Fetal Pattern of Aldolase in Transplantable Hepatomas

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With the technical assistance of Claudine Gregori

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SUMMARY

Solid, transplantable, well-differentiated (slow-growing) Hepatomas 122, 175, and 189, and poorly differentiated (fast-growing) Hepatoma 178 were studied for aldolase activity. Aldolase A (muscle type), which is relatively abundant in fetal liver, was increased in slow-growing rat hepatomas. In addition to aldolase A, aldolase C (brain type) hybridized with aldolase A was present in the fast-growing hepatoma. Aldolase C was present in fetal liver and transplanted hepatoma, but it was absent in normal adult liver, as shown by kinetic, electrophoretic, and immunological studies (action of antiserum anti-aldolase C). The resurgence of transplanted hepatoma, but it was absent in normal adult liver, as shown by kinetic, electrophoretic, and immunological studies (action of antiserum anti-aldolase C). The resurgence of fetal forms has been described for other enzymes in cancerous tissues, and it is not specific in carcinogenesis. We propose that, in some pathological states, the biosynthesis of the adult form of enzymes is preferentially repressed, while the fetal form would be derepressed.

INTRODUCTION

Aldolases A, B, and C are true isozymes. Their synthesis is determined by different genes, and they may be synthetized by the same cell, as shown by the formation of hybrids. In the brains of mammals, aldolases A and C are hybridized and form 5 isozymes. Aldolases A, B, and C appear to be tetrameric molecules (17).

Aldolase A (muscle type) acts primarily on FDP and slightly on F1P. Its activity ratio is >50. Aldolase B has the same activity for the 2 substrates (FDP/F1P = 1). Aldolase C has an activity ratio for FDP/F1P between 5 and 8, depending on the species. By electrophoresis, aldolase C, the most negatively charged, migrates towards the anode. Migration of aldolase B varies with the species.

Substrate specificity of aldolases (EC 4.1.2.13 and EC 4.1.2.7) is abnormal in human and experimental hepatomas.

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4 The abbreviations used are: FDP, fructose 1,6-diphosphate; FIP, fructose 1-phosphate; DEAE-cellulose, diethylaminoethyl cellulose; 3'MeDAB, 3'-methyldimethylaminoazobenzene.

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The aldolase activity ratio was between 3 and 13 in human hepatoma and between 20 and 30 in rat ascitic hepatoma, while it is 1.0 or very close to 1.0 in normal liver (29, 33). One of us had suggested that hepatoma contains not only liver-type aldolase [termed aldolase B by Penhoet et al. (18)] but also muscle-type aldolase (aldolase A). The abnormal aldolase in hepatomas was also compared to the fetal aldolase. These studies have been continued, and the results have been confirmed (11, 25).

Recently, evidence for the presence of aldolase A in Novikoff hepatoma was reported by Brox et al. (2, 7) on tryptic fingerprints of the tumor enzyme. A preliminary report described the presence of aldolase C (brain type) in fetal liver and poorly differentiated hepatoma 178 (32). The purpose of this report is to describe new studies concerned with aldolases A and C in hepatomas.

MATERIALS AND METHODS

Hepatomas were induced in ACI/N rats ingesting 0.025% N-2-fluorenyldiacetamide in the diet (22). They were poorly differentiated (fast-growing) or well-differentiated (slow-growing) hepatocellular carcinomas (23).

Hepatoma 178. This poorly differentiated carcinoma developed in an 8-week-old male rat given the carcinogenic diet for 4-week periods, alternated with 1 week on the basal diet, until 12 weeks of carcinogen administration were completed. The animal was killed at 52 weeks.

Hepatoma 122. The well-differentiated carcinoma was from a 12-week-old female rat ingesting the carcinogen-containing diet continuously for 52 weeks.

Hepatoma 175. The primary carcinoma was observed in a male rat 12 weeks of age receiving the carcinogen-containing diet for a total of 16 weeks (alternated with the basal diet as described for Hepatoma 178). The animals were killed 65 weeks after the start of the diet. Histologically, the carcinoma was well differentiated.

Hepatoma 189. This well-differentiated hepatoma came from a castrated 4-week-old rat given carcinogen as for Hepatoma 175. The animal was killed at 52 weeks.

Normal liver was obtained from control adult ACI/N rats without transplants. Host liver from animals with Hepatoma 178 was also used. Fetal livers were removed between the 16th and 21st day of gestation, and fetal age was estimated from the length of the fetus.

Rats were decapitated. Tissues were extracted in the cold in
Aldolase activity was measured according to the colorimetric method of Sibley and Lehninger (35), which we have adapted to the use of F1P as substrate (24) (the concentration of F1P was 6.5 x 10^{-2} M). Aldolase A was prepared from rabbit muscle by the method of Taylor (37). Aldolase B was prepared from rabbit liver, by the method of either Morse and Horecker (12) or Penhoet et al. (15). Aldolase C was prepared from bovine brain by the method of Rensing et al. (21) with slight modifications. After fractionation by ammonium sulfate, the precipitate was dissolved in 5 x 10^{-2} M Tris-HCl buffer, pH 7.5, with 10^{-3} M EDTA and 5 x 10^{-4} M FDP. The solution was applied to a column of Sephadex G-100; the elution was performed with the same buffer (with substrate FDP), and the pooled fractions were applied to a column of DEAE-cellulose 11. Elution was performed with a linear gradient of NaCl (0 to 0.35 M); aldolase C is collected at the end of the elution. Nevertheless, this peak still contains hybrids AC with pure tetramer C4; consequently, a 2nd chromatographic purification on DEAE-cellulose column was performed, with the same NaCl gradient for elution.

Preparation of aldolase C from hepatomas was performed according to the same technique, but with only 1 chromatography on DEAE-cellulose column.

Antisera to A, B, and C aldolases were prepared in chickens by repeated injections of crystalline aldolases A, B, or C, with or without complete Freund adjuvant. Antisera show a strict type specificity (16) but no significant species specificity; for example, antisera against aldolase C, and prepared from bovine brain, react with rat aldolase C but not with bovine aldolases A or B. The antisera were tested by the immunodiffusion test of Ouchterlony in agar with 4% NaCl.

Inhibition tests were performed on tissues extracted in 0.01 M Tris-HCl with 10^{-3} M EDTA and 4% NaCl solution. Extracts at final comparable concentration were incubated at 37°C for 1 hr and then at 4°C for several hours with 100 µl antisera. After centrifugation at high speed, supernatants were estimated for aldolase activity by comparison with the same extracts with normal chicken serum.

In experiments of inactivation by anti-C, antisera anti-C were tested not only directly with the extract but also with the supernatant obtained after action of anti-A (because aldolase C is hybridized with aldolase A).

Electrophoresis was performed on starch gel with phosphate citrate buffer containing 0.01 M β-mercaptoethanol (pH 7.0) for 18 hr at 4°C with a potential gradient of 3 V/cm.

After migration, isozymes were stained for aldolase activity by reduction of tetrazolium salts, coupling with glyceraldehyde-3-P dehydrogenase, with the coenzyme NAD. The reaction specificity was tested by omitting the substrate in the mixture. In some experiments, the isozymes were revealed by defluorescence of NADH in an α-glycero-P dehydrogenase-coupled reaction (31). This procedure eliminates possible errors due to some nonspecific dehydrogenases with NAD as coenzyme.

RESULTS

Table 1 summarizes the results for aldolase activities with the 2 substrates (FDP and F1P) of normal rat liver, fetal rat liver, and transplantable hepatomas of different types. The

### Table 1

Aldolase activities in normal and fetal liver and in transplantable hepatomas (mean ± S.E.)

<table>
<thead>
<tr>
<th>Rat tissues</th>
<th>(I.u./g fresh tissue at 37°C)</th>
<th>Ratio (FDP/F1P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver [12]</td>
<td>16.1 ± 2.2</td>
<td>16.1 ± 2.2</td>
</tr>
<tr>
<td>Muscle [8] (gastrocnemius)</td>
<td>168 ± 12</td>
<td>4.76 ± 0.26</td>
</tr>
<tr>
<td>Brain [8]</td>
<td>19.0 ± 2.2</td>
<td>0.76 ± 0.1</td>
</tr>
<tr>
<td>Fetal liver</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15th–17th day [6]</td>
<td>5.95</td>
<td>1.52</td>
</tr>
<tr>
<td>18th–19th day [8]</td>
<td>5.42</td>
<td>2.17</td>
</tr>
<tr>
<td>20th–21st day [6]</td>
<td>5.75</td>
<td>4.0</td>
</tr>
<tr>
<td>Slow-growing hepatomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma 122 [6]</td>
<td>10.0</td>
<td>8.1</td>
</tr>
<tr>
<td>Hepatoma 175 [10]</td>
<td>7.65 ± 0.66</td>
<td>6.44 ± 0.9</td>
</tr>
<tr>
<td>Hepatoma 189 [14]</td>
<td>13.0 ± 0.68</td>
<td>7.6 ± 0.56</td>
</tr>
<tr>
<td>Fast-growing hepatomas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hepatoma 178 [16]</td>
<td>18.8 ± 1.58</td>
<td>0.59 ± 0.04</td>
</tr>
<tr>
<td>Host liver [8]</td>
<td>14.9 ± 1.56</td>
<td>14.5 ± 1.65</td>
</tr>
</tbody>
</table>

*Muscle and brain included for comparison.

*No. in brackets, no. of experiments for each group.
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aldolase activities of rat muscle and brain and the aldolase activity ratio for pure aldolases (of rabbit or cattle) are also given for comparison. The mean ratio of normal rat liver is 1.0; this ratio of 1.0 or close to 1.0 is constant in normal liver.

The FDP/F1P aldolase activity ratios found in brain (24.5) and in muscle (36.2) fit well with the data of other authors.

In fetal liver, the aldolase activity toward FDP was relatively high with an aldolase activity ratio different from that of adult liver. This ratio decreases with the length of gestation: it is between 3.0 and 6.0 at the 16th to 17th day; between 2.0 and 3.0 at the 18th to 19th day; and between 1.2 and 1.5 at the 20th to 21st day, i.e., near term. The FDP activity remains constant, while F1P activity increases.

The most interesting results were concerned with the aldolase activities of the hepatomas. The contrast between the 2 types of hepatomas was striking. In the slow-growing hepatomas, the aldolase activity ratio, although not absolutely normal, was nevertheless changed very little: 1.76 ± 0.08 for Hepatoma 189; 1.28 ± 0.15 for Hepatoma 175; and 1.25 for Hepatoma 122. Hepatoma 189 was the most abnormal among the slow-growing hepatomas. The total aldolase activity was lowered in Hepatoma 122 (mean 10.0) and particularly in Hepatoma 175 (mean 7.65). The difference from the aldolase activity of normal liver was significant (p < 0.01).

Hepatoma 178 (fast-growing) was quite abnormal. The total aldolase activity was close to the normal; but the aldolase activity ratio was considerably increased, at 32.2 ± 2.3 instead of 1.0 ± 0.02. The aldolase activities appeared to be normal in host liver, with an aldolase activity ratio of 1.03.

Electrophoresis experiments strengthened the results by demonstrating the abnormal isozymic pattern of aldolase in fast-growing hepatomas (Fig. 1). In muscle, there was a unique band, anodic but near the origin, corresponding to aldolase A. In brain, 5 bands of activity were discernible. According to the work of Penhoet et al. (18), they correspond successively (from the origin to the most anodic band) to the pure tetramer A4; the 3 hybrids A3C1, A2C2, and A1C3; and the pure tetramer C4. The band corresponding to the aldolase of normal liver, aldolase B, is cathodic.

Both cathodic and anodic bands were seen in fetal liver. The cathodic isozyme corresponded to aldolase B. It is possible to hypothesize that the 3 anodic isozymes are hybrids of C and A aldolases.

Moreover, in the fast-growing hepatomas, there was an isozymic pattern quite different from the normal one. No cathodic band was observed. There were instead 5 anodic bands (of which the last 2 were quite weak, but nevertheless distinct). Their migration was identical to the migration of the brain aldolase, with the exception that there were more slow bands in the hepatoma than in the brain.

Fig. 2 gives another example of the pattern of fast-growing Hepatoma 178, with the same anodic bands. By contrast, the aldolase patterns of 2 slow-growing hepatomas, Hepatoma 122 and Hepatoma 175, were quite similar to that of the normal liver.

Fig. 3 shows hybrids between aldolase B and aldolase A in slow-growing Hepatoma 189.

The results of the immunological studies are summarized in Fig. 1. The pattern of aldolase isozymes in muscle, brain, normal liver, fetal liver, and Hepatoma 178.
Aldolase Fetal Pattern in Transplantable Hepatomas

Table 2. The degree of inhibition by each specific antiserum corresponded roughly to that expected from the electrophoresis pattern of each organ. Aldolase activity of brain was almost completely inhibited by anti-A, although the brain contains aldolase C with aldolase A (as seen with electrophoresis and as is generally accepted). It must be remembered, however, that these 2 aldolases are hybridized together in brain and that antiserum anti-A generally reacts with hybrids of 2 types as well as with the pure type. Fetal liver actually contains the 3 types of aldolases, A, B, and C, as shown by the percentage of inhibitions by the 3 antisera: 40 to 80% by anti-A; 40 to 60% by anti-B; and 17 to 40% by anti-C.

Hepatoma 178 contained practically no hepatic aldolase (aldolase B). Instead, this fast-growing hepatoma did contain aldolase A and C, as shown by the strong inhibition caused by the specific antisera. There was 85 to 90% inhibition of aldolase activity with antiserum anti-A. With antiserum anti-C, there was a mean percentage of inhibition of 30 when the antiserum reacted directly with the tissue extract. When anti-C was tested on supernatants after precipitation by anti-A, the mean percentage of inhibition was increased to 75.

There was 50 to 70% inhibition of FDP activity with antiserum anti-aldolase A and 70 to 80 with anti-B, in the slow-growing hepatomas. The turnover number of aldolase A is about 10 times that of aldolase B (16) so that the greatest part of FDP activity in these hepatomas is due to the aldolase A, although the real amount of aldolase B is greater (as shown by the relative F1P activity). For example, an aldolase activity ratio FDP/F1P) equal to 2 corresponds approximately to an amount of aldolase B 10 times that of aldolase A. This fact explains why antiserum anti-aldolase A inhibits 50 to 70% of FDP activity in Hepatoma 189, although the amount of aldolase A protein in these hepatomas is probably only about 10%. Also, these antisera act against the hybrids A-B.

The results of the partial purification of aldolase C in fast-growing Hepatoma 178 are given in Table 3. The total aldolase activity of the hepatoma corresponds not only to aldolase C, but also to aldolase A, the amount of which is probably higher than that of aldolase C. From the data of Penhoet and Rutter (18, 19) on the distribution of the hybrids A-C and on the high specific activity of aldolase A, we may estimate that the FDP aldolase activity of the aldolase C nonhybridized with aldolase A (tetramer C4) corresponds to about 2% of the total FDP activity and to about 10% of the total F1P aldolase activity.

On this approximate basis, the yield of our preparation of pure aldolase C in hepatoma would be about 5%. After isolation of aldolase C, the aldolase activity ratio FDP/F1P is lowered from 27 to about 5 (which is the ratio of pure aldolase C) and antiserum anti-C chiefly inhibits this purified fraction to an extent of 95%.

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Table 2

<table>
<thead>
<tr>
<th>Rat tissues</th>
<th>Anti-A (%)</th>
<th>Anti-B (%)</th>
<th>Anti-C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal liver</td>
<td>5–10</td>
<td>80–90</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Muscle</td>
<td>90–95</td>
<td>&lt;2</td>
<td>0 &lt; 2</td>
</tr>
<tr>
<td>Brain</td>
<td>85</td>
<td></td>
<td>95</td>
</tr>
<tr>
<td>Fetal liver (16th to 19th day)</td>
<td>40–80</td>
<td>40–60</td>
<td>17–40</td>
</tr>
<tr>
<td>Slow-growing hepatoma (Hepatoma 189)</td>
<td>50–70</td>
<td>70–80</td>
<td>30b</td>
</tr>
<tr>
<td>Fast-growing hepatoma (Hepatoma 178)</td>
<td>85–90</td>
<td>&lt;5</td>
<td></td>
</tr>
</tbody>
</table>

Inhibition tests were performed by incubating different extracts (at final comparable concentrations) with 100 µl of antiserum at 37° for 1 hr and then at 4°. After centrifugation, supernatants were estimated for aldolase activity by comparison with the same extracts treated by normal chicken serum.

DISCUSSION

The presence of aldolase A in well-differentiated, slow-growing hepatomas has been demonstrated by kinetic, electrophoretic, and immunological methods. These findings are comparable to the previous findings on hepatomas induced by 3’MeDAB (14). In studying 3’MeDAB-induced hepatomas, one of us has found a mean aldolase activity ratio of 2.5 and a marked inhibition by antiserum anti-aldolase A. The present electrophoretic results with Hepatoma 189 were also comparable to those obtained earlier. In slow-growing hepatomas, electrophoresis revealed the presence of hybrids between the normal adult aldolase B and the muscle-type aldolase A, providing evidence for the synthesis of the 2 kinds of subunits in the same cell. In Hepatomas 122 and 175, there was less aldolase A.

In the fast-growing Hepatoma 178, electrophoresis showed anodic bands, the migration of which is the same as in the brain. There were similar bands, although less distinct, in fetal livers (from the 16th to 20th day).
Aldolase A is >50. The low ratio of our fraction cannot be the fast-growing hepatoma (after action of anti-A). After aldolase, inhibited a mean 70% of the supernatant extract of a-fetoprotein in some hepatomas (1).

Therefore, 2 types of aldolases in Hepatoma 178 were well as the finding of a "kidney-type" glutaminase (10) in carcinomas have been reported. These include the fetal forms of hexokinase (5, 34) and lactate dehydrogenase (26), as well as in muscular dystrophy (3, 9). Therefore, it seems likely that, in some pathological states, the biosynthesis of the adult form of enzymes if preferentially repressed, while the fetal, less-differentiated form would be derepressed.

It is felt that the study of the fetal forms of enzymes in carcinomas will be a new approach to cancer enzymology at the molecular level.

The evidence for the presence of brain-type aldolase C is strengthened by the results of the immunological studies. Antiserum anti-C, which inhibits less than 2% of normal liver aldolase, inhibited a mean 70% of the supernatant extract of the fast-growing hepatoma (after action of anti-A). After isolation of a purified fraction, the aldolase activity ratio was about 5 instead of 26 before purification. This ratio corresponds to that of pure rat aldolase C. The ratio of pure aldolase A is >50. The low ratio of our fraction cannot be caused by aldolase B because it is totally absent, as shown by antiserum anti-B. The best evidence is provided by antiserum anti-C, which completely precipitates the purified fraction. Therefore, 2 types of aldolases in Hepatoma 178 were completely (aldolase C) of almost completely (aldolase A) absent from normal liver.

In the well-differentiated Hepatomas 189, 122, and 175, and also in primary hepatomas induced by 3'dMeDAB, aldolase A is found. On the other hand, there were 2 aldolases, A and C, in solid, poorly differentiated, transplantable hepatoma and also in ascitic hepatomas (30, 36). Both of these aldolases were present in the fetal liver, in a relatively abundant amount.

These findings confirm the previous hypothesis of Schapira et al. (29) of a "derepression" of fetal forms of enzymes in hepatomas. This hypothesis was strengthened by the findings of Nordmann (13), who has shown by immunological methods that the amount of aldolase A is increased when it is compared with the amount of aldolase A present in normal adult liver. The comparison must be made with the presence of α-fetoprotein in some hepatomas (1).

More recently, several examples of fetal patterns of enzymes in carcinomas have been reported. These include the fetal forms of hexokinase (5, 34) and lactate dehydrogenase (26), as well as the finding of a "kidney-type" glutaminase (10) in hepatomas. Potter (20) presented the concept of "ontogeny as blocked ontogeny," and Fishman et al. (6) offered the hypothesis of the derepression of the genome in carcinomas. Uriel (38) proposed the term of "retrodifferentiation" for a stepwise process enabling the adult cell to synthesize proteins which are normally produced only by the immature cell. Although the control mechanism in higher animals is probably different from that described in bacteria by Jacob and Monod (4, 8), we agree with the suggestion by Weinhouse et al. (39) that "impairment of gene control, rather than alteration of gene structure may be a crucial factor in the neoplastic transformation."

It is also known that this "fetal pattern" is not specific for carcinogenesis. There is a fetal type of lactate dehydrogenase, aldolase, and creatine kinase in atrophied muscle (27, 28), as well as in muscular dystrophy (3, 9). Therefore, it seems likely that, in some pathological states, the biosynthesis of the adult form of enzymes if preferentially repressed, while the fetal, less-differentiated form would be derepressed.

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### REFERENCES


### Table 3

<table>
<thead>
<tr>
<th>Hepatomas before purification</th>
<th>Total aldolase activity (i.u.)</th>
<th>Specific aldolase activity (i.u./mg)</th>
<th>% inhibition by</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment 1</td>
<td>800</td>
<td>0.2</td>
<td>4</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>840</td>
<td>0.21</td>
<td>20</td>
</tr>
<tr>
<td>Chromatographic peak of elution</td>
<td>0.4</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>0.9 mg</td>
<td>1.3</td>
<td>95</td>
</tr>
</tbody>
</table>
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