Lymphoma affects non-lymphatic tissues

Abstract
Dalton’s Lymphoma (DL) is a type of Non-Hodgkin's lymphoma that begins in the lymphatic system. It is also reported to be transplantable T-cell lymphoma that can be induced through ascite fluid in murines. There are several reports showing effects of ascite fluid on lymphatic tissues but its impact on other organs are limiting. It was intended to study the impact of ascite fluid introduction and development of lymphoma on non-lymphatic tissues, brain and liver. A significant modulation was observed on the activity of lactate dehydrogenase (LDH) isoforms, super oxide dismutase (SOD) and catalase in non-lymphatic tissues. The LDH isoforms, LDH4 and LDH5 in brain and a predominant LDH5 in liver increased significantly in lymphoma bearing mice. The activity of catalase reduces significantly both in brain and liver of DL mice. However, the Mn-SOD isoform in brain and liver increases significantly while the activity of Cu-Zn-SOD decreases in lymphoma bearing mice. The denaturing poly-acrylamide gel-electrophoresis (SDS-PAGE) shows lymphoma-mediated changes in protein profile of liver as well as brain. We could observe modulation of two major polypeptides of about 22kDa and 50kDa in brain and 18kDa in liver of mice bearing lymphoma.

Keywords: Dalton's Lymphoma (DL), LDH, SOD and Catalase.

INTRODUCTION
The biochemical alterations are highly sensitive and mostly preferred for the diagnosis of pathological conditions over studies on morphological aberrations. It has also been demonstrated that stress modulates transcription factors, acute-phase proteins, antioxidant enzymes, and structural proteins (Kim J, et al., 2005; Muller JM et al., 1997). The tumor or cancerous cells primarily use glucose as their main energy source and the enzymes involved in glucose metabolism pathway serve as an important marker to diagnose the pathological conditions. The lactate dehydrogenase, LDH (EC 1.1.1.27) is one of the most susceptible glucose metabolizing enzymes and a known enzymatic marker of metabolic adaptations (Prabhakaram M. et al., 1984, 1987), pathological conditions (Mishra and Shukla, 1999; Aniscow EK et al., 2000; Niakan B, 2001; Sandqvist MM et al., 2001) and therapeutic monitoring (Mishra L. et al., 2004; Trigun SK et al., 2007). It is also recognized as potential tumor marker in assessing the progression of the proliferating malignant cells (Engan T et al., 1990). The reactive oxygen species (ROS) are normal by-products of cellular metabolism and are regulated by cellular defense mechanism provided by antioxidant enzymes (Mirmomeni MH et al., 1979). The antioxidant enzymes are inducible and the level of the antioxidant enzymes reflects the level of their substrate, the ROS. It is also reported that a balance between ROS produced and antioxidant defense mechanism is essential for normal cellular function (Guyton KZ et al., 1993; Srivastava A et al., 2006). The ROS are responsible for cellular damage, tissue damage, DNA modifications, and many human diseases (Cross CE et al., 1987). The SOD and Catalase are one of the major enzymatic antioxidant defenses in the body. The mice lacking SOD2 die several days after birth due to massive oxidative stress (Li Y et al., 1995) and the mice lacking SOD1...
Lymphoma affects non-lymphatic tissues and develop a wide range of pathologies, including hepatocellular carcinoma (Elchuri et al., S.,2005), an acceleration of age-related muscle mass loss (Muller FL, 2006; Han ES, 2008) an earlier incidence of cataracts and a reduced lifespan. However, the mice lacking SOD3 do not show any obvious defects and exhibit a normal lifespan (Sentman ML et al., 2006). The involvement of oxidative stress in neurodegenerative disease is also suggested where the increased formation of ROS leads to neuronal damage (Seif-El-Nasr M et al., 2008). The antioxidant enzymes such as super oxide dismutase (SOD) and catalase are also reported to be up-regulated during carcinogenesis. They are generally up-regulated in the presence of oxidative stress (Teixeira KC et al., 2008).

Among the SOD enzymes, MnSOD plays an important role in neo-vascularization and angiogenesis (Masuko Ushio-fukai et al., 2008). The catalase, a major peroxisomal enzyme, is involved in a number of important cellular metabolic processes as well as detoxification of H$_2$O$_2$ (Ilan Y et al., 1981; Prince VS et al., 2004). The reports also indicate that the energy production in cancerous cells increases over fourfold (Koukourakis M et al., 2003) than the normal cells. Thus growing cancerous cells up-regulate anaerobic glycolysis vis-à-vis LDH activity (Newsholme EA et al., 1991) even in the presence of oxygen (Pathak C et al., 2005). In cases of lymphoma the production of ROS increases due to hypoxic condition but the production of antioxidant enzymes does not increase concomitantly. The stress of hypoxia also induces the gene Hif-1α that induces production of LDH in cascade (Firth JD et al., 1995; Semenza GL et al., 1996). Since there is a relation between antioxidants and enzymes of glucose metabolism, the impact of lymphoma is likely on non-lymphatic tissues. The brain and liver as non-lymphatic organs are intended to study because of known primary organ affected with hypoxic condition. The brain and liver are highly sensitive to oxygen deprivation and can begin to die within the five minute after oxygen supply has been cut off. It is also suggested that hypoxic conditions are more stressful for these organs because they need oxygen for their metabolism in order to avoid excessive metabolic accumulation. In this report, the enzymatic and polypeptide pattern of non-lymphatic tissues like brain and liver is described.

**MATERIALS AND METHODS**

**Animals**

AKR strain of mice was used for all the experiments because of their short life span and high susceptibility for tumor development. The mouse colony was maintained under standard mice feed and drinking water at 25±2 °C in animal house facility of the department. The adult mice (15-20 weeks old) were selected for this study.

**Chemicals**

All chemicals used in the experiment were of molecular and analytical grade purchased from companies (Sigma-Aldrich chemicals, E-Merck, Himedia, Amersco, Biogene and SRL) of repute.

**Experimental procedure**

Dalton’s lymphoma (DL) was induced in adult mice (15-20 weeks old) by serial transplantation of live lymphoma ascite cells. For induction of lymphoma, each mouse received ~5×10$^6$ cells/ml in normal saline through intra-peritonial injection. The life span of DL mice was 20±2 days however life span of normal mouse is about 18 months as also reported earlier (Pathak C et al., 2005). The control and DL bearing mice were sacrificed to collect brain, and liver. The tissues were washed in ice-cold normal saline and 20% homogenate (w/v) was prepared in Tris-Cl buffer pH 7.4 containing 0.15M NaCl, 0.2mM PMSF and 5mM EDTA. The homogenate was centrifuged and supernatant was used as crude extract of tissue. The protein content of the sample was determined according to standard method (Lowry OH et al., 1951).

**In-gel enzyme activity assay**

Native polyacrylamide gel (8%) electrophoresis was performed to analyze LDH, SOD and Catalase enzyme activity from brain and liver of control and DL bearing mice. Electrophoresis was carried out at 4°C for three hours, applying a current of 100mV. The gels were stained for specific enzymes. The procedure for LDH specific staining was based on method of Worthington with modifications (Mishra R, Shukla SP, 1999). The staining solution for LDH comprised 0.1M Tris-Cl buffer (pH 8.4), 1mg ml$^{-1}$ Nicotinamide adenine dinucleotide (NAD$^+$), 0.5mg ml$^{-1}$ Nitro blue tetrazolium (NBT), 0.1mg ml$^{-1}$ Phenozine metho sulphate (PMS) and 0.05M lithium lactate. The Catalase specific staining
Lymphoma affects non-lymphatic tissues

Figure 1a: Native PAGE (6.0%) showing enzyme activity of lactate dehydrogenase (LDH) in brain and liver of control and lymphoma (DL) bearing mice.

Figure 1b: Percent Relative Density of LDH isoforms in brain and liver of control and lymphoma (DL) bearing mice.

Figure 2a: Native PAGE (6.0%) showing catalase activity in liver and brain of control and lymphoma (DL) bearing mice.

Figure 2b: Percent Relative Density of Catalase in liver and brain of control and lymphoma (DL) bearing mice.

Figure 3a: Native PAGE (8.0%) showing SOD activity in liver and brain of control and lymphoma (DL) bearing mice.

Figure 3b: Percent Relative Density of SOD in brain and liver of control and lymphoma (DL) bearing mice.

was observed with 0.03% H₂O₂, 0.2% potassium ferricyanide and 0.2% ferric chloride (Woodbery W et al., 1971). The staining mixture for SOD consists of phosphate buffer 0.1M (pH 7.4), 2.34mM (NBT), 28 µM riboflavin and 28mM NNN'N' Tetramethylethylenediamine (TEMED) (Beuchamp C and Fridovich I, 1971).

SDS-PAGE analysis
The SDS-PAGE (10%) was performed to analyze protein profile in brain and liver of control and lymphoma bearing mice using standard method (Laemmli UK et al., 1970). The concentration of acrylamide was 10% and serial
Lymphoma affects non-lymphatic tissues

The concentration of crude extract was loaded in each lane of the gel. The electrophoresis was carried out for three hours, applying a current of 100mV. The gel was stained overnight in 0.1% Coomassie Brilliant blue R-250 (Wilson CM, 1983). The gel was also analyzed through silver staining (Sammons DW et al., 1981).

**Statistical analysis**

The percent relative density of bands showing enzyme activity was estimated using Alpha Ease FC (Alpha Innotech, USA) software. Student’s t-test was applied to determine the difference between the means of control versus Dalton's lymphoma sample. The analyzed data of active fraction of enzymes are presented as histograms. The histograms show values of means of percent relative densities of three separate experiments. The standard deviation (SD) of evaluation of data is shown as error bars on the histograms. The $P<0.001$ and $P<0.01$ were adopted as a criterion of significance.

**RESULTS**

It was interesting to observe effects of lymphoma on non-lymphatic tissues. The brain of lymphoma bearing mice shows high activity of LDH4 and LDH5 isoforms as compared to control mice (Fig. 1a and 1b). The liver also shows elevated activity of its predominant isoform LDH5 as compared to its respective control tissue (Fig. 1a & 1b). Lymphoma bearing mice show reduced activity of antioxidant enzymes like Catalase (Fig. 2a and 2b). The activity of catalase was significantly low in both brain and liver as compared to the control mice. The activity of Mn-SOD was found to be elevated in both liver and brain but the activity of Cu-Zn-SOD was reduced in liver as well as in brain of lymphoma bearing mice (Fig. 3a and 3b). The SDS-PAGE profile of brain and liver tissue of Dalton’s lymphoma bearing mice shows absence of two polypeptides of nearly 50kDa and 22kDa in brain and 18kDa in liver (Fig 4).

**DISCUSSION**

The modulation of activity of LDH isoforms (LDH4 and LDH5) in non-lymphatic tissues like brain and liver (Fig.1a and 1b) may be due to induced hypoxic condition through lymphoma.
hemophagocytic syndrome. It resulted due to massive infiltration of activated macrophages with hemophagocytosis in the spleen, liver, bone marrow and perisplenic lymph nodes (Iyama S et al., 2008). However, specific activation of the LDH4 and LDH5 isoforms is critically implicated during lymphoma.

The reduced activity of Catalase (Fig. 2a and 2b) is likely to favour accumulation of superoxide radicals (O$_{2}^-$) and H$_2$O$_2$. The finding is in agreement that if the O$_{2}^-$ is not rapidly neutralized it can trigger lipid peroxidation (Kellog EW and Fridovich I 1975, 1977). It also influences oxidation of the SH groups that are of prime importance in stimulating cell multiplication (Apffel CA and Walker JE, 1973; Asada K, and Kanamatsu S, 1976). The capacity of catalase that favours cell multiplication may also be seen as a protective action against H$_2$O$_2$ (Fridovich I, 1982; Peters JW and Foote CS, 1976). The isoforms of SOD, like Mn-SOD and Cu-Zn-SOD in liver and brain show differential modulation (Fig. 3a and 3b) induced by ascite cells. It was interesting to observe that the level of MnSOD is increases and the level of CuZnSOD decreases (Fig. 3a and 3b). It is likely that MnSOD promote mitochondrial H$_2$O$_2$ production, thereby stimulating extracellular sprouting and neovascularization (Masuko Ushio-fukai et al., 2008). Thus SOD serve as H$_2$O$_2$ generating enzyme and it is also involved in neo-angiogenesis rather than antioxidant enzymes (Teixeira KC et al., 2008). The reduced activities of catalase and SOD are also reported in Dalton's lymphoma ascites transplanted (DLAT) mouse liver compared to normal (Pathak C et al. 2008). The reports suggest that decreased level of antioxidant enzymes and elevated level of LDH (LDH4 and LDH5) isoforms in cancerous cell favour activation of enzymes like guanylate cyclase and cyclic 3'-5' GMP that acts as regulators of cell division (Mittal CK et al., 1977). The results also indicate that ascite fluid influences host detoxification process even in non-lymphatic tissues. It is likely that the rate of production of free radicals is higher than the production of antioxidant enzymes. Since the brain and liver are the more susceptible organs for the free radicals, enzymes like SOD and Catalase were also detected in brain and liver. The results of this study and previous studies show that hypoxia and activation of Hypoxia inducible factors are important pathways that contribute to tumorigenesis, angiogenesis, increased glycolysis and tumor cell survival (Young CD et al., 2008; Keith B et al., 2007; Gorden JD et al. 2007).

It was also interesting to observe DL mediated modulation of protein profile in non-lymphatic tissues like brain and liver. It is appreciable to observe absence of two polypeptides of nearly 50kDa and 22kDa in brain and 18kDa in liver (Fig. 4). The finding is in agreement with reports that show various types of stress induced activation or silencing of genes encoding for regulatory transcription factors, acute-phase proteins, antioxidant enzymes, and structural proteins (Muller JM et al., 1997; Dalton TP et al., 1999). The reports (Muller JM, and Rupec RA et al., 1997) also suggest that NF-$\kappa$B and AP-1 activate Ras-Raf mediated pathways in response to reactive oxygen intermediates. Although there are several reports related to stress mediated response on enzymatic profile but information about the modulation of proteomic profile in non-lymphatic tissues are limiting. These unique and novel stress polypeptides are under characterization.

In conclusion, the results of this study demonstrate the influence of asciite fluid on dehydrogenase and antioxidant enzymes in non-lymphatic tissues. The elevated level of LDH4 and LDH5 indicates anaerobic condition induced by lymphoma. The results also provide DL-induced changes in brain and liver polypeptides and are presumed to influence proteomics of non-lymphatic tissues.

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References

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Lymphoma affects non-lymphatic tissues


