Effect of Cisplatin on the Lactate Dehydrogenase Activity and its Isozyme Pattern in Dalton’s Lymphoma Bearing Mice

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Summary Lactate dehydrogenase (LDH) activity and its isozyme patterns were determined in various tissues of normal, Dalton’s lymphoma (DL) bearing and cisplatin treated tumorous mice. Tumor-bearing hosts showed about two fold higher serum LDH activity than that in the normal animals and following cisplatin treatment (8 mg/kg body wt, i.p.) for 1–4 days, serum LDH activity further increased. In kidney, as compared to normal mice, there was no significant change in the enzyme activity in tumor bearing hosts, but liver LDH activity increased in tumorous condition. After cisplatin treatment overall 20–30% decrease in the activity was noted in kidney and liver, with slight increase on the day 2 of treatment. LDH isozyme analysis revealed that in serum and kidney, all the five isozyme constituents were present, whereas, in liver and ascites tumor supernatant only LDH-3, -4 and -5 were observed with the predominance of LDH-5. In liver, after cisplatin treatment LDH-3 and -4 expression gradually decreased. In DL cells, LDH-5 was the only isozyme form present and after cisplatin treatment its activity increased.

Thus, it is suggested that LDH activity is definitely affected in the tissues of tumor bearing hosts and during tumor regression after cisplatin treatment. The changes in LDH activity could be very useful parameter in malignancy and cisplatin-mediated chemotherapy against murine Dalton’s lymphoma in particular and cancer in general. LDH isozyme patterns revealed the presence of tissue specificity of different isozymes, with only LDH-5 in tumor cells and appearance of some specific isozyme variant, named here as LDH-T, in the serum of tumor bearing hosts.

Cis-diamminedichloroplatinum (II), commonly known as cisplatin, is a leading chemotherapeutic drug being used effectively against a variety of malignancies (Kociba et al. 1970, Rosenberg 1985, Prasad and Giri 1994). However, its therapeutic efficacy is limited due to its side effects which include nephrotoxicity, neuroxicity, gastrointestinal toxicity, ototoxicity (Prestayko et al. 1979, Roberts et al. 1988), embryotoxicity (Keller and Aggarwal 1983), mutagenicity (Giri et al. 1998) etc. Many of the properties and biological effects of cisplatin have been well documented (Rosenberg 1985, Chu 1994, Pil and Lippard 1997) with numerous reports indicating that cellular DNA could be the primary target in its anticancer activity (Pinto and Lippard 1985, Zamble and Lippard 1995).

In addition to its interaction with cellular DNA, the changes in various biochemical/ enzymatic parameters, immune response, cell surface etc. have also been observed which led to propose the involvement of multistep and multilevel effects of cisplatin in the tumor cells/host during cisplatin-mediated chemotherapy against cancers (Giri 1995). Enzymatic changes have also been implicated in the mechanism of action of cisplatin (Aggarwal 1993). Aggarwal and Meara (1996) reported the cisplatin induced inhibition of variety of dehydrogenases (isocitrate dehydrogenase, β-hydroxybutyrate dehydrogenase, glutamate dehydrogenase, malate dehydrogenase, succinate dehydrogenase and lactate dehydrogenase) in liver and kidney of rats, and suggested that the inhibition of dehydrogenase activity may be involved as a mechanism behind cisplatin-induced toxicities. Stefanini

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Cytologia 64 (1985) has discussed the changes in various enzymes in patients with malignancies and suggested that enzymes, isozymes and enzyme variants could be very useful in the detection and evaluation of biomarkers in the malignancies. Enzymes involved in the synthesis of nucleic acid (thymidine kinase, uridine kinase), of essential amino acids (phosphoserine phosphatase), or in glycolysis (hexokinase, aldolase) have been reported to be elevated in malignant tissue (Yesher 1978).

Lactate dehydrogenase (LDH, EC 1.1.1.27), the terminal enzyme in the anaerobic glycolysis, catalyses a reversible reaction of pyruvate to lactate. Increased activity of LDH has been reported in testicular cancer (Lippert and Javadpour 1981), cancerous breast (Stefanini 1985) and pulmonary tissue (Yesher 1978). Isoenzyme or isozyme determinations of the particular enzyme are becoming widely used means of detecting specific tissue specificity/disturbances. LDH exists in five isozyme forms, controlled by two genes which synthesize unique peptides (Cahn et al. 1962, Markert 1963). A single peptide combines with itself forming a tetramer representing the native LDH molecule. The two LDH tetramers, thus formed, are the electrophoretic extremes, i.e. LDH-1 moves farthest toward the anode during electrophoresis (most anodic) and LDH-5 is the extreme cathodic enzyme. These enzyme forms of LDH have been denoted as H4 and M4 where H represents the peptides characteristic of heart muscles and M for the skeletal muscles (Appella and Markert 1961, Cahn et al. 1962). The intermediate isoenzymes LDH-2, LDH-3 and LDH-4 are hybrids formed by random association of H and M subunits into tetramers. Thus, LDH-2, -3 and -4 would contain H3M, H2M2 and HM3 subunits respectively. The accepted nomenclature for LDH isoenzymes is LDH-1, LDH-2, LDH-3, LDH-4 and LDH-5. Rajadhyaksha et al. (1986) reported that LDH isozyme distributions were different in the thymocytes of normal and leukemic mice. The thymocytes from leukemic animals displayed very low values of H : M ratio and they suggested that it may serve as a sensitive marker parameter to probe leukemic transformation in murine systems.

The biochemical events that accompany cisplatin-induced growth inhibition and/or cytotoxicity, besides binding of the drug to nucleic acids, are largely unknown. Seeing the importance of LDH in malignancy, the present studies were undertaken to investigate the LDH activity and analyse its isozyme patterns in the tissues of normal, tumor bearing and cisplatin treated tumorous mice.

Materials and methods

Chemicals

All the biochemicals used in the assay of LDH, polyacrylamide gel electrophoresis and detection of LDH isozymes were purchased from Sigma Chemicals Co., U.S.A. Other chemicals utilized in the experiments were of analytical grade. Cisplatin, obtained from Prof. C. L. Litterst of NIH, U.S.A. as a gift, was thoroughly mixed in 0.89% NaCl in darkness 10–15 min before use. Glass double distilled water was always used in the preparation of various solutions.

Tumor maintenance

Ascites Dalton’s lymphoma is being maintained in vivo in 10–12 weeks old inbred Swiss albino mice by serial intraperitoneal (i.p.) transplantations of $1 \times 10^7$ tumor cells per animal (0.25 ml vol. in phosphate buffered saline, PBS, 0.15 M NaCl, 0.01 M sodium phosphate buffer, pH 7.4). Tumor transplanted hosts survive for 22–24 days.

Cisplatin treatment and LDH assay

Therapeutic dose of cisplatin against malignant tumors has been established to be 8–10 mg/kg body weight (Rosenberg 1985). In present studies single dose of cisplatin (8 mg/kg body wt, i.p.) was administered to four tumor bearing mice on the 10th day post-tumor transplantation which is the log phase of tumor growth. We have noted that with the regression of the Dalton’s lymphoma
very little ascites was recoverable from the hosts after 6–7 days of cisplatin treatment (Prasad et al. 1998), so the treatment schedule for 1 to 4 days only was used. After 1, 2, 3, and 4 days of cisplatin treatment (i.e. on 11, 12, 13 and 14th day after tumor transplantation) liver, kidney, blood and tumor were collected. The same tissues were collected from the control tumor-bearing hosts also which had been injected with the same volume of 0.89% NaCl. Sera were separated from the blood samples. Ascites tumor was centrifuged (2500 rpm for 10 min, at 4°C) to separate the tumor cell pellet and ascites tumor supernatant. Liver, kidney and blood serum were also collected from the normal animals without tumor, treated with 0.89% NaCl only. The cisplatin treatment and enzyme assay was repeated independently four times.

LDH activity was measured in the tissues using Sigma diagnostics enzyme determination kit following the method of Cabaud and Wroblewski (1958). Briefly, to 1 ml of supplied pyruvate substrate containing NADH (1 mg/ml), 0.1 ml of six fold diluted serum or tumor supernatant or tissue homogenate (10% in PBS, in case of tumor cells, liver and kidney) was added. It was mixed gently and incubated for 30 min at 37°C. Then, 1 ml of “Sigma colour reagent” (2,4-dinitrophenylhydrazine, 20 mg/dl in 1 N HCl) was added, mixed by swirling and allowed to stand at room temperature. After 20 min, 10 ml of 0.4 N NaOH solution was added and mixed properly. After 10 min the absorbance was read at 525 nm in the spectrophotometer. The change in absorbance (activity) in various treated groups was expressed as a percentage of control. Values given are the average of samples from 4 separate determinations.

Determination of LDH isozyme patterns

The homogenates were centrifuged at 8,000 g for 20 min at 4°C. The supernatant was used for determination of the isoenzyme patterns. LDH isozymes were separated by electrophoresis on vertical disc polyacrylamide gels following the method of Davis (1964). 50 µl of the tissue sample was mixed with equal volume of glycerol and two drops of the marker dye (bromophenol blue, 0.01%), and it was loaded on the top of the polymerized gel in the tube with the help of a syringe. After loading of the samples, the electrode buffer (0.05 M Tris-glycine, pH 8.3) was added gently in the tubes. The electrophoresis was carried at 4°C with the current of 1.5 mA/tube for 15 min, and continued later with 3 mA/tube till the marker dye reached to the bottom of the tubes. The gels were carefully removed and stained for LDH following the method of Rosalki (1974). The staining solution contained 2.5 ml of 1 M Tris-HCl buffer, pH 8.3, 0.5 ml of 1 N lithium lactate, 80 mg of nicotinamide adenine dinucleotide (NAD), 1.2 mg of phenazine methosulphate (PMS), 0.8 mg of p-nitroblue tetrazolium chloride (NBT) and 47 ml of water. The gels in the test tubes were completely immersed in the staining solution and incubated for 15 min at 37°C. Violet colour LDH bands appeared after incubation. The gels were then rinsed once with fixative (7% acetic acid), stored in fixative and photographed.

Results

LDH activity

Serum LDH activity in tumor-bearing animals was found to be more than two fold higher than that of normal animals. Cisplatin treatment of the tumor bearing hosts for 1 to 4 days resulted in further significant increase in the serum enzyme activity (Fig. 1A). In the liver of tumor-bearing hosts also LDH activity increased by about 40% of the normal animals (Fig. 1B), whereas, in the kidney of normal and tumor-bearing mice no significant variation in the enzymatic activity (Fig. 1C) was noted. Following cisplatin treatment, an overall decrease in the activity was noted in liver and kidney, although, on day 2 of treatment slight increase in the enzymatic activity was also observed. (Fig. 1B, C). In the ascites tumor supernatants of 1–2 days cisplatin-treated hosts, LDH activity was noted to be more than 2-fold higher than that of control (untreated tumorous hosts). How-
Fig. 1. Bar charts showing the changes in lactate dehydrogenase (LDH) activity in various tissues of Dalton’s lymphoma (DL)-bearing mice treated with or without cisplatin. A) Serum; note more than two fold increase in the enzymatic activity in the tumor bearing hosts than that of normal animals. Cisplatin treatment results further increase of serum LDH. B) Liver; as compared to normal mice higher LDH activity was observed in tumor bearing hosts. Note an overall decrease in LDH activity following cisplatin treatment. C) Kidney; no significant variation in the enzymatic activity was noted between the normal and tumor bearing mice. Following cisplatin treatment, overall decreased LDH activity was observed, except on the day 2 of treatment. D) Ascites supernatant; contrary to DL cells, enzymatic activity increased in the ascites supernatant following cisplatin treatment of the DL. Note the presence of about 2 fold increased activity on the day 1–2 of cisplatin treatment. E) Tumor (DL) cells; note the steady decrease in LDH activity following cisplatin treatment. N=normal mice; TB=Tumor bearing mice (control); TB cisplatin treated=Tumor bearing mice treated with cisplatin (8 mg/kg body wt); TS=Ascites supernatant; Numbers 1, 2, 3 and 4 denote the number of days after cisplatin treatment. Statistical analysis: student’s t-test, n=4, *=p<0.05; **=p<0.02; ***=p<0.01; ****=p<0.001; compared to respective untreated control TB, TS or DL.
ever, following 3–4 days of the treatment the enzyme activity in the ascites supernatant decreased (Fig. 1D). DL cells on the other hand, showed the decrease in LDH activity following 1–4 days of cisplatin treatment (Fig. 1E).

**LDH isozyme analysis**

Serum: LDH isozyme patterns showed that in serum all the five isozymes of LDH (i.e. LDH-1, 2, 3, 4, 5) were present in varying intensities. LDH-5 was noted to be maximally intense while LDH-1 was the least (Fig. 2A). In the serum of the normal animals, LDH-2 and 3 seem to be more prominently expressed than that of tumor-bearing hosts. Following cisplatin treatment of the tumor-bearing control, and 1, 2, 3, 4 days of cisplatin treated tumorous mice respectively. A) Serum, Note the presence of all the five isozymes with the predominance of LDH-5 and its increased activity after cisplatin treatment. Note the presence of new isozyme variant, named LDH-T (arrow head), in the serum of tumor bearing hosts. B) Kidney, also showing the presence of all the five isozymes with poor LDH-5 isozyme and relatively higher activity of LDH-2 and -3 isozymes. C) Liver, showing the predominance of LDH-5. LDH-4 and -3 were other other isozymes noticeable which disappeared after cisplatin treatment. Ref.-C shows the reference sample showing all isozyme bands. D) Ascites supernatant, high activity of LDH-5 and very weak activity of LDH-2, -3 and -4 is notable. Also note the appearance of very darkly stained diffused LDH-5 isozyme in cathodic region in 1 and 2 days cisplatin treated group which may suggest the presence of increased activity of LDH-5 and/or the appearance of the additional isozyme(s) variant. Ref.-D shows the reference sample showing all isozyme bands. E) Dalton's lymphoma (DL) cells, showing the presence of very darkly stained only LDH-5 isozyme. Note the increased activity after cisplatin treatment. 1–2 faint isozyme variants were also observed in the cathodic side of LDH-5. Ref.-E shows the reference sample showing all isozyme bands.
bearing hosts, LDH-5 activity (intensity) was increased. The important notable feature was the presence of one extra isozyme band, named here as LDH-T, near the cathodic position in the serum of tumor bearing hosts treated with or without cisplatin (Fig. 2A).

Kidney: In the kidney also all the five isozymes were observed with LDH-2 and 3 having more intense staining and hence reflecting comparatively higher activity. Following cisplatin treatment, no significant variation in the intensities of isozymes was observed (Fig. 2B).

Liver: In the liver, only three isozymes-3, 4 and 5 were observed of which LDH-5 is noted to be the predominant form. Interestingly, after cisplatin treatment LDH-3 and 4 intensities (activity) decreased and by the 4th day of treatment, LDH-5 appeared to be the only isozyme form present in liver (Fig. 2C VI).

Ascites supernatant: Dalton’s lymphoma ascites tumor collected on the day 4 of cisplatin treatment was very viscous, thus, the cells and supernatants collected only upto 3 days of treatment have been used for LDH isozyme analysis. In the ascites supernatant (Fig. 2D) LDH-5 was seen as the most active isozyme form. LDH-2, -3 and -4 were very faintly present. On the day 1 and 2 of cisplatin treatment the LDH-5 intensity increased very dense towards cathode region of the gel which suggests the presence of increased activity of LDH-5 and/or the appearance of the additional isozyme band (Fig. 2D III, IV). The LDH-4 isozyme activity also increased after cisplatin treatment.

Tumor cells: In the Dalton’s lymphoma cells LDH-5 was the only isozyme form observed. The activity of the isozyme increased after cisplatin treatment (Fig. 2E IV). The additional 1–2 faint isozyme bands were also noted near LDH-5 towards cathode. (Fig. 2E).

Discussion

Enzymatic changes may reflect the overall changes in metabolism that occur in malignancy (Stefanini 1985). In present study, as compared to normal animals, more than two fold increase in serum LDH activity has been observed in Dalton’s lymphoma-bearing mice (Fig. 1A) which may suggest the release of LDH in the tissues of tumor-bearing hosts. In general a higher rate of glycolysis has been reported in tumor cells (Warburg 1956, Yesher 1978), and it has been suggested that the rapid turnover of malignant cells should release ecto- and endo-enzymes into the blood stream (Stefanini 1985). The higher LDH activity in tumor-bearing mice was observed in liver also, the main site of glycolysis, (Fig. 1B) which in turn support the earlier findings. It has been suggested that the enzymatic/metabolic changes in biochemical processes aiding cancerous cells over normal surrounding tissue may be related to the aggressiveness of the tumor, as in the case of β-hexosaminidase activity in ovarian cancer and of glucose-6-phosphate dehydrogenase in carcinoma of the prostate (Zampella et al. 1982). Cisplatin treatment of the tumor bearing hosts resulted in an overall decrease in LDH activity in liver, kidney and tumor cells (Fig. 1B, C, E) which may indicate decreased synthesis and/or increased leakage of the enzyme from the cells due to injury. The second possibility may be more implicated here, since, simultaneously a sustained increase of LDH level/activity in the serum of cisplatin-treated mice was also noted (Fig. 1A). Enzymes getting into the blood after cell necrosis of certain organs have been used to indicate the degree of tissue damage. The effect of some pesticides (copper sulphate, paraquat and methidation) on fish causing tissue necrosis has been demonstrated by increased levels of LDH in blood sera (Asztalos and Nemcsok 1985). Following cisplatin treatment, the decrease in LDH activity in tumor cells was noted (Fig. 1E) with the parallel elevation of LDH in ascites supernatant which was very high on the day 1–2, but slightly lower on the day 3–4 of treatment (Fig. 1D). This may fairly indicate cell injury and/or altered membrane permeability of DL cells. It has been reported that cisplatin treatment brings about definite changes in the arrangement of surface membrane ruffles/blebs of DL cells with the appearance of membrane vesicles, alongwith a decrease in ascites supernatant protein con-
tent by 3–4 days of cisplatin treatment (Prasad and Giri 1994). It has been observed that cisplatin treatment of cultured renal epithelial cells and renal cortical slices in vitro causes the leakage of LDH from the cells into the medium and it may indicate the cell injury (Gemba and Fukuishi 1991, Kim et al. 1997).

The LDH isozymes has been used as a diagnostic aid (Rambotti and Davis 1981, Rajadhyaksha et al. 1986). Results from the present investigations showed that in the serum of normal and tumor bearing mice all the five LDH isozymes (or isoenzymes) were present with LDH-5 showing more intensity/activity than others. Also, serum LDH-2 and -3 appeared to be more expressed in normal animals than those of tumor-bearing hosts (Fig. 2A). On the other hand, in kidney, also showing all isozymes, LDH-5 was found to be the least active isozyme form whereas LDH-2 and -3 were noted to be relatively more active forms. Interestingly, in other three tissues, i.e. liver, tumor cells and ascites supernatant, distinct variations were notable in relation to number and nature of various isozymes. In the liver, only three isozymes, LDH-3, -4 and -5 were noticed with the LDH-5 being the main isozyme form. After cisplatin treatment, LDH-3 and -4 isozymes reduced markedly and by the 4th day of treatment LDH-5 was the only isozyme form noticeable in liver (Fig. 2C). In ascites supernatant also LDH-5 was observed to be the most prominent isozyme (Fig. 2D) while in tumor cells LDH-5 was the only isozyme form present (Fig. 2E). Thus, the comparative analysis of isozyme patterns very clearly showed the predominance of LDH-5 in the tumor. It has been reported that human malignant cells, both lymphoid (Rambotti and Davis 1981) and from other organs (Fleischner et al. 1981) possess LDH isozyme patterns distinguished by lower H : M ratios (i.e. predominance of LDH-5) and present observation of the predominance of LDH-5 in DL cells is in this accordance. Tumor cells have higher glycolytic activity (Warburg 1956, Yesher 1978) and it is known that LDH 5 is highly active in anaerobic glycolysis (Dawson et al. 1964). Thus, the predominance of LDH-5 may reflect the anaerobic metabolism of these tissues, since hypoxic conditions have been shown to exist in the tumors (Kallman 1972). In the serum of tumor bearing animals cisplatin treatment resulted the increased activity of LDH-5 (Fig. 2A III–VI). The increased expression of LDH-5 in DL cells and serum following cisplatin treatment (Fig. 2E, A), therefore, suggests that cisplatin treatment may further increase the hypoxic condition in the tumor. This is supported from the observations of the significant decreased oxygen consumption by DL cells treated with cisplatin in vivo (Giri 1995). In the ascites supernatant also after cisplatin treatment for 1–2 days, the LDH-5 intensity appeared very dense towards cathode region of the gel which suggests the presence of increased release of LDH-5 from the tumor cells and/or the appearance of the additional isozyme bands. It has been postulated that anaplasia is accompanied by molecular adjustments, which precede morphologic changes in malignancy and may manifest themselves through changes in enzyme patterns, thus, there is predominance of LDH-1 in normal colonic mucosa and of LDH-5 in colonic cancer tissue (Carda-Abella et al. 1982).

Another important notable feature was the presence of additional band(s) near the LDH-5 isozyme towards cathode in the serum of tumor-bearing hosts (Fig. 2A) which is also seen faintly in tumor supernatant/tumor cells, but absent in kidney and liver. This may indicate the presence of new isozyme variant and it has been tentatively designated as LDH-T (Fig. 2A) for being present in tumorous condition. The exact physiological significance of this new isozyme variant in serum of tumor bearing hosts cannot be clearly understood at present, but it could be the consequence of a physiological adaptation of tumor cells to the general hypoxic condition. It may serve as an useful marker for Dalton’s lymphoma in vivo. It has also been suggested that oncogenes may have a role in the production of variants by malignant cells (Stefanini 1985). LDH has been suggested to be a fairly sensitive marker for most solid tumors with the presence of some isozyme variants of LDH in the serum of these patients (Lippert et al. 1981).

Thus, the results from the present biochemical studies indicate that LDH activity is definitely affected in the tissues of tumor-bearing hosts and during tumor regression after cisplatin treatment.
It suggests that changes in LDH activity could be very useful parameter in malignancy and cisplatin-mediated chemotherapy. LDH isozyme patterns revealed the presence of tissue specificity of different isozymes, with only LDH-5 in tumor cells and appearance of some isozyme variants in the serum of tumor-bearing hosts.

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References


