Histochemical and Ultrastructural Study of Lactic Dehydrogenase in Chemically Induced Lung Cancer

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ABSTRACT

Light and electron microscopy studies of lactic dehydrogenase activity were carried out in embryonic, neonatal, and adult mouse lungs and in lungs undergoing chemically induced carcinogenesis. Embryonic mouse lungs were collected on the 6th, 12th, and 18th days of gestation; 1-day-old lungs were used for the neonatal model. These were compared with adult normal mouse lung and lungs of the animals treated with 4-nitroquinoline 1-oxide at a monthly interval until cancer developed. Enzymatic activity was seen in the embryonic, precancerous, and malignant lung tissues and was found diffusely in the cytoplasm of the epithelial cells.

INTRODUCTION

LDH activity is predominantly found in the skeletal muscle, heart muscle, liver, and lung of mammals (2, 6). This enzyme can be grouped into 5 isoenzymes; however, histochemical stain can effectively distinguish only the skeletal and the heart muscle types. Although lung cancer is one of the most common forms of human neoplasia, little is known of the histochemical or biochemical characteristics of lung parenchyma when cancer is developing or has developed.

To our knowledge, in spite of wide interest in LDH activity in lung cancer, no systematic study has been undertaken either on the ontogeny of LDH activity in the experimental mammalian lung or on the role of LDH in the lung parenchyma during carcinogenesis. This histochemical study was designed to investigate the lactic dehydrogenase activity in the pneumocytes in embryos, in neonates, and in adult mice and to compare these findings with cancer cells and cells undergoing carcinogenesis.

MATERIALS AND METHODS

Animals

This study was begun with 40 pregnant inbred white A/J mice (The Jackson Laboratory, Bar Harbor, Maine). Ten mice were killed on the 6th day, 10 on the 12th, and 10 on the 18th day of gestation to provide embryonic lung tissue. One-day-old mouse lung was used as the neonatal model. Mice up to the age of 6 months were divided into 2 groups of 100 each. Group 1 received injections of 4-nitroquinoline 1-oxide (K & K Laboratories, Plainview, N. Y.) as a suspension of lecithin (50 mg of the chemical, 20 ml of water, and 2 g of egg lecithin) in a dose of 0.1 ml each week for 6 weeks s.c. in the back. The total dose of nitroquinoline in each animal was 1.5 mg. Group 2 served as controls. All mice were given solid food and water.

Ten animals from Group 1 and 10 animals from the control Group 2 were sacrificed, and autopsy was performed at monthly intervals after the last injection of the carcinogen. The incidence of cancer, its metastasis, and the activity of the LDH in the pneumocytes were checked. The LDH activity in early carcinogenic lung and in the tumor tissue was compared to the normal pulmonary epithelium in the control animals.

Total LDH Activity

Reaction Medium. The reaction medium consisted of 100 mM L-lactate, 25% (3 mM) dextran (Sigma Chemical Co., St. Louis, Mo.), 18 mM NAD in 0.05 M sodium cacodylate buffer (pH 7.2). Nitro blue tetrazolium salt (0.61 mM) and phenazine methosulfate (0.10 mM) were added for light microscopy histochemistry. Thiocarbamyl nitro blue tetrazolium salt (0.50 mM; Polysciences, Inc., Warnington, Pa.) (2) and 0.10 mM phenazine methosulfate were used for electron microscope histochemistry (6). The reaction was carried out in the dark, with constant agitation for 30 min.

Selective Staining for Isoenzymes. A modified McMillan technique (7) was used for selective staining of heart and muscle LDH; 4 M urea was added to the reaction medium for inhibition of muscle LDH, and a combination of 8 mM pyruvate and 80 mM lactate was used to inhibit heart LDH.

Preparation of Tissue. The pregnant mice were anesthetized with Nembutal (40 mg/kg of body weight) injected i.p. Under sterile conditions the uteri were opened, the fetuses were removed, and the embryonic lung tissue was dissected out. Lung tissue from neonates and adult mice was obtained by standard thoracotomy.

Fresh 1- to 3-mm cubes of lung tissue were placed immediately in the cold fixative for 3 hr and were then washed for 20 hr in 0.05 M cacodilate-buffered sucrose solution containing 0.3 mM sucrose solution and 1% CaCl₂ at 4°C (pH 7.2). The blocks were then deep-frozen in liquid nitrogen, and sections 8 to 10 μm thick were cut in a cryo-
stat. For light microscopy the sections were mounted on albumin-coated glass slides, incubated in reaction medium, washed in 0.05 M cacodylate buffer (pH 7.2) containing 0.3 M sucrose, and mounted in glycerin jelly.

For electron microscopy the prefixed tissues were sectioned at 50 μm (Sorvall T. C. tissue sectioner). After incubation in the reaction medium for 3 hr, the sections were washed briefly in distilled water and then treated with 5% osmium tetroxide in 0.05 M cacodylate buffer at pH 7.2 (equal parts) for 2 hr. The sections were then dehydrated and embedded in Araldite.

Thin sections (600 Å) were cut with a diamond knife and mounted on uncoated 300 mesh copper grids. The sections were stained with an alcoholic solution of 2% uranyl acetate for 10 min and with lead citrate for 3 min. Micrographs were obtained with the RCA EMU-4 microscope at an acceleration voltage of 50 kV.

RESULTS

Light Microscopy

The total LDH activity was studied in frozen sections under the light microscope. The site of enzyme activity appeared as cytoplasmic granules. In 6-day embryos the enzymatic activity was marked (Fig. 1). In 12- and 18-day embryonic lungs, the reaction was less intense. One-day-old and adult pulmonary epithelium showed a few particles distributed throughout the cytoplasm (Fig. 2).

In lungs undergoing carcinogenesis, the first signs of muscle LDH were noted 3 months after injection of the carcinogen, at which time cellular atypism was observed. Five months after injection lung cancer developed. The LDH activity at this time increased, as in embryonic lungs (Fig. 3). The granules were scattered throughout the cells and stained intensely dark.

Electron Microscopy

Embryonic Lung. In embryonic lung the LDH reaction was observed in cells resembling the granular pneumocytes. These cells were large with prominent vesicular nuclei and undulating cell membranes. The cytoplasm contained mitochondria, ribosomes, and a few endoplasmic reticula. The LDH activity was distributed throughout the cytoplasm along with glycogen granules (Fig. 4). It is also seen scattered inside the mitochondria (Fig. 5).

Neonate Lung. The degree of LDH activity was relatively less in neonates, and the cytoplasm was rich in rough endoplasmic reticula. Occasional phagosomes and myelin bodies were also seen.

Control. The sections from both embryonic and neonatal lung tissue were studied without LDH reaction and showed moderate amount of intracytoplasmic glycogen.

Lungs of Carcinogen-treated Mice. In the precancerous state of the lung, 3 months following injection, at which time cellular atypism was confirmed by light microscopy, LDH activity was also found diffusely in the cytoplasm. Reactions were noted around the mitochondria and in the rough endoplasmic reticulum. This LDH activity became more intense when undifferentiated carcinoma developed (Fig. 6). Intramitochondrial granules were also noted in carcinomatous cells (Fig. 7). In control adult lung this enzymatic activity was almost absent (Fig. 8).

DISCUSSION

Fahimi and Karnovsky (4) stressed the importance of the fixative in the histochemistry of LDH, which is loosely attached, readily soluble enzyme. Baba and Sharma (2) tested various fixatives and concluded that 2% buffered glutaraldehyde fixative was able to reproduce the localization of LDH as reported by others (2). The observations made herein were consistently reproducible in several sets of experiments.

The cellular localization of LDH activity was intense in 6-day embryos, and frequently details of cytoplasmic localization of LDH activity could not be properly ascertained. However, in sections where the localization could be defined, it was commonly found in the perinuclear portion of the cytoplasm of the epithelial cells.

With gradual maturity of the fetus, at 12 and 18 days, the degree of localization of LDH diminished and enzyme activity was sparse. This decrease continued throughout neonatal and adult life, and in fully adult mice there was almost no discernible activity.

From 12 days onward the cells could be identified as granular pneumocytes, and it was apparent that the enzyme activity was found primarily in granular pneumocytes of developing lung. This observation was confirmed in neonatal and adult animals inasmuch as all enzyme activity was found in granular pneumocytes, although localization was sparsely visible. It seemed that, as the endodermal cells began to acquire features characteristic of type 2 pulmonary epithelial cells (3, 5), LDH activity decreased.

The animals that received 4-nitroquinoline 1-oxide for production of pulmonary cancer showed similarly increased muscle LDH activity from 3 months onward; after carcinoma developed the muscle LDH activity was found to be intense.

The degree of LDH isoenzyme activity is of considerable interest in the study of carcinogenesis of the lung. In a well-controlled biochemical study, Yamane et al. (7) determined the LDH isoenzyme in mouse lung cancer produced by 4-nitroquinoline 1-oxide. They found that LDH activity in the lung increased to 1.7 times that of normal. In the tumor itself the activity was 2.5 to 3.5 times higher than in control lung tissue. LDH activity was high in the undifferentiated state of fetal lung tissue and in the immature granular pneumocytes, as well as in precancerous and cancerous pulmonary epithelium. This observation closely corresponds to the biochemical study of Yamane et al. (7) in lung cancer.

It has been shown that muscle LDH is usually present in tissues that are more active in anaerobic metabolism. Heart LDH is found largely in tissues utilizing aerobic pathways, which utilize fatty acids as a source of energy. Muscle LDH may be associated with greater conversion of pyruvate to lactate and may allow less pyruvate for the Krebs cycle than does heart LDH. This observation suggests that chemically induced lung carcinoma perhaps utilizes anaerobic glycolytic pathways similar to those of very embryonic cells.
with predominant muscle LDH activity. This observation might be useful for further studies in this line.

REFERENCES

Fig. 1. Six-day-old embryo. LDH showing densely stained granules. Frozen section, × 280.
Fig. 2. Adult pulmonary tissue. Very little LDH activity. Frozen section, × 280.
Fig. 3. Mouse lung 5 months after injection of 4-nitroquinoline 1-oxide. Increased LDH activity. Frozen section, × 280.
Fig. 4. Electron micrographs of 6-day-old embryo. Glycogen (Gly) and LDH granules are seen. LDH granules, as seen (arrows), are darker around perinuclear portion and around mitochondria. G, Golgi; m, mitochondria. × 19,624.4.
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Figs. 5-7. All unidentified arrows are LDH.

Fig. 5. Electron micrograph of 6-day-old embryo. M, mitochondria; Er, endoplasmic reticulum. × 59,220.

Fig. 6. Electron micrographs of lung tumor induced by 4-nitroquinoline 1-oxide. M, mitochondria; Er, endoplasmic reticulum; LB, lamellar body; G, Golgi; MvB, microvesicular body. × 19,524.4.

Fig. 7. Electron micrographs of mouse lung tumor. M, mitochondria; G, Golgi. × 60,480.

Fig. 8. Electron micrographs of adult mouse lung. Nuc, nucleus; M, mitochondria; Er, endoplasmic reticulum; Mv, microvilli; TJ, tight junction; Pinocytosis vesicles. × 21,000.
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