ISSN: 0975-766X
CODEN: IJPTFI
Available Online through
www.ijptonline.com
Research Article

BIOCHEMICAL ALTERATIONS DUE TO MEMBRANE DAMAGE IN BENZO (a)PYRENE INDUCED EXPERIMENTAL LUNG CANCER

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Received on 19-05-2012 *Accepted on 31-05-2012*

Abstract

Cancer is an important cause of morbidity and mortality worldwide. Each year 9 million new cancer cases are detected and 5 million people die of it. Three cancer sites the lung, breast and liver still account for most of our treatment failures and these cancers are targeted as the today's therapeutic battle ground. The present study is focused on assessing the biochemical alterations in the male swiss albino mice on induction with Benzo(a)pyrene [B(a)P] which is considered to be a potential carcinogen primarily affecting the lungs.The lung has the potential of metabolizing many foreign chemicals to a vast array of metabolites with different pharmacological and toxicological properties. Inspite of greater capacity for metabolizing harmful compounds, there are still a large number of chemicals that have damaging effects on the lung. The use of B(a)P, which is a chemical carcinogen, causes the generation of free radicals, which is responsible for the initial assaults that occur in the cell that leads to prolonged carcinogenesis. Hence assessment of membrane damage with respect to glycoproteins and ATPases would help in measuring the degree of impact on the cells. Further analysis of levels of nucleic acid content, activities of marker enzymes would indicate the extent of membrane damage in lung carcinoma bearing animals. The current study is an attempt to understand the assaults caused to the membranes due to B(a)P induced lung carcinogenesis during varied induction periods. Hence the present investigation has been focused to evaluate the damage caused by B(a)P in the initiation and promotion events leading to cancer. Since glycoproteins play an important role in cell recognition and membrane ATPases reflect the membrane integrity, these above said parameters play a very important role in the assessment of membrane damage of a cell which is said to be an early event taking place during the oxidative insult caused by B(a)P induced free radicals.

Key words: Lung cancer, Benzo(a)pyrene.

Introduction

Lung cancer is the leading cause of cancer death worldwide. Epidemiological and laboratory animal model studies have demonstrated that smoking and environmental exposure to carcinogens is closely linked to increased lung cancer risk (1, 2, 3, 4). Tobacco exposure has been implicated in 90% of lung carcinomas, and smokers have a 20-fold greater risk of developing lung cancer compared with persons who have never smoked (5, 6, 7, 8). Benzo(a)pyrene (B[a]P) is one of the polycyclic aromatic hydrocarbons a chemical carcinogen, present in tobacco smoke causes lung cancer in humans and in experimental systems (9, 10). The radical cationic forms of B[a]P may be involved in both the mechanism and metabolic activation leading to the formation of DNA adducts, which are key components for tumor initiation process (11). B[a]P activates oxidative stress-induced cell proliferation and carcinogenesis by transcriptional elevation of several genes including *c-jun, c-fos, c-myc* and *iNOS*. These genes are activated by stress signals through the stimulation of tyrosine kinase, which in turn modulates downstream events including the expression of nuclear proto oncogenes (12). The metabolic activation of B[a]P is mediated through cytochrome P_{450}. Although chemically almost inert, it is metabolically converted into highly reactive electrophile. The current study is an attempt to understand the assaults caused to the membranes due to B[a]P induced lung carcinogenesis during varied induction periods. Hence the present investigation has been focused to evaluate the damage caused by B[a]P in the initiation and promotion events leading to cancer. Since glycoproteins play an important role in cell recognition and membrane ATPases reflect the membrane integrity, these above said parameters play a very important role in the assessment of membrane damage of a cell which is said to be an early event taking place during the oxidative insult caused by B[a]P induced free radicals. The aim of the current study is to assess the membrane damage in the target tissue with respect to glycoproteins alteration and ATPases activity that would help in measuring the degree of the impact of the carcinogen on the cells. Hence the present study is an effort undertaken to see the progression of cancer with prolonged induction time period.

Materials and Methods

Chemicals: Benzo(a)pyrene was purchased from sigma chemical company St. Louis, MO, USA. All other chemicals used were of analytical grade.

Animals

Healthy male Swiss Albino Mice (6-8 weeks old) weighing 25-30g were used throughout the study. The animals were purchased from Central Animal House Facility, Dr.ALM PG IBMS, University of Madras, Taramani, Chennai-600113 and were maintained in polypropylene cages in a controlled environmental condition of temperature and humidity on alternatively 12 h light/dark cycles. All animals were fed standard pellet diet (Gold Mohor rat feed, Ms. Hindustan Lever Ltd., Mumbai) and water ad libitum. The experimental designs were approved by the institutional animal ethical committee. (IAEC No.02/025/09).

Experimental design

The animals were divided into three groups

Group I - Control animals.

Group II - 50mg/Kg body weight of Benzo[a]pyrene in corn oil was given orally for an induction period of 60 days.

Group III - 50mg/Kg body weight of Benzo[a]pyrene in corn oil was given orally for an induction period of 90days.

After the experimental period the animals were sacrificed by cervical decapitation and lung tissues were immediately excised, weighed, and processed for homogenization with motor driven Teflon coated homogenizer in ice-cold 0.1 M Tris-HCl buffer pH 7.4 to get 10% homogenate. The lung tissue homogenate was used for the following biochemical estimations.

Biochemical analysis

Total protein was estimated by the method of Lowry et al, 1951(13).The marker enzyme, γ–glutamyl transpeptidase (GGT) was estimated according to the method of Orlowski and Meister, 1965 (14) modified by Rosalki and Rao, the aryl hydrocarbon hydroxylase (AHH) was estimated by Mildred et al., 1981 (15).Membrane bound enzymes Na^+ K^+ ATPases (16), Mg^{2+} ATPases (17), and Ca^{2+} ATPases (18). The inorganic phosphorus was estimated according to the method of Fiske and Subbarow (19).

To the weighed amount of the defatted tissue, 2ml of 4N HCl was added and the mixture was refluxed at 100 degree Celsius for 4 hours in a test tube with suitable marble lids. The hydrolysate was neutralized with sodium

hydroxide. Aliquots of the neutralized samples were taken for the analysis of membrane bound glycoproteins. Hexose level was estimated by the method of Neibes, 1972 (20), hexosamine by the method of Wagner, 1974 (21) and for Sialic acid a weighed amount of defatted tissue was hydrolyzed with 1.0 ml of 0.1 N sulphuric acid at 80 degree Celsius for 60 minutes to release sialic acid bound to the proteins. The solution was then neutralized with sodium hydroxide. Sialic acid was estimated by the method of Warren, 1975 (22).The nucleic acids were extracted by the method of Schneider, 1957 (23); DNA was estimated by the method of Burton (1956) and RNA by the method of Rawa et al (1977).

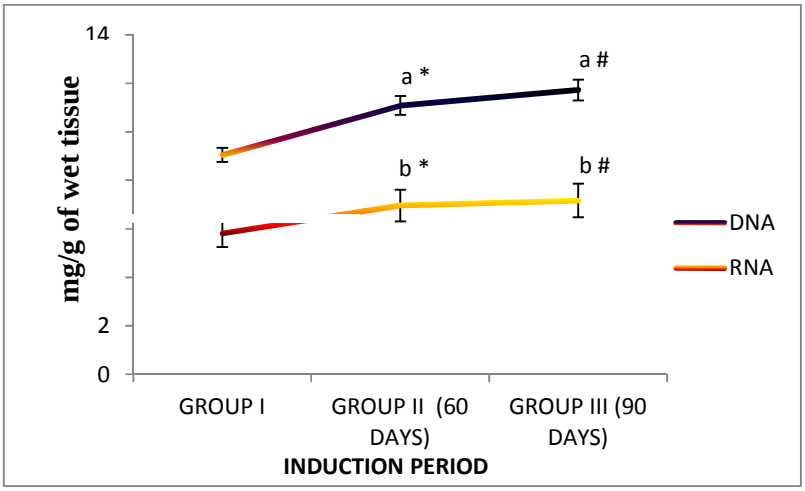
Statistical analysis

Results are expressed as mean ± S.D (n=6). Comparisons between the means of the control and treated groups were made using one way analysis of variance (ANOVA) using the SPSS software package for windows.

Results

Figure 1 shows the levels of DNA and RNA in plasma of control and experimental animals. Elevated levels of DNA and RNA were observed in lung carcinoma bearing animals, which may be due to a chemical interaction between the carcinogen and nucleic acids. The graph shows an increase in the level of nucleic acids when the three groups taken under consideration are compared. There is a notable increase in the levels of DNA and RNA from 60 days to 90 days when compared with that of the control animals.

Figure 1: **The Level of Nucleic Acids (DNA & RNA) In Plasma of Control and Experimental Animals.**

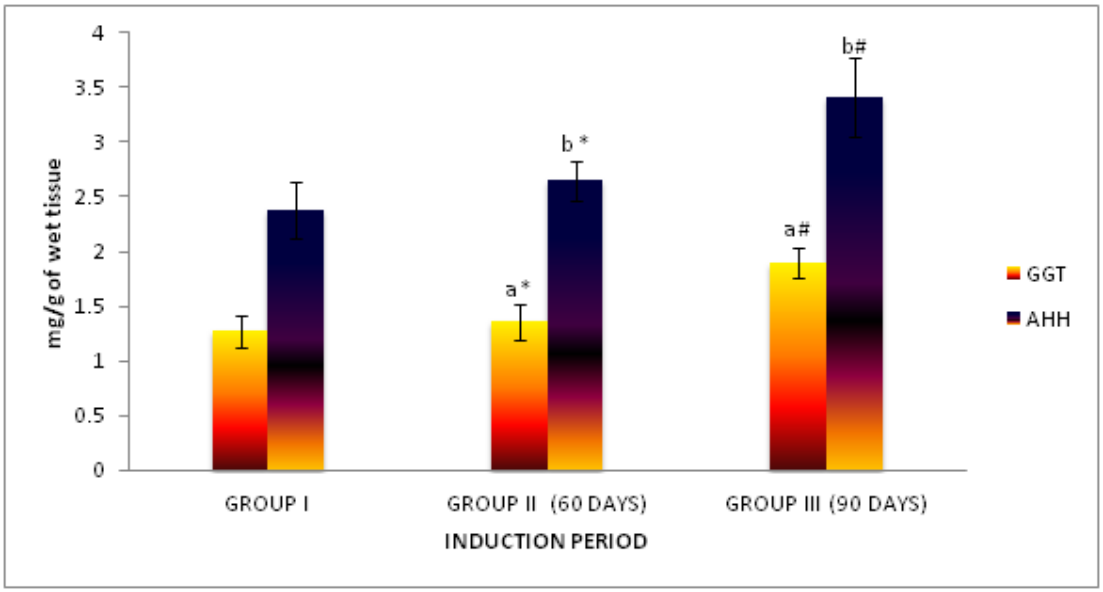


Values are expressed as mean ± SD for six mice in each group
Lung DNA and RNA-mg/g of wet tissue.
a-as compared with group I
b-as compared with group I

Statistical significance: *p<0.05;#p<0.01

Figure 2 shows the activities of marker enzymes in lung of control and experimental animals. In the present study we have observed increased activities of marker enzymes such as AHH and GGT in lung cancer of B[a]P induced lung cancer bearing mice. The toxicity and destructive effects of carcinogen may result in the death of the cells of the lung and release of intracellular enzymes into the circulation, thereby showing a rapid and significant increase of these enzymes in tissue of cancer bearing cells. Increased activities of these enzymes could be due to alteration in cancer cell morphology, lung-cell damage and leaking of the enzymes.

Figure 2: Activities of Marker Enzymes in Lung of Control and Experimental Animals.



Each value is expressed as mean ± S.D. for six mice in each group.

a: compared with Group I; b: compared with Group II; NS: compared with Group I.

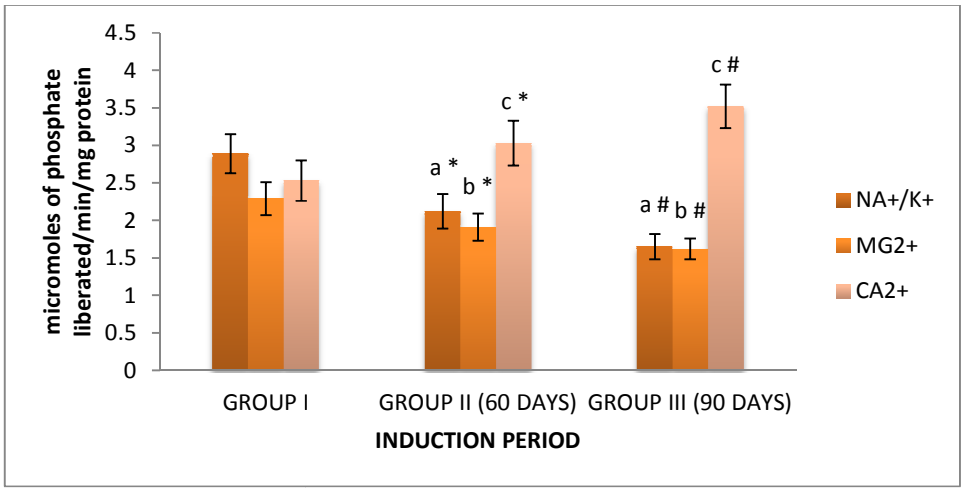
Units: AHH – micro-moles of fluorescent phenolic metabolites formed/min/mg protein;

GGT – nano-moles of p-nitroaniline formed/min/mg protein.

Statistical significance: *p<0.01, #p<0.05, NS-Not significant

Figure 3 shows the activities of membrane bound ATPases in the lung of control and experimental animals. Decrease in activities of Na^+/K^+ and Mg^{2+} and Ca^{2+} ATPase were found in cancer bearing animals. This shows that the direct damage of plasma membrane occurs through the interaction with the membrane components as with the ion dependent ATPases and ion channels.

Figure 3: Activities of Membrane Bound ATPases In Lung of Control and Experimental Animals.



Values are expressed as mean ± SD for six mice in each group

$Na+/K^+$, Mg^{2+} and Ca^{2+} ATPases -µmoles of phosphate liberated/min/mg protein.

a-as compared with group I

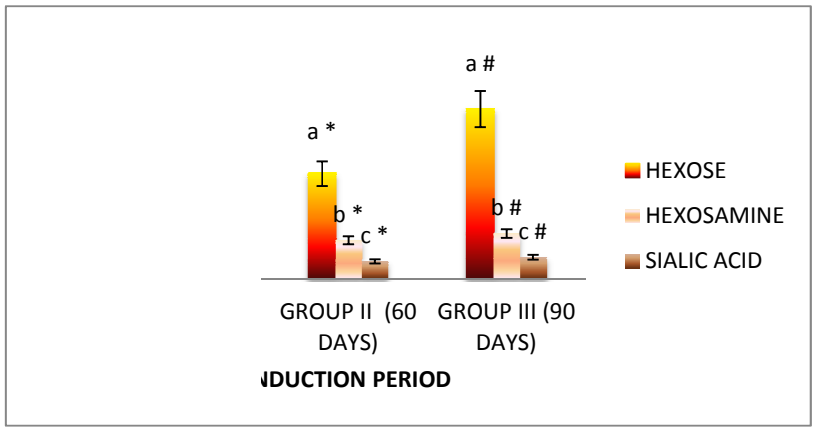
b-as compared with group I

c-as compared with group I

Statistical significance: $^{*}p<0.05$;$^{\#}p<0.01$

Figure 4 shows the level of glycoproteins in the lung of control and experimental animals. Increased levels of hexose, hexosamine and sialic acid are observed in lung cancer bearing animals.Glycoconjugates are very important compounds that consist of different categories such as glycopeptides, glycolipids, and lipopolysaccharides. Aberrant glycans, or oligosaccharide moieties attached to lipids and proteins, are often expressed during disease development, including cancer progression to a metastatic stage.

Figure 4: Level of Glycoprotein In Lung of Control and Experimental Animals.



Values are expressed as mean ± SD for six mice in each group

Hexose, hexosamine and sialic acid –mg/g of defatted tissue

a-as compared with group I

b-as compared with group I

c-as compared with group I

Statistical significance: $^{*}p<0.05$; $^{\#}p<0.01$

Discussion

Carcinogenesis is a multistage process characterized by continuous change in specific heredity and phenotype. The whole process of carcinogenesis is accompanied by subsequent activation of a group of protooncogenes and inactivation of cancer suppressor genes, leading to continuous accumulation of controlled proliferation of cells.

In our present study of using B[a]P as the chemical carcinogen, the mice lung underwent different stages of carcinogenesis and progression. The incidence of rate of lung cancer was 100%.

The presence of nucleic acids in plasma of cancer patients has been recognized since 1970s.Later it was reported that the circulating extracellular DNA exhibits tumour-related alterations, such as decreased strand stability.DNA content of tumor is found to be an important indicator of prognosis, because it is well correlated with the size of the tumor in the cancerous condition. The RNA level in lung cancer bearing animals was also increased but not as significant as DNA. The increase in DNA content may lead to an increase in transcription, which might have resulted in moderately elevated RNA content in cancer cells.

Measurement of marker enzyme levels can be useful in the detection and diagnosis of cancer. Its level reflects the extent (stage) of the disease and can be useful in predicting how well the disease will respond to treatment. The activities of marker enzymes were found to be elevated in tissues of lung carcinoma bearing animals, which could be due to the destruction of the neoplastic tissue. The abnormal variations in the marker enzymes reflect the overall change in metabolism that occurs during malignancy (24).

The marker enzymes such as AHH, ADA, GGT, and LDH are specific indicators of lung damage (25). The increase in the activities of these enzymes may be due to the increased tumour incidence. The markers of our interest AHH and GGT activities were increased in lung cancer animals.GGT is a broad specificity transferase that catalyses the transfer of gamma glutamyl groups from a large variety of peptide donors to a wide range of aminoacids and peptide receptors (26). γ -Glutamyl transpeptidase activity serves as a marker for the progress of carcinogenic events. The transfer of γ - glutamyl groups from peptide donors to peptide receptors and aminoacids

is the catalytic function of GGT. GGT is not only useful in diagnosis but also has prognostic value in malignancies such as lung cancer and malignant melanoma. The enzyme is membrane bound and its active site is oriented on the outer surface of cell membrane. Y-Glutamyl transpeptidase is a cell surface enzyme that cleaves extra cellular glutathione thereby providing the increased intracellular glutathione synthesis (27). This deviation shows the progress of carcinogenic process, since its ability correlated with growth rate, histological differentiation and survival time ofthe host (28).

AHH converts polycylic hydrocarbons to phenols, dihydrodiols, quinines and epoxides. This enzyme system that is highly inducible in mouse skin as well as in most mammalian tissues is positively correlated with susceptibility to benzo(a)pyrene cytotoxicity leading to carcinogenesis.High AHH levels were induced in pulmonary tissues of animals exposed to benzo(a)pyrene.

ATPases are membrane bound enzymes and are mostly present on the basolateral membrane. They help in maintaining the ionic gradients between aqueous intra and extracellular phases. Na^+/K^+ ATPase activity pumps Na^+ ions out of the cell. The cytosol of animal cells contains a concentration of potassium ions twenty times higher than that in the extra cellular fluid. Conversely, the extra cellular membrane contains a concentration gradient of sodium ions 10 times greater than that within the cells. These concentration gradients are established by the active transport called the Na^+/K^+ ATPase. As a result, the intracellular concentration of Na^+/H^+ exchange is established across the brush border membrane which splits up the ATP for energy purpose. The activity of Na^+/K^+ ATPase can be regulated by hormones, G-proteins, and secondary messengers.

In malignancy, the cell membrane plays a crucial role in the stimulation and control of the cell adhesiveness, mortality, and proliferation in a much damaged condition. Protection of membranes is of potential usefulness in the treatment of disease processes. The membrane bound enzymes such as Na^+/K^+ ATPase, Mg^{2+} ATPase and Ca^{2+} ATPase are responsible for the transport of sodium/potassium, magnesium and calcium ions across the cell membranes at the expense of ATP by hydrolysis. Ca^{2+} ATPase activity is mainly impaired due to oxidative modification of thiol group present in the enzyme. Thiol modification has been recognized as critical event in cytotoxicity. Damage to these thiol moieties may result in inhibition of Ca^{2+} ATPase function. The decrease in the activity of cancer bearing animals suggests that there is an alteration in the fluidity of the membrane due to the

toxicity created by B[a]P.

Mg^{2+} ATPases is poised to regulate the flow of potential energy from the mitochondria and from the cytoplasm. The decreased activities of Na+/K+ ATPase and Mg2+ ATPase in lung cancer bearing animals may be due to increase in the production of free radicals that leads to cell injury. The inhibiting function of ion dependent ATPases leads to disturbances in ion homeostasis. Disturbances in ion homeostasis results in impaired signal transduction, altered cellular metabolism, changes in cell membrane permeability and integrity and an elevation in membrane fluidity and disturbances of vital function. Thus the activities of all three ATPases in lung tissue have been found to be inhibited in carcinoma bearing animals.

Glycoproteins are one of the many acute phase proteins present in the plasma. They are found in greatest concentration in animal fluids like plasma and urine and also in the connective tissues. Spiro in 1959 established that liver is the major organ involved in the normal synthesis of glycoproteins. Elevation of glycoprotein contents are useful indicators of carcinogenic process and these changes alter the rigidity of the cell membranes.

In our study, glycoproteins, hexose, hexosamine and sialic acid levels were found to be significantly elevated in lung cancer bearing animals.Sialic acid is widely distributed in nature as terminal sugars in glycoproteins or glycolipids impart a net negative charge to cell surface and are reported to be important to maintain cell-to-cell and cell-to-matrix interactions. Serum sialic acid levels can be used as laboratory markers in a variety of pathological conditions. Marked elevation of serum sialic acid concentration that correlates with the clinical activity of the disease has been documented in many malignancies. Malignant cells have been reported to have more sialic acid in their cell membrane than normal cells. In the present study increased levels of sialic acid were observed in tissue of cancer bearing animals.

Conclusion

To conclude, many biochemical and molecular changes were observed in the tissues of lung cancer bearing animals of different periods of induction. Lung cancer bearing animals showed a significant increase in lung weight and a reduction in body weight. The biochemical alterations were evidently seen from the results of the estimation of the levels of nucleic acids, glycoproteins, activities of marker enzymes and membrane bound ATPases in lung of control and experimental animals. The increase in levels of nucleic acids, glycoproteins and the

inhibitory action of membrane bound enzymes suggests the production of free radicals (oxidative stress).This oxidative stress might act as a threat in causing many diseases one of which is lung carcinoma.

Acknowledgement

One of the authors, M.S.Veena wishes to acknowledge the effort and support provided by Mr. V.Vipin and M.S.Shyam Kumar in manuscript compilation.

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