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Lack of amino acids in mouse hepatocytes in culture induces the selection of preneoplastic cells

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ABSTRACT

Protein malnutrition occurs when there is insufficient protein to meet metabolic demands. Previous works have indicated that cycles of protein fasting/refeeding enhance the incidence of early lesions during chemical carcinogenesis in rat liver. The general objective of this work was to study the effect of aminoacids (Aa) deprivation on the proliferation and survival of hepatocytes, to understand its possible involvement in the generation of pre-neoplastic stages in the liver. Lack of Aa in the culture medium of an immortalized mice hepatocyte cell line induced loss in cell viability, correlating with apoptosis. However, a subpopulation of cells was able to survive, which showed a more proliferative phenotype and resistance to apoptotic stimuli. Escaping to Aa deprivation-induced death is coincident with an activated mTOR signaling and higher levels of phospho-AKT and phospho-ERKs, which correlated with increased activation of EGFR/SRC pathway and overexpression of EGFR ligands, such as TGF-α and HB-EGF. Lack of Aa induced a rapid increase in reactive oxygen species (ROS) production. However, cells that survived showed an enhancement in the levels of reduced glutathione and a higher expression of γ-GCS, the regulatory enzyme of glutathione synthesis, which can be interpreted as an adaptation of the cells to counteract the oxidative stress. In conclusion, results presented in this paper indicate that it is possible to isolate a subpopulation of hepatocytes that are able to grow in the absence of Aa, showing higher capacity to proliferate and survive, reminiscent of a preneoplastic phenotype.

1. Introduction

In mammals, protein malnutrition causes weight loss, decreased growth, high susceptibility to infection diseases and cancer. In liver, both caloric and proteic malnutrition cause severs metabolic changes [1–4]. However, how recurrent protein deprivation affects the liver function has not been intensively examined. Protein calorie malnutrition arises when there is no sufficient energy or protein available to cover the metabolic demands due to disease and increased nutrient losses [5,6]. The rapidity of protein–calorie malnutrition development is determined by factors, such as nutritional adequacy, underlying diseases and physiologic states [7]. Caloric restriction has been associated with a delay in the development of both spontaneous and induced neoplasia. In contrast, cycles of fasting/refeeding were shown by us and others to enhance the incidence of early lesions during chemical carcinogenesis in rat liver [1–4]. The frequent amino acid deprivation settles in liver metabolic and ultrastructure changes distinctive of a preneoplastic profile [1,4].

Oxidative stress, viewed as the overproduction of reactive oxygen species (ROS), the failure of the antioxidant defense of the organism, or both, plays a major role in human pathology [8]. Chronic exposure to ROS and the resulting oxidative stress are considered central components of the onset of cell transformation and proliferation in cancer [9,10]. Importantly, malnutrition has a deleterious effect on the liver, with a marked increase in ROS production and changes in the contents of oxidative stress enzymes in the same direction as reported in the development of tumor [11]. However, in healthy cells ROS are not just a by-product of oxygen metabolism, but have physiological specific roles, serving to regulate key signaling pathways. They appear to be required for cellular processes as fundamental as proliferation, intracellular signaling, and gene expression [10]. ROS can affect the

Abbreviations: Aa, aminoacids; EGFR, Epidermal growth Factor Receptor; FBS, fetal bovine serum; CM, complete medium; PM, aminoacid-starved medium; HCC, Hepatocellular carcinoma; H_2DCFDA, 2′,7′-dichlorodihydro-fluorescein diacetate; γ-GCS, gamma glutamylcysteine syntethase; mTOR, mammalian Target Of Rapamycin; PTKs, Protein Tyrosin Kinases; ROS, Reactive Oxygen Species; TGF-β, Transforming Growth Factor-beta.

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activity of signaling pathways by direct modification of regulatory proteins via disulphide bond formation, nitrosylation, carbonylation or glutathionylation [11–13]. Extracellular stimuli transduce signals through a variety of intracellular pathways including protein tyrosine kinases (PTKs), serine/threonine kinases, phospholipases and Ca^{2+}. When cells are stimulated with ROS, signals are transferred through the same signaling pathways as those triggered by growth factors. Oxidants activate PTKs and the downstream signalling components including mitogen-activated protein (MAP) kinases, protein kinase C (PKC), phospholipase C and Ca^{2+} in the cells [14].

Currently, apoptosis or programmed cell death is a focus of intense research. This process must be finely regulated because an excess or a defect in apoptosis could cause disorders in development, autoimmune diseases, neurodegenerative diseases and cancer. Several studies indicate that during the early stage of hepatocarcinogenesis, there is a gradual increase in apoptotic cells related to the development of preneoplastic foci [15,16]. Preneoplastic liver cells are more susceptible than normal hepatocyte to cell death, suggesting that the initiation of the tumor can be reversed by the elimination of these cells. In fact, carcinogens and tumor promoters inhibit apoptosis and increase the amount of preneoplastic cells, which accelerate the development of cancer [17,18].

The signaling pathways of cancer cells can operate in an independent way and/or through interconnections, often involving the activation of receptor tyrosine kinases, which promote cancer development. One of the most important is the phosphoinositide 3-kinase (PI3K)/Akt (PKB) pathway [19]. Downstream PI3K/Akt there are several serine/threonine kinases, one of them mTOR (mammalian Target Of Rapamycin). The TOR proteins family is involved in regulation of mRNA transcription initiation and protein translation in response to essential amino acids intracellular concentrations. mTOR regulates primary signaling pathways, determines the growth and proliferation of cancer cells and regulate the apoptotic process [20]. Hence, mTOR is a nutrient sensor and it has a fundamental role in cell metabolism. In addition, mTOR is an inducer of protein translation and phosphorylates both the ribosomal p70S6 kinase and the inhibitor of translation initiation 4E-BP1 (eIF4E-binding protein) in response to nutrients and hormone signals [21].

The general objective of this work was to study the effect of the deprivation of aminoacids in the culture medium on the proliferation and survival of hepatocytes, to better understand its possible involvement in the generation of pre-neoplastic stages in the liver.

2. Experimental procedures

2.1. Cell culture conditions

Non tumorigenic cell line derived from newborn mice hepatocytes immortalized with SV40 virus (Parental cells = Par cells) [22] were cultured in DMEM (Lonza, Basel, Switzerland) medium with 10% fetal bovine serum (FBS), antibiotic–antimycotic (1%) and maintained in a humidified atmosphere of 37 °C, 5% CO_2. For experiments (cells at 60% confluence), medium was replaced by DMEM (complete medium: CM) or by aminoacid-starved medium (PM = private medium), which contained HANKS' BALANCED SALTS (H9269, Sigma-Aldrich, Madrid, Spain), glucose and vitamins (M6895 Sigma-Aldrich, Madrid, Spain), as similar concentrations as found in the complete DMEM. To restore the concentrations of essential (eAa) and nonessential aminoacids (neAa), supplements M5550 (eAa) and M7145 (neAa) (Sigma-Aldrich, Madrid, Spain) were used.

2.2. Analysis of cell number

Cell number was analyzed after crystal violet staining (0.2% in 2% ethanol), as previously described [23].

2.3. Proliferation measurement by [3H]-thymidine incorporation

Cells were treated during 48 h in the presence of 1 μCi/ml, 1 μM Methyl-[^{3}H]-thymidine (Hartmann Analytic GmbH, Braunschweig, Germany). Then, cells were incubated for 20 min at 4 °C with 10% trichloroacetic acid, washed twice with 70% ethanol and let them dry for at least 1 h. Finally, the acid precipitated material is suspended in a buffer containing 2% Na_2CO_3, 0.1 N NaOH, 0.5% SDS and radioactivity was measured in a scintillation counter 1209 Rackbeta (Wallac, Turku, Finland) diluting 100 μL of acid precipitated material in 5 ml of scintillation liquid.

2.4. Cell viability analysis

Cells were plated in 96-well plates. Multitox-Fluor Multiplex Cytotoxicity Assay kit (Promega, Madison, USA) reagents were added after treatments as indicated by manufacturer's protocol. Fluorescence was measured in a Microplate Fluorescence Reader FLUOstar Optima (Biogen Científica S.L., Madrid, Spain). Viability was calculated as the ratio between live cells fluorescence and dead cells fluorescence, and expressed as percentage of untreated cells.

2.5. Analysis of caspase-3 activity

Caspase-3 activity was analyzed fluorimetrically as described previously [24]. Protein concentration of cell lysates was determined by using the Bio-Rad protein assay kit (Hercules, CA, USA). Results are calculated as units of caspase-3 activity per microgram of protein.

2.6. Measurement of intracellular redox state

The oxidation-sensitive fluorescent probes 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA) (from Invitrogen, Carlsbad, CA, USA) and Monochlorobimane (from Sigma-Aldrich, Madrid, Spain) were used to analyze the total intracellular content of ROS and GSH content, respectively. After treatment, cells were incubated with 2.5 μM H_2DCFDA (30 min, 37 °C), or 2 mM Monochlorobimane (1 h, 37 °C) in HBSS without red phenol, lysed with a buffer containing: 25 mM HEPES pH 7.5, 60 mM NaCl, 1.5 mM MgCl, 0.2 mM EDTA and 0.1% Triton X-100 (10 min, 4 °C) and transferred in duplicate into a 96-well plate. Fluorescence was measured in a Microplate Fluorescence Reader FLUOstar Optima and expressed as percentage to control after correction with protein content [25]. Carbonylated proteins were detected by using the OxyBlot™ Protein Oxidation Detection Kit (Millipore, Billerica, MA, USA) according to supplier protocols. Briefly, the carbonyl groups in the protein side chains were derivatized to 2,4-dinitrophenylhydrazone (DNP-hydrazone) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by polyacrilamide gel electrophoresis followed by Western blotting, using a primary antibody specific to DNP. Coomassie Brilliant Blue R-250 staining was used to monitor equal loading.

2.7. Western blot analysis

Total protein extracts were obtained using a lysis buffer containing 30 mM Tris–HCl pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycolate, 0,1% SDS and 10% glycerol. Pellets were incubated during 1 h in lysis buffer at 4 °C, sonicated 5 s, boiled 5 min and centrifuged at 13,000 rpm during 10 min at 4 °C. Western blot procedure was carried out as described previously [26]. The antibodies used were: mouse anti-β-actin (clone AC-15; Sigma-Aldrich, Madrid, Spain); rabbit anti-gamma glutamylcysteine syntethase (γ-GCS) (Abcam, Cambridge, UK), anti-Akt (CS-9272), anti-phospho Akt (Ser473) (CS-9271), anti-Phospho-p44/42 MAPK (Thr202/Tyr 204) (pERK1/2) (CS-9101), anti 44/42 ERK Kinase (CS-9122), anti-

Phospho-Src (CS-2101) and anti-phosho-EGF receptor (Tyr1068) (CS-2236) antibodies were from Cell Signalling (Beverly, MA, USA). Antibodies were used at 1:1000, except β-actin (1:3000). Protein concentration was measured with BCA™ Protein Assay kit (Pierce, Rockford, USA). Antibody binding was visualized using Amersham ECL (GE Healthcare, Madrid, Spain).

2.8. Analysis of gene expression

RNeasy mini kit (Qiagen, Valencia, CA, USA) was used for total RNA isolation. Reverse transcription (RT) was carried out using the High Capacity Reverse Transcriptase kit (Applied Biosystems, Foster City, CA, USA), with 500 ng of total RNA from each sample for complementary DNA synthesis. Semiquantitative PCRs were performed using specific primers for mice samples:

EGFR: F 5'AAACTCTTCGGGACGCCCAATC3', R5'TGGCGATGGATGGGATCTTTG3'
HBEGF: F 5'CGGTGGTGCTGAAGCTCTTTC3', R5'TGGTAACCAGGGAGGCAGTG3'
TGF-α: F 5'TGGTGCAGGAAGAGAAGC3', R5'TGACAGCAGTGGATCAGC3'
18S: F 5′-GCGAAAGCATTTGCCAAGAA-3′, R5′-CATCACAGACCTGTTATTGC-3′

Products were obtained after 30–35 cycles of amplification and 59–65 °C of annealing temperature and were electrophoresed in 1.5% agarose gels.

2.9. Immunocytochemistry

Cells were plated on gelatin-coated glass coverslips. The monolayer was washed with PBS, cells were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature and incubated for 2 min with 0.1% Triton X-100. For F-actin staining, cells were subsequently incubated with 100 nM Rhodamine-Phalloidin (Fluka) at room temperature; for E-Cadherin, anti-cadherin E (BD Biosciences Pharmingen, San Diego, CA, USA; 1:50) was diluted in 1% bovine serum albumin and incubated for 2 h at room temperature. After several washes with PBS, the samples were incubated with fluorescent-conjugated secondary antibody (1:200 for Alexa Fluor 488–conjugated antimouse, Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature. Cells were visualized in an Olympus BX-60 microscope with the appropriate filters.

2.10. Statistical analysis

All the measurements were subjected to one-way analysis of variance followed by the Dunnett test of mean comparison with references (InStat, Graph Pad software). P values lower than 0.05 were considered as significant. Data from at least three separate experiments were analysed.

3. Results

3.1. Effect of amino acid deprivation on hepatocyte proliferation and survival

To know the capacity of hepatocytes to progress in culture in the absence of extracellular aminoacids (Aa), we cultured these cells in complete medium (CM) or in Aa-deprived medium (private medium: PM), which might be supplemented with essential (eAa) or non-essential Aa (neAa). Hepatocytes were shown to be sensitive to the lack of amino acids, being more sensitive to lack of essential amino acids than nonessential, as shown in Fig. 1A. Cell number observed at 12, 24 and 48 h was significantly lower when incubated without Aa, as compared with the proliferation in complete medium. To know whether cells might be dying, we analyzed the appearance of apoptotic features, such as activation of caspase-3, which was increased at 12 and 24 h in cells incubated in the absence of Aa (Fig. 1B). We confirmed the cytotoxic effects through the analysis of cell death (Fig. 1C), observing that the appearance of dead cells increases with time of incubation without Aa. Supplementation with essential amino acids showed a slight rescue on the apoptotic and cytotoxic effects, particularly when compared to that produced by incubating with non-essential Aa, which had no effect. Interestingly, pro-survival signaling pathways, such as pAKT and pERKs, appeared to be activated in those cells that survived to 24 h of incubation in the absence of Aa, particularly in the absence of non-essential or without Aa (Fig. 1D).

To mimic the effect of the lack of Aa, we proceeded to incubate hepatocytes in the presence of rapamycin, a macrolide antibiotic with antifungal and immunosuppressive properties, which binds to cytoplasmic FK506-binding protein-12 (FKBP12) and in complex with FKBP12 inhibits the mTOR family kinases. As it can be seen in Fig. 2, rapamycin decreased the number of viable cells and increased caspase-3 activity in cells incubated in complete medium, in a similar way to that observed when cells were cultured in the absence of aminoacids.

3.2. Isolation and characterization of a hepatocyte population that survives in aminoacid-deprived medium

As we have shown, when hepatocytes are cultured in the absence of Aa, 50–60% of the cells detaches and dies through apoptosis after 48 h. After 72 h of culture with PM the few surviving cells were incubated with complete medium, thus leading to the selection of the population of cells that appear to resist to the acute deprivation of Aa. After changing the medium, cells began to proliferate and expanded. We call "Sel" to this selected population of cells. In phase contrast microscopy, increase in cell number/field and more cytoplasmic connections, as a network, were observed (Fig. 3A upper panel). Labeling with phalloidin (F-actin) or immunostaining with anti-E cadherin antibodies, revealed no significant differences in cytoskeleton organization and cell–cell contacts between the parental and the selected cells (Fig. 3A middle and lower panel respectively). However, Sel cells showed a significant higher proliferation rate than the parental ones, as can be seen in Fig. 3B, where we show the number of cells after 24 and 48 h of plating. This conclusion was confirmed by studying the incorporation of [^{3}H]-Thymidine as an analysis of DNA synthesis (Fig. 4). Increase in cell proliferation in Sel cells versus the parental ones was observed regardless the initial cell density (Fig. 4A) and was more significant in the presence of high concentrations of FBS (Fig. 4B), indicating that response to extracellular mitogens is enhanced in these cells. In addition, Sel cells revealed a certain capability to survive in the absence of aminoacids, since submitting them to a new acute treatment with Aa-depleted medium stimulated the activity of caspase-3 but in much lower extent than that observed in the parental cells (Fig. 4C). Interestingly, Sel cells were also more resistant to extracellular stimuli of apoptosis. Indeed, the response to the Transforming Growth Factor-beta (TGF-β), a physiological inducer of hepatocyte apoptosis whose concentration is elevated in liver tumors to counteract the abnormal growth of preneoplastic cells [26–28], is altered in Sel cells (Fig. 4D). Both TGF-β-induced decrease in cell viability and caspase-3 activation were attenuated in Sel when compared to the parental cells. In summary, the population of cells that have survived to the cytotoxic effect of Aa deprivation show higher proliferative response and less sensitivity to apoptosis than the parental cells.

3.3. Relationship between aminoacid deprivation and oxidative stress

As we introduced above, ROS production and oxidative stress are important cellular hallmarks for apoptosis and/or transformation processes. Furthermore, malnutrition has a deleterious effect on the liver,

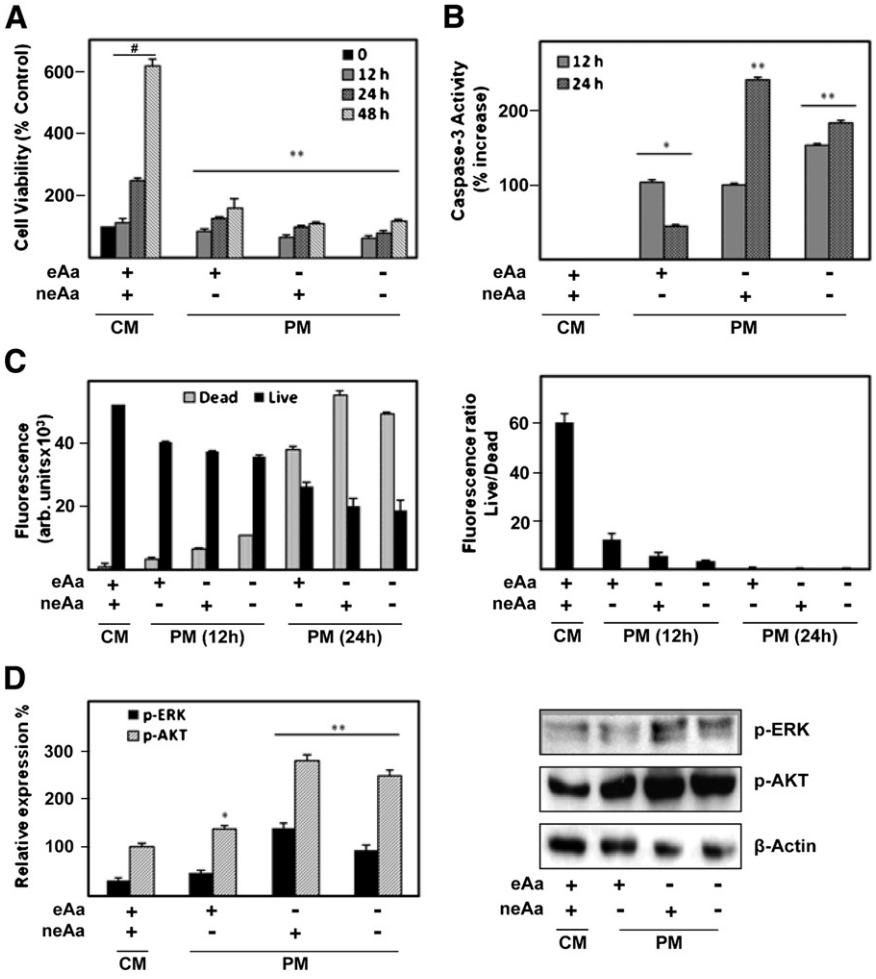

Fig. 1. Aminoacid withdrawal induces death of hepatocytes. Hepatocytes were cultured for 12, 24 and 48 h in complete medium (CM) or in the absence of aminoacids (PM = private medium). Essential (eAa) or nonessential (neAa) aminoacids were added to the PM in separate experiments. (A) Analysis of the cell viability. (B) caspase-3 activity. (C) cytotoxicity assay with Multitox-Fluor Multiplex, live or death fluorescence (Left panel) and cell fluorescence live/dead ratio (Right panel). (D) Western blot for the analysis of p-ERKs and p-AKT. β-actin was used as loading control. In A, results are expressed as percentage of initial number of cells; in B and D are expressed as percentage of the control (cells cultured in CM = control medium); in C, the values show arbitrary fluorescence units (left panel) and the ratio of cell fluorescence live / dead (right panel); in D a representative Western blot of at least three different experiment is shown. Mean ± S.E.M. Data are from three independent tests. Statistical differences from PM vs CM under the same conditions: *$p<0.05$, ** $p<0.005$; in A, #$p<0.05$ when compared the different times of treatment vs the zero time condition.

with a marked increase in ROS production and changes in the contents of oxidative stress enzymes [4,29]. Accordingly, we decided to measure different oxidative stress markers in cells cultured in the presence or in the absence of Aa, comparing the parental and the Sel cells. When both the parental and Sel cells were cultured in the absence of Aa, a significant increase in ROS production was observed (Fig. 5A). Analysis of protein carbonylation (Fig. 5B), revealed protein oxidation at 12 h after Aa deprival, decreasing at longer times. Interestingly, in parallel to ROS production, an increase in reduced GSH concentration was observed (Fig. 5C), which can be interpreted as an intend of the cells of counteracting the oxidative stress. In this line of evidence, we observed that absence of Aa induced the expression of γ-GCS, the rate-limiting enzyme for GSH biosynthesis, in the parental cells, which correlated with the increased basal levels of GSH observed in such conditions. This fact might explain why protein oxidation decreases at long times after treatment. It is worthy to note that Sel cells expressed basal higher levels of γ-GCS than the parental cells when cultured in complete medium (Fig. 5D).

3.4. Activation of different signaling pathways in response to aminoacid withdrawal

As we have previously shown in Fig. 1D lack of Aa induced ERKs (p42/44 MAPK) and AKT phosphorylation in Par cells. Here, we wanted to compare p-AKT and p-ERK levels of Sel cells with their parental cells, incubated in the presence or in the absence of Aa. As shown in Fig. 6A, both p-ERKs and p-AKT levels were much higher in Sel cells than in the Par ones. Downstream AKT signals, such as p70S6 and GSK3 were also higher phosphorylated in Sel cells (Fig. 6B). Interestingly, after Aa withdrawal, hepatocytes showed increased levels of p-SRC, which correlated with increase in the levels of phosphorylation of the EGF receptor (EGFR) (Fig. 6B). These results indicate that the EGFR/SRC pathway might be overactivated in Sel cells. In agreement with this hypothesis, Sel cells expressed higher levels of EGFR ligands, such as TGF-α and HB-EGF. Semiquantitative RT-PCR analysis clearly revealed that both ligands were overexpressed in Sel cells, when compared with normal hepatocytes (Fig. 6C), and interestingly, Aa withdrawal also induced the expression of both ligands. Finally, the absence of Aa induced an increase in the phosphorylation of the stress MAPK proteins c-JNK and p38, which remained increased in Sel cells (Fig. 6D).

4. Discussion

Hepatocellular carcinoma (HCC) is the third most important cause of cancer death worldwide. It is often diagnosed at an advanced stage, when it is not amenable to curative therapies. Advance in cancer biology has suggested that a number of pathways are responsible

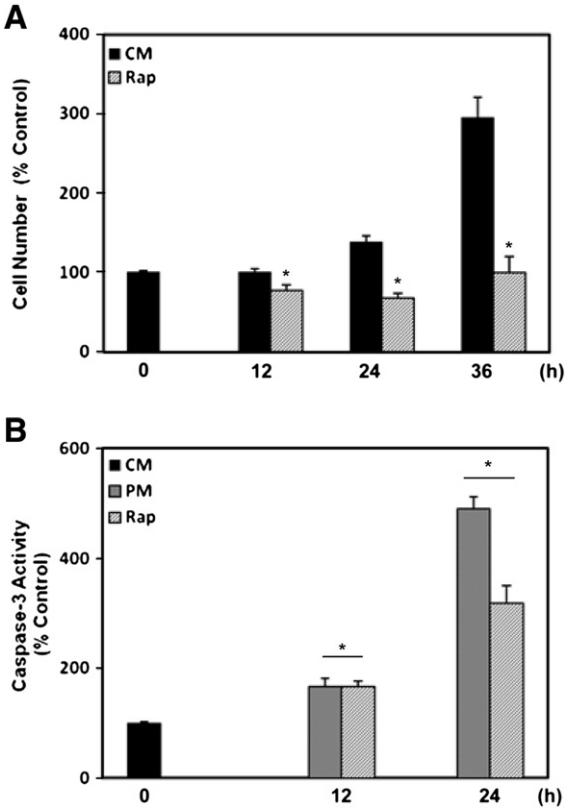

Fig. 2. Rapamycin mimics the effect of Aa withdrawal. Cell number (A) and caspase-3 activity (B) at the indicated times of culture of hepatocytes in complete medium (CM) or in the absence of aminoacids (PM = private medium) or in CM + 10 μM rapamycin (Rap). Data are calculated as percentage relative to control cells at zero time and represent the mean ± S.E.M of three independent experiments. (*p<0.05, **p<0.005 vs the CM condition).

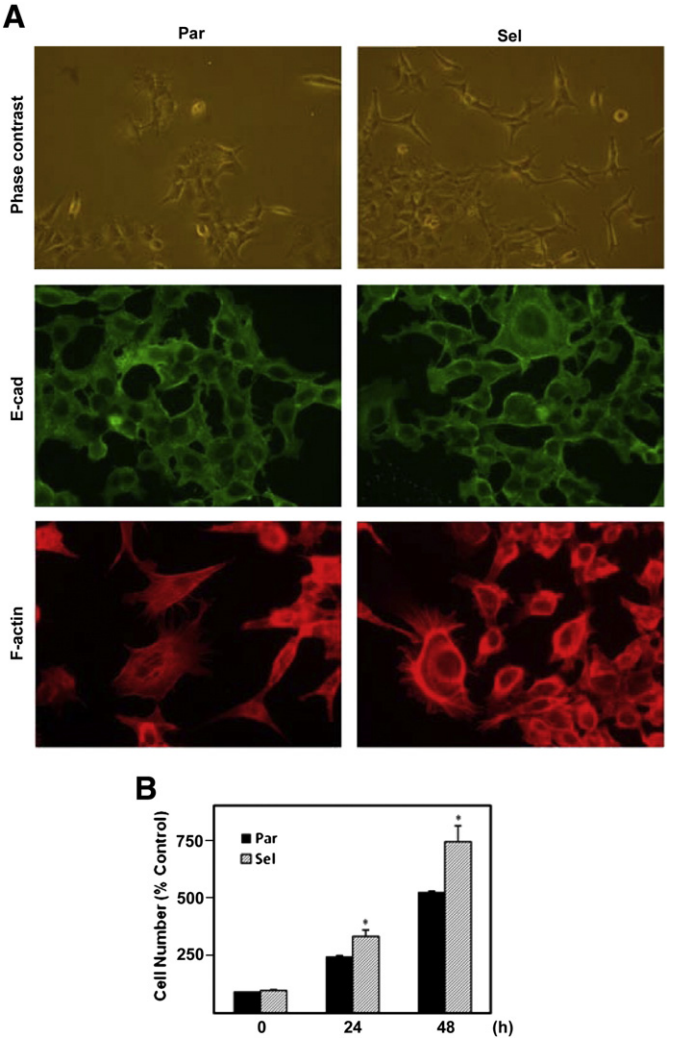

Fig. 3. Selection of a population of cells that survive in Aa-deprived medium. Comparative analysis between the parental cell line (Par, left) and the selected cells (Sel, right): (A) phase contrast microscope pictures (upper panel), immunofluorescence study of the basal expression of E-cadherin (middle panel) and F-actin (bottom panel). (B) Cell number after 24 and 48 h of culture under basal conditions (10% FBS), expressed as percentage of their respective controls at initial time (0 h). Mean ± S.E.M. Data are from three independent tests. Statistical differences from Sel vs Par cells under the same conditions: *p<0.05.

for initiating and maintaining deregulated cell proliferation and increased survival [30–33]. Metabolic pathways are currently receiving enormous attention, due to the fact that tumors have a special metabolism which makes them more susceptible to lack of specific nutrients [33]. Previous studies from our and other groups have indicated that cycles of fasting/refeeding might have an incidence in the generation of early lesions during chemical carcinogenesis in rat liver [1–3]. In fact, frequent amino acid deprivation settles in liver metabolic and ultrastructure changes reminding a preneoplastic profile [1,4]. In this work we show that, although lack of Aa might inhibit growth and induce apoptosis in hepatocytes (Fig. 1), its absence, directly or indirectly, has an influence on the selection of a population of cells with higher capacity to proliferate and survive, expressing autocrine growth factors and survival signals, which might play relevant roles in liver differentiation and, particularly, in hepatocarcinogenesis.

The isolation and subculture of the subpopulation of cells that are able to survive to the absence of Aa-proapoptotic effects (Sel cells) allowed us to prove that they show a more proliferative phenotype (Figs. 3 and 4) and resistance to apoptosis. The higher proliferation was observed both in the presence and in the absence of FBS, this last result indicating that the Sel cells might show activation of autocrine signals that confers them proliferative autonomy. In this line of evidence, these cells showed higher levels of p-AKT and p-ERKs under basal conditions, when compared with the parental ones (Figs. 1 and 6), which correlated with higher activation of the EGFR/SRC pathway and overexpression of EGFR ligands, such as TGF-α and HB-EGF, which are well-known EGFR ligands produced by rat hepatic cells. TGF-α is proved to be a physiological regulator of liver regeneration by means of an autocrine mechanism [26–27] and HB-EGF, although barely expressed in normal rat hepatocytes, is highly expressed in liver tumor cells [28], or in hepatocytes from fibrotic rat liver [27]. It is well known that production of EGFR ligands might confer both increase in proliferation and apoptosis resistance in hepatocytes and hepatoma cells [8,18,24,28]. EGF protects hepatocytes from the apoptosis induced by TGF-β, which might explain why Sel cells show attenuated apoptotic response to this cytokine, when compared with the parental cells (Fig. 4D) [34–37].

In this study, we also show that the lack of Aa induced significant changes in the oxidative status of the cells. A rapid increase in ROS production is observed, concomitant with a later enhancement in the levels of reduced glutathione and a higher expression of γ-GCS, the regulatory enzyme of glutathione synthesis. These data indicate that the adaptation of the cells to changes in metabolism produces increase in ROS production. Cell that survive to this stress process have adapted to counteract oxidative damage through increasing its antioxidant defenses, particularly GSH. Indeed, protein oxidation decays as soon as GSH increases (compare data from Fig. 5B and C). Released ROS might play a role in the process of selection of cells that survive to the complete lack of Aa. Different intracellular signals are controlled by oxidative stress, with later effects in gene transcription.

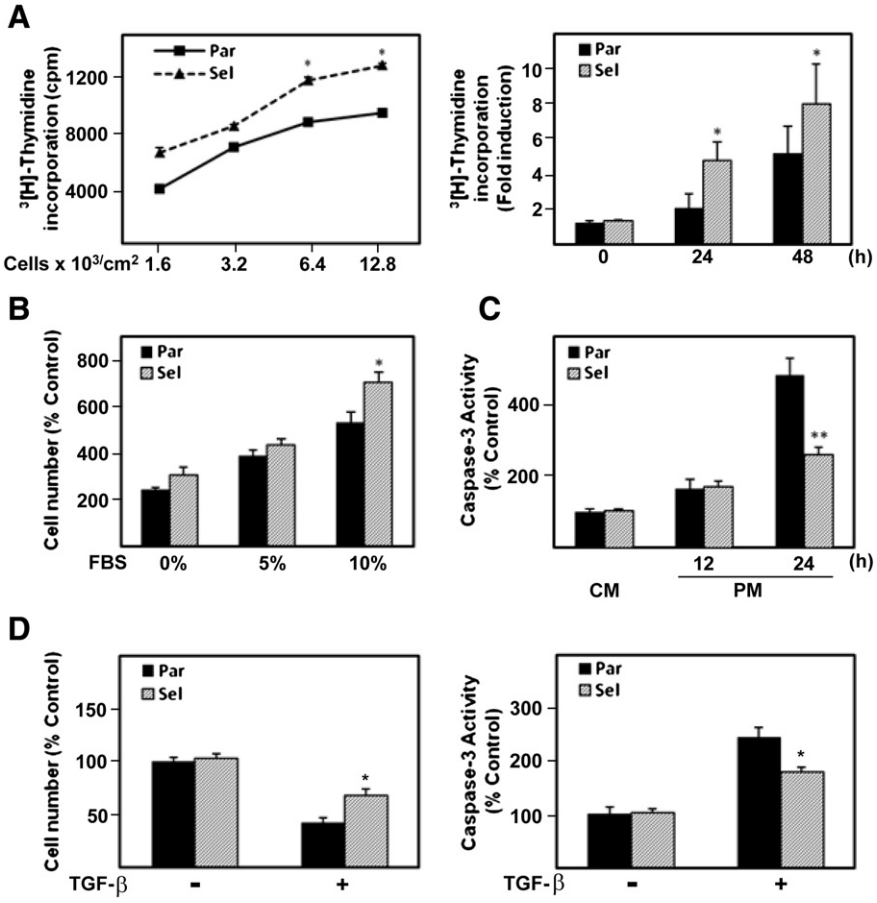

Fig. 4. Hepatocytes that survive to Aa withdrawal show higher proliferative capacity and lower sensitivity to apoptosis. (A) Cell growth was determined after 48 h through analysis of DNA synthesis by [3H]-Thymidine incorporation, at different cell plating (left panel) and different times of culture (right panel). (B) Comparison of cell number of Par and Sel cells, after 48 h at different percentages of FBS in control medium culture; (C) caspase-3 activity upon incubation of Par and Sel cells for 12 or 24 h without Aa in the culture medium; and (D) response in cell viability (left panel) and caspase-3 activity (right panel) to 2 ng/ml TGF-β for 24 h in culture medium. Mean ± S.E.M. Data are from at least three independent tests. Statistical differences from Sel vs Par cells under the same conditions: *$p<0.05$, **$p<0.05$.

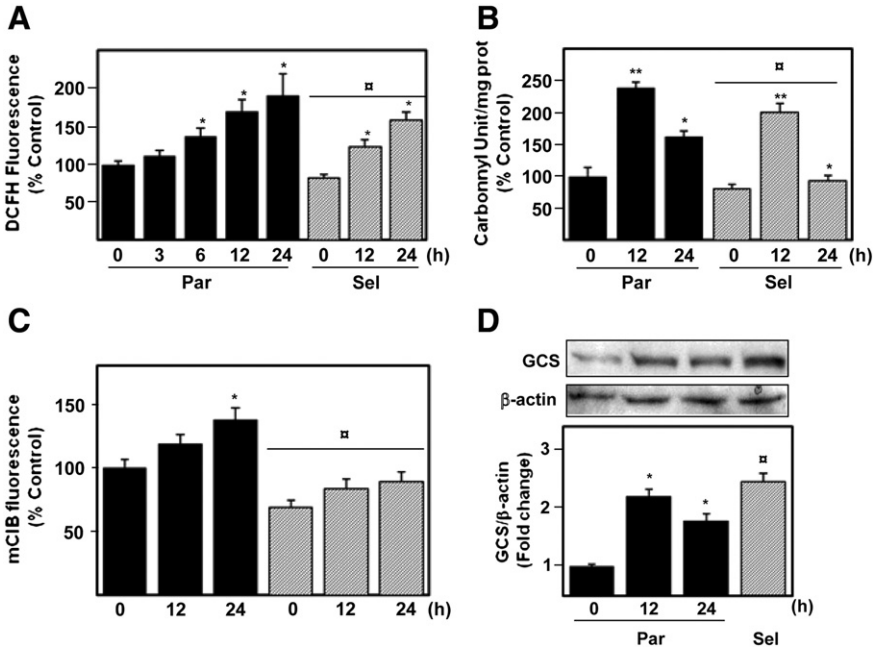

Fig. 5. Aminoacid withdrawal induces changes in the oxidative status of the cells. Par and Sel cells were incubated for the indicated times in medium without Aa and it was measured: Total ROS production (A), percentage of carbonylated proteins (B), GSH content (C) and γ-GCS (GCS) levels by Western blot (D). In A, B and C, data represent the mean ± SEM of three independent experiments. In D, a representative experiment is shown (upper panel), and mean ± S.E.M of densitometric analysis (lower panel) of three different western blots are shown. Statistical differences: *$p<0.05$, **$p<0.005$ when compared in the same cell line the Aa deprival effect at different times vs zero time; #$p<0.05$ when compared Sel cells vs Par cells under the same experimental conditions.

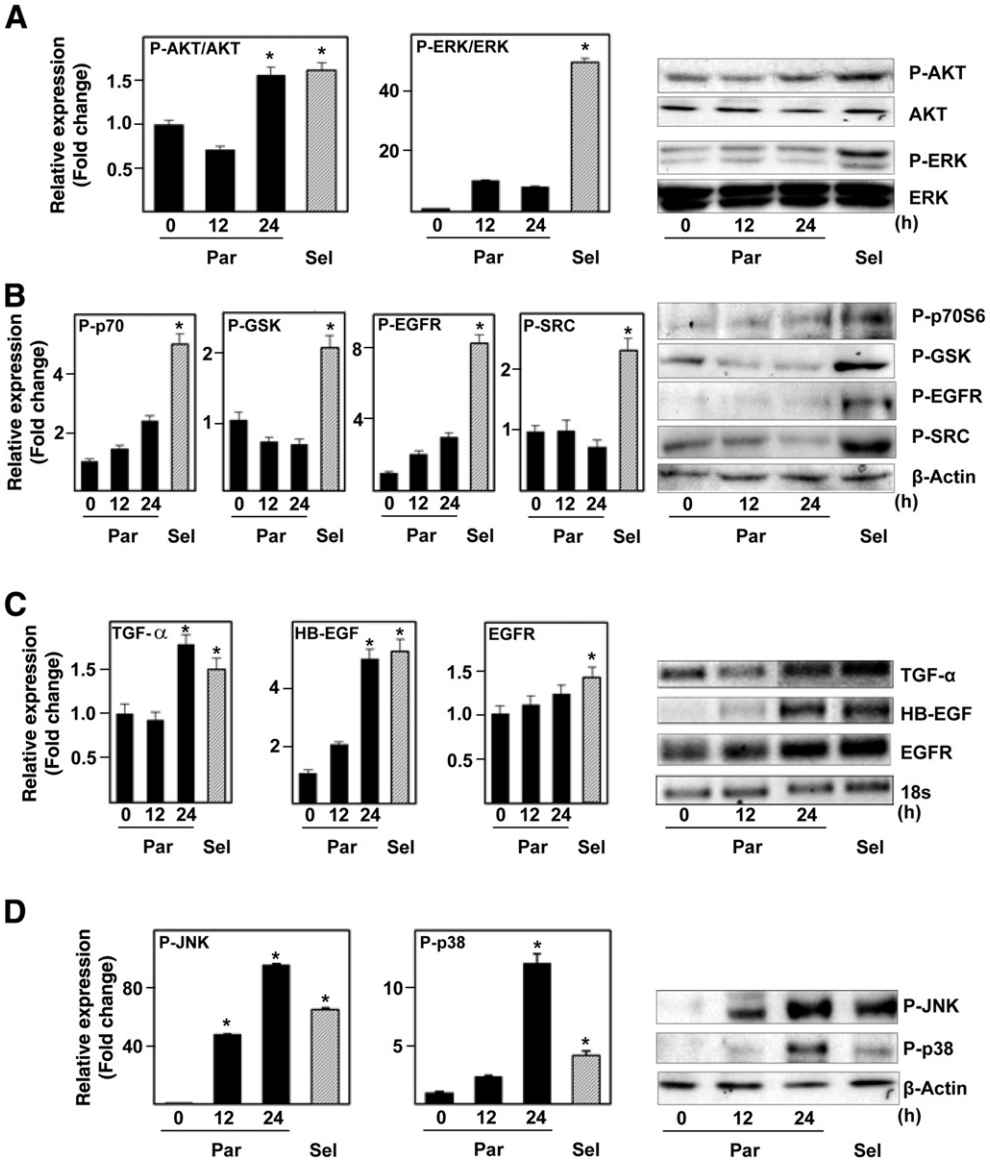

Fig. 6. Activation of different signaling pathways in response to aminoacid withdrawal. Hepatocytes (Par) were cultured during 24 and 48 h in Aa-free medium. In parallel, the selected cells that survived in Aa-depleted medium (Sel) were cultured under basal conditions. In both cases, protein extracts were collected for Western blot analysis of the proteins indicated in each panel (A, B and D) or RNA was collected for semiquantitative PCR analysis of the expression of growth factors TGF-α and HB-EGF and their receptor EGFR (C). β-actin was used as loading control for western blot and the ribosomal RNA 18S for PCR. Right panels show a representative experiment and left panels indicate the densitometric analysis of at least three independent experiments. Mean ± S.E.M. *$p<0.05$ vs Par cells at zero time.

Among them, $p38^{MAPK}$ has been proposed to be activated by ROS [38]. JNK is another signal under redox regulation which might contribute to regulate gene expression through the AP-1 transcription factor [39]. Our results indicate that both p38 and JNK are activated after Aa deprivation in hepatocytes (Fig. 6). Interestingly, intracellular signals that respond to oxidative stress might be regulating the transcription of EGFR ligands, either directly or indirectly by acting on NF-κB target genes whose product are required for TGF-α or HB-EGF transcriptional induction [16].

A key element for the maintenance of energy homeostasis is TORC1, which is a multiprotein complex that includes mTOR. In all eukaryotic cells, mTOR integrates nutrient signals and coordinates cell growth, proliferation, and metabolism [31,40]. Under normal circumstances, in the presence of growth factor and nutrients, mTOR is constitutively activated. This activation is achieved via a cascade that involves the activation of the PI3K/Akt-mediated phosphorylation of mTOR [41]. An emerging view is that the mTOR complex protein kinase is a critical mediator of the cellular response to many stresses, including DNA damage as well as drops in the levels of energy, glucose, amino acids, and oxygen. These stress responses impact a wide variety of both pathological and physiological states, such as aiding in the resistance of tumor cells to conventional therapy and modulating life span [42]. Recent studies indicate that mTOR is a sensor for the presence of aminoacids, although it is not yet well established whether it is a direct or an indirect effect [43]. Results presented here indicate that rapamycin, a macrolide that inhibits the mTOR-dependent downstream signaling pathways, mimics the effects of lack of aminoacids, inhibiting proliferation and inducing cell death in hepatocytes (Fig. 2). Interestingly, the Sel cells show higher levels of p70S6, the main TORC1 target and p-GSK, its downstream substrate, which might indicate that the capacity of the cells to escape from Aa deprivation-induced death is coincident with an activated mTOR signaling. It has been suggested that increased mTOR activity confers a preneoplastic phenotype to hepatocytes, by altering the translation of genes vital for establishing normal hepatic energy homeostasis and moderating hepatocellular growth [44]. As a

PI3K/Akt downstream target, mTOR is overactivated in a relevant percentage of HCC [30] and rapamycin has revealed as a promising therapeutic drug, alone or in combination with other targeted therapies, such as sorafenib [45].

5. Conclusions

In conclusion, results presented in this paper indicate that it is possible to isolate in vitro a population of hepatocytes that are able to grow in the absence of Aa, which has higher capacity to proliferate and survive, showing a preneoplastic phenotype. This could explain why the alternately deprivation of proteins in diet could induce hepatocarcinogenesis susceptibility. A process like that is continuously happening in developing countries, in which dietary proteins are ingested irregularly, due to their high cost. A final consideration to be done from these results is that, although it is well established that calorie restriction prevents oncogenic processes, it is necessary the right equilibrium among all the diet components.

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