteins retain characteristics determining destructive rate when transplanted to hepatocyte target cells.

Materials and Methods

Collagenase was obtained from Boehringer-Mannheim, insulin from Boots, Nottingham, U.K. and Fisofluor from Fisons, Loughborough, U.K. Cell culture materials was supplied by Gibco Paisley Scotland, L-[4,5-^{3}H]leucine and [^{3}H] sodium borohydride were from Amersham, U.K. All other chemicals were obtained from Sigma, Poole, U.K.

Preparation of hepatocytes

Tissue culture dishes (60 mm) were coated with an adsorbed collagen layer (2 mg/ml). Hepatocytes were prepared[6] and washed three times in sterile calcium free Krebs - Henseleit bicarbonate solution and once in Leibovitz L-15 medium, pH 7.4. For further details see legends to Figures.

Preparation and reductive methylation of OMM

Rat livers were perfused *in situ*[6] with 150 ml of calcium free Krebs Henseleit bicarbonate buffer. Outer mitochondrial membranes (OMMs) were prepared from the blood free livers[11]. Approximately 4 mg of the OMMs were resuspended in 150 µl of 0.2 M sodium borate buffer pH 9.0. The preparation was cooled on ice. 20 µl of 20 mM formaldehyde were added and the contents mixed by means of a Pasteur pipette. After 30s one crystal of [^{3}H]sodium borohydride was added and the preparation quickly mixed. One minute later 150 µl of 0.4 M sodium phosphate buffer pH 5.8 containing 100 mM glycine was added. The methylated preparation was dialysed against fresh changes of phosphate buffered saline for 15 h prior to use.

Hepatocyte - OMM fusion

Hepatocytes (35×10^6) were pelleted by centrifugation (50 x g for 3 min). Reductively methylated OMM (2 mg) was added in 150 µl of phosphate buffered saline and the tube gently rotated to ensure thorough mixing. 500 µl of 50% PEG 1500 in serum and antibiotic free L-15 medium pH 7.4 was added and the tube gently rotated for 90s. 10 ml of medium alone was then slowly added. The cells were then pelleted and washed three times in 10 ml of the L-15 culture medium supplemented as in figure 1 but without hormones. Finally the cell pellet was resuspended in 10 ml of the same medium.

Analyses

Lactate dehydrogenase activity and protein were determined [6].
Radioactivity in the cell pellet was determined by trichloroacetic acid precipitation on 2.5 mm GF/C glass fibre discs [9]. Trichloroacetic acid (10% w/v) soluble radioactivity was measured with Fisofluor as scintillant.

Results and Discussion

Hepatocyte mitochondrial proteins are generally long-lived: mitochondrial protein has an average $t_{\frac{1}{2}}$ of 84-104 h and OMM protein a $t_{\frac{1}{2}}$ of 60-70 h[3]. Therefore it is essential to establish culture conditions so that

hepatocyte monolayers can be maintained in a stable metabolic state for 4-6 d if meaningful measurements of degradation of transplanted mitochondrial proteins are to be made. Freshly isolated hepatocytes are in a protein catabolic state[5]. Hepatocytes obtained from animals previously given intravenous [^{3}H]-valine (150 μCi) 24 h before preparation of the cells have an endogenous rate of protein degradation of 3.5 ± 0.7%/h (3) in suspension culture[6]. This increased rate of endogenous proteolysis compared to *in vivo* rate (1.6%/h[7]) is the result of activation of a supplementary lysosomal pathway of protein degradation[8]. In preliminary experiments, therefore, we systematically established culture conditions whereby *in vivo*-like protein metabolism could be achieved and maintained over long periods in hepatocyte monolayers (see Fig. 1). The rate of endogenous protein degradation in the cells under these conditions was 1.3%/h and 0.9%/h measured following an *in vitro* or *in vivo* pulse labelling of hepatocyte proteins with [^{3}H]-leucine and [^{3}H]-valine respectively. The rate of endogenous protein degradation does not oscillate before or after Medium change during 4-6 d in culture. Protein synthesis measured by incorporation of [^{3}H]-valine or [^{3}H]-leucine is constantly maintained in the monolayers. Therefore since protein metabolism is stabilized in the cells at *in vivo*-like rates, no cell loss from the plates occurs as measured by total protein and no lactate dehydrogenase is lost from the cells (Fig. 1) meaningful measurements of the fate of transplanted mitochondrial proteins can be carried out.

Mitochondrial and OMM preparations from rat liver were radiolabelled by mild reductive methylation[10] with sodium [^{3}H]-borohydride so that the very high specific radioactivity needed for subsequent monitoring of the destruction of transplanted proteins could be achieved. Reductive methylation of OMM-preparations gave the distribution of radiolabel in membrane hydrolysates on subsequent amino acid analyses shown in Fig. 2. Approximately 75% of the radiolabel in OMM-preparations is associated, as

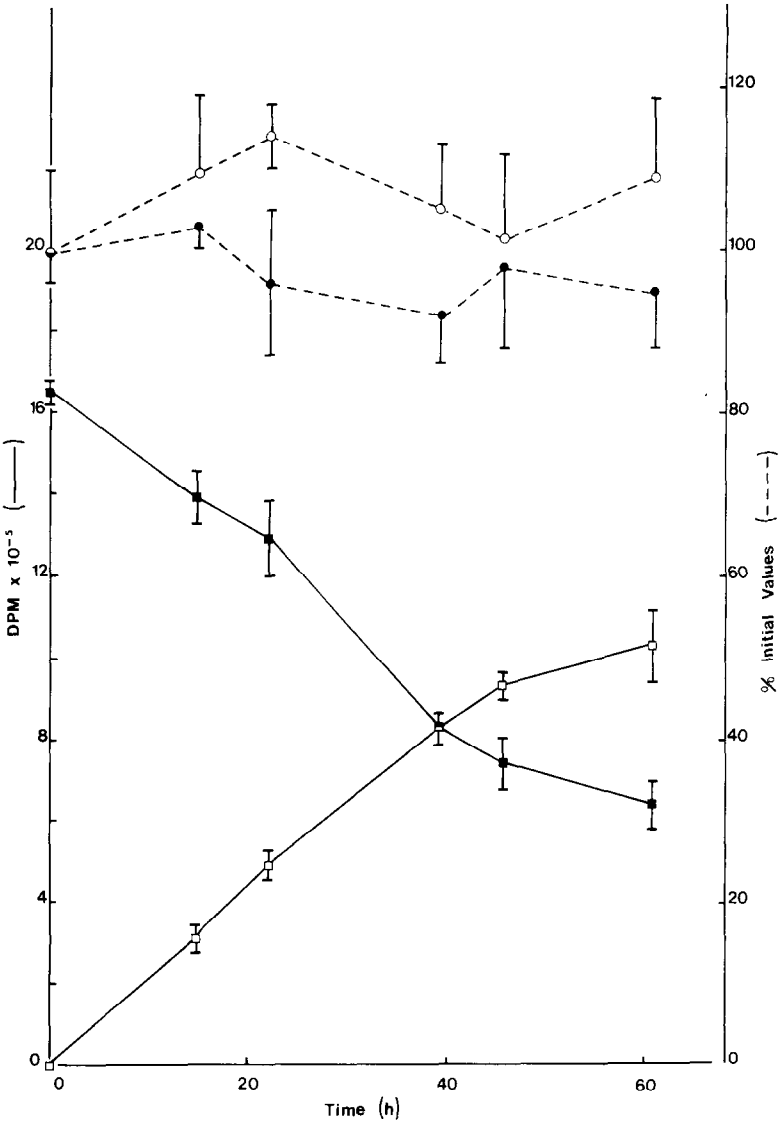

Figure 1. Endogenous protein degradation in stabilised hepatocyte monolayers

Cells (2.0×10^6) were plated out in 3 ml leucine free L-15 medium, pH 7.4 supplemented with glucose (8.3 mM) hepes(25 mM), penicillin-streptomycin (1 unit/ml), insulin (0.8 µg/ml), dexamethasone (10^{-6}M) and 10% heat inactivated new born calf serum. After 2.5 h the medium was replaced by medium supplemented with 3 µCi/ml L-[4,5-^{3}H]leucine. The cells were cultured in this medium for 15 h. Two 4 h chases were conducted at 37° in culture medium containing 10 mM leucine to allow the degradation of short lived proteins. The medium (containing 10 mM leucine) was changed after this period (zero time) and then at 24 h intervals. The medium was removed at the times indicated and retained for analyses. The cells were harvested by trypsinization (0.5% trypsin, 0.2% EDTA in 0.9% NaCl). This procedure took at least 20 min. The cells were washed twice with medium containing 10% serum and the cell pellet was frozen. Lactate dehydrogenase activity (O), protein (●), TCA insoluble counts in cell pellet (■), TCA soluble counts in medium (□). The values are the means ± of duplicate determinations on a minimum of four separate plates.

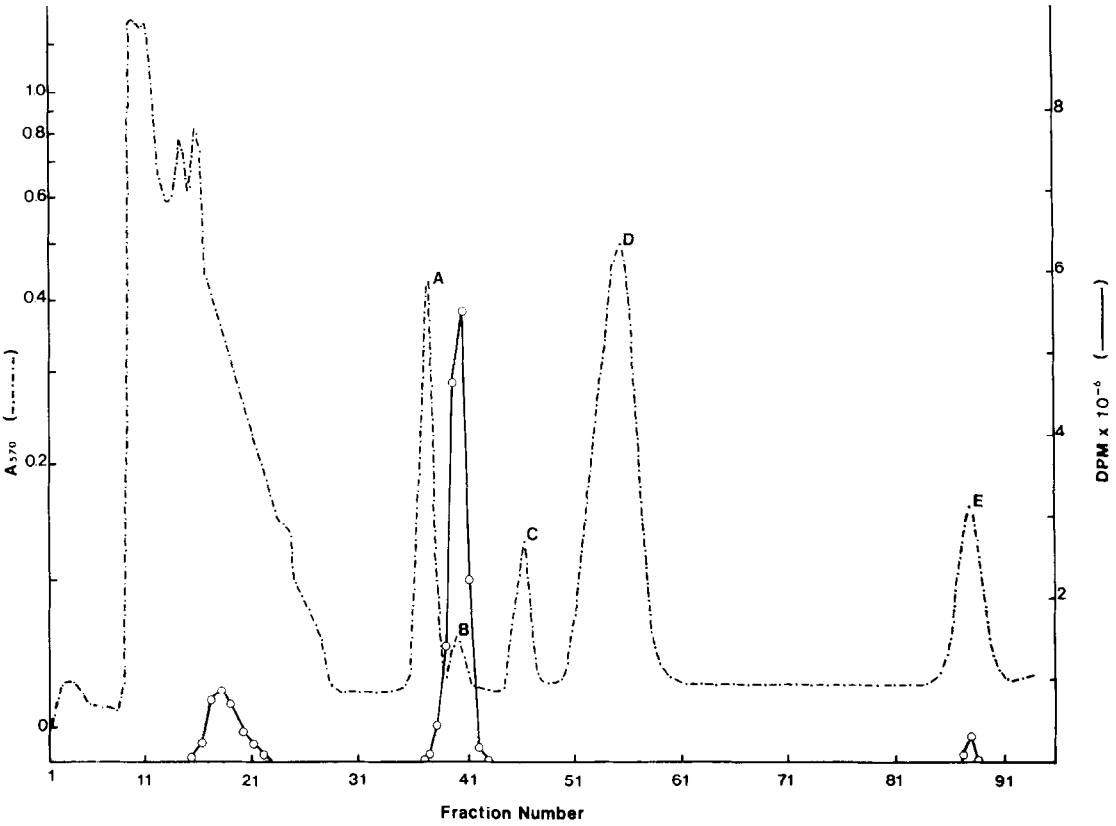

Figure 2. Amino acid analysis of reductively methylated OMMs
A sample of OMM was hydrolysed (6 N HCl at 110° for 24 h). The hydrolysate was analysed on a Locarte amino acid analyser. Another sample of the hydrolysate, together with a small amount of L-[5(n)-^{3}H]arginine as marker, was similarly analysed but fractions collected for measurement of radioactivity. (A) lysine, (B) methylated lysine, (C) histidine, (D) ammonia, (E) arginine.

expected[10] with methylated lysine: the affiliation of the remainder is unknown but may be due to phospholipid modification[12]. During the degradation of transplanted [^{3}H]-reductively methylated proteins in hepatocyte monolayers [^{3}H]-dimethyl lysine is progressively released into the medium.

Polyethylene glycol mediated organelle membrane-cell fusion is routinely achieved with mitochondrial and OMM preparations in order to deliberately miscompartmentalize the organelle preparations. In preliminary experiments where mitochondrial-cell fusion was carried out label is lost from mitochondrial proteins in a monophasic manner giving a $t_{\frac{1}{2}}$ of 69.2 ± 5 h (3) for the half-life of mitochondrial protein which is slightly faster

than the in vivo rate[3]. Clearly reductive methylation of mitochondrial preparations and miscompartmentalization does not appreciably alter mitochondrial properties so that the organelle preparation (or OMM preparation, see below) is signalled out for rapid destruction (cf "abnormal" reticulocyte proteins[13]). Subsequent experiments were carried out with OMM vesicle preparations since they constitute a morpologically simpler system facilitating interpretation of the data on the degradation of transplanted proteins.

The destruction of transplanted [^{3}H]-OMM proteins in hepatocyte monolayers is shown in Fig. 3. A single monophasic destruction of OMM proteins occurs with a $t_{\frac{1}{2}}$ of 70.5 ± 9 h (4). This figure is remarkably similar to the average destruction rate of OMM proteins in vivo ($t_{\frac{1}{2}}$, approx. 60 h[3]). In the absence of polyethylene glycol less than 10% of [^{3}H]-OMM radioactivity is associated with hepatocytes compared to when the fusogen is present. The slow in vivo-like rate of destruction of transplanted [^{3}H]-OMM protein cannot be due to heterophagy (endocytosis) as recently suggested for polyethylene glycol independent mitochondrial entry into cultured cells[14] since the destruction rate of endocytosed proteins is very rapid indeed (e.g. asialofetuin in hepatocytes approx. $t_{\frac{1}{2}}$ 40 min[15]). The [^{3}H]-OMM vesicles do not adhere adventitiously to the outside of the hepatocytes since cell harvesting is routinely performed by prolonged (20 min) trypsinization of the monolayers (cf. removal of adsorbed liposomes by trypsinization[16]).

If the [^{3}H]-OMM proteins are internalized into hepatocytes for destruction at in vivo-like rates then it should be possible to modulate the rate of the destructive process by agents known to alter intracellular proteolysis. Agents known to modulate intracellular autophagy (insulin, high concentations of amino acids and serum) are essential for the metabolic stabilization of the hepatocyte monolayers (Fig. 1). We therefore decided to investigate the effect of chloroquine, which inhibits the destruction of proteins segregated in autophagosomes[15], on the

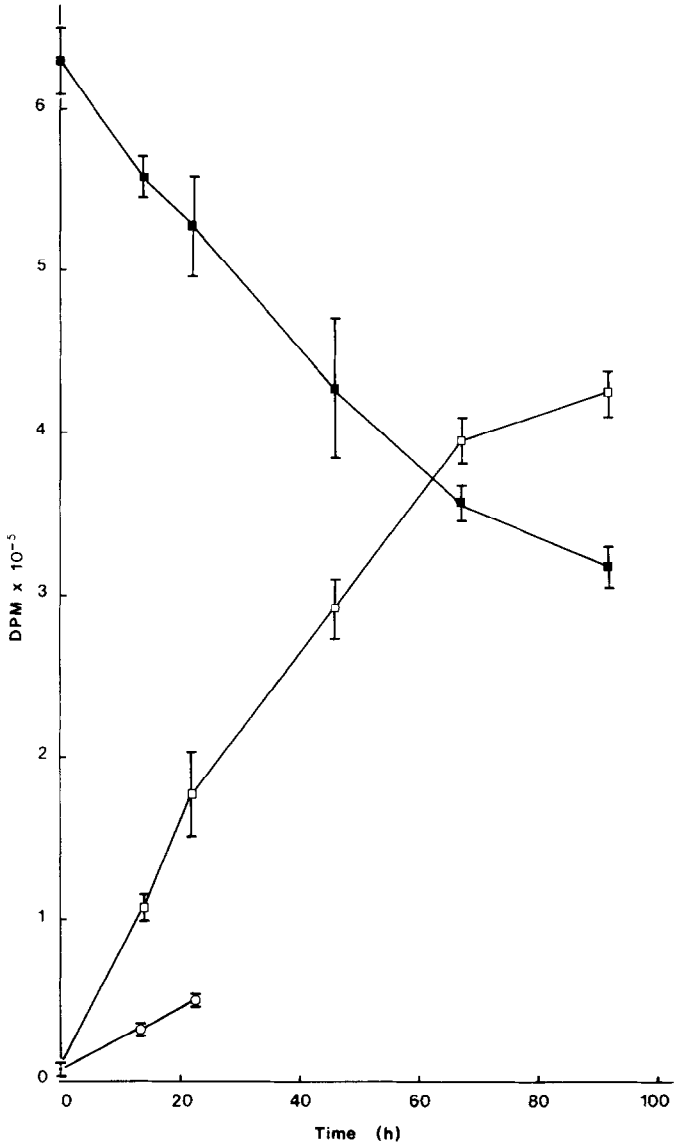

Figure 3. Destruction of transplanted [^{3}H]-OMM proteins in hepatocyte monolayers
The cells were plated out at a density of 1.2 x 10^6 cells following the fusion process as described in the Methods. After 2.5 h the medium was replaced (zero time) and changed at 24 h intervals. At the times indicated media were retained and cells harvested by trypsinization. TCA insoluble counts in cell pellet (■), TCA soluble counts in medium (□), TCA soluble counts in medium of cells cultured in the presence of 200 uM chloroquine for 24 h (○). The values are the means ± of duplicate determinations on a minimum of four separate plates.

destructive fate of transplanted OMM proteins. In preliminary experiments we showed that 200 μM-chloroquine was concentrated in hepatocytes (80 nmol/mg protein) and that it did not appreciably alter protein synthesis ([^{3}H]-leucine incorporation into protein) or affect cell viability

(trypan blue exclusion and lactate dehydrogenase release) over a 24 h period. At this concentration chloroquine inhibits the destruction of transplanted [^{3}H]-OMM proteins by 57% (Fig. 3). This result supports the concept of a dual pathway of protein degradation in hepatocytes[2] extended to the destruction of transplanted OMM proteins. The demonstration with chloroquine that OMM proteins are destroyed in part by a lysosomally mediated mechanism also confirms that the [^{3}H]-OMM proteins are inside the cells. Destruction of transplanted [^{3}H]-OMM proteins by lysosomal and presumably non-lysosomal processes would create two populations of degradation rates for transplanted OMM proteins in hepatocytes. We have recently shown that two such populations of degradation rates can be delineated for OMM proteins in liver in vivo[4].

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