ABSTRACT

In benign and malignant ovarian tumor patients, human placental alkaline phosphatase (HPLAP) was determined in serum and extracts from surgical tumor biopsies using a highly specific enzyme-antigen immunoassay based on a mouse monoclonal antibody (E6) to HPLAP. Serum HPLAP levels >0.1 unit/liter were found in 58% of ovarian cancer patients. Serum carcinoembryonic antigen levels were positive (>5.4 ng/ml) in 17% of these patients. HPLAP was detected in extracts from 13 of the 14 tumors investigated (range, 2.4 to 557 milliunits/g). Only the mixed heterologous Müllerian sarcoma was negative. The highest HPLAP content of normal ovarian tissue was 1.1 milliunits/g. The amount of heat-stable and β-lactamase-insensitive alkaline phosphatase was in all cases much higher than the fraction recognized by E6. The neoplastic origin of HPLAP was confirmed immunohistochemically on paraffin sections by an indirect avidin-biotin-peroxidase staining procedure using E6. The staining pattern was compared to the histochemical distribution of total alkaline phosphatase on adjacent sections. A consistency was found between the amount of HPLAP in tissue extracts and its immunohistochemical distribution. In all the tumors, staining for HPLAP was observed mainly on the plasma membranes of carcinoma cells. In 9 of the 10 carcinomas, the histological distribution of HPLAP and also of total alkaline phosphatase was heterogeneous. HPLAP staining, present in one of five normal ovaries, was restricted to germinal inclusion cysts. The present results support the hypothesis that serious ovarian tumors originate from these cysts.

INTRODUCTION

Despite progress in clinical oncology, ovarian carcinoma remains near the top in the mortality statistics of all gynecological cancers (17). Late diagnosis and understaging severely impair the possible success of therapy. Therefore, there is an urgent need for a clinically useful tumor marker, enabling early serological recognition of the disease. A number of potentially valuable markers for ovarian tumors have been reported: CEA (46); CA 125 (22); NB70K (4); HPLAP (2); CSAp (38); an ovarian cytidenoacarcinoma-associated antigen (3); an ovarian carcinoma antigen (25); a human milk fat globule antigen (12); galactosyltransferase (47); human chorionic gonadotropin; and human placental lactogen (34).

In healthy adults, 6 different organ-specific isoenzymes of AP (EC 3.1.3.1.) are known to exist (29). The placental type is present in the placental trophoblast. In 1968, an ectopically expressed placental-like form of AP (the Regan type) was observed in a patient with lung carcinoma (14). Subsequently, HPLAP has been reported in mammary, testicular, gastrointestinal, hematopoietic, cervical, and ovarian tumors (8, 21, 33, 35), using electrophoretic techniques, polyclonal antibodies, and more or less specific enzyme inhibitors. The recent introduction of a monoclonal antibody with a high specificity for HPLAP allowed the discrimination of low concentrations of HPLAP from the other AP isoenzymes, especially the intestinal type (10).

In the present study, the occurrence of HPLAP in serum and tissue extracts and its histological distribution in malignant and benign ovarian tumors and in normal ovaries were investigated using a specific monoclonal antibody (10). The data were analyzed in function of the stage of dissemination and the histological type of the tumor. The final report of the NIH 1980 Consensus Development Conference (36) on CEA as a cancer marker was a useful guideline in the experimental setup of this study.

MATERIALS AND METHODS

Patients and Tissues. Fourteen patients with different types of ovarian tumors were studied. In all of them, a sample of the tumor was obtained during surgical intervention. Five normal ovaries were obtained on the occasion of total hysterectomies. All tissues were cut in 1.5-mm-thick slices, which were sequentially allotted for biochemical analysis and for histology. Samples for biochemical analysis were frozen in liquid nitrogen, and samples for histology were fixed within 1 hr after surgical removal.

Materials. 3-Amino-9-ethylcarbazole, tetranitro blue tetrazolium, and trypsin (type III, 11,250 units/mg) were obtained from Sigma Chemical Co., St. Louis, MO; biotinylated horse anti-mouse immunoglobulin serum, avidin, and biotinylated horseradish peroxidase were obtained from Vector Laboratories, Inc., Burlingame, CA; 5-bromo-4-chloro-3-indoxylphosphate-p-toluidine salt was obtained from Serva Feinbiochemica GmbH, Heidelberg, Federal Republic of Germany; the CEA radioimmunoassay kit was obtained from Abbott Diagnostics Division, Antwerp, Belgium; rabbit anti-mouse IgG2b serum was purchased from Nordic Immunology, Tilburg, The Netherlands; 96-well microplates (4-39454 immunoassays) were obtained from Nunc, Roskilde, Denmark.

Monoclonal Antibody. The preparation of HPLAP from full-term human placenta, immunization of BALB/c mice, hybridization of spleen cells with SP2/0 myeloma cells, and selection of hybridoma E6, producing a highly specific monoclonal antibody (of the IgG2b, k type) to HPLAP, have been described elsewhere (10). The E6 culture supernatant containing 10% fetal calf serum was used without further purification for both the EAIA and the immunohistochemical staining of HPLAP.
Tissue Extraction of HPLAP. Approximately 0.5 g of tissue was homogenized in 4.5 ml 50 mM Tris-HCl buffer (pH 6.8), using a Potter-Elvehjem Model S homogenizer. Two ml n-butyl alcohol were added over a 20-min period under continuous magnetic stirring. After centrifugation of the homogenate for 20 min at 3000 x g, the aqueous phase was collected. A 1.5-ml portion was chromatographed on a 1.3- x 6.5-cm Sephadex G-25 column in 50 mM Tris-HCl buffer (pH 6.8). The AP-containing fractions were pooled and stored at -20° until assayed for AP and HPLAP.

Determination of Total AP. The total AP activity in tissue butanol extracts was determined by the method of Van Belle et al. (44). One unit of enzyme activity is defined as the liberation of 1 ßtritol 4-nitrophenol per min of enzyme activity is defined as the liberation of 1 ßmol 4-nitrophenol from 4-nitrophenyl phosphate per min at 37° when incubated in a solution containing 5 mM 4-nitrophenyl phosphate in 0.1 M N-ethylaminoethanol buffer (pH 10.2). Temperature inactivation was performed at 65° for 5 min (13). Inhibition with 1.25 mM L-p-bromotetramisole was performed as described elsewhere (45).

EIAA for HPLAP. Standard HPLAP was prepared and purified from full-term placenta as described by Lehmann (27). The absence of liver, bone, and kidney AP was verified using a comparable EIAA based on our monoclonal antibody (AP 230) produced against liver AP and reacting exclusively with these AP isoenzymes. Diluted to an activity of 250 units/liter in PBS containing 0.5% BSA, it was frozen and stored in liquid nitrogen.

Wells of a flat-bottomed, 96-well microplate were coated with 0.2 ml rabbit anti-mouse IgG2b serum (1/1000) in 10 mM Tris-HCl buffer (pH 8.5) containing 10 mM NaCl and 10 mM NaN3. The remaining protein adsorption sites were blocked with 1% BSA and 1% fetal calf serum in PBS. After being washed with PBS containing 50 mM NaCl and 0.05% Tween 20, 0.2 ml spent E6 culture supernatant (1/100 in PBS) was added to the wells and incubated for 3 hr. After another washing, 0.2 ml of a standard series of HPLAP or samples (serum or tissue extracts), undiluted or diluted in PBS containing 0.5% BSA was added and incubated overnight at 4°. Prior to developing, the plates were washed 3 times in PBS/NaN3/Tween 20. Two hundred ßl 5 mM p-nitrophenyl phosphate in 0.1 M N-ethylaminoethanol buffer (pH 10.2) were added to the plates; after 4 hr incubation at 37°, the reaction was stopped by the addition of 0.05 ml 3 M NaOH. The absorbance was measured at 405 nm. Calculation of sample concentrations was performed using a spline function program. Units of HPLAP activity are defined in the same way as for total AP. The lower detection limit was 0.02 unit/liter. The P98 value for serum HPLAP levels, which was determined on 1650 samples on an average hospital population, was 0.1 unit/liter. Further details, performance characteristics, and quality control experiments have been published elsewhere (39).

Immunohistochemical Localization of HPLAP. Tissue slices (1.5 mm thick) were fixed for 1.5 hr at room temperature in buffered 4% formaldehyde [0.1 mM sodium cacodylate buffer (pH 7.4) containing 1% CaCl2]. Fixed tissues were washed for 15 min in the same buffer solution without formaldehyde and embedded in Paraplast. Sections (5 µm) were mounted on goat serum/formaldehyde-coated glass slides, hydrated, and treated for 20 min with 0.003% trypsin in 10 mM Tris-HCl buffer (pH 7.3) containing 0.9% NaCl and 1 mM CaCl2. After equilibration in TBS and treatment with normal horse serum (1/5) for 20 min, spent E6 culture supernatant (1/50) was applied without washing, and incubation was performed overnight. The sections were then washed and treated with biotinylated affinity-purified horse anti-mouse immunoglobulin serum for 30 min followed by the avidin/biotin/peroxidase complex for 1 hr. All dilutions were made in TBS. After extensive washing, peroxidase was revealed with 0.02% 3-amino-9-ethylcarbazole and 0.002% H2O2 in 20 mM acetate buffer (pH 5.2) containing 9.5% dimethyl sulfoxide. The sections were counterstained with methyl green and mounted in Kaiser’s glycerin/gelatin mounting medium. Control staining was performed on adjacent sections by replacing E6 with culture supernatant containing a mouse monoclonal antibody of the same immunoglobulin class (IgG2b) but of irrelevant specificity. Full-term human placenta, processed identically, was used as a positive control in each staining session. No positive staining was obtained in normal human liver, kidney, stomach, or intestine.

Histochemical Localization of AP. Paraffin sections adjacent to those used for the immunohistochemical demonstration of HPLAP were used to localize AP by the method of Gossrau (16) with 0.025% 5-bromo-4-chloro-3-indoxylphosphate-p-toluidine salt as the substrate and 0.05% tetranitro blue tetrazolium as the color reagent, in 0.1 mM Tris-HCl buffer (pH 9.4). The sections were counterstained with methyl green and mounted in glycero/gelatin mounting medium. Full-term human placenta was used as a positive control.

With this procedure, liver, kidney, intestinal, and placental AP could be demonstrated histochemically. The localization of liver AP is identical to the pattern on frozen sections, as we have shown elsewhere (9).

Routine Histology. For anapomorphathological tumor identification, adjacent sections were routinely stained with hematoxylin/eosin or periodic acid-Schiff reagent.

Radioimmunoassy of CEA. The serum levels of CEA were determined using the Abbott CEA radioimmunoeassay kit. The detection limit was 0.5 ng/ml serum. The samples were thawed only once, immediately prior to assay. CEA values above 5.4 ng/ml were considered to be pathological (5).

Electrophoretic Separation of AP Isoenzymes. Before electrophoresis, tissue extracts were incubated with E6, AP 230, or neuraminidase (0.2 unit/ml) or heat inactivated at 65° for 5 min. Starch gel electrophoresis was performed as described by Poulik (40). The results were compared to the electrophoretic pattern of the untreated extracts.

RESULTS

Patients, Serological Assays, Tissue Extracts, and Electrophoretic Studies. The pertinent clinical and histopathological data of the patients are summarized in Table 1.

In 7 of the 14 tumor patients (Patients 1, 3, 5, 7, 8, 9, and 12), the serum HPLAP level (Table 2) was ≥0.1 unit/liter, which is the P98 value for an average hospital population [n = 1650; Pollet et al. (39)]. None of these tumor patients were cigarette smokers. Five had a HPLAP level ≥0.2 unit/liter. This level was always associated with neoplasia in the reference population studied. In these 5 patients, the tumor was malignant, bilateral and, in general, metastasized. The histopathology of the tumors was: 2 papillary serous cystadenocarcinomas; an endometrioid carcinoma; a poorly differentiated adenocarcinoma; and a granulosa cell carcinoma. Cystic fluid collected from the latter tumor contained 1.21 units HPLAP/liter. Ascitic fluid from Patients 2, 7, and 8 contained 0.18, 0.36, and 3.5 units HPLAP/liter, respectively, which is 3 times higher than their corresponding serum levels. Serum CEA (Table 2) determined in the same blood samples was positive in 2 of the 14 patients (Patients 1 and 7).

The tissue contents of AP and HPLAP are presented in Table 3. The highest contents of AP were found in 2 papillary serous cystadenocarcinomas and in the mixed heterologous Müllerian sarcoma. The HPLAP content of carcinomas varied between 0.3 and 19% of the AP content. The amount of HPLAP as well as the HPLAP/AP ratio in the 2 benign tumors were in the same range as for carcinomas. The lowest HPLAP levels for carcinomas (Tumors 6 and 7) were 2 times greater than the highest content of normal ovaries. The elevated AP level in Biopsy 18 (normal ovary) was due to a follicular cyst.

The results from heat inactivation and L-p-bromotetramisole inhibition are summarized in Table 4. In all cases, the amount of heat-stable or L-p-bromotetramisole-insensitive AP was greater...
Table 1
Patient clinical data and tumor histopathology

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<tr>
<th>Patient</th>
<th>Age (yr)</th>
<th>Histopathology</th>
<th>Laterality</th>
<th>Stage</th>
<th>Grade</th>
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<td>Bc</td>
<td>ic</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>58</td>
<td>Papillary serous cystadenocarcinoma</td>
<td>B</td>
<td>U</td>
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<tr>
<td></td>
<td>3</td>
<td>77</td>
<td>Papillary serous cystadenocarcinoma</td>
<td>B</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>57</td>
<td>Endometrioid carcinoma</td>
<td>U</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>57</td>
<td>Endometrioid carcinoma</td>
<td>B</td>
<td>U</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>59</td>
<td>Endometrioid carcinoma</td>
<td>B</td>
<td>la</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>47</td>
<td>Endometrioid carcinoma</td>
<td>B</td>
<td>IV</td>
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<tr>
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<td>50</td>
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<td>IV</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>39</td>
<td>Granulosa cell carcinoma</td>
<td>B</td>
<td>III</td>
</tr>
<tr>
<td></td>
<td>11</td>
<td>53</td>
<td>Mixed heterologous Müllerian sarcoma</td>
<td>U</td>
<td>la</td>
</tr>
<tr>
<td>Borderline tumor</td>
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<td>34</td>
<td>Borderline papillary serous cystadenocarcinoma</td>
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<tr>
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<td>19</td>
<td>44</td>
<td>Normal ovary</td>
<td></td>
<td></td>
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Table 2
Serum concentrations: corresponding HPLAP levels (determined by the EAIA) and CEA levels in preoperative serum samples obtained from the patients listed in Table 1

<table>
<thead>
<tr>
<th>Patient</th>
<th>HPLAP (units/liter)</th>
<th>CEA (ng/ml)</th>
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<tr>
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<td>1.36</td>
<td>4.5</td>
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<td>BDL</td>
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</tr>
<tr>
<td>5</td>
<td>0.36</td>
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</tr>
<tr>
<td>6</td>
<td>BDL</td>
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</tr>
<tr>
<td>7</td>
<td>0.12</td>
<td>5.9</td>
</tr>
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<td>8</td>
<td>1.23</td>
<td>2.1</td>
</tr>
<tr>
<td>9</td>
<td>0.29</td>
<td>2.9</td>
</tr>
<tr>
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<td>0.07</td>
<td>0.6</td>
</tr>
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<td>BDL</td>
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<td>0.04</td>
<td>1.9</td>
</tr>
<tr>
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<td>BDL</td>
<td>BDL</td>
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<td>BDL</td>
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</tr>
<tr>
<td>16</td>
<td>BDL</td>
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</tr>
<tr>
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<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>18</td>
<td>BDL</td>
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<td>19</td>
<td>0.05</td>
<td>ND</td>
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<td>ND</td>
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Table 3
Tissue levels, histochemical data, and immunohistochemical data

<table>
<thead>
<tr>
<th>Patient</th>
<th>AP (milliunits/g)</th>
<th>HPLAP (milliunits/g)</th>
<th>% of HPLAP of total AP</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1,016</td>
<td>170</td>
<td>17</td>
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<tr>
<td>2</td>
<td>10,034</td>
<td>373</td>
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<tr>
<td>3</td>
<td>10,182</td>
<td>557</td>
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<tr>
<td>4</td>
<td>2,833</td>
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</tr>
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</tr>
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<td>483</td>
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<td>8.3</td>
<td>++</td>
</tr>
<tr>
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<td>593</td>
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<td>10,843</td>
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<tr>
<td>12</td>
<td>2,758</td>
<td>517</td>
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<tr>
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<td>372</td>
<td>4.3</td>
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<td>+</td>
</tr>
<tr>
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<td>184</td>
<td>2.3</td>
<td>1.3</td>
<td>+</td>
</tr>
<tr>
<td>16</td>
<td>18</td>
<td>&lt;0.2</td>
<td>&lt;0.2</td>
<td>+</td>
</tr>
<tr>
<td>17</td>
<td>131</td>
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<td>0.09</td>
<td>+</td>
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<td>19</td>
<td>77</td>
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<td>&lt;0.5</td>
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</tr>
<tr>
<td>20</td>
<td>50</td>
<td>0.2</td>
<td>0.4</td>
<td>+</td>
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Table 4
Biochemical characteristics of tissue AP

<table>
<thead>
<tr>
<th>Patient</th>
<th>% of residual AP activity after Heat inactivation</th>
<th>L-P-Bromotetramisole inhibition</th>
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<tr>
<td>1</td>
<td>64</td>
<td>66</td>
</tr>
<tr>
<td>2</td>
<td>11</td>
<td>3</td>
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<tr>
<td>3</td>
<td>84</td>
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<td>6</td>
<td>66</td>
<td>69</td>
</tr>
<tr>
<td>7</td>
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<td>1.5</td>
</tr>
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<td>0</td>
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<td>9</td>
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<td>11</td>
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<td>0</td>
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<tr>
<td>12</td>
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</tr>
<tr>
<td>16</td>
<td>0.1</td>
<td>0</td>
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</table>

**Note:** According to the International Federation of Gynecology and Obstetrics. 1, well differentiated; 2, moderately well differentiated; 3, poorly differentiated; 4, C, bilateral; u, unilateral.

Table 3 (continued)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Tissue contents</th>
<th>Grading</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>AP+ HPLAP++</td>
<td>++</td>
</tr>
<tr>
<td>2</td>
<td>AP+ HPLAP++++</td>
<td>++++</td>
</tr>
<tr>
<td>3</td>
<td>AP+ HPLAP+++++</td>
<td>++++</td>
</tr>
<tr>
<td>4</td>
<td>AP+ HPLAP++++++</td>
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</table>

* * *

Tumor 5 (endometrioid carcinoma). The electrophoretic mobility of heat-stable AP from Tumor 3, which amounted to 84% of the total AP content, was slower than that of HPLAP from full-term placenta. In contrast, the heat-stable fraction from Tumor 5 (18% of total AP) had the same mobility as did placental HPLAP. Incubation with monoclonal antibody AP 230 to liver AP revealed that Tumor 3 contained only a small fraction of liver AP, whereas for Tumor 5 a large fraction of the total AP content was recognized by this antibody. Neuraminidase treatment demonstrated that no detectable amount of intestinal-type AP was present in either tumor.

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limiting the fixation time to 1.5 hr, sufficient activity of AP was preserved on paraffin sections for its histochemical staining while obtaining good histomorphology. Pretreatment of sections with trypsin significantly improved the sensitivity of the HPLAP immunohistochemical staining without affecting its specificity. In full-term placenta, heavy HPLAP staining (Fig. 2) was present on the apical plasma membrane of the syncytiotrophoblast. Moderate staining was observed on its basal plasma membrane, mainly in the larger vili. Patches of granular staining were present in the villous connective tissue. The histochemical localization of AP in placenta was identical to the immunohistochemical pattern for HPLAP.

Germinal inclusion cysts were present in one of the 3 normal postmenopausal ovaries (Patient 17). Only the epithelial cells lining these cysts together with the cystic content were strongly positive for AP (Fig. 3A) and HPLAP (Fig. 3, B and C). In the normal premenopausal ovaries (Patients 18 and 19), no cysts were found, and no HPLAP staining was detectable.

In all tumoral biopsies, the stroma was devoid of HPLAP staining, while a diffuse staining for AP was present, varying in intensity. The luminal plasma membranes of endothelial cells in small blood vessels and capillaries were also stained for AP.

In the first serous cystadenofibroma (Patient 13), numerous flattened cysts lined with a cuboid epithelium were present. Their apical epithelial membrane with bulging cytoplasmic extensions was weakly stained for AP and more strongly stained for HPLAP (Fig. 5).

The second serous cystadenofibroma (Patient 14) contained a number of cysts up to 0.5 mm in diameter. Their content and the luminal plasma membrane of the lining epithelium were stained for AP and for HPLAP (Fig. 4).

In the borderline papillary serous cystadenoma (Patient 12), strong and uniform AP (Fig. 6A) and HPLAP staining (Fig. 6, B and C) was present on the apical plasma membrane of the epithelial cells lining the papillae.

In the 2 well- to moderately well-differentiated papillary serous cystadenocarcinomas (Patients 1 and 2), HPLAP staining (Fig. 8B and Fig. 9B, respectively) was observed on approximately 50% of the carcinoma cells, forming monolayered papillae. The staining was membranous and predominantly localized at the luminal side. The pattern for AP (Fig. 8A and Fig. 9A, respectively) was comparable to that for HPLAP.

The less-differentiated papillary serous cystadenocarcinoma (Patient 3) was composed of glandular and acinar structures and multilayered papillae. Intense staining for AP (Fig. 7A) and HPLAP (Fig. 7, B and C) was present on almost all carcinoma cells; a diffuse staining was observed in the necrotic parts. HPLAP staining of carcinoma cells was exclusively membranous and in general present on the entire cell surface (Fig. 7C).

In the well-differentiated endometrioid carcinoma (Patient 4), HPLAP staining was observed on the entire cell surface (Fig. 10B) or only the luminal side (Fig. 10C) of the carcinoma cells in some of the glandular structures. Heavy staining for AP (Fig. 10A) was present in the tumoral stroma; the glandular structures were occasionally positive, comparable to the HPLAP staining.

In the first well- to moderately well-differentiated endometrioid carcinoma (Patient 5), HPLAP staining was focal (Fig. 11, B and C). Some glandular structures were strongly positive; many were weakly positive and some were negative. HPLAP was localized on the entire surface of carcinoma cells (Fig. 11C). Staining for AP (Fig. 11A) was present in all glandular structures.

In the second well- to moderately well-differentiated endometrioid carcinoma (Patient 6), uniform AP staining (Fig. 12A) was present on the luminal plasma membrane of unstratified carcinoma cells lining the cysts and also in the cystic content. HPLAP staining was very scarce (Fig. 12B) and was restricted to the luminal membrane of isolated or a few neighboring carcinoma cells (Fig. 12C).

In the moderately well-differentiated endometrioid carcinoma (Patient 7), AP staining (Fig. 13A) was present on the luminal plasma membrane of carcinoma cells lining pseudolumina and in the content of these lumina. HPLAP staining (Fig. 13B) was also membranous and was present only in a few glandular structures.

In the poorly differentiated adenocarcinoma (Patient 8), AP staining (Fig. 14A) varied in intensity. HPLAP staining (Fig. 14B) was observed only in some of the conglomerates of carcinoma cells and was predominantly membranous (Fig. 14B, inset).

In the moderately well-differentiated granulosa cell carcinoma (Patient 9), HPLAP (Fig. 15, B to D) was rarely present in acinar and glandular structures. In vesicular structures, HPLAP staining was absent or weak and was restricted to Call-Exner bodies (Fig. 15D). Weak staining was sometimes observed in the necrotic center. Small solitary or clustered trabeculae embedded in the larger stromal septa were, in general, heavily stained for HPLAP (Fig. 15B). Negative or weakly positive cells were sometimes observed between the strongly positive cells (Fig. 15C). HPLAP was present on the entire surface of carcinoma cells (Fig. 15C) except in the Call-Exner bodies where it was exclusively luminal. The pattern observed for total AP (Fig. 15A) was different. Numerous heavily stained glandular and acinar structures were adjacent to moderately positive or negative ones. Vesicles, their necrotic center, and Call-Exner bodies were moderately to strongly positive. AP and HPLAP staining of trabeculae was comparable.

In the poorly differentiated granulosa cell carcinoma (Patient 10), AP (Fig. 16A) and HPLAP staining (Fig. 16, B and C) resembles that observed in Tumor 7.

In the mixed heterologous Müllerian sarcoma (Patient 11), staining for HPLAP was negative; staining for AP was strong in all conglomerates of epithelial cells (Fig. 17).
DISCUSSION

Until recently, all investigations on HPLAP as a tumor marker have been based on heat stability, enzyme inhibition characteristics, electrophoretic techniques, and polyclonal antisera. All these methods suffer from a lack of sensitivity and specificity, especially in the discrimination between placental and intestinal AP. Using a specific mouse monoclonal antibody to HPLAP, we have demonstrated by EAIA and by immunohistochemistry the presence and histological distribution of HPLAP in malignant ovarian neoplasia, in benign ovarian tumors, and in normal ovaries. Our observations confirm data obtained by Benham et al. (2), who detected HPLAP in butanol extracts from benign and malignant ovarian tumors using a rabbit polyclonal antisera and electrophoretic mobility, heat stability, and inhibition criteria for identification. HPLAP has also been determined recently in benign and malignant ovarian tumors and in normal ovaries using heat stability and \( \gamma \)-phenylalanine sensitivity (11). Our HPLAP values for ovarian tissues samples, obtained during surgery, are in the same range as reported by these authors but are lower than the values determined by McLaughlin et al. (32) in postmortem ovaries using 2 specific monoclonal antibodies.

To the best of our knowledge, this is the first report on the immunohistochemical localization of HPLAP in normal ovarian tissue and in benign and malignant ovarian tumors. The well-documented specificity of the monoclonal antibody (10) and the concurrence between the immunohistochemical (HPLAP) and the histochemical (AP) staining in all HPLAP-positive sites underline the specificity of the present staining results. A broad correlation was found between the immunohistochemical grading and the tissue HPLAP levels.

The mixed heterologous Müllerian sarcoma apparently contained 2 milliunits HPLAP/g. However, a 0.02% nonspecific interference of total AP in the EAIA determination of HPLAP is able to account for the observed HPLAP level. The absence of HPLAP staining in this tumor confirms this explanation. Likewise, Takeda et al. (43) were unable to demonstrate positive histochemical staining of AP after heat inactivation on the membrane surface of the carcinomatous cells in a mixed mesodermal tumor of the ovary. Elevated serum levels of HPLAP have been observed, however, in an ovarian carcinoma (17).

All remnants of the germinal epithelium and all constituents of normal pre- and postmenopausal ovaries and also the follicular cyst in Biopsy 18 were negative for HPLAP staining. The only positive staining for HPLAP in normal ovarian tissue was found in germinal inclusion cysts. The presence of HPLAP in these cysts and in all serous tumors, from the benign cystadenofibromas over the borderline papillary cystadenoma to the papillary cystadenocarcinomas, reinforces the hypothesis that the serous ovarian tumors originate from the ovarian surface epithelium and, more in particular, from germinal inclusion cysts (7). In addition, the appearance of HPLAP in the germinal inclusion cysts reveals that the formation of these cysts is accompanied by some biochemical alterations in the germinal epithelial cells. The germinal epithelium is also considered the source of endometrioid carcinoma (7). The presence of HPLAP in these tumors fits also with the formulated hypothesis. By analogy, the presence of HPLAP in gastrointestinal premalignant states has been reported using a polyclonal antiserum (42) or polycyclamide gel electrophoresis and inhibition techniques (26). Our results also suggest that the presence of HPLAP in ovarian or tumor tissue is not an indicator for cancer as such.

The histological distribution patterns for HPLAP and for total AP demonstrate the pronounced heterogeneity of ovarian tumors in their expression of HPLAP and other forms of AP. Using the present monoclonal antibody, 9 of the 10 carcinomas studied contained an important number of carcinoma cells in which HPLAP could not be demonstrated immunohistochemically. The proportion of HPLAP-positive cells varied strongly between the different cases investigated. That ovarian and other tumors are not uniform populations of cancer cells is becoming more and more evident, and several mechanisms have been proposed for the explanation of this phenomenon (19, 20). Also, in mammary, testicular, and gastrointestinal tumors, the distribution of HPLAP staining varied greatly when monoclonal (30, 37) or polyclonal antibodies (42, 44) were used. Analogous observations have been made on the histological distribution of other markers in ovarian tumors, i.e., NB 70K (4) using a polyclonal antiserum and CA 125 (22) using a monoclonal antibody.

Further study will be necessary to determine if other forms of HPLAP are present in these tumors, some of which may not be recognized by the present monoclonal antibody. The occurrence of such forms of HPLAP is supported by our heat inactivation and \( \gamma \)-phenylalanine inhibition results. Indeed, the heat-stable and inhibitor-insensitive fraction of AP in the tumoral extracts is in all cases studied much greater than the fraction recognized by the present monoclonal antibody in the EAIA. The presence of intestinal-type AP can be excluded since the electrophoretic mobility of all tumoral AP was altered after neuraminidase treatment.

The unusual electrophoretic mobility of heat-stable HPLAP from Tumor 3 provides further evidence in favor of the existence of different types of neoplastic HPLAP. Differences in electrophoretic mobility of HPLAP from ovarian tumors compared to the 6 common placental HPLAP phenotypes have been reported (2). In addition, the presence of several variants of HPLAP in single testicular tumors has been demonstrated using 6 different monoclonal antibodies to HPLAP (37). In ovarian tumors, the coexistence of Regan and non-Regan HPLAP has been reported, even in single tumor cells, on the basis of histochemical techniques (15).

In all tumors investigated, the major part of HPLAP was localized at the level of the plasma membrane. In some cases, only the luminal cell surface was stained. This is partially in agreement with light microscopic and ultrastructural data from the paper of Sasaki and Fishman (41), who found predominant intracellular histochemical staining for HPLAP in one, combined intracellular and plasma membrane staining in another, and predominant plasma membrane staining in a third case of unspecified ascitic and tumoral ovarian cancer cells using \( \gamma \)-phenylalanine and \( \gamma \)-homoarginine inhibition. There are, however, no immunohistochemical data available on ovarian tumors based on a specific monoclonal antibody or a polyclonal antiserum to HPLAP.

Serum levels of HPLAP were elevated [\( \geq 0.1 \) unit/liter: the P99 value of our average hospital population (39)] in 7 of 11 (64%) ovarian carcinoma patients [including the borderline case]. This is more than the 35% reported by McLaughlin et al. (31) in an analogous study also using a specific monoclonal antibody. If the 100% cancer specific cutoff value of 0.2 unit/liter is taken as...
the limit for positivity in our study, 46% of the ovarian carcinoma patients still had elevated serum HPLAP levels. Raised serum levels of placent-al-intestinal-like AP have also been reported in 40% of patients with ovarian carcinoma (18) using a rabbit antiserum cross-reacting with intestinal AP. In another study based on heat inactivation and polyclonal antibodies, placent-al-intestinal-like AP was detected in 47% of serum samples from ovarian cancer patients (15). Finally, elevated heat-stable serum AP levels \( \geq 0.3 \) IU/liter were observed in 15% (28), 17% (6), and 18% (23) of ovarian carcinoma patients. In our study, the occurrence of elevated circulating HPLAP levels was in general associated with bilaterality and dissemination. This may limit the usefulness of HPLAP as a tool for the early detection of ovarian cancer. However, the HPLAP concentration in ascitic fluid was, in the 3 cases studied, 3 times higher than the corresponding serum levels, as has also been reported by heat inactivation and L-phenylalanine inhibition (11, 15). Consequently, the EIAA determination of HPLAP levels in ascitic fluid samples instead of serum might help in recognizing ovarian carcinomas at an earlier stage, thereby increasing the clinical relevance of the test.

In the present study, the serum CEA values were positive (\( \geq 5.4 \) ng/ml) in 17% of ovarian cancer patients. This frequency is comparable to the 14% positivity (\( \geq 5 \) ng/ml) reported by Bast et al. (1) and the 19% positivity (\( \geq 4 \) ng/ml) reported by Malkin et al. (28). In contrast, in the studies reviewed by Van Nagell et al. (1) and the 19% positivity (\( >4 \) ng/ml) reported by Bast et al. (46) and the study of Haije et al. (18), 50 and 44% of the patients were positive, respectively (values \( \geq 2.5 \) ng/ml are considered positive). Differences in assay methodology and criteria for positivity may be responsible for these discrepancies, which illustrate the need for standardization. In our series, HPLAP is a far better serological marker for ovarian cancer than is CEA.

At present, CA 125 seems to be the most promising tumor marker for ovarian serous carcinomas, with 74% of ovarian carcinoma patients having serum CA 125 levels higher than the \( P_{95} \) value observed in normal healthy blood donors and patients with benign disease (1, 24). However, as reported by Ruibal et al. and according to our own unpublished observations, several other nontumoral clinical conditions such as peritonitis, pancreatitis, and chronic liver disease are associated with high serum CA 125 levels in the range observed for ovarian and other tumors. Apparently, this is not the case for HPLAP. The serum level of 0.2 unit/liter, which is reached by 46% of the present ovarian carcinoma patients, was never observed in the absence of neoplasia in a sample of 1650 hospital patients including the clinical conditions mentioned above.

In conclusion, HPLAP was found and localized immunohistochemically in 2 of 2 benign, 1 of 1 borderline, and 10 of 11 malignant ovarian tumors. In normal ovaries, HPLAP was detected only in germinal inclusion cysts. The appearance of HPLAP in germinal inclusion cysts and its presence in the serous neoplasms support the hypothesis that these cysts may be the origin of the serous ovarian tumors. Most malignant tumors were very heterogeneous in their expression of HPLAP which, in all cases, was predominantly membranous. HPLAP, as determined by the present EIAA, is a better serological marker for ovarian cancer than is CEA.


PLACENTAL ALKALINE PHOSPHATASE IN OVARIAN CANCER

Fig. 2. Normal full-term placenta. HPLAP staining, x 150.

Fig. 3. Germinal inclusion cysts in normal ovary (Patient 17). A, AP staining, x 150; B and C, HPLAP staining. B, x 150; C, x 60.

Fig. 4. Serous cystadenofibroma (Patient 14). HPLAP staining. A, x 40; B, x 150; C and D, x 610.

Fig. 5. Serous cystadenofibroma (Patient 13). HPLAP staining, x 40.

Fig. 6. Borderline papillary serous cystadenoma (Patient 12). A, AP staining, x 60. B and C, HPLAP staining. B, x 60; C, x 610.
Fig. 7. Poorly to moderately well-differentiated papillary serous cystadenocarcinoma (Patient 3). A, AP staining, x 150. B and C, HPLAP staining. B, x 150; C, x 610.

Fig. 8. Well- to moderately well-differentiated papillary serous cystadenocarcinoma (Patient 1). A, AP staining, x 150. B, HPLAP staining, x 150.

Fig. 9. Well- to moderately well-differentiated papillary serous cystadenocarcinoma (Patient 2). A, AP staining, x 150. B, HPLAP staining, x 150.

Fig. 10. Well-differentiated endometrioid carcinoma (Patient 4). A, AP staining, x 150. B and C, HPLAP staining. B, x 150; C, x 240.
Fig. 11. Well- to moderately well-differentiated endometrioid carcinoma (Patient 5). A, AP staining, x 60. B and C, HPLAP staining. B, x 60; C, x 240.

Fig. 12. Well- to moderately well-differentiated endometrioid carcinoma (Patient 6). A, AP staining, x 60. B and C, HPLAP staining. B, x 60; C, enlargement of rectangle indicated in B, x 610.

Fig. 13. Moderately well-differentiated endometrioid carcinoma (Patient 7). A, AP staining, x 150. B, HPLAP staining, x 150.

Fig. 14. Poorly differentiated adenocarcinoma (Patient 8). A, AP staining, x 60. B, HPLAP staining, x 60; inset, x 360.
Fig. 15. Moderately well-differentiated granulosa cell carcinoma (Patient 9). A, AP staining, ×60. B to D, HPLAP staining. B, ×60; C, ×240; D, ×150.

Fig. 16. Poorly differentiated granulosa cell carcinoma (Patient 10). A, AP staining, ×60. B and C, HPLAP staining. B, ×60; C, ×610.

Fig. 17. Mixed heterologous Müllerian sarcoma (Patient 11). AP staining, ×150.
Human Placental Alkaline Phosphatase in Benign and Malignant Ovarian Neoplasia


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