Location of Adult and Fetal Aldolases A, B, and C by Immunoperoxidase Technique in LF Fast-growing Rat Hepatomas

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ABSTRACT

The resurgence of aldolase isozymes in cancerous tissues is a well-known but poorly understood phenomenon. This resurgence poses the problem of whether or not adult and fetal aldolase isozymes are produced by the same cells.

For clarification of this question, the immunoperoxidase technique was used to locate aldolases A, B, and C in one type of fast-growing hepatoma, the LF hepatoma and, by comparison, in normal adult liver. Under optical microscopy, aldolases A and C were located in the cytoplasm of almost all of the cancerous cells. An isozyme antigenically identical with aldolase B was also demonstrated to be present in almost all of the cells, but the reaction indicating the presence of this isozyme was weaker. In normal adult liver, only aldolases A and B were demonstrated to be present in almost all the hepatocytes. Under electron microscopy in LF hepatoma, the three isozymes were found to be present mainly in the cytoplasm.

These facts suggest that the three types of aldolase are very present in the same cells at the same time, and they provide indirect arguments leading us to think that the resurgence of fetal aldolase isozymes in cancer is not the consequence of cellular selection but is due to a disturbance at the gene control level.

INTRODUCTION

The resurgence of fetal isozymes in cancer is a well-known phenomenon demonstrated first for aldolase (33) and later for several other enzymes (25, 32, 35). This resurgence poses a problem. Do the cells that normally synthesize the adult isozyme become capable of synthesizing the fetal type during carcinogenesis? In this case genes not expressed normally would become expressed in the cancerous state. On the other hand, are the cells that synthesize fetal isozymes different from the cells that synthesize the adult type? In this case these cells would be fetal cells selected during carcinogenesis.

It is difficult to demonstrate whether both adult and fetal proteins are synthesized by the same cells or by different cells. First, we need an adult counterpart to the fetal enzyme. This is the case with aldolase, in contrast to α-fetoprotein for instance. Aldolase B is the predominant enzyme in adult liver, and aldolases A and C are plentiful in fetal liver (18) and in fast-growing hepatomas (34). Secondly, histological methods specific for aldolase must be used. Several of these methods have already been described (1, 22, 29). However, they demonstrate only that aldolase is present in the cells; they do not give any information on the nature of the isozyme. Immunoenzymatic techniques are probably superior because of their specificity; in addition, they provide information on the ultrastructural location of these isozymes.

In this study, we demonstrate that in 1 type of solid fast-growing hepatoma, the LF hepatoma, aldolases A, B, and C are present in almost all of the cells. Using the immunoenzymatic technique (immunoperoxidase), we were able to infer that the 3 isozymes are probably synthesized by the same cell.

MATERIALS AND METHODS

Material

The LF hepatoma cells of Lafarge and Frayssinet stemmed from a clone of fast-growing tumor cells obtained from a 4-dimethylaminobenzene-induced hepatoma (5, 11). Injection of these cells i.p. into 8-day-old Wistar-Commery rats produced tumors within 4 days. The animals were killed 6 to 7 days after inoculation, and their tumors were removed at once. Normal adult liver was obtained from adult Wistar C-Commery rats. A fragment from each tissue was taken for immunological studies; another was fixed for morphological studies.

Immunological Methods

Purification of Enzymes. Aldolase A was prepared from rat muscle according to the method of Gracy et al. (13). The extracted supernatant was brought to 45% saturation with saturated ammonium sulfate. This solution was centrifuged at 37,000 x g for 1 hr, and the precipitate was dissolved in 10 mM Tris-HCl buffer, pH 7.5, with 1 mM EDTA-10 mM β-mercaptoethanol. The solution was then applied to a cellulose phosphate Whatman column equilibrated with the same buffer. Elution was performed with a linear NaCl gradient (0 to 350 mM) in 10 mM Tris-HCl buffer, pH 7.5-1 mM EDTA-2.5 mM fructose 1,6-diphosphate. The purity of the protein was checked by acrylamide gel
Aldolase B was prepared from rat liver. It was purified in the same manner as was aldolase A with the following differences. The 45 to 65% ammonium sulfate fraction was applied to a Whatman CM 52 cellulose column. The elution was performed with 2.5 mM fructose 1,6-diphosphate in 10 mM Tris-HCl buffer, pH 7.5-1 mM EDTA. The protein was completely purified by isoelectrofocusing in a LKB 8101 column filled with 1% ampholines (pH 9 to 11). The purified protein showed only 1 band on polyacrylamide gel electrophoresis with or without sodium dodecyl sulfate. The specific activity with fructose 1,6-diphosphate was 2.5 IU/mg; with fructose 1-phosphate, it was 2.5 IU/mg; thus the activity ratio of both substrates was 1.0.

Aldolase C was prepared from rat brain (15). It was purified as for aldolase A with the following differences. The 45 to 65% ammonium sulfate fraction was applied to a Whatman DEAE 52 cellulose column. The elution was performed with 0.5 mM fructose 1,6-diphosphate-150 mM NaCl in 10 mM Tris-HCl buffer-1 mM EDTA, pH 7.5. The enzyme was further purified by electrophoresis on a potato starch block and then by isoelectrofocusing in a LKB column 8101 filled with 1% ampholines (pH 4 to 6). The purity of the protein was checked by electrophoresis and by determination of the NH2-terminal amino acid. The specific activity with fructose 1,6-diphosphate as substrate was 6.0 IU/mg; with fructose 1-phosphate, it was 0.8 IU/mg. The aldolase C activity ratio was 7.5 (15).

Preparation of Antisera. Antisera to aldolases A, B, and C were prepared in rabbit by immunization with pure crystalline enzymes. The first injection of purified aldolase [1 mg in 1 ml of phosphate-NaCl buffer with an equal volume of complete Freund’s adjuvant (Difco Laboratories, Detroit, Mich.)] was made into the hind footpads. Ten days later, i.m. and s.c. injections were made, followed 10 days after by an i.m. injection. After the last injection, antisera were tested by immunodiffusion against antialdolase antibodies, and C antisera were absorbed by normal rat serum and brain.

After a washing, the immunodiffusion and immunoelectrophoresis plates were stained by the method of Penhoët et al. (30). Monospecific antisera were obtained by absorption with the use of normal rat tissue extracts (7). Anti-aldolase A and C antisera were absorbed by normal rat serum and liver; anti-aldolase B antiserum was absorbed by normal rat serum and brain.

Morphological Methods

We used a method similar to that used to demonstrate albumin in human liver cells (9). Rat tumor and normal adult liver fragments were fixed for 10 hr at 4° in a solution of 2.75% paraformaldehyde with 0.2% picric acid buffered with 100 mM phosphate buffer, pH 7.4 (37). The conservation of the immunological structure of fixed aldolase was demonstrated in vitro by adding this fixative to purified aldolase under the same conditions as those used in tissue fixation. When, after dialysis, the fixed preparation was tested by immunodiffusion against antialdolase antibodies, no difference in immunological reactivity between it and unfixed aldolase was detectable.

After fixation, tissue fragments were washed 2 times in phosphate buffer with 250 mM sucrose for 48 hr at 4°. One hr before cutting, the fragments were immersed in a 10% glycerol solution with 100 mM phosphate buffer, pH 7.4. Sections (8 μm thick) were cut with a cryostat and divided into 4 groups, 3 groups being incubated for 1 hr at room temperature with a different aldolase antiserum (1/25 dilution) and the fourth group acting as control. The sections were then washed 3 times, 20 min each, and incubated with sheep anti-rabbit γ-globulin antibodies labeled with peroxidase (Institut Pasteur, Paris, France). Sections were washed again (3 times, 20 min each), and the presence of the peroxidase was revealed by the technique of Graham and Karnovsky (14). Some sections (LF hepatoma and normal adult liver) were studied on optical microscopy. Others (LF hepatoma only) were postfixed in a 1.5% osmium tetroxide solution buffered with Veronal, pH 7.2-50 mM buffer for 1 hr and embedded in Epon. Ultrathin sections were examined in a Siemens Elmiskop IA electron microscope without further staining.

Control reactions were prepared as follows. Some sections were incubated with normal rabbit serum and then incubated with labeled sheep anti-rabbit γ-globulin antibodies. Other sections were incubated in the Graham and Karnovsky solution only (14), and still others were incubated with anti-aldolase antiserum absorbed by the corresponding antigen and then incubated with labeled sheep anti-rabbit γ-globulin antibodies.

Identification of Tumor Aldolases

Tissue aldolases in the supernatant of hepatomas were detected by immunodiffusion. Enzymes were extracted with either 0.5 or 4 ml of 150 mM NaCl in water per g of tissue and centrifuged at 37,000 × g for 30 min; the supernatants were put into wells around a central well containing monospecific antiserum. After the appearance of precipitation lines, immunodiffusion plates were washed and then stained by the method of Penhoët et al. (30).

RESULTS

Morphological Results

Light Microscopy. In LF hepatomas both aldolases A and C appeared as dark brown deposits located in the cytoplasm of the cells, whereas the nucleus remained unstained (Figs. 1 and 2). Almost all the cells contained aldolases A and C. However, stain intensity varied from cell to cell. No aldolase was detected in the connective tissue. Similar results were obtained for the intracellular distribution of aldolase B; however, the intensity of the staining was weaker than that observed for aldolases A and C (Fig. 3). Because of the thickness of the sections (8 μm) and the
size of the cells, the superposition of 2 cells cut at different levels can sometimes give the appearance of a "positive" nucleus for aldolase; hepatoma cells have an average diameter of about 10 μm and their nucleoplastic ratio is high. The control reactions were negative (Fig. 4).

In normal adult liver, aldolase B was detected in the cytoplasm of almost all the hepatocytes. The reaction appeared very strongly as dark brown granulated deposits (Fig. 5). Aldolase A was also located in the hepatocyte cytoplasm, but the intensity of the reaction was very much weaker than that for aldolase B (Fig. 6). By contrast, no reaction was detectable in the cytoplasm of the hepatocytes with anti-aldolase C antisera (Fig. 7). The control reactions were negative (Fig. 8).

Electron Microscopy. Results for aldolases A, B, and C in LF hepatomas were similar; the electron-dense products of the reaction indicating the presence of aldolase were located in the whole of the cell cytoplasm; a positive reaction was sometimes observed on the membranes of the rough endoplasmic reticulum; however, in LF hepatomas these organelles are not abundant. No reaction was visible in the nucleus or in the mitochondria, except for a slight reaction on the external membranes (Figs. 9 to 11). All the control reactions were negative (Fig. 12).

Immunological Results

Immunodiffusion plates showed precipitation lines between normal rat tissues and hepatoma aldolases A and C and the corresponding aldolase antiserum. As demonstrated in a previous study (19), this confirms the identity between aldolases A and C from LF fast-growing hepatoma and from normal rat tissue (muscle, brain, and fetal liver). The precipitation lines given by fetal liver (with 4 ml of 150 mM NaCl per g of tissue) proved the presence of aldolase A and C in this tissue. On the contrary, adult liver extract at the same concentration and at a stronger concentration (0.5 ml of 150 mM NaCl per g of tissue) did not show any precipitation lines with anti-aldolase A and C antisera (Fig. 13).

With anti-aldolase B antiserum, no precipitation line was obtained with hepatoma extracts at a dilution identical with that used for other normal rat tissues (with 4 ml of 150 mM NaCl per g of tissue) (Fig. 14). A precipitation line was obtained only with concentrated LF hepatoma supernatant extracts (with 0.5 ml of 150 mM NaCl per g of tissue). Between the precipitation lines of normal rat adult liver andLF hepatoma, a spur was visible. This line could not be specifically stained by tetrazolium salt reduction (30).

DISCUSSION

This is the first time that the 3 types of aldolase have been located in the cytoplasm of cells of a fast-growing hepatoma, the LF hepatoma.

Immunofluorescence (8, 20, 42) and immunoperoxidase techniques (10, 31, 42) with the use of monospecific antibodies to locate cellular antigen are already in use. The immunoperoxidase technique is very sensitive since it enables detection of an enzyme by electron microscopy, whereas the histochemical techniques used particularly for aldolase do not (1, 22, 29). However, as pointed out by several authors (3, 42), one of the main problems of the immunoperoxidase technique is to preserve protein antigenicity. Indeed, our tests for the immunological characteristics of the fixed protein showed that antigenicity is conserved.

With optical microscopy, aldolases A, B, and C were identified in the cytoplasm of almost all the cancerous cells. By contrast, only aldolases A and B were identified in normal adult liver; moreover, the reaction for aldolase A is much weaker than that for aldolase B. The small quantity of aldolase A in rat adult liver, already described by other authors (24, 26) and estimated as less than 1%, was not detected by the immunodiffusion technique. This fact emphasizes the sensitivity of the immunoperoxidase technique.

Electron microscopy demonstrates clearly that aldolases A, B, and C are always located in the cytoplasm, not in the nucleus. The fact that a small amount of aldolase was found on the endoplasmic reticulum membranes could be due to its passive absorption on these membranes. The occasional presence of aldolase on the mitochondrial membranes could be similarly explained. Consequently, our results in LF hepatomas disagree with the results obtained by Foemmel et al. (10), who found that aldolase B was mainly associated with the endoplasmic reticulum in normal adult rat liver. However, we confirm the more recent experiments of Arion and Lange (2), who have demonstrated that aldolase B is not associated with the endoplasmic reticulum but is located in the soluble supernatant fraction obtained after ultracentrifugation of the rat adult liver homogenate.

Morphological results indicate that aldolase B is present probably in a small amount in LF fast-growing hepatomas. This is shown by the weak labeling with anti-aldolase B antiserum. Immunodiffusion experiments confirm these results and show the loss of enzymatic activity. The spur between normal adult liver and hepatoma extract indicates that some antigenic determinants present in normal adult liver aldolase B are not present in the hepatoma enzyme. It seems that a "cross-reacting material" for aldolase B occurs in hepatoma tissues. This could be the result of a very low rate of synthesis. The few synthesized molecules would be partly degraded before their disappearance and could be the result of a very low rate of synthesis; they could be detected because they had kept a part of their antigenic determinants. Other authors have described the appearance of a spur when normal and degraded proteins were studied with the same antiserum (23, 27, 29).

Our results provide indirect evidence that leads us to think that some cells contain aldolases A, B, and C at the same time and that the resurgence of fetal aldolases A and C is not the consequence of stem cell selection. The cellular origin of fetal antigens that appear during carcinogenesis has been much disputed especially for α-fetoprotein (4, 6, 12, 28, 29, 40). Using immunofluorescence and radioactive labeling techniques, some authors have found α-fetoprotein in specialized cells (6, 40), whereas some others do not distinguish these cells from hepatocytes (12, 28). The results recently obtained by Kuhlman (21), who used immunoperoxidase, do not point out any difference...
between the labeled and unlabeled cells.

Are the genes of fetal aldolases expressed in fetal liver by fetal hepatocytes only? We still cannot completely exclude the possibility that fetal aldolases are also synthesized by hematopoietic cells. Indeed the study of fetal liver sections by the immunoperoxidase technique is difficult because of the presence of endogenous peroxidase in the hematopoietic cells. It has been reported that it is difficult to inhibit this peroxidase completely (38). The question could be clarified by separating the different fetal liver cell types, but this procedure was beyond the aim of this study.

In conclusion, our results support the view that the same cells synthesize both the fetal types of aldolases A and C and the normal adult type B; consequently, in hepatoma there is an abnormal expression of normally "silent" genes. Thus our findings provide new arguments for a "dysfunction of gene control" in cancer (41).

REFERENCES


Figs. 1 to 3. Light microscopy; immunoperoxidase; LF hepatomas. The products of the reaction indicating the presence of aldolase are represented by dark brown deposits located in the cytoplasm of almost all the cancerous cells. Fig. 1. With anti-aldolase A antiserum. × 570. Fig. 2. With anti-aldolase C antiserum. × 570. Fig. 3. With anti-aldolase B antiserum. The intensity of the reaction is weaker for this isozyme. × 570.

Fig. 4. Light microscopy; immunoperoxidase; LF hepatomas. Control reaction. No dark brown deposits are visible in the cytoplasm of the cells. × 570.

Figs. 5 to 7. Light microscopy; immunoperoxidase; normal adult liver. The products of the reaction indicating the presence of aldolase are visible in almost all the cells. Fig. 5. With anti-aldolase B antiserum. × 340. Fig. 6. With anti-aldolase A antiserum. × 340. Fig. 7. With anti-aldolase C antiserum. No reaction is visible in the cytoplasm of the hepatocytes for this isozyme. × 340.

Fig. 8. Light microscopy; immunoperoxidase; normal adult liver. Control reaction. No reaction is visible in the cytoplasm of the hepatocytes. × 340.

Figs. 9 to 11. Electron microscopy; immunoperoxidase; LF hepatomas. The products of the reaction indicating the presence of aldolase are represented by electron-dense deposits, which are exclusively located in the cytoplasm of the cells and not in the nucleus. Fig. 9. With anti-aldolase A antiserum. × 20,000. Fig. 10. With anti-aldolase B antiserum. × 23,000. Fig. 11. With anti-aldolase C antiserum. × 16,000. The reaction is homogeneous for aldolases A and C and heterogeneous for aldolase B. N, nucleus; M, mitochondria; ER, endoplasmic reticulum.

Fig. 12. Electron microscopy; immunoperoxidase; LF hepatomas. Control reaction. No electron-dense products are visible in the cytoplasm of the cells. N, nucleus; M, mitochondria; ER, endoplasmic reticulum. × 23,000.

Fig. 13. Ouchterlony double-diffusion with anti-aldolase A antiserum (a) and anti-aldolase C antiserum (b). Center well: anti-aldolase A antiserum (a); anti-aldolase C antiserum (b). Well 1, adult brain; Well 2, LF fast-growing hepatomas; Well 3, muscle; Well 4, adult liver; Well 5, adult liver; Well 6, fetal liver. Extracts 1, 2, 3, 5, and 6, 4 ml of 150 mM NaCl in water per g of tissue. Extract 4, 0.5 ml of 150 mM NaCl in water per g of tissue.

Fig. 14. Ouchterlony double-diffusion with anti-aldolase B antiserum. Center well, anti-aldolase B; Wells 1, 3, and 5, normal adult liver; Well 2, fetal liver; Well 4, normal adult brain; Well 6, LF fast-growing hepatomas; Extracts 1 to 5, 4 ml of 150 mM NaCl in water per g of tissue; Extract 6, 0.5 ml of 150 mM NaCl in water per g of tissue.
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