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The Bioenergetic Signature of Cancer: A Marker of Tumor Progression[1]

José M. Cuezva,[2] Maryla Krajewska, Miguel López de Heredia, Stanislaw Krajewski, Gema Santamaría, Hoguen Kim, Juan M. Zapata, Hiroyuki Marusawa, Margarita Chamorro, and John C. Reed

Departamento de Biología Molecular, Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid-Consejo Superior Investigaciones Científicas, Universidad Autónoma de Madrid, 28049 Madrid, Spain [J. M. C., M. L. d. H., G. S., M. C.]; Department of Pathology, College of Medicine, Yonsei University, Seoul, Korea [H. K.]; and The Burnham Institute, La Jolla, California 92037 [J. M. C., M. K., S. K., J. M. Z., H. M., J. C. R.]

ABSTRACT

Mitochondrial H^+-ATP synthase is required for cellular energy provision and for efficient execution of apoptosis. Almost one century ago, Otto Warburg proposed the hypothesis that mitochondrial function might be impaired in cancer cells. However, his hypothesis was never demonstrated in human carcinomas. In this study, we have analyzed the expression of the β-catalytic subunit of the H^+-ATP synthase (β-F1-ATPase) of mitochondria in carcinomas of the human liver, kidney, and colon. We show that carcinogenesis in the liver involves a depletion of the cellular mitochondrial content, as revealed by reduced content of mitochondrial markers, whereas in kidney and colon carcinomas, it involves a selective repression of the expression of the β-F1-ATPase concurrent with an increase in the expression of the glycolytic glyceraldehyde-3-phosphate dehydrogenase. Both mechanisms limit mitochondrial cellular activity in cancer, strongly supporting Warburg's hypothesis, and suggest a mechanism for the resistance and compromised apoptotic potential of tumor cells. Furthermore, we show that the metabolic state of the cell, as defined by a bioenergetic mitochondrial index relative to the cellular glycolytic potential, provides a signature of carcinogenesis of prognostic value in assessing the progression of colorectal carcinomas.

INTRODUCTION

Mitochondria play an essential role in providing energy to the eukaryotic cell. The molecular machine involved in the synthesis of cellular ATP is the H^+-ATP synthase, a molecular rotatory-engine complex located in the inner mitochondrial membrane (1, 2). In recent years, mitochondria have become a central subject of study because of their role as sensors and executioners of apoptosis (3–5). Apoptosis is an energy-dependent, genetically encoded program for cell death that is indispensable for the normal development of the organism (6). Alterations in the cellular program of apoptosis contribute to the progression of various human pathologies, including cancer and neurodegenerative diseases (7, 8).

The study of the energy metabolism of cancer cells was a central issue of cancer research until the era of molecular biology. As early as 1930, Otto Warburg proposed the hypothesis that cancer cells may have impaired mitochondrial function and that this alteration would result in the elevated rate of glycolysis that is a common feature of most tumors (9). Although the glycolytic phenotype of many cancer cells and tumors has been demonstrated at both the biochemical and molecular levels (10–12), the presumed impairment of mitochondrial function was never established in cancer biology (13). In fact, to this day, we still do not know the role that mitochondria play in neoplastic transformation and in maintaining or promoting the transformed state. Only in the case of the highly glycolytic hepatoma cell lines is their abnormal energetic phenotype ascribed to a marked reduction in the cellular content of mitochondria (13, 14). In this case, the mitochondrial phenotype of hepatomas mimics the phenotype of the fetal hepatocyte (15), in which a program of organelle biogenesis limits the number of mitochondria/cell (14, 16).

These findings, together with the observation that efficient execution of apoptotic cell death requires the molecular components of the H^+-ATP synthase (17, 18), in addition to adequate supplies of ATP (19, 20), led us to investigate the mitochondrial phenotype of human solid carcinomas. To this end, we examined the expression of β-F1-ATPase[3] relative to the expression of the mitochondrial Hsp 60 chaperone in liver, kidney, and colon carcinomas. The findings obtained reveal two alternative pathways by which cancer cells down-regulate the activity of mitochondria. In the case of liver cancer, we showed that there is a general down-regulation of mitochondrial components that is consistent with a repression of the program of mitochondrial proliferation (16). In contrast, in kidney and colon carcinomas, we observed a specific down-regulation of the expression of the β-F1-ATPase that is consistent with a selective repression of the expression of the components involved in mitochondrial bioenergetic function. Along with the limitation of mitochondrial oxidative phosphorylation in kidney and colon carcinomas, we observed an up-regulation of the glycolytic GAPDH. Thus, we proceeded to develop a bioenergetic index of the cell (BEC index) that could be used for classification and prognostic purposes in certain types of cancers. Here we show that the BEC index is drastically reduced in kidney and colon carcinomas, providing a bioenergetic signature of the cell. This index has prognostic value in assessing clinical outcome for patients with early-stage colorectal carcinomas. We further suggest that the BEC index provides a general target for therapeutic intervention in many types of cancer.

MATERIALS AND METHODS

Antibodies. The anti-β-F1-ATPase used in this study was generated in rabbits using the recombinant rat liver β-F1-ATPase-His_6 protein as immunogen. The full-length β-F1-ATPase cDNA from rat liver (21), cloned in pRSET B plasmid, was expressed in BL21 cells by induction with 0.5 mM isopropyl β-D-thiogalactopyranoside. After cell growth and lysis, the clarified cell lysate was applied to TALON resin (Clontech, Palo Alto, CA), and the β-F1-ATPase-His_6 protein eluted with 50 mM imidazole. The eluted protein was dialyzed against 0.5× PBS and freeze-dried. Antibodies were raised in New Zealand White rabbits (22). Commercially available antibodies for mitochondrial Hsp 60 (Stressgene, Victoria, British Columbia, Canada), GAPDH, and HKs I and III (Biogenesis, Poole, United Kingdom) were purchased.

Tissues and Patient Specimens. Normal tissues for immunohistochemical analysis were derived either from human biopsy and autopsy material. All reports of the human tissue samples used in this study were received in a coded

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[2] To whom requests for reprints should be addressed, at Centro de Biología Molecular "Severo Ochoa," Universidad Autónoma de Madrid, 28049 Madrid, Spain. Phone: 34-91-397-4866; Fax: 34-91-397-4799; E-mail: jmcuezva@cbm.uam.es.

[3] The abbreviations used are: β-F1-ATPase, β subunit of the mitochondrial H^+-ATP synthase; Hsp, heat shock protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; BEC index, bioenergetic cellular index; HCC, hepatocellular carcinoma; FNH, focal nodular hyperplasia; A6-8, mitochondrial ATPase subunits 6 and 8; mtDNA, mitochondrial DNA; HK, hexokinase; DFS, disease-free survival; ROS, reactive oxygen species; HRP, horseradish peroxidase.

form so that the identities of the patients were not known. Tissues were fixed in either neutral-buffered formalin, zinc-buffered formalin, B5, or Bouin's solution (Sigma Chemical Co., Inc., St. Louis, MO), and embedded in paraffin. Archival paraffin blocks were obtained for five specimens each of HCCs, hepatoblastomas, and FNHs. For part of the analysis, we constructed tissue microarrays containing specimens acquired from paraffin blocks of normal and human carcinomas, which were sectioned at 4–5-μm thickness. Colon carcinoma specimens were obtained from the Department of Pathology, Yonsei University, College of Medicine, Seoul, Korea. Tissue samples included 104 primary tumors derived from patients who presented between 1986 and 1996 with Dukes' stage B (stage II disease, as defined by American Joint Committee on Cancer and Union Internationale Contre le Cancer criteria). Patients with Dukes' stage B2 ($T_3N_0M_0$) constituted 91% of the cohort, whereas 9% represented Dukes' B3 ($T_4N_0M_0$) cancer. All patients were treated by surgical resection of the involved segment of colon. No postoperative adjuvant chemotherapy was performed initially in all cases. However, chemotherapy was administered for some patients after relapse. Clinical data represent a median follow up of 60 months. The vital parts of tumor specimens free of necroses were selected for preparation of tissue microarray chips. Vascularization status was assessed morphologically with histological staining with H&E and special stain of Masson-Trichrome. The construction of colon cancer tissue microarrays has been described elsewhere (23). Analysis of the expression of common molecular markers in the cohort of colon cancer cases revealed that 20% of them were microsatellite instability positive, 54% were p53 positive, and 58% were MIB1 positive. Immunopositivity for these proteins was defined when immunoreactivity scored >20%. The association of these markers with novel proteins involved in the signaling of apoptosis will be described elsewhere.

Immunohistochemistry. Tissue sections and microarrays were immunostained using a diaminobenzidine-based detection method using the EnvisionPlus-horseradish peroxide system (DAKO, Carpinteria, CA), using an automated immunostainer. The dilutions of the sera used were 1:3000 for anti-β-F1-ATPase, 1:800 for anti-Hsp 60, and 1:1000 for anti-GAPDH, anti-HK I, and anti-HK III. Nuclei were counterstained with hematoxylin. The immunostaining procedure was performed in parallel using preimmune serum to verify the specificity of the results. The immunostaining results for each marker were scored independently by two investigators using different approaches: (*a*) by an expert pathologist according to the intensity (0, negative; 1^+, weak; 2^+, moderate; and 3^+, strong) and percentage of immunopositive cells, yielding scores of 0–300; and (*b*) by densitometric scanning of the stain deposition per unit of cytoplasm of the cell. The unit of cytoplasm of the cell was defined as the area equivalent to ~30 μm^2 of the perinuclear region of the cytoplasm of the cell. For this purpose, digital pictures of the liver sections and plugs of the tissue microarrays were taken at ×1000-fold magnification with a Spot 3.1 camera (Diagnosis Instruments, Inc., Starling Heights, MI) and converted into gray scale, and the absorbance of the unit of cytoplasm was measured in 5–30 different cells using the Image-Pro plus 4.1 program (Media Cybernetics LP, Silver Spring, MD). In immunocytochemistries, several precautions have to be taken in the preparation of the sample and in the quantification of the stain deposition because of intratumoral heterogeneity and/or the subcellular localization of the marker being assessed: (*a*) the cores used in the study should be obtained from representative areas of the vital part of the tumors and free of necroses; (*b*) because of the perinuclear localization of mitochondria, the cellular area chosen for quantification of the immunostaining of mitochondrial markers has to be selected close to the nuclei of the cells; and (*c*) in tumors that reveal intratumoral heterogeneity for the expression of the glycolytic GAPDH marker (*i.e.*, see Fig. 4), the quantification of the cellular expression level of the marker has to be done in several fields and in a larger number of cells that, on average, represent the mean of the tumor sample analyzed. Remarkably, the quantification of β-F1-ATPase immunostaining of the colon tissue microarrays by scoring and densitometry revealed that both approaches provided essentially the same findings ($P < 0.001$; data not shown).

Western Blotting. Human liver and colon tissue biopsies were obtained from the Banco de Tejidos y Tumores, IDIBAPS, Hospital Clinic, Barcelona and from Red de Banco de Tumores, Centro Nacional de Investigaciones Oncológicas Carlos III, Madrid, Spain, coded for anonymity. Whole-cell lysates were prepared from frozen tissue biopsies. Protein concentrations were determined with the Bradford reagent (Bio-Rad Protein Assay) using BSA as a standard. Lysates were subjected to SDS-PAGE/immunoblot analysis, using

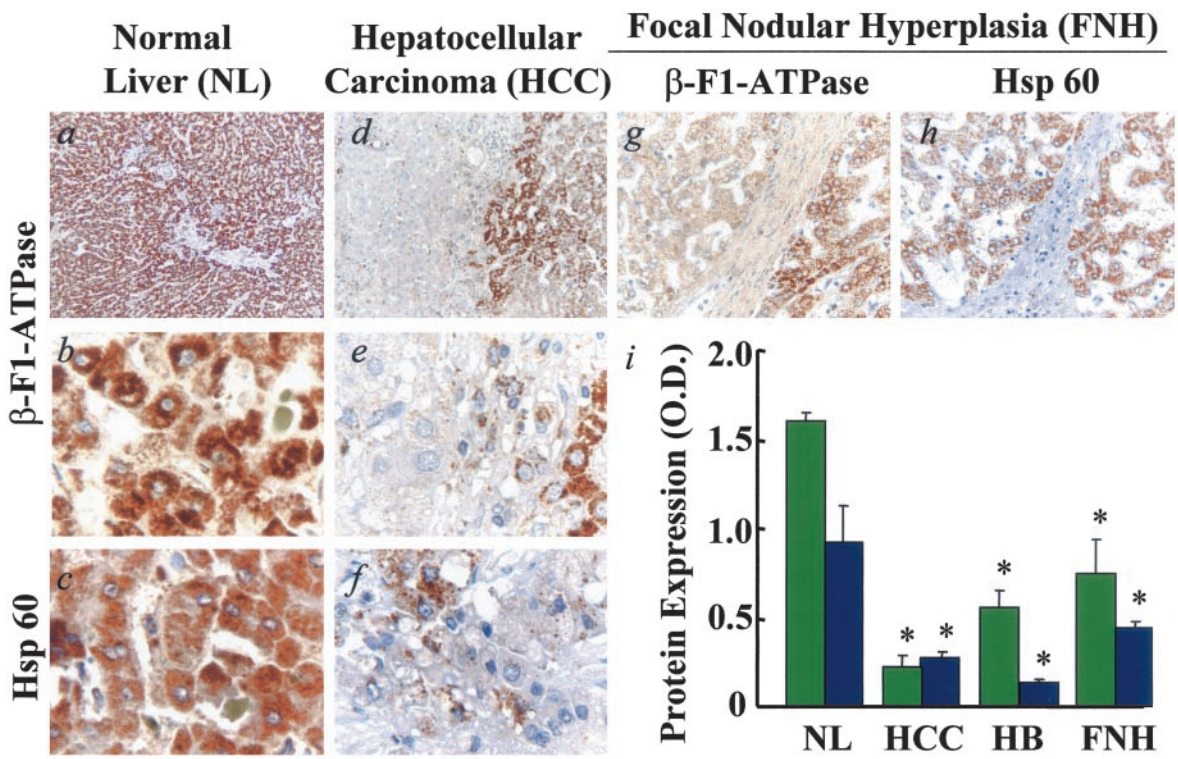

Fig. 1. Immunohistochemical analysis of the mitochondrial β-F1-ATPase and Hsp 60 in normal, precancerous lesions and carcinomas of the human liver. Representative photomicrographs provide examples of the immunostaining for normal liver (*NL, a–c*), HCCs (*d–f*), and FNHs (*g* and *h*) taken at ×10, ×20, ×40, and ×60. The histogram summarizes the expression level of cellular β-F1-ATPase (*green*) and Hsp 60 (*blue*; means; *bars*, SE.) in randomly selected samples from three normal livers (*NL*), five hepatocellular carcinomas (*HCC*), three hepatoblastomas (*HB*), and three FNHs (*FNH*), as determined by densitometric scanning of the stain deposition in the cytoplasm of the cells. *, $P < 0.001$ or less, when compared with NL by Student's *t* test.

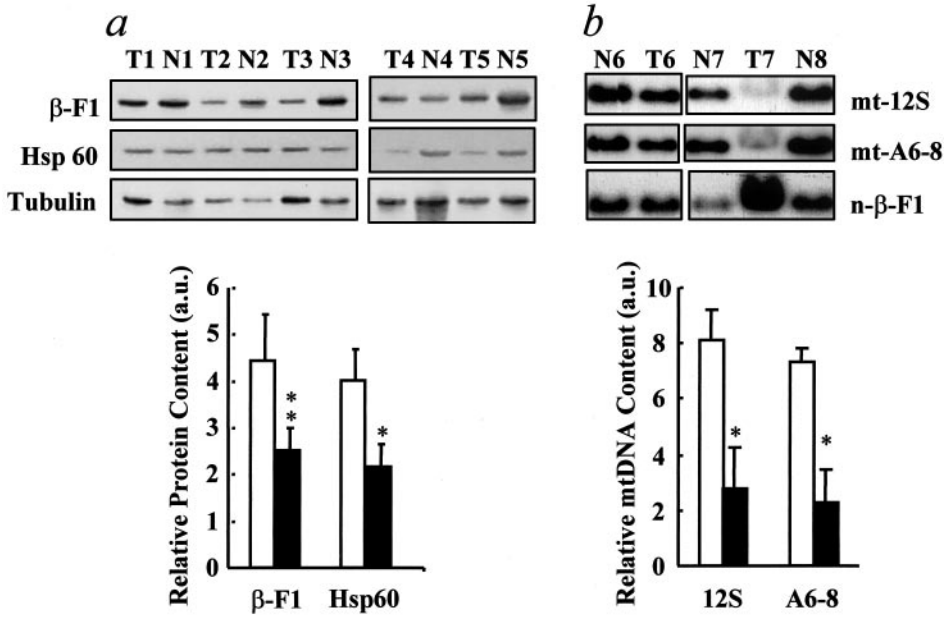

Fig. 2. Repression of mitochondrial biogenesis in human HCCs. *a*, Western blot analysis of the expression levels of β-F1-ATPase, Hsp 60, and tubulin in SDS-PAGE fractionated proteins from five normal human livers (*N1–N5*) and from five HCCs (*T1–T5*). The histogram shows the relative cellular content of each mitochondrial marker relative to the expression level of tubulin (β-F1-ATPase:tubulin and Hsp 60: tubulin ratios) in 9 normal livers (□) and 13 HCCs (■). *, $P < 0.03$; **, $P < 0.02$ when compared with the expression of normal liver by Student's *t* test. *Bars*, SE. *b*, Southern blot analysis of the mtDNA copy number in cellular DNA extracted from three normal human livers (*N6–N8*) and from two HCCs (*T6–T7*). The histogram shows the relative mtDNA content, as assessed by the hybridization signals of the mitochondrial genes (*mt-12S* and *mt-A6-8*) relative to the representation of the nuclear encoded β-F1-ATPase gene (*n-β-F1-ATPase*), in normal liver (□) and HCCs (■). *, $P < 0.01$ when compared with normal by Student's *t* test. *Bars*, SE.

the appropriate dilution of the antisera, and secondary horseradish peroxidase-conjugated goat-antirabbit or antimouse antibody (1:3000 v/v dilution) with detection accomplished using an enhanced chemiluminescence detection method.

DNA Isolation and Southern Blot Hybridization. Total DNA was extracted from human liver samples after digestion with RNase and proteinase K (14). Total cellular DNA (10 μg) was digested with *Bam*HI. The digested DNAs were resolved on 0.8% agarose gels, transferred, and fixed onto nylon membranes (Gene-Screen, NEN-Life Science Products, Boston, MA). The membranes were incubated with [^{32}P]dCTP-labeled DNA probes. The DNA probes used in this study were human β-F1-ATPase cDNA, for a nuclear-encoded gene, and specific DNA probes for the mitochondrial encoded ATPase 6-8 and 12S rRNA genes. Conditions for hybridization and membrane washing have been described in detail previously (14). For stripping labeled DNA probes, membranes were incubated in sterile water at 90–100°C for 20 min. Membranes were exposed to X-ray films and analyzed by laser densitometric scanning.

Electron Microscopy. Small pieces (~1 mm^3) of human liver samples were fixed by immersion in freshly prepared 4% paraformaldehyde in 0.1 M Sörensen phosphate buffer (pH 7.2) and supplemented with 6% sucrose for 2 h at 4°C. Samples were rinsed in buffer, and the free-aldehyde groups were quenched with 50 mM ammonium chloride in PBS for 60 min at 4°C. Afterward, the samples were rinsed in PBS, dehydrated in acetone, and finally processed for embedding in Lowicryl K4M (Polysciences Europe, Eppelheim, Germany) according to the manufacturer's instructions. Gold interferential color ultrathin sections were collected in collodion/carbon-coated nickel grids. For the simultaneous immunocytochemical localization of β-F1-ATPase and Hsp 60, the grids were incubated for 5 min with PBS containing 1% BSA and then incubated with a 1:50 dilution of anti-β-F1-ATPase for 60 min in the same buffer. After three washes with PBS, grids were incubated for 45 min with protein A-gold complex (10 nm). Afterward, the grids were incubated with 0.1 mg/ml of protein A in PBS for 30 min. After this step, the procedure was repeated using the anti-Hsp 60 antibody (1:25 dilution) and the protein A-gold complex (15 nm). Fixation was carried out with 1% glutaraldehyde in PBS. Counterstaining was performed with 2% uranyl acetate (7 min) and 1% lead citrate (45 s). The grids were observed in a Jeol 1010 electron microscope under 80 kV accelerating voltage.

Statistical Analysis. Statistical analysis for comparison of the expression levels of the markers in normal *versus* cancerous tissues was performed by the Student's *t* test. Data were analyzed using the STATISTICA software package (StatSoft). An unpaired *t* test method (data not shown) and log-rank test were used for correlation of immunostaining data with the patient survival. Survival

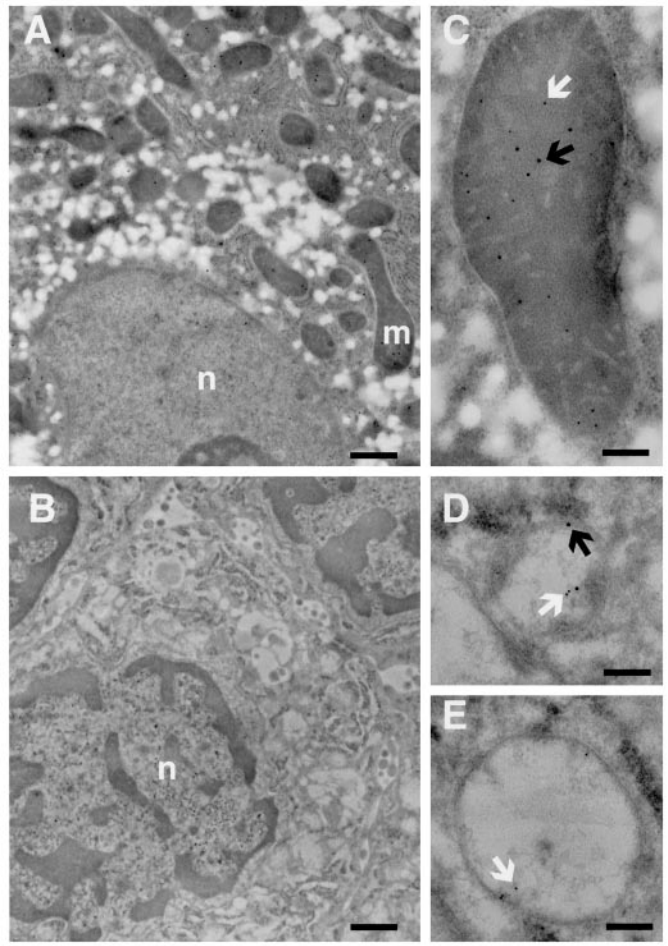

Fig. 3. Electron microscopy reveals alterations of mitochondria in liver cancer. Mitochondria of the hepatocytes in normal human liver showed characteristic high electron density and inner membrane crests (*A* and *C*), whereas mitochondria are barely identifiable in HCC (*B*). Identification of mitochondrial remnants in HCCs (*D* and *E*) required immunodetection of the mitochondrial β-F1-ATPase (*white arrow*, 10-nm gold particles) and Hsp 60 (15-nm gold particles, *black arrow*) proteins (*C–E*). *n*, nucleus; *m*, mitochondrion. *Scale bars: A* and *B*, 1 μm; *C–E*, 200 nm.

distributions were estimated using Kaplan-Meier curves. Multivariate Cox proportional hazards models were fitted to the data to assess which biomarkers were independently associated with DFS and overall survival. Ninety-five % confidence intervals for the hazard ratio were calculated by a formula $\exp[\beta \pm 1.960\ \mathrm{SE}(\beta)]$, where $\mathrm{SE}(\beta)$ denotes the SE of the estimated regression coefficient.

RESULTS

The Mitochondrial Phenotype in Liver Carcinogenesis. Immunohistochemistry of the normal human liver with antibodies against the β-F1-ATPase (Fig. 1, *a* and *b*) and Hsp 60 (Fig. 1*c*) proteins showed the specific recognition of mitochondria in the hepatocytes. Immunostaining of liver sections derived from HCCs and hepatoblastomas indicated a drastic reduction of the expression level of both the β-F1-ATPase (Fig. 1, *d*, *e*, and *i*) and Hsp 60 (Fig. 1, *f* and *i*) in cancer cells, either when compared with the expression found in normal livers (Fig. 1, *a–c*) or when compared with that of the hepatocytes of the adjacent noncancerous tissue in the same sample (Fig. 1, *d–f* and *i*). Furthermore, we also studied the expression of HKs I and III in normal liver and in the hepatic neoplasms. We did not obtain any significant differences in the expression level of HKs I and III between the normal tissue and the carcinomas (data not shown). Altogether, these findings indicated that the observed differences in β-F1-ATPase and Hsp 60 expression between normal and cancer cells do not result from artifacts of tissue fixation. Expression of both β-F1-ATPase (Fig. 1, *g* and *i*) and Hsp 60 (Fig. 1, *h* and *i*) was also significantly reduced in liver sections derived from patients diagnosed with FNH. This finding might suggest that the alteration of mitochondrial cellular activity is an early event in the development of the malignant state of the liver.

Immunoblot analysis further confirmed that the relative cellular expression of the mitochondrial markers, as assessed by the β-F1-ATPase/tubulin and Hsp 60/tubulin ratios, is significantly reduced in HCCs ($P < 0.02$ and $P < 0.03$, respectively) when compared with normal human livers (Fig. 2*a*). The parallel reduction in the bioenergetic (β-F1-ATPase) and structural (Hsp 60) markers of mitochondria (Figs. 1 and 2*a*) strongly suggested that the biogenesis of mitochondria is repressed in human liver carcinogenesis. Therefore, we quantified the relative cellular content of mitochondrial DNA by assessing the representation of two genes encoded in the mtDNA (12S and A6-8) relative to the representation of one encoded in the nucleus (β-F1-ATPase). The results obtained confirmed that the biogenesis of mitochondria is impaired in human HCCs, because a significant reduction in mtDNA levels was observed in HCCs when compared with normal livers ($P < 0.01$; Fig. 2*b*).

Electron microscopy revealed that HCC cells were devoid of or-

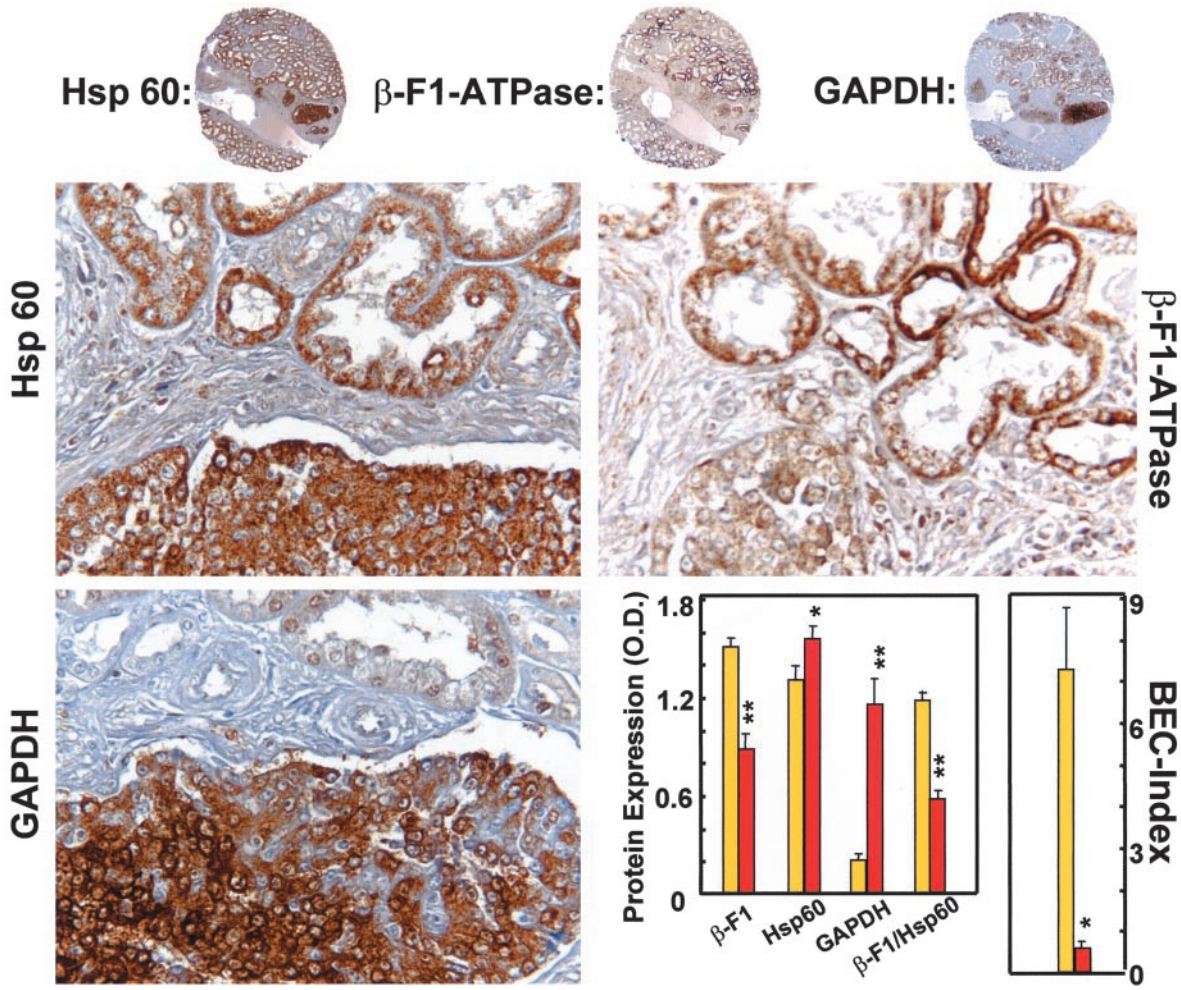

Fig. 4. Immunohistochemical analysis of β-F1-ATPase, Hsp 60, and GAPDH in human renal cell carcinomas. Representative photomicrographs provide examples of the immunostaining for each marker at ×10 and ×60 in the carcinoma and in the proximal normal epithelial cells of the same kidney biopsy. The histograms summarize the expression level of each marker (means; *bars*, SE.), as well as of the calculated β-F1-ATPase:Hsp 60 and β-F1-ATPase:Hsp 60:GAPDH (BEC index) ratios, in normal (*yellow*) and cancer (*red*) cells of four different patients. ∗ and ∗∗, $P < 0.05$ and $P < 0.01$, respectively, when compared with normal by Student's *t* test. *O.D.*, absorbance.

ganelles with the ultrastructure of normal human liver mitochondria (Fig. 3, compare *A* and *C versus B*). High-resolution immunoelectron microscopy, using anti-β-F1-ATPase and anti-Hsp 60 sera, allowed the identification of positive immunoreactive structures in the cytoplasm of the HCC (Fig. 3, *D* and *E*). These structures corresponded to double membrane organelles that lacked the electron density and infolds of the inner membrane (Fig. 3, *D* and *E*) that characterize normal liver mitochondria (Fig. 3*C*), most likely representing the remnants of mitochondria or mitochondrial "ghosts." Altogether, we showed that the protein (Figs. 1 and 2) and DNA (Fig. 2) markers, as well as the organelles themselves (Fig. 3), are very much reduced and altered in human hepatocarcinogenesis. These results suggest that the development of cancer in the liver might be intimately related to the repression of the cellular program responsible for proliferation of mitochondria.

Reduced Mitochondrial β-F1-ATPase Expression Is a Marker of Tumor Progression. Contrary to the findings in hepatocarcinogenesis, analysis of expression Hsp 60 in human kidney (Fig. 4) and colon (Fig. 5) carcinomas did not reveal a decrease in this structural mitochondrial protein relative to normal tissues, as assessed by immunohistochemical (see Fig. 4 for kidney carcinomas) and immunoblotting (see Fig. 5 for colon carcinomas) procedures. In contrast, a highly significant down-regulation of the β-F1-ATPase protein was found when compared with the normal tissue. The specific reduction of β-F1-ATPase was evidenced by the decrease of the β-F1-ATPase: Hsp 60 ratio (Figs. 4 and 5). This finding indicates that in these types of carcinomas, no repression of the biogenesis of mitochondria occurs, but rather a selective inhibition of the expression of the bioenergetic H^+-ATP synthase relative to other structural components of mitochondria is operative.

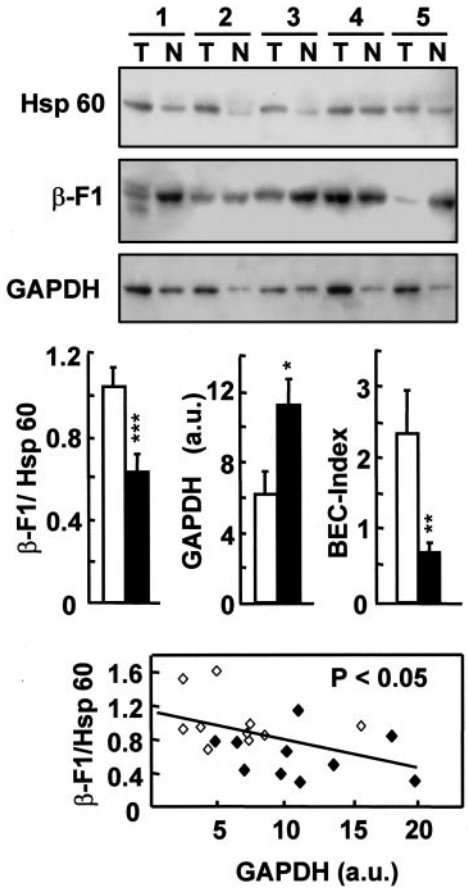

Fig. 5. Western blot analysis of the expression levels of β-F1-ATPase, Hsp 60, and GAPDH in human colonic carcinomas. Protein samples from normal (*N*) and tumor (*T*) whole-cell lysates were fractionated by SDS-PAGE and then blotted with the corresponding antibodies. For illustration purposes, the results of five patients (*Lanes 1–5*) assayed in the same gel are shown. The histograms show the relative cellular content of GAPDH, as well as of the calculated β-F1-ATPase:Hsp 60 and β-F1-ATPase:Hsp 60:GAPDH (BEC index) ratios (means; *bars*, SE) in normal (□) and tumor (■) samples derived from 10 different patients. *, **, and ***, $P < 0.02$, $P < 0.01$, and $P < 0.006$, respectively, when compared with the expression of normal by Student's *t* test. A significant linear correlation ($P < 0.05$) was observed between the phosphorylation capability of mitochondria (β-F1:Hsp 60 ratio) and the glycolytic potential of the cell (GAPDH) in samples derived from normal (◇) and cancerous (◆) tissue of the colon.

Loss of β-F1-ATPase expression would be expected to create a bottleneck in mitochondrial oxidative phosphorylation. Consistent with a diminished phosphorylation capability of the cancer cell and with the high net glucose uptake and lactate release observed in substrate balance studies across colonic carcinomas (24), we observed that kidney (Fig. 4) and colon (Fig. 5) carcinomas expressed a significantly higher amount of the glycolytic GAPDH. In fact, a significant inverse correlation ($P < 0.05$) was observed between the β-F1-ATPase:Hsp 60 ratio and the expression level of GAPDH in colon samples (Fig. 5), suggesting that the potential for cellular energy provision by the function of mitochondrial oxidative phosphorylation is inversely correlated with that of anaerobic glycolysis. In line with these observations, a BEC index was derived to define the metabolic state of the cell. The BEC index is a nondimensional ratio that expresses the mitochondrial activity, as indirectly assessed by the β-F1-ATPase:Hsp 60 ratio, relative to the cellular glycolytic potential, as assessed by the amount of the GAPDH marker. This index provides a bioenergetic signature of cellular status. The BEC index in kidney (Fig. 4) and colon (Fig. 5) carcinomas was significantly lower than in the corresponding normal tissues.

The potential impact of down-regulation of β-F1-ATPase protein, and thus of the bioenergetic signature of the cancer cell in tumor progression, was further examined by analyzing the expression of the mitochondrial (β-F1-ATPase and Hsp 60) and glycolytic (GAPDH) markers in tissue microarrays of colorectal carcinomas for which the clinical follow-up of 104 patients was documented (23). The comparison of these biomarkers in tumor *versus* normal cells was also possible because 58 of the 104 tumor specimens contained adjacent normal colonic epithelium in the same section. The results obtained from this immunohistochemical analysis (Fig. 6 and additional data not shown) confirmed and extended the findings obtained by immunoblotting (Fig. 5). In this regard, both the adenocarcinoma and the adjacent normal epithelium expressed high levels of Hsp 60, with no significant differences observed in the expression level of Hsp 60 between normal and tumor cells (Fig. 6). In contrast, immunohistochemical analysis of β-F1-ATPase revealed a highly significant down-regulation of expression of this protein in malignant tissue. Furthermore, reductions in β-F1-ATPase were more pronounced in tumors derived from patients with progressive disease (Fig. 6). In contrast to β-F1-ATPase, densitometric analysis of the cytosolic immunostaining for GAPDH revealed a significant increase in adenocarcinomas when compared with the normal epithelium (Fig. 6), although this difference was not statistically significant when assessed by immunoscore (data not shown). Consistent with the aforementioned findings, the BEC index of the tumors was significantly lower than that of the normal epithelium (Fig. 6). It is interesting to note that the BEC indexes calculated from Western blot data of colon samples (Fig. 5) provided essentially the same values as those obtained by immunocytochemistry (Fig. 6), suggesting the practical and wide potential use of the BEC index.

The Kaplan-Meier survival curves (Fig. 6), derived from data obtained by immunoscore of the tissue microarrays, showed the association of each of the markers analyzed with overall survival and DFS. Overall patient survival was calculated from the date of tumor

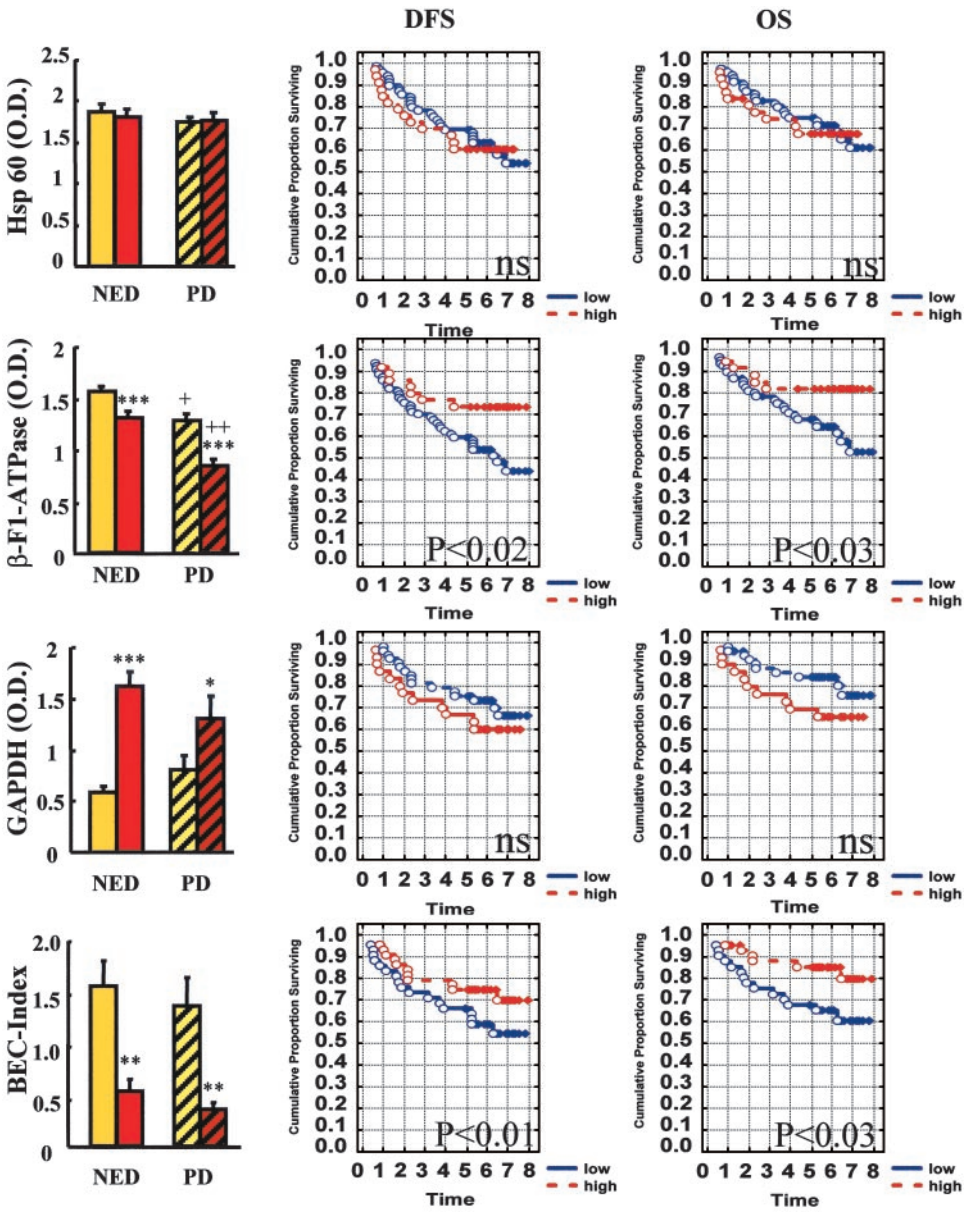

Fig. 6. Immunohistochemical analysis and Kaplan-Meier survival analysis of colon tissue microarrays. The histograms show the densitometric analysis of the immunoreactivity of the markers and of the calculated BEC index in 104 primary colorectal adenocarcinomas (*red*) and 58 nonneoplastic epithelium (*yellow*). Patients were divided into two groups based on clinical outcome: the *NED* (no evidence of disease) group, those who survived without disease ($n = 59$; ■) and the *PD* (progressive disease) group, those who died from disease ($n = 31$) plus those surviving with disease recurrence ($n = 10$; ▨). *,**, and ***, $P < 0.03$, $P < 0.002$, and $P < 0.001$, respectively, when compared with the expression of normal colonic epithelium in the same group by Student's *t* test. +, $P < 0.002$ and ++, $P < 0.001$, when compared with the expression of normal and cancerous colonic epithelium in the NED group, respectively, by Student's *t* test. The Kaplan-Meier survival analysis shows the association of the expression level of the markers, as assessed by immunoscore, in colorectal carcinomas with overall survival (*OS*) and DFS. High (*red*) and low (*blue*) expression levels are defined. *ns,* nonsignificant.

diagnosis and DFS from the date of surgery and until the date of tumor recurrence. Hsp 60 and GAPDH levels in the tumors revealed no significant correlation with the prognosis of the patients, although those patients with progressive disease tended to have higher expression of the GAPDH marker (Fig. 6). In contrast, the expression level of β-F1-ATPase in the tumors revealed a significant correlation with both the survival of the patients ($P < 0.03$) and the time of recurrence of the disease ($P < 0.02$). Likewise, the BEC index of the tumors revealed a significant correlation with both the survival of the patients ($P < 0.03$) and the time of recurrence of the disease ($P < 0.01$). This observation suggests that the potential for cancer metastases and recurrence is linked to the down-regulation of oxidative phosphorylation and concurrent enhancement of glycolysis, in agreement with the recent observation that tumor lactate concentration predicts an increased metastatic risk in head and neck cancer (25).

DISCUSSION

Recent studies suggest that mitochondrial DNA mutations may impair oxidative phosphorylation in cancer (26). The data presented here demonstrate, for the first time, that the expression of a critical molecule required for synthesis of mitochondrial ATP is repressed in human carcinomas. This constitutes the first molecular evidence of a general alteration of mitochondrial bioenergetic function in cancer. These findings support Warburg's hypothesis, irrespective of the existence of mutations in mitochondrial DNA. It would be interesting to know whether the repression of β-F1-ATPase in cancer is also accompanied by a parallel down-regulation of other protein complexes involved in mitochondrial energy transduction.

The overall activity of oxidative phosphorylation in the cell is the result of both the bioenergetic competence of the organelles and of the cellular mitochondrial content. The content of mitochondria in the cell is regulated both during development and by cell type-specific programs (16). This study shows that in cancer of the human liver, a parallel down-regulation of the bioenergetic (β-F1-ATPase) and structural (Hsp 60 and mtDNA) components of mitochondria occurs, strongly suggesting that liver carcinogenesis is accompanied by repression of the program of mitochondrial biogenesis that is

responsible for the proliferation of mitochondria in the hepatocyte. Peroxisome proliferator-activated receptor γ coactivator-1 is a transcriptional coactivator that is required for mitochondrial biogenesis in brown adipose tissue (27) and for the cellular differentiation of the hepatocyte (28). It is possible that carcinogenesis of the liver could be associated with alterations of the function of this coactivator. In contrast, in kidney and colon carcinomas, we show a specific down-regulation of the expression of the bioenergetic marker of oxidative phosphorylation, suggesting that oncogenesis in these tissues only affects the mechanisms that control the program of differentiation of mitochondria (16, 29, 30), which is linked to the control of the translation of oxidative phosphorylation mRNAs (14, 21, 31, 32).

Independently of the mechanism by which the H^+-ATP synthase is down-regulated in liver, kidney, and colon carcinomas, it is reasonable to suggest that both a low bioenergetic competence of the mitochondria (kidney and colon) and a low mitochondrial cellular content (liver) contribute to the expansion of cancer cells and, perhaps, to their resistance to chemo- and radiotherapy, because the overall oxidative phosphorylation capability of the cell is diminished, and thus, the apoptotic potential of the cancer cell is hampered (19, 20). In this regard, it has been shown that defects in the H^+-ATP synthase suppress Bax-induced lethality in *Saccharomyces cerevisiae* (17) and that the inhibition of the mammalian H^+-ATP synthase with oligomycin reduces cell death triggered via the mitochondrial pathway for apoptosis (17). It might be speculated, therefore, that a "normal" cellular phenotype with low H^+-ATP synthase or a low BEC index, as observed in the hepatocytes of premalignant FNHs (Fig. 1) and in the normal epithelium adjacent to the colon carcinomas in some patients (Fig. 6), provides the cellular bioenergetic background of diminished apoptotic potential that is required for deregulated proliferation and oncogenesis.

The glycolytic reprogramming of tumor metabolism has been recently elucidated and explained on the grounds of a combined action of oncogenic mutations in c-*myc* (33) and up-regulation of hypoxia-inducible factor-1α (11, 34–37). In contrast, the repression of β-F1-ATPase expression in carcinomas of the liver, kidney, and colon is produced in a situation where the cellular abundance of β-F1-ATPase mRNA is increased in the tumor when compared with normal tissues, as revealed by Virtual Northern blot analysis (38). This finding strongly suggests, in agreement with previous findings in developing liver (15, 21, 30) and in rat hepatomas (14), that regulation of the expression of β-F1-ATPase in cancer is exerted at the level of mRNA translation. Indeed, the β-F1-ATPase mRNA is subjected to stringent translational control by cell type-specific RNA binding proteins (21, 31), the RNA binding activity of which is regulated during development (21) as well as in carcinogenesis (14).

Mitochondrial electron transport is the major endogenous source of ROS (39). The generation of ROS is a physiological process that depends on the cellular activity of mitochondrial respiration, determining the life span of cells and organisms (40). ROS promote the activation of the intrinsic pathway of apoptosis. The mechanism of participation of the H^+-ATP synthase in apoptosis is a current subject of study (18), and it is also possible that its contribution in apoptosis could be mediated via ROS. In this regard, and because of the coupling between mitochondrial respiration and oxidative phosphorylation, the down-regulation of the H^+-ATP synthase in cancer cells would limit the flux of electrons down the respiratory chain, and therefore, the generation of the superoxide radical, a promoter of DNA damage and likely signal for induction of the mitochondrial cell-death pathway (39). We anticipate, therefore, that cells with a low BEC index, as a result of a low mitochondrial content and/or activity, would be prone to establishing a transformed phenotype and become more resistant to programmed cell death in response to oxidative stress.

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