Developmental Phase-specific Alkaline Phosphatase Isoenzymes of Human Placenta and Their Occurrence in Human Cancer

Lillian Fishman, Haruhiko Miyayama, Shirley G. Driscoll, and William H. Fishman

INTRODUCTION

Antigens normally present in early embryonic and fetal life are produced by malignant cells. For instance, α-fetoprotein of the yolk sac (11) is detectable in yolk sac tumors (17) and in human and experimental hepatoma and teratocarcinoma (1). Also, human chorionic gonadotropin is produced as one would expect in choriocarcinoma, but also by nontrophoblastic neoplasms such as cancers of the lung, testes, etc. (2). Another developmental protein antigen, placental-type alkaline phosphatase [Regan isoenzyme, (8, 9)] has been reported in bronchogenic carcinoma, cancer of the ovary, and other malignant neoplasms.

When such proteins appear in tumors, they may indicate the reexpression of a gene set characteristic of a particular event in early development. This information may be valuable in classifying stages in neoplastic progression.

The original purpose of this investigation has been to identify the phase in trophoblast development that elicits isoenzymes of the term placental type such as Regan isoenzyme. However, in the earliest (8 to 10 weeks) placentas, the isoenzyme pattern as examined by biochemical, electrophoretic, and immunological criteria revealed the presence of a species that we had observed earlier as non-Regan (nonplacental) alkaline phosphatase in ovarian cancer (15). This isoenzyme species was localized on the microvilli of the early placentas on the basis of enzyme histochemical and immunohistochemical studies. Subsequently, the term placental isoenzyme appeared and increased in activity while the early placental type diminished. Most interesting was the appearance in early placenta of an unusual alkaline phosphatase isoenzyme that did not have any of the antigenic determinants known for placental, intestinal, liver, and bone preparations.

From this information, it is now possible to show the patterns characteristic of 3 phases in trophoblastic development with respect to alkaline phosphatase. In each phase, we present an example of its occurrence in human cancer that provides a guide for interpreting tumor alkaline phosphatases as reflections of the activation of phase-specific trophoblastic genes.

SUMMARY

Alkaline phosphatase electrophoretic patterns characteristic of three phases in early human trophoblast development are described in this preliminary communication. Phase 1 (6 to 10 weeks) consists entirely of two heat-sensitive, L-homoarginine-inhibited bands, the slower one of which possesses antigenic determinants of liver-bone-type alkaline phosphatase, whereas the fast band lacks any of the known alkaline phosphatase antigenic determinants. Phase 2 pattern (11 to 13 weeks) is that of a mixture of Phase 1 and Phase 3 isozyme components, the latter exhibiting two isozyme bands with the characteristics of term placental alkaline phosphatase. These three patterns of developmental phase-specific placental alkaline phosphatases correspond in order to non-Regan isoenzyme, a mixture of Regan and non-Regan isozymes and Regan isoenzyme in a variety of human cancer tissues. The biochemical profile characteristic of trophoblast developmental Phase 1 alkaline phosphatase is expressed as 78.5% heat-sensitive inhibition (5 min at 65°), 66.3% L-homoarginine inhibition, and 17.3% L-phenylalanine inhibition where n = 12. It is hypothesized that the alkaline phosphatase of human tumor tissues reflects the expression of placental genes corresponding to one or more phases of trophoblastic development.

MATERIALS AND METHODS


Early human placentas from 8 to 16 weeks of gestation were washed free of blood with cold 0.9% NaCl solution and
were homogenized individually by hand in 0.05 M Tris-HCl buffer, pH 8.6 (approximately 30% w/v), using a Duall homogenizer. The homogenates were centrifuged in an International clinical centrifuge for 2 min at 2000 rpm. The supernatant was assayed for alkaline phosphatase (pH 9.8) activity according to the methods of Fishman and Ghosh (7) and Lin and Fishman (13). Conditions for measuring inhibition by L-phenylalanine and L-homoarginine are those used previously (3). Heat inactivation was carried out for 5 min at 65°. Units are defined as µg phenol per ml per 15 min released from phenyl phosphate. Isoenzyme electrophoresis was done on microzone, cellulose acetate membranes according to the method of Inglis et al. (12) using Gelman high-resolution buffer, pH 8.8, as the chamber buffer and naphthol AS-MX phosphoric acid as the substrate. The uniform range of enzyme units applied in the experiments illustrated in Figs. 3, 4, and 5 are shown in the tabulated portion of Fig. 3.

Histochemistry. The conditions for evaluating the nature of the alkaline phosphatases in placental tissue sections were those of Sasaki and Fishman (15) who used the L-phenylalanine inhibitor for confirming Regan isoenzyme and L-homoarginine for non-Regan, except that the substrate concentration was 4.4 mM and variamine blue was substituted for fast blue BBN.

Immunohistochemistry. Peroxidase-labeled antiserum to term alkaline phosphatase was applied to fixed frozen sections of early placenta as described by Miyayama et al. (14). These were then examined for the presence of the term placental isoenzyme.

RESULTS

Histochemical Evidence. The objective was to demonstrate the cell locus of the liver-bone-type alkaline phosphatase in an 8-week placenta that had no biochemical indication of term placental alkaline phosphatase but did exhibit heat-sensitive, homoarginine-inhibited activity. Fig. 1 shows that the isoenzyme activity is exclusively associated with the microvilli by azo staining technique and that it exhibited complete inhibition by L-homoarginine, while still exhibiting substantial activity in the presence of L-phenylalanine. No evidence in this placenta of term placental alkaline phosphatase was obtained with peroxidase-labeled antibody to term placental alkaline phosphatase under conditions in which this reagent gave positive results with term placenta (Fig. 2).

Biochemical Studies. From 8 to 16 weeks of gestation (n = 8), the concentration of total placental alkaline phosphatase increases linearly from 150 to a value of approximately 1800 units/g, wet weight, which is about one-twenty-fifth the concentration value of term placenta. Data on 6- to 10-week placental tissue in Table 1 are given for heat stability and L-phenylalanine and L-homoarginine inhibition.

Electrophoretic Migration. In Fig. 3, the results of microzone cellulose acetate electrophoresis showing alkaline phosphatase zones can be correlated with biochemical information on the inhibition by heat and amino acids obtained from early placental sonic extracts arranged in order of duration of gestation.

At 8 weeks, 2 bands were visible, A and B, representing predominantly heat- and L-homoarginine-sensitive alkaline phosphatase with low inhibition by L-phenylalanine (Specimen 1). This pattern is classified as Phase 1.

The later placentas of 10, 12, and 13 weeks (Specimens 2, 3, and 4) exhibited a 3rd band, C, which migrated most slowly. (Band A in Specimen 2 appeared to have 2 components.) Biochemically, the total alkaline phosphatase is made up of components of intermediate heat stability and L-phenylalanine and L-homoarginine inhibition, of which Band B is presumed to be entirely heat sensitive and L-homoarginine inhibited (see below). Such patterns are representative of Phase 2.

Phase 3 was characterized by a migration pattern of 2 bands (Specimen 5, 15.5 weeks), typical of the molecular weight Variants A and B of mature placenta and exhibiting their heat stability and high inhibition by L-phenylalanine.

Immunoelectrophoresis (Figs. 4 and 5; Tables 1 and 2).

Table 1

<table>
<thead>
<tr>
<th>Wk of gestation</th>
<th>Total (µg phenol/ml/15 min)</th>
<th>Heat inactivation for 5 min at 65° (%)</th>
<th>Inhibition by 0.008 M L-homoarginine (%)</th>
<th>Inhibition by 0.005 M L-phenylalanine (%)</th>
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<tr>
<td>6</td>
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<td>87</td>
<td>74</td>
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<tr>
<td>10</td>
<td>350</td>
<td>80</td>
<td>60</td>
<td>10</td>
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</table>

Mean ± S.D. 188.5 ± 76.6 78.5 ± 8.6 66.3 ± 8.5 17.3 ± 7.4

* Containing 5 mM L-phenylalanine. Each of these 12 extracts exhibited Phase 1 isoenzyme pattern on microzone electrophoresis. All homogenates were sonically disrupted in the cold for 1 minute with the Biosonic set at "Lo," 60%.
The principle of retardation of antigen in the presence of specific antibody was used as illustrated for Phase 1 alkaline phosphatase in Fig. 4. Thus, enzyme-containing supernatants of centrifuged placental homogenates were mixed in the proper proportion with specific antiserum raised against various species of alkaline phosphatase (3, 4, 10). The combination of antigen and antibody was marked by the formation of a complex the migration of which was retarded as compared with that of the antigen alone. One notes that Band B was retarded by antiserum to liver alkaline phosphatase but not by antiserum to intestinal or placental alkaline phosphatase.

In Phase 1 and Phase 2 preparations (Table 2), the fast band did not react with antiserum produced against alkaline phosphatase of human liver, bone, intestine, and mature placenta as well as antiserum to alkaline phosphatase of bone sarcoma and to 2 HeLa cell lines, TCRC-1 and TCRC-2, which were cloned from the wild type; the former contained Regan and the latter contained non-Regan alkaline phosphatase (14).

The B band that appeared in extracts of Phase 1 and Phase 2 placenta cross-reacted with antiserum to bone, liver, bone sarcoma, and TCRC-2.

In Phase 1, no cross-reaction appeared with antiserum to mature placental alkaline phosphatase. In Phase 2, the C band was retarded by this antiserum and antiserum to TCRC-1. Thus, it was possible to characterize Bands B and C immunologically in Phases 1 and 2, but Band A did not appear to be immunologically similar to any type of alkaline phosphatase that we have tested thus far.

In Phase 3, all the alkaline phosphatase is retarded by antiserum to mature placental alkaline phosphatase, the fast and slow bands now corresponding to the molecular weight variants A and B described by Fishman and Ghosh (7). (Similar findings were seen with antiserum to TCRC-1.)

A summary of the findings relating developmental phase with the biochemical and antigenic properties of placental and human tumor alkaline phosphatase appears in Table 2.

**DISCUSSION**

Using biochemical, immunological, and electrophoretic techniques on extracts of 8 early placentas, we found the alkaline phosphatase of 8- to 10-week placentas to be different from that of mature placenta. At about 11 to 13 weeks, the latter isoenzyme type appears and by about 14 to 16 weeks all the alkaline phosphatase exhibits the properties of term placenta. The earliest placental isoenzyme on electrophoresis contained a fast-migrating moiety which did not resemble immunologically the liver, intestinal, bone, or term placenta isoenzymes. Also, the total enzyme activity was all heat sensitive and was inhibited by L-homoarginine, but not by L-phenylalanine, indicating nonterm placental type. By the end of the 1st trimester, the enzyme activity had become heat stable and could be inhibited by L-phenylalanine, properties of term placental-type alkaline phosphatase.

The initiation of alkaline phosphatase production in the early trophoblast and the subsequent change to another form characteristic of mature placenta could be interpreted as the expression of 2 separate genes, one being "turned-off" while the other was being "turned-on," or the metabolic modification of a protein controlled by 1 gene. Regardless of the ultimate explanation, we can profit from our ability to distinguish definite phases in the development of this isoenzyme during gestation.

Placental alkaline phosphatase is produced by certain neoplasms. An isoenzyme of alkaline phosphatase indistinguishable immunologically and biochemically from term placenta was first detected in a cancer of the lung of a male patient named Regan. Subsequently, Regan alkaline phosphatase (term placenta) has been found in a variety of tumors, mainly of the ovary, testis, pancreas, etc.

Many other tumors produce alkaline phosphatase with properties that are unlike the isoenzyme of mature placenta, the so-called non-Regan isoenzymes. For instance, choriocarcinoma (5), some ovarian tumors (15), etc., contain alkaline phosphatase resembling the liver-bone type which is L-homoarginine and heat sensitive and cross-reacts with antiserum raised against the liver isoenzyme. In addition, these tumor isoenzymes react with antiserum to TCRC-2 HeLa cells (non-Regan), and as well as to antiserum to bone sarcoma.

This similarity of the early placental alkaline phosphatase to that liver-bone type was noted in that it reacts with antiserum to liver and is L-homoarginine sensitive. In addition, the former contains antigen sites in common with

### Table 2

**Immunological characteristics of 1st-trimester placental alkaline phosphatase**

<table>
<thead>
<tr>
<th>Microzone bands</th>
<th>Intestine</th>
<th>Liver&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Term placenta</th>
<th>HeLa cell line TCRC-1&lt;sup&gt;1&lt;/sup&gt;</th>
<th>HeLa cell line TCRC-2&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Bone sarcoma</th>
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<tbody>
<tr>
<td>Phase 1, n = 3</td>
<td>8-10 wk</td>
<td>A 0 0 0 0 0 0</td>
<td>B 0 + 0 0 0 +</td>
<td>C 0 0 + + 0 0</td>
<td>B 0 + 0 0 + +</td>
<td>C 0 0 + + 0 0</td>
</tr>
<tr>
<td>Phase 2, n = 3</td>
<td>11-13 wk</td>
<td>A 0 0 0 0 0 0</td>
<td>B 0 + 0 0 0 +</td>
<td>C 0 0 + + 0 0</td>
<td>B 0 + 0 0 + +</td>
<td>C 0 0 + + 0 0</td>
</tr>
<tr>
<td>Phase 3, n = 2</td>
<td>14-15 wk</td>
<td>A 0 0 0 0 0 0</td>
<td>B 0 + 0 0 0 +</td>
<td>C 0 0 + + 0 0</td>
<td>B 0 + 0 0 + +</td>
<td>C 0 0 + + 0 0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Antigen retardation electrophoretic test.

<sup>b</sup> The antigenic determinants for liver alkaline phosphatase cannot be distinguished from bone-type alkaline phosphatase in this system (3).

<sup>c</sup> See Ref. 16.
some tumors as it combines with antiserum to TCRC-2 HeLa cells and antiserum raised against bone sarcoma. The above evidence leads us to believe that tumor alkaline phosphatase which shows liver-bone-like characteristics is of the early placental type in contrast to the Regan or mature placental type.

Although early placental alkaline phosphatase is similar to liver immunologically and in its sensitivity to inhibition by L-homoarginine, it is clear from Table 1 that, unlike bone and liver alkaline phosphatases, a minor component is resistant to heating for 5 min at 65°C. Rabbit antisera to 'liver' alkaline phosphatase cross-reacts with bone-type isoenzyme.

From our limited experiences thus far, it is reasonable to expect a degree of biological variation from placenta to placenta, in part due to the difficulty of fixing placental age with precision and to the variable rate of maturation of the placenta. It is also possible that the isoenzyme manifested at 8 weeks may later be shown to have appeared at an earlier stage of development, just as α-fetoprotein usually attributed to fetal liver has now been shown to have first appeared in the yolk sac, an organ which is the precursor to liver. Therefore, for the time being, we define early placental type as the heat-sensitive, L-homoarginine-inhibited activity which migrates in 2 bands, the fast-moving band on "microzone" electrophoresis lacking known antigenic determinants and the slower band possessing liver (bone) antigenic sites.

Ultimately, when it is proven that non-Regan alkaline phosphatase is the early placental form of the enzyme, then the production of alkaline phosphatase in human tumors would have a single interpretation. This postulates that trophoblastic genes for alkaline phosphatase are activated in cancer cells which may be those of the 6- to 10-week placenta, of the term placenta, or of a mixture of both.

ACKNOWLEDGMENTS

We thank Dr. Julien Van Lancker for extending us the hospitality of his laboratory. We also thank Dr. R. M. Singer for making available antisera to HeLa TCRC-1 and TCRC-2.

REFERENCES

Fig. 1. Cytochemical staining for alkaline phosphatase of an 8-week (Phase 1) placenta. a, microvillar staining in the presence of L-phenylalanine, b, lack of reaction in the presence of L-homoarginine in the incubating medium.

Fig. 2. Positive immunohistochemical localization of term placental alkaline phosphatase (a) and negative reaction in an 8-week placenta (b).
Fig. 3. Microzone cellulose acetate electrophoresis of alkaline phosphatase in homogenates of placentas arranged in order of gestational age. From left to right they are 8, 10, 12, 13, and 16 weeks. The pattern at 8 weeks is defined as Phase 1; at 10, 12 and 13 weeks, Phase 2; and at 16 weeks, Phase 3.

Fig. 4. Antigen-retardation tests of a Phase 1 placental homogenate alkaline phosphatase. From left to right, the 1st slot is Phase 1 homogenate control; the 2nd slot is a combination of control plus antiserum to human liver alkaline phosphatase; the 3rd slot, control plus antiintestinal alkaline phosphatase; and the 4th slot, control plus antiserum to human placental alkaline phosphatase.

Fig. 5. Antigen retardation tests of a Phase 2 placental alkaline phosphatase pattern in which bands A and B overlap. From left to right, the 1st pattern is Phase 2 homogenate control; the 2nd pattern represents the mixture of control with antiserum to human liver alkaline phosphatase; the 3rd pattern, control plus antiserum to term placental alkaline phosphatase; and the 4th pattern, control plus antiserum to bone sarcoma alkaline phosphatase.

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Gestational age (wk)</th>
<th>Alkaline phosphatase activity (μg/ml)</th>
<th>L-phenylalanine (5 mM)</th>
<th>L-homoaarginine (8 mM)</th>
<th>Heat (5 min at 65°)</th>
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<tbody>
<tr>
<td>1</td>
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