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Oxidative Phosphorylation Enzymes in Normal and Neoplastic Cell Growth

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Cancer cells, despite growing aerobically, have the propension to utilize the glycolytic pathway as energy source. This biochemical phenotype is accompanied by a decreased content of mitochondria and, paradoxically, by enhanced transcription of nuclear and mitochondrial-encoded genes for the enzymes of oxidative phosphorylation (OXPHOS). The role of OXPHOS enzymes in normal and neoplastic cell growth has been studied in liver regeneration and human hepatocellular carcinoma. In early liver regeneration characterized by active mtDNA replication, a decrease in the content and activity of ATP synthase occurs while transcription of the ATPsynβ nuclear gene is activated. Translation of ATP synthase subunits seems, on the contrary, to be less effective in this phase. In the second replicative phase of liver regeneration, the repression of ATPsynβ translation is relieved and normal cell growth starts. In this replicative phase the recovery of the liver mass appears to be directly related to the recovery of the OXPHOS capacity. Mitochondria isolated from biopsies of human hepatocellular carcinoma exhibit a decreased rate of respiratory ATP synthesis (OXPHOS) and a decreased ATPase activity. The decline in the activity of the ATP synthase is found to be associated with a decreased content of the ATPsynβ in the inner mitochondrial membrane. In neoplastic tissue the ATPase inhibitor protein (IF_1) is overexpressed. This could contribute to prevent hydrolysis of glycolytic ATP in cancer cells. A peptide segment of IF_1 (IF_1-(42-58)-peptide), constructed by chemical synthesis, proved to be equally effective as IF_1 in inhibiting the ATPase activity of the ATP synthase complex in the mitochondrial membrane deprived of IF_1. The synthetic peptide might turn out to be a useful tool to develop immunological approaches for the control of neoplastic growth.

KEY WORDS: Cell growth; mitochondrion; oxidative phosphorylation; F_0F_1 ATP synthase; liver regeneration; hepatocellular carcinoma; adenosine triphosphatase-inhibitor protein.

Oxidative phosphorylation (OXPHOS), driven by mitochondrial respiration, covers more than 90% of the energy demand of most differentiated cells. In contrast, in rapidly growing cancer cells (Nakashima *et al.*, 1984), as well as in some normal tissues like fetal tissues or retina (Pedersen, 1978), considerable amounts of ATP can be derived from the glycolytic pathway. This biochemical phenotype of cancer cells seems to be related to the observed changes of carbohydrate metabolizing enzymes (Taketa *et al.*, 1988) and to the alteration of number, shape, and function of mitochondria (Pedersen, 1978).

Biochemical and molecular biology investigations have revealed an apparent paradox in the bioenergetics of cancer cells. The decreased content of mitochondria in these cells, rather than reflecting a reduced expression of genes coding for mitochondrial OXPHOS enzymes, has been found to be associated with an enhanced transcription of nuclear DNA and mitochondrial DNA-encoded OXPHOS genes, including those of subunits of the ATP synthase. Luciakova and Kuzela (1992) have reported that rapidly growing

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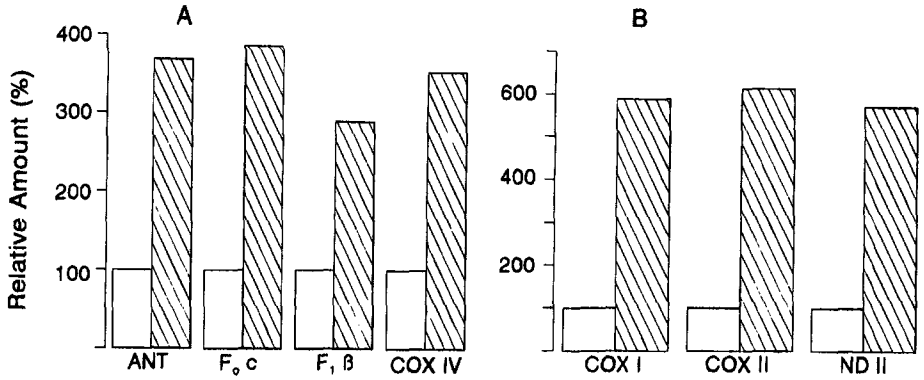

Fig. 1. Relative levels of nuclear gene transcripts in poly (A)-rich RNA (A) and of mtDNA transcripts (B) in mitochondria from normal liver and Zajdela hepatoma. Empty bar, normal liver; dashed bar, Zajdela hepatoma. ANT, ADP/ATP translocase; F_0c, N,N'-dicycloexylcarbodiimide-binding protein; $F_1\beta$, β-F_1 subunit; COX IV, cytochrome oxidase subunit IV; COX I, cytochrome oxidase subunit I; COX II, cytochrome oxydase subunit II; ND II, NADH-ubiquinone reductase subunit 2. The values of normal liver were set to 100%. Data from Table 1 and Table 2 of Luciakova and Kuzela (1992).

rat hepatoma cells contain five times less mitochondria than hepatocytes from normal liver, while the transcripts of nuclear genes coding for some subunits of the ATP synthase and of cytochrome *c* oxidase, as well as the transcripts of mt-genes for subunits of respiratory complexes, were higher in the hepatoma cells than in normal hepatocytes (Fig. 1). The translation efficiency of the hepatoma transcripts was reported to be unaltered as compared with normal liver transcripts. The half-life of the translated proteins was, however, found to be reduced in the hepatoma mitochondria. Luciakova and Kuzela concluded that the reduction in the number of mitochondria in hepatoma cells might derive from enhanced protein degradation by endogenous mitochondrial proteases (Goldberg, 1992).

Enhanced proteolytic degradation of mitochondrial constituents might be related to the proposed role of mitochondrial proteases in apoptosis (Petit *et al.*, 1996). Increased levels of mtDNA transcripts have also been observed in chemically induced hepatomas (Corral *et al.*, 1989), familial polyposis coli intestinal polyps (Yamamoto *et al.*, 1989), as well in viral and oncogene-transformed fibroblasts (Glaichenhaus *et al.*, 1986).

It has been suggested that the alterations in the expression of the OXPHOS gene could be associated with the dilution of the cellular content of mtDNA following the rapid replication of nuclear DNA (Torroni *et al.*, 1992). Furthermore, one might wonder whether the OXPHOS enzymes produced in cancer cells undergoing alterations in the expression of their genes are structurally and functionally competent.

Mitochondrial biogenesis, involving the expression of the nuclear and mitochondrial genomes regulated by a variety of transcription factors, is likely to be particularly susceptible to mutagenic factors and other harmful agents. Among the nuclear encoded proteins controlling the mt genome (Nagley, 1991; Shadel and Clayton, 1993; Daga *et al.*, 1993) a primary role seems to be played by the mitochondrial transcription factor 1 (mt TFA). This binds to the heavy- and light-strand promoters in the displacement (D-loop) region of mtDNA and controls its transcription and replication (Shadel and Clayton, 1993). The expression of mtTFA is in turn controlled by the nuclear transcription factors NRF-1 and NRF-2 which also control the expression of genes encoding cytochrome *c*, subunits for three of the five complexes of OXPHOS, including the ATP synthase, the mitochondrial RNA processing RNA (which generates primers for mtDNA replication), and 5-aminolevulinate synthase (the rate-limiting enzyme in the heme synthesis) (Scarpulla, 1997). Thus, there exists in eukaryotic cells a network of communicating nuclear genes and proteins which control the concerted expression of at least some of the nuclear genes and of the mitochondrial genes coding for OXPHOS enzymes (Fig. 2). There are two features of the nuclear factors NRF-1 and NRF-2, which might be of particular relevance for cancer. The first is the apparent existence of functional NRF-1 sites in genes encoding rate-limiting enzymes and proteins involved in cell growth

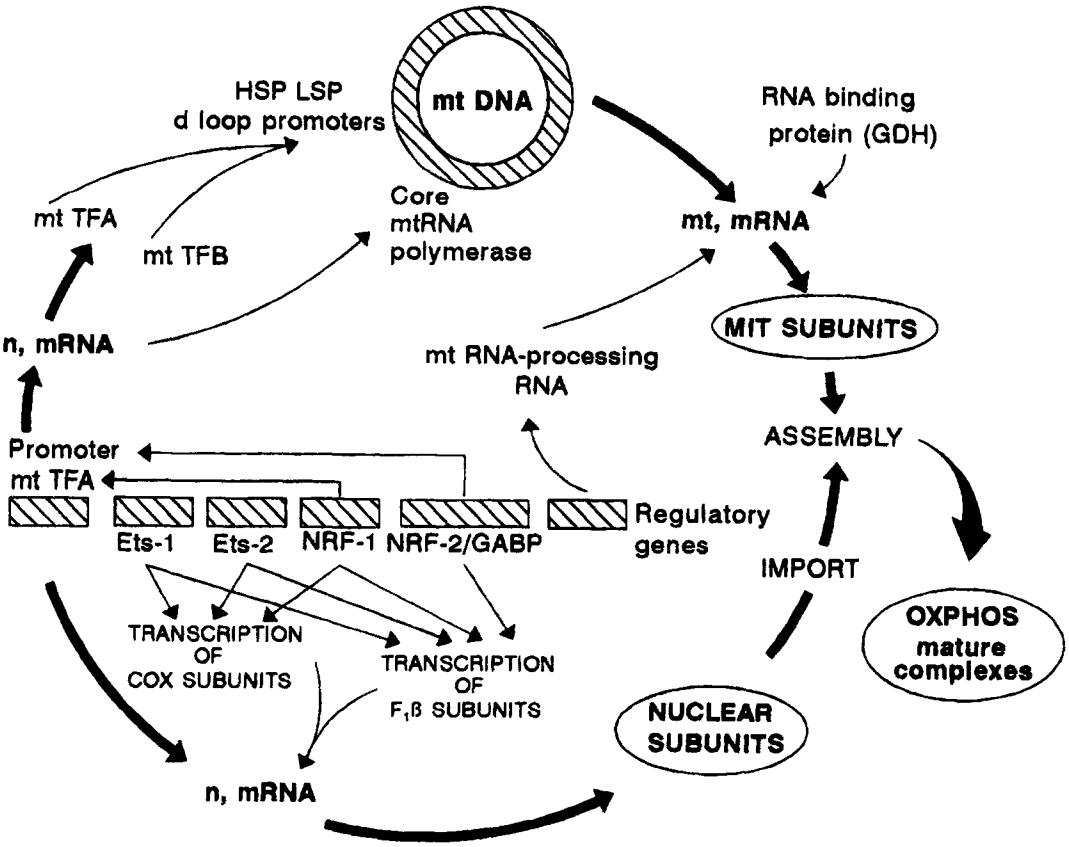

Fig. 2. Interplay of regulatory genes coding for transcription factors of nuclear and mitochondrial OXPHOS genes. mtTFA and mtTFB, nuclear transcription factors of mt genome; HSP and LSP, heavy and light strand promoters in the mt genome; Ets-1 and Ets-2, nuclear genes of the Ets family coding for transcription factors of cytochrome *c* oxidase (COX) and $F_1\beta$ subunit of F_0F_1 ATP synthase; NRF-1 and NRF-2/GABP, nuclear genes coding for transcription factors of COX, other respiratory enzymes, $F_1\beta$ subunit, and mtTFA. For references see Papa (1996).

(Scarpulla, 1997). This would indicate a role for NRF-1 in linking the expression of subunits of OXPHOS enzymes with cell growth. NRF-2 shares, on the other hand, homology with the mouse GA-binding protein (GABP), an Ets-domain transcription factor, related to the v-ets oncogenes (Wasylyk *et al.*, 1993). Ets proteins have been reported to regulate the expression of the β subunit of the F_1-ATP synthase (ATPsynβ) (Villena *et al.*, 1994).

OXIDATIVE PHOSPHORYLATION IN LIVER REGENERATION

An experimental model that can contribute to elucidate the role of energy metabolism and OXPHOS enzymes in normal and neoplastic cell growth is provided by liver regeneration. In this process, hepatocytes undergo a transitory retrodifferentiation shift toward a fetal phenotype (Uriel, 1979), associated with a decrease in the content and activity of ATP synthase and the capacity of OXPHOS.

Work by Papa *et al.* (Buckle *et al.*, 1985a, b, 1986; Guerrieri *et al.*, 1994, 1995) illustrates the changes of OXPHOS in the course of liver regeneration. After a lag of some 24 hours after hepatectomy the mass of liver starts to recover so as to reach the original value in about 4 days (Fig. 3A). In the initial retrodifferentiation phase of regeneration glycolysis seems to be preferentially utilized for ATP supply by the surviving hepatocytes as shown by the decrease in hepatic glycogen and glycemia (Lai *et al.*, 1992). In fact, during the initial retrodifferential phase of liver regeneration, the capacity of mitochondrial ATP synthesis decreases (Fig. 3A). The recovery in the liver mass, which takes place in the retrodifferention phase of regeneration,

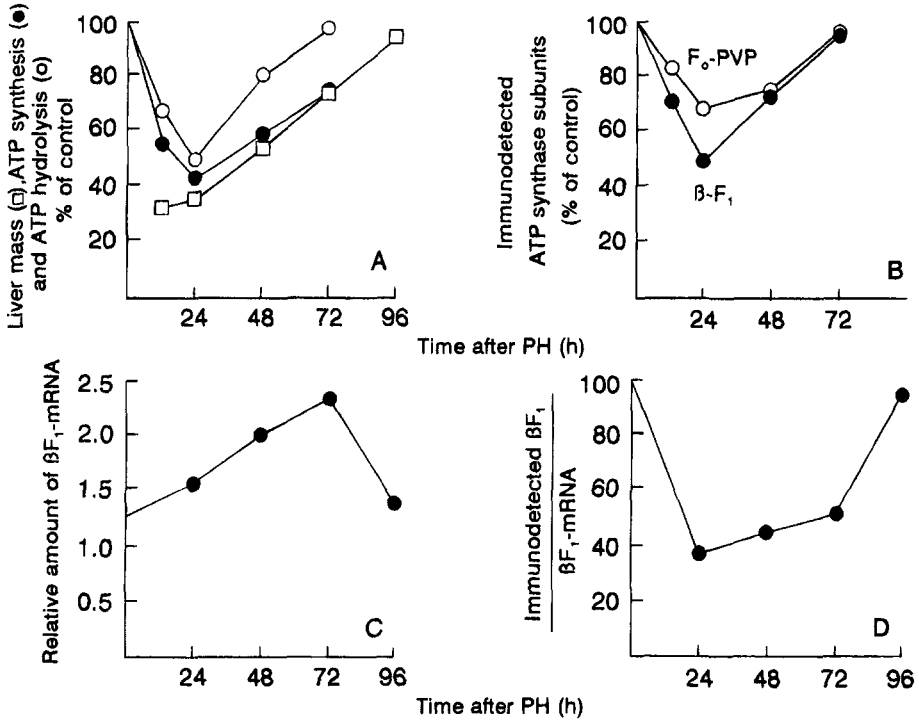

Fig. 3. Time course of changes in liver mass, ATP synthesis, and ATP hydrolysis (A), amount of immunodetected subunits of ATP synthase (B), relative amount of βF_1-mRNA (C), and ratio between βF_1 polypeptide and its mRNA (D) after partial hepatectomy. From Guerrieri *et al.* (1994, 1995).

was found to be associated with return of both the ATPase and ATP synthase activity to the control values measured at the time of hepatectomy. The mitochondrial ATPase activity changed during liver regeneration with a time course similar to that of ATP synthesis (Fig. 3A). The decline and the subsequent recovery in the ATPase and ATP synthase activity were accompanied by corresponding, similar changes in the content of F_0 and F_1 subunits (Fig. 3B). The cellular content of the mRNA for the nuclear-coded ATPsynβ subunit was found to rise, parallel to the increase in the content of mtDNA (Nagino *et al.*, 1989) also in the initial retrodifferentiation phase of liver regeneration (Fig. 3C), during which the content of the ATPsynβ and subunit F_0I decreased. The translational efficency of the accumulating ATPsynβ mRNA appeared, in fact, to decrease in the first 24 hours after hepatectomy to a low level which persisted for about 2 days, after which it regained the control value (Fig. 3D).

To summarize, the observations reported indicate that in the early retrodifferentiation phase of liver regeneration there occurs an activation of mtDNA replication, as well as of transcription of the ATPsynβ nuclear gene. Translation of ATP synthase subunits seems, on the contrary, to be less effective in this phase of liver regeneration, a situation which resembles that of fetal liver in which mRNA for ATPsynβ accumulates in a translationally repressed state (Izquierdo *et al.*, 1995). In the second replicative phase of liver regeneration, the repression of ATPsynβ translation is relieved, as it also occurs after birth in the postnatal liver (Izquierdo *et al.*, 1995), and normal cell growth starts. In this replicative phase, the recovery of liver mass appears to be directly related to the recovery of the capacity of OXPHOS. It can be concluded that the replicative hepatocyte burst in liver regeneration, as in postnatal liver development, requires concerted expression of a fully assembled functional ATP synthase. This concerted activation of biogenetic events is apparently altered in neoplastic cell replication in which the expression burst leads only to unproductive enhanced level of mRNAs for OXPHOS genes.

In liver regeneration there occurs, immediately after hepatectomy, transitory induction in the expression of proto-oncogenes like myc and ets-2 (Bhat *et al.*, 1987). The levels of the mRNAs of these factors reach a transitory peak around 2 and 4 hours after hepatectomy, respectively. The transcription of these

two proto-oncogenes thus precedes DNA replication and might contribute to activate it. The ets-2 proto-oncogene controls the transcription of the ATPsynβ and cytochrome *c* oxidase nuclear genes (Villena *et al.*, 1994). It will be interesting to verify if the altered expression of OXPHOS enzyme genes in hepatomas and transformed cell cultures is associated with activation of the expression of the ets-2 proto-oncogene.

OXIDATIVE PHOSPHORYLATION IN HUMAN HEPATOMAS

The reduction in the number of mitochondria in cancer cells has been found to be associated with alterations in their bioenergetic function. These include: (i) a decreased capacity to conserve the aerobic transmembrane ΔpH due to enhanced cyclic flow of Na^+ across the mitochondrial membrane (Papa *et al.*, 1983); (ii) impaired ATP/ADP (Barbour and Chan, 1983; Lau and Chan, 1984) and pyruvate/OH^- exchanges (Paradies *et al.*, 1983); (iii) a decreased ATP hydrolase activity (Capuano *et al.*, 1989; Chernyak *et al.*, 1991); (iv) a high hexokinase activity associated with the mitochondrial outer membrane (Arora and Pedersen, 1988). Model systems, widely used for these studies, have been transplantable hepatomas, ascites cells, and cultured cell lines. Studies carried out on the ATP synthase and OXPHOS capacity in biopsies from human hepatocellular carcinoma (Capuano *et al.*, 1996) showed that, when compared with normal human liver, mitochondria isolated from the tumor exhibit a significantly depressed respiratory activity in state 3 coupled (plus ADP and Pi) with three different oxidizable substrates. This was accompanied by a marked decrease in both the rate of ATP synthesis and P/O ratios. Moreover, submitochondrial particles isolated from hepatocellular carcinoma showed, as compared with control particles, considerably lower values of the V_{max} for ATP hydrolysis, which was associated with a decrease in the amount of immunodetected β-F_1 in both submitochondrial particles and mitochondria (Capuano *et al.*, 1996). The decrease in the content and activity of OXPHOS enzymes in hepatoma mitochondria thus seems to contribute, together with the reduced number of mitochondria, to a severe impairment of the OXPHOS capacity in tumor cells.

THE ATPASE INHIBITOR AND TUMOR GROWTH

The ATPase inhibitor (IF_1) is a protein of 10 kDa which associates with the ATP synthase of mitochondria, inhibiting ATP hydrolysis by the complex but not respiratory ATP synthesis (Harris and Das, 1991). IF_1 is overexpressed in neoplastic tissues (Luciakova and Kuzela, 1984). This overproduction, by saturating the inhibitory site on the F_0F_1 complex (1 mole per complex), could play a specific critical role in sparing ATP supply (from glycolysis) for the highly energy-demanding growth of cancer cells. If this is the case, it should be possible to control or suppress the growth of cancer cells by neutralizing the ATP sparing effect of IF_1 with competing synthetic peptide analogues or antibodies raised against IF_1.

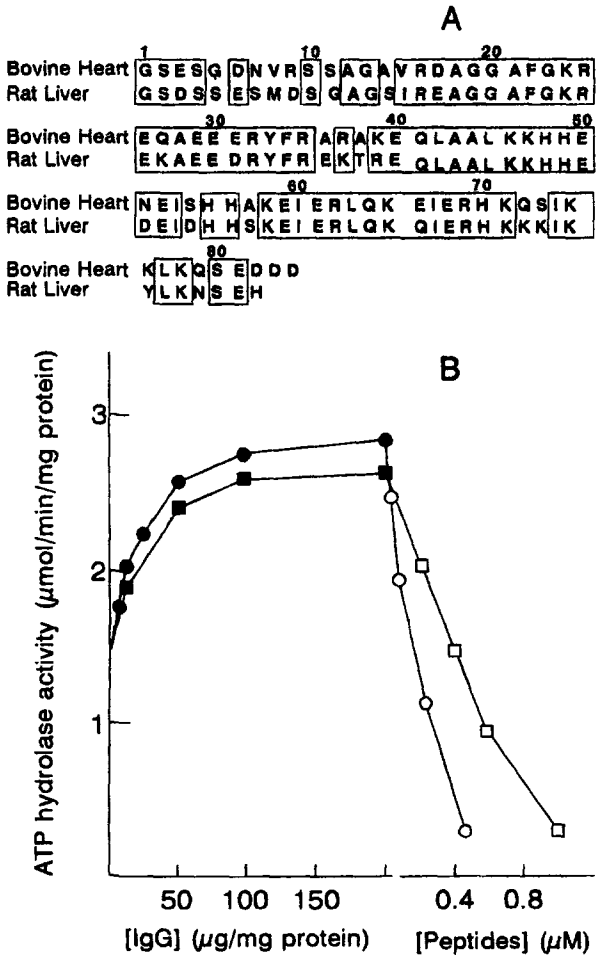

Fig. 4. F_1 inhibitor protein sequences (A) and effect of polyclonal antibodies against IF_1 and the synthetic IF_1-(42-58)-peptide on ATP hydrolase activity in EDTA submitochondrial particles (ESMP) from bovine heart (B). (■) ESMP incubated with IgG fraction of the antibody against IF_1 and (□) subsequently treated with IF_1; (●) ESMP incubated with IgG fraction of the antibody against the IF_1-(42-58)-peptide and (○) subsequently treated with the IF_1-(42-58)-peptide. From Papa *et al.* (1996).

It has been suggested (Harris, 1984; Jackson and Harris, 1986) that an α-helical rod encompassing residues 22–79 in the bovine heart protein represents a critical segment of IF_1. At positions 48, 49, 55, and 56 there are, in the mammalian IF_1, histidine residues (Fig. 4A) (Lebowitz and Pedersen, 1993). Treatment with diethylpyrocarbonate, a histidine modifier, causes loss of the inhibitory activity of IF_1 (Schnizzer *et al.*, 1996).

Peptide segments with the same sequence as the bovine-heart IF_1 as well as with amino acid replacements have been constructed by chemical synthesis and tested for their inhibitory activity on the mitochondrial ATPase activity (Papa *et al.*, 1996). The synthetic IF_1-(42-58) peptide exerted the same inhibitory activity on ATP hydrolysis by F_1 as well as by the F_0F_1 complex in inside-out vesicles of the inner mitochondrial membrane devoid of IF_1, as compared with the overall IF_1. The inhibitory affinity for ATP hydrolysis of the IF_1-(42-58) peptide exhibited the same pH dependence as that of the natural IF_1. These results show that the IF_1-(42-58) segment of IF_1 represents a critical domain for the inhibition of ATP hydrolysis in the F_0F_1 complex (Papa *et al.*, 1996). This is further documented by the finding that the IgG fraction of a rabbit antisera against the synthetic IF_1-(42-58) peptide exhibited the same marked stimulatory effect, as the antibody against IF_1, on the rate of ATP hydrolysis in vesicles of the inner mitochondrial membrane, retaining the natural complement of IF_1 (Fig. 4B).

The synthetic IF_1-(42-58) peptide should thus prove useful in producing polyclonal or monoclonal antibodies which can be used to develop approaches for the diagnostic evaluation and possibly treatment of tumors.

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