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Full Length Research Paper

Cytoplasmic, peroxisomal and mitochondrial membrane phospholipid alteration in 3[1]-diamino-azo-benzene (3[1]-DAB)-induced hepatocellular carcinoma

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Alteration in the cytoplasmic, mitochondrial and peroxisomal membrane phospholipid has been investigated in 3[1]- diamino-azo-benzene (DAB) -induced hepatocellular carcinoma in rats. The cytoplasmic membrane of tumour group shows 27.5, 19.7, 38.7 and 34.7% increase in phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin respectively compared with the control group, mitochondrial membrane shows 46.4, 67.2, 64, and 73.4% decrease in phosphatidylserine, phosphatidylcholine, phosphatidyletholamine, and sphingomyelin respectively in tumour group compared with the control. Peroxisomal membrane phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin composition of the tumour decreased by 20.0, 44.4, 79.2 and 78.6% compared with the control. The altered phospholipid is associated with altered cellular enzyme distribution. The soluble fraction of the cytoplasm shows specific activities of catalase and D-amino acid oxidase in soluble fraction of tumour group are 23.3 and 23.0% lower respectively compared to that of the control while Succinate dehydrogenase in tumour group is 42.2% higher than control group, the mitochondrial fraction shows specific activities of catalase, Succinate dehydrogenase and D-amino acid oxidase are 33.3, 33.3, and 58.3% higher respectively in control than the tumour group. The specific activities of catalase, Succinate dehydrogenase and D-amino acid oxidase are 61.5, 50.3, and 56.7% higher respectively in control compared with the tumour group. In the cytoplasmic, mitochondrial and the peroxisomal fractions of the control group, the phospholipid/protein ratio increased by 18.8, 35.7 and 48.6% respectively above the tumour group. These alterations might have implications on the metabolism, functions, morphology, metastasis and apoptosis in transformed cells.

Key words: Tumour, phospholipids, enzymes, phospholipid/protein ratio.

INTRODUCTION

The biochemical events that lead to the transformation of normal cell into tumour or cancer cells are complex (Liotta, 1992). Although these events are initialized by factors which alter the normal sequence of genetic material of cells (Flaks and Flaks, 1982; Devita et al., 1996; Lowry et al., 1951), the downstream consequences of such genetic alteration have not been fully elucidated (Devita et al., 1996). Some of the changes well documented in transformed cells include: uncontrolled cell division (Liotta, 1992), decreased requirement for growth factors, loss of contact (Devita et al., 1996), inhibition on growth, changes in cellular morphology and surface properties of transformed cells (Solomon et al., 1991; Folkman and Moscona, 1978; Morris, 1963) . The integration of all these properties confers on cancerous cells their metastatic ability (Liotta, 1992; Solomon et al., 1991). Also, there are research evidences establishing

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changes in intracellular composition of cells sequel to transformation (Morris, 1963). Organelles with reduced distribution have been reported in tumour cells, well documented ones include: mitochondria, lysosomes (Morris, 1963) and peroxisomes (Flaks and Flaks, 1982). To corroborate this claim, reduced activities of biomarker enzymes have been observed in these organelles in tumour and cancerous tissue samples (Rechcigl and Wollman, 1963). Also, recent findings about the involvement of lysosomes and peroxisomes in addition to the already discovered mitochondria (Chacon and Acosta, 1991; Kagan et al.,1992; Schulze-osthoff et al., 1992) in apoptotic cell death has raised questions about the exact biochemical reasons underlying the decreased distribution of these organelles in cancerous and tumour tissues.

This research is aimed at investigating distribution of marker enzymes and membrane phospholipids alteration in selected organelles (peroxisomes, mitochondria and cytoplasmic fraction) associated with 3^1-diamino-azo-benzene (3^1-DAB)-induced hepatocellular carcinoma in rats.

MATERIALS AND METHODS

Experimental animals

Female Sprague rats maintained in a closed colony of two rats per cage were fed rat cubes *ad libitum.* The water of the tumour group (experimental) contained 0.06% 3^1-diamino-azo-benzene (3^1-DAB). This treatment lasted for twenty four (24) weeks.

Tissue fractionation

All animals were killed under light anesthesia (chloroform). The liver was removed and rinsed in phosphate buffer saline solution (pH 7.2). The organ was then homogenized via up-down strokes of Teflon pestle rotated at 120rpm in a smooth glass homogenizer in a solution containing 0.25M sucrose solution containing 0.01M tris-HCL and 1mM $MgCl_2$ (pH 7.5) maintained at 4^oC. Differential centrifugation procedure was carried out at 4^oC on serval S-2 centrifuge equipped with SS-34 rotor for isolating mitochondrial and peroxisomal fractions as described previously (Ghosh et al., 1986).

Membrane phospholipids

The membrane phospholipids were extracted using the method described in Schulze-osthoff et al. (1992). Total phospholipids were estimated using the phospho-vanillin method as described in Wang et al. (1989). Individual phospholipids were first resolved on silica H gel plate, developed in chloroform/methanol/water/acetic acid (65:25:4:1) and run alongside with standard phospholipids. Each identified class of phospholipid was scrapped off the plate and dissolved in chloroform/ methanol (2:1). After decantation, the phospholipid was then estimated using the phospho-vanilin method.

Enzyme activity and protein estimation

Protein estimation was performed using method described by Lowry et al. (1951). The specific activities of catalase, Succinate dehydrogenase, and D-amino acid oxidase were determined as biomarkers and to estimate the distribution of these enzymes in the cytoplasmic (whole liver or soluble fractions as indicated), peroxisomal (peroxisomes) and mitochondrial fractions of normal and tumour cells using the methods described by Folkman and Moscona (1978).

RESULTS

I. For the whole liver: The tumour group shows 27.5, 19.7, 38.7, and 34.7% increase for phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin respectively compared with the normal group.
II. For peroxisomal fraction: When compared with the normal group, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin composition of the tumour peroxisomal fraction decrease by 20.0, 44.4, 79.2 and 78.6% respectively.
III. For mitochondrial fraction: There is 46.4, 67.2, 64 and 73.4% decrease in phosphatidylserine, phosphatidylcholine, phosphatidyletholamine and sphingomyelin respectively in tumour group compared with the normal.
IV. Soluble fraction: The specific activities of catalase and D-amino acid oxidase in soluble fraction of tumour group are 23.3 and 23.0% lower respectively compared to that of the normal group. While the specific activity of succinate dehydrogenase in tumour cells is 42.2% higher than soluble fraction of normal group compared to the normal group.
V. Mitochondrial fraction: the specific activities of catalase, succinate dehydrogenase and D-amino acid oxidase are 33.3, 33.3 and 58.3% higher respectively in normal group compared with the tumour group.
VI. Peroxisomal fraction: the specific activities of catalase, succinate dehydrogenase and D-amino acid oxidase are 61.5, 50.3 and 56.7% higher respectively in normal group compared with the tumour group.
VII. Whole liver: The phospholipid: protein ratio of the normal group is 18.8% higher than that of the tumour group
VIII. Mitochondrial fraction: The phospholipid: protein ratio of the normal group is 35.7% higher than that of the tumour group
IX. Peroxisomal fraction: The phospholipid: protein ratio of the normal group is 48.6% higher than that of the tumour group.

DISCUSSION

The data obtained for phospholipids component of membrane in a given cell and its organelles is only valid when such membranes are isolated and purified free of other components of the cell possessing phospholipids. The purity of the hepatic cytoplasmic, peroxisomal and mitochondrial membranes was ascertained by the specific activities of the marker enzymes (urate oxidase

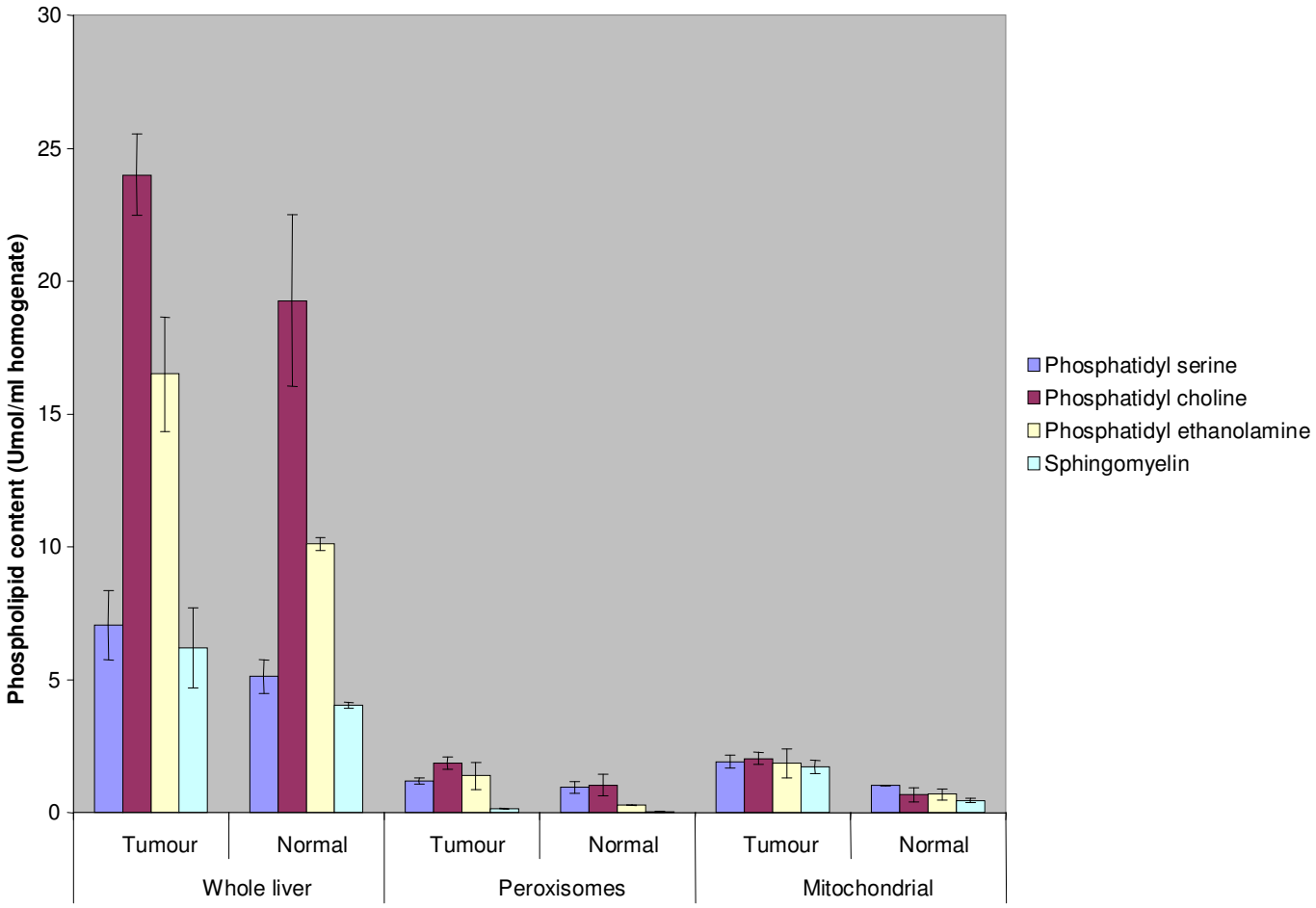

Figure 1. Shows the membrane phospholipid distribution in whole liver and liver peroxisomes and mitochondrial in normal and tumour groups.

for peroxisomal fraction, succinate-cytochrome C-dehydrogenase for mitochondrial fraction, data not shown) in their respective fractions.

The phospholipids of the whole liver and selected organelles (peroxisomes and mitochondria) of normal and 3^1-diamino-azo-benzene (3^1-DAB) treated rats were investigated (Figure 1). The data show that for the whole liver, the tumour group showed 27.5, 19.7, 38.7 and 34.7% increase for phosphatidylserine, phosphatidyl-choline, phosphatidylethanolamine, and sphingomyelin respectively compared with the normal group.

Biochemically, cells synthesize phospholipids for maintenance of membrane integrity during turnover, expansion of cytoplasmic volume in cell growth, cell division and differentiation.

Therefore, the quantitative pattern of phospholipids reported in the data possibly explains the source of phospholipids needed for the formation of membranes of daughter cells in the rapidly dividing hepatocytes of tumour group.

Also, the individual class of phospholipids showed unique quantitative pattern, which can improve our general understanding of phospholipid metabolism and membrane formation in tumour cells. Sphingomyelin has the highest percentage compared to other classes of phospholipids estimated. In nervous tissues this finding would not be unusual, considering its role as the major lipid of the myelin sheath. In tumour cells of hepatic origin, this can be explained however, as a deliberate biochemical strategy to reduce the concentration of ceramide in the cell. Ceramide is the immediate precursor of sphingomyelin in a single step involving transfer of phospho-choline from phosphatidylcholine to ceramide to form 1, 2-diacylglycerol and sphingomyelin respectively (Perry et al., 2000). This process shifts the equilibrium from ceramide to sphingomyelin. Ceramide and its catabolite sphingosine, not sphingomyelin are implicated in linking cell surface receptors, such as tumor necrosis factor alpha (TNF-α) and the Fas receptor, to apoptotic pathways. To maintain this equilibrium, the synthesis of phosphatidylcholine is increased to 38.7% above the normal cells. This process may also be accompanied by the decrease in sphingomyelinase activity in tumour cells.

The data also reveal that phosphatidylcholine has the lowest percentage. This contrasts the published work of Podo et al. (2007) that reported high phosphatidylcholine in breast and ovarian cancer. It has been biochemically proven that phosphatidylcholine is the donor of phosphocholine during the synthesis of sphingomyelin. Therefore, an initial increase in the intracellular concentration of phosphatidylcholine is followed by sphingomyelin synthesis. This is consistent with reported increase in phosphocholine-containing compounds in tumour cells by Glunde et al. (2004). While ceramide induces apoptosis, sphingomyelin induces cell proliferation which aids tumorigenesis (Perry et al., 2000; Radin, 2003).

Peroxisomes have been reported to participate in reactions which involved oxidation of D and L-amino acid, metabolism of long chain fatty acids and most recently, induction of apoptosis. When compared with the normal group, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine and sphingomyelin composition of the tumour peroxisomal fraction decrease by 20.0, 44.4, 79.2 and 78.6% respectively. To explain this finding, two assumptions would be made: assuming there is no significant difference in the number of peroxisomes of the tumour and the normal group respectively, this means that the peroxisomes of the normal group is more stable and dynamic compared to the tumour group (since the peroxisomes of the tumour group has lowered phospholipid distribution). The alternative assumption would be that given that the peroxisomes in the tumour and the normal groups have the same phospholipid composition, but the data indicate quantitative difference in the number and distribution of peroxisomes with the normal group having more peroxisomes compared with the tumour group. We agree that both assumptions may be pivotal to tumourigenesis depending on the nature of the carcinogen and the stage of its progression. Also, the role of peroxisomes in the apoptosis during tumourigenesis might be the biochemical force driving the alteration of their cellular distribution and modification of their phospholipid composition in tumour groups. Sphingomyelin in tumour peroxisomes was 78.6% lower than the estimated value for the normal group. The probable explanation for this is likely repression of key enzymes in synthesis of sphingosine, ceramide and sphingomyelin. Sphingosine, which is the immediate precursor of ceramide, has been reported to release cytochrome C and activate caspase (Radin, 2003), though the mechanism by which this occurs is still unknown. While ceramide strongly activates the c-Jun N-terminal kinase signaling pathway (JNK/SAPK pathway) leading to the phosphorylation of the N-terminus of c-Jun, c-Jun and c-Fos then heterodimerize to form AP-1, which is capable of inducing apoptosis. It can therefore be concluded that the lowered sphingomyelin in the peroxisomes is aimed at reducing its intracellular composition and subsequently nullifying its role in apoptosis (Radin, 2003; von Haefen et al., 2002; Kroesen et al., 2001).

Mitochondrion is the power-house of the cells due to their ATP-generating ability. This metabolic feat depends on the electron transport chain and the oxidative phosphorylation which is also dependent on the integrity of mitochondrial membranes. Recently, other membrane-dependent processes that determine the life-span of a cell have been traced to mitochondrial membrane integrity and function (Kroesen et al. 2001; Zhuang et al., 2000). These processes include: maintenance of low cytosolic calcium concentration (Chacon and Acosta, 1991), regulation of production of highly reactive oxygen species and the regulation of the release of cytochrome C in response to tumour necrosis factor (Kagan et al., 1992; Ghosh et al., 1986; von Haefen et al., 2002). Although, early reports claimed that the mitochondrial ATP generation proceeds at such a low rate such that it does not meet the metabolic demands of a fast dividing cells Hinkle et al., 1991), studies have also confirmed that mitochondria have reduced occurrence in tumour cells (Morris, 1963). Reduced enzyme activities and altered structures have also been well documented (Morris, 1963). The alteration in the structure and the composition of mitochondria can be traced to the alteration of the cell membrane and this can be traced to altered transportation of protein from the cytoplasm to the mitochondrial matrix sequel to possible altered mitochondrial phospholipid/protein composition. Our data show 46.4, 67.2, 64 and 73.4% decrease in phosphatidylserine, phosphatidylcholine, phosphatidyl-etholamine, and sphingomyelin respectively in tumour group compared with the normal. The reduced incorporation of phospholipids in the mitochondria might be biochemically tailored by the tumour cells to reduce mitochondrial functions thereby avoiding apoptosis (Kroesen et al., 2001; Zhuang et al., 2000). Sphingomyelin has the lowest percentage distribution (73.4%) which is consistent with our previous findings in peroxisomes. This can be ascertained that tumour cells inhibit the pathway for the synthesis of sphingosine in order to inhibit apoptosis (Perry et al., 2000).

Figure 2 shows the distribution of the key enzymes in the soluble fraction of the liver, mitochondria and peroxisomes.

For the soluble fraction of the liver, the specific activities of catalase and D-amino acid oxidase in soluble fraction of tumour group are 23.3 and 23.0% lower respectively compared to that of the normal group while the specific activity of succinate dehydrogenase in tumour cells is 42.2% higher than soluble fraction of normal group compared to the normal group.

Catalase is a peroxisomal enzyme, its synthesis takes place in the cytosol in form of apocatalase. Peroxisomes have a single membrane (Perry et al., 2000), such that under agitation, the membrane breaks and catalase appears in the soluble fraction during fractionation. The activity of this enzyme depends on availability of iron III

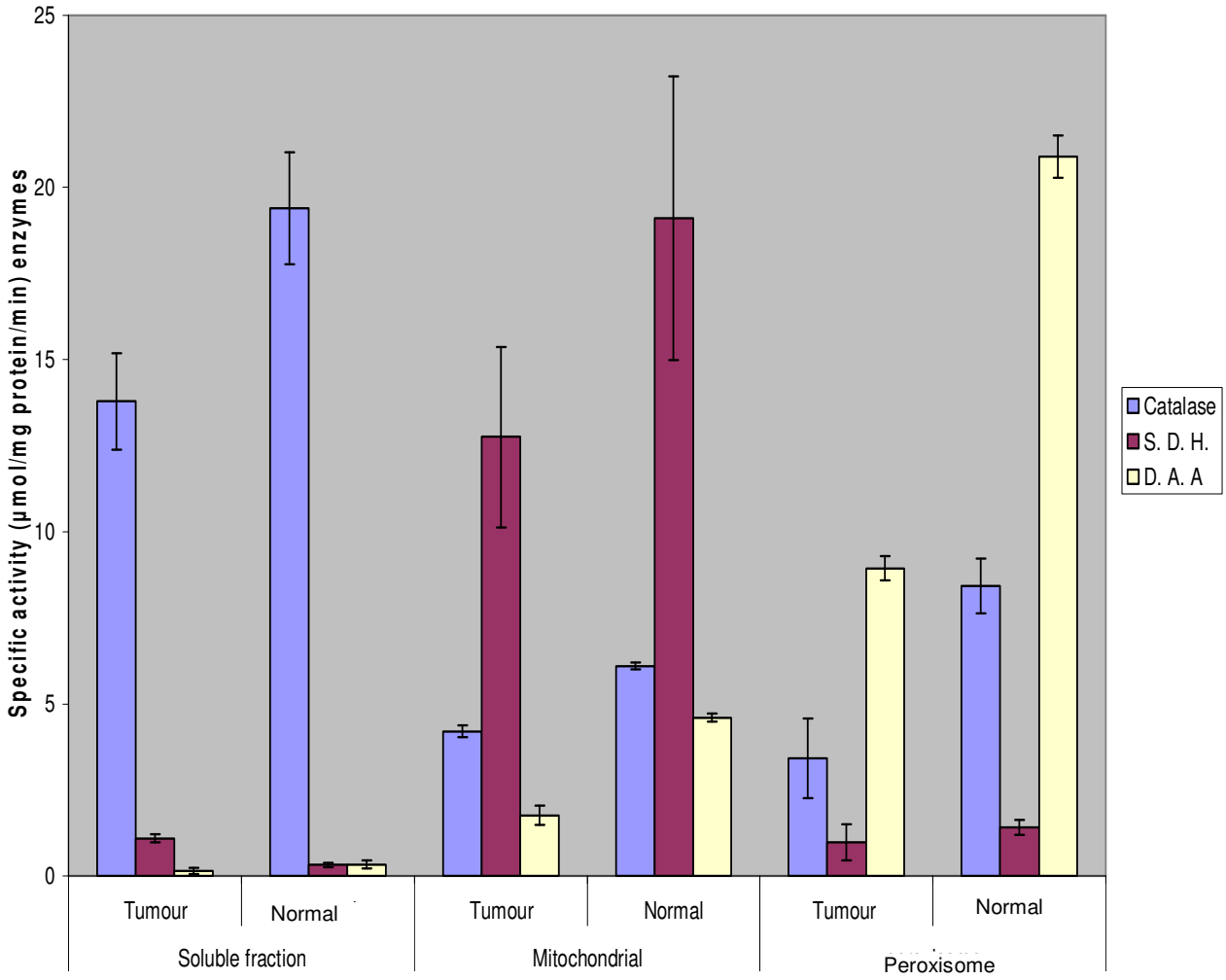

Figure 2. Shows the specific activities(µmol/mg protein/min) of marker enzymes in fractions of normal and transformed liver of rats.

ion (De-Hoop and Ab, 1992). Its lowered activity in the soluble fraction can then be traced to three factors: reduced synthesis in tumour cells, reduced transportation of apocatalase into the peroxisomes for conversion into catalase and the unavailability of iron III ion for incorporation into apocatalase to form catalase (Perry et al., 2000; De-Hoop and Ab, 1992), the crescendo of events that summarizes into lowered specific activity of catalase in tumour cells might be more complex. Orally administered 3^1-DAB chelates iron and other metals, interacts with cells of duodenum, and proximal jejunum-the portion of the small intestine responsible for iron absorption. This interaction possibly lowers their iron-absorbing capacity (Brittenham, 2000; Hoelzer, 1997), which in turn reduces the availability of iron in the liver for incorporation into apocatalase (De-Hoop and Ab, 1992). In fast dividing cells, there is an absolute necessity for metabolic economy. Metabolic emphasis would rather be on angiogenesis and erythropoiesis which require iron rather than catalase formation (Dupont et al., 1998). Another plausible explanation is the progressive loss of liver function associated with hepatocellular carcinoma, there is progressive reduction in the synthesis and secretion of proteins involved in the absorption, transportation and storage of iron. For D-amino acid oxidase, its synthesis can be repressed due to its function as generator of hydrogen peroxide-a well documented oxidant (Konno and Yasumura, 1992; Katagiri et al., 1991), capable of inducing cell injury and death. Also, tumour cells might direct FAD prosthetic group to other enzymes of anabolic importance geared towards cell division rather than activating D-amino acid oxidase whose activity may be lethal to the cell especially deficient in catalase (Konno and Yasumura, 1992).

The high specific activity of succinate dehydrogenase in the soluble fraction of the tumour group is a strong indication of a reduced stability of membrane in the mitochondria of tumour cells (Kagan et al., 1992), since the membrane is double and they are less likely to respond to agitation by breaking as compared with

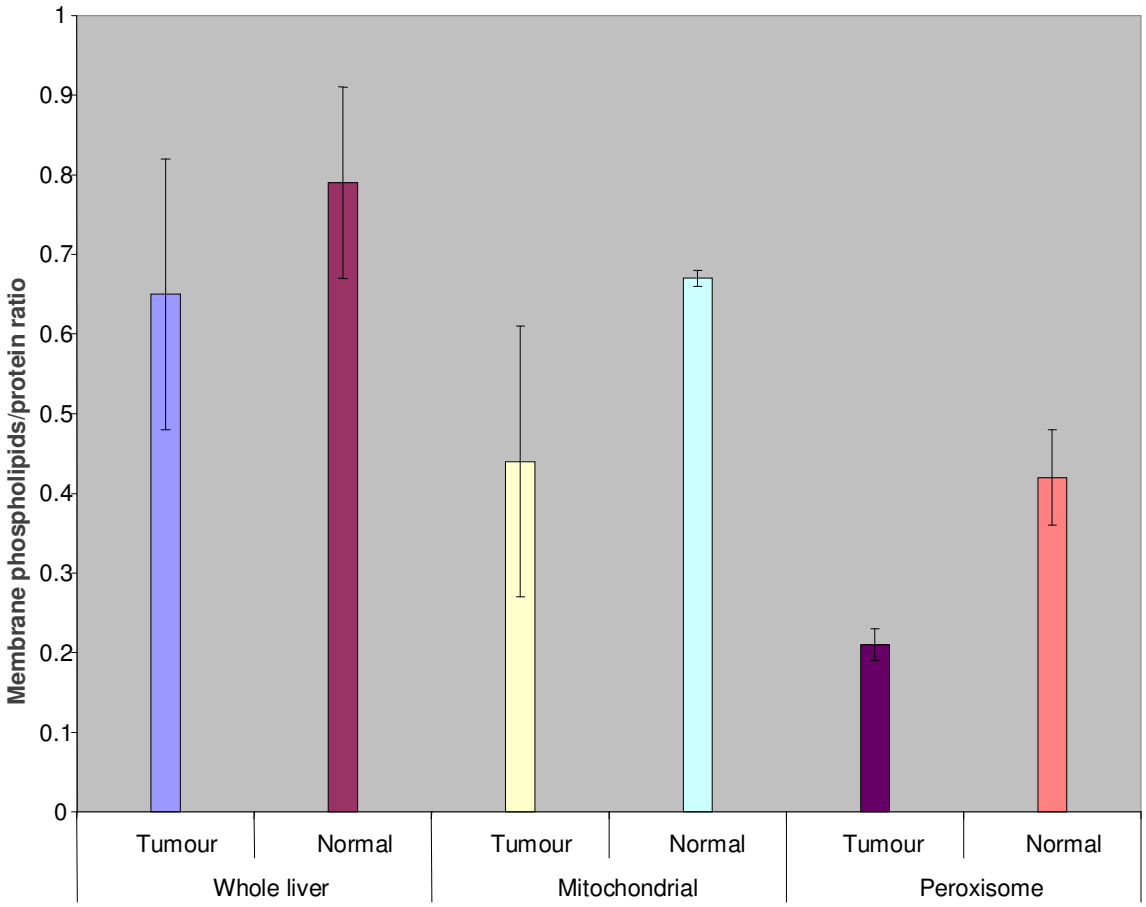

Figure 3. Shows total phosphol lipids to protein ratio in the whole liver, liver peroxisomal and mitochondrial fraction of normal and tumour group rats.

peroxisomal membrane. This finding agrees with the data generated for the mitochondrial membrane phospholipid distribution in tumour cells (Figure 1). It is well reported that permeability of mitochondria is increased with progression of tumour (Schulse-Osthoff et al., 1992).

Peroxisomal fraction of the tumour cells also exhibited 56.7% loss of D-amino acid oxidase specific activity compared with the normal group. This can be accounted for by assuming reduced distribution of peroxisomes, tumour-specific channeling of energy into metabolic processes aimed at cell division rather than expression of peroxisomal proteins which in turn might participate in death via apoptosis and hydrogen peroxide induced cell death.

The phospholipid/protein ratio for whole liver, mitochondria, and peroxisomes of the normal group is 18.8, 35.7 and 48.6% higher than that of the tumour group. This can be explained as the result of reduced phospholipid distribution in the membranes (Figure 3).

In conclusion, although the biochemical event that ultimately leads to tumourigenesis starts from the alteration of cellular DNA, the transformed cells and their organelles have registered biochemical foot-prints during the entire process. Altered membrane phospholipid distribution, key enzyme distribution and the phospholipid-to-protein ratio in the whole liver, mitochondria and the peroxisomal fraction are some of such foot-prints with dire implication on the metabolisms, cell/tissue functions, morphology and metastasis in tumour and cancerous cells.

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