Influence of O.C.T. Embedding Compound on Determinations of Estrogen and Progestin Receptors in Breast Cancer

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Estrogen receptor (ER) and progestin receptor (PR) concentrations in tumor biopsies are important predictive indicators of a clinical response to endocrine therapy of breast cancer. To assess interference of O.C.T. (optimum cutting temperature) embedding compound in assays of ER and PR by radioligand binding, we determined specific binding capacities and affinities of ER and PR in cytosols by a multipoint iteration method, using split samples of 14 breast-tumor biopsies, one portion serving as untreated control, the other treated with O.C.T. There was no statistically significant difference between these two groups. We then compared these data with those of historical controls analyzed both in the presence and absence of sodium molybdate (10 mmol/L). Eighty breast-tumor specimens (mean ± SD patients' ages, 59 ± 14 y) embedded in O.C.T. compound and analyzed without molybdate gave ER and PR values that differed insignificantly from those for 306 samples (patients' ages, 61 ± 14 y) untreated with O.C.T. Thirty-nine specimens (patients' ages, 58 ± 15 y) embedded in O.C.T. compound were analyzed in the presence of molybdate and compared with the results for 288 specimens (patients' ages, 31 ± 14 y) untreated with O.C.T. Again, there was no significant difference in the concentrations and affinities of receptors in the two groups. Evidently O.C.T. compound does not alter the receptor status of tumor biopsies.

Additional Keyphrases: embedding medium · radioligand binding · effect of molybdate

Increased public awareness and intensified screening methods such as breast self-examination and mammography have improved the diagnosis of breast cancer at earlier stages. As a fortunate consequence, the average size of breast-cancer lesions at biopsy and mastectomy has been smaller. Often the only tissue available for hormone-receptor analyses may be the biopsy that was embedded in O.C.T. (optimum cutting temperature) compound for the frozen-section diagnosis. O.C.T. compound is composed of polyvinyl alcohol, benzalkonium chloride, and polyethylene glycol in distilled water according to the manufacturer (Miles Laboratories, Elkhart, IN). Although Muench and Maslow (1) had suggested that O.C.T. embedding compound had a deleterious influence on the measurement of steroid receptors in breast cancer, their conclusion was based upon an artificial situation in which O.C.T. compound was added directly to cytosol. With the implementation of steroid receptors as a mandatory clinical chemical test (2, 3), in some instances the selection of patients for therapy of breast cancer has been made without benefit of these valuable tests because of that previous report (1).

We examined the effect of O.C.T. compound on the concentrations of estrogen receptors (ER) and progestin receptors (PR) in human breast-tumor specimens that had been embedded and frozen immediately for pathological examination. ER and PR concentrations and affinities were evaluated by the radioligand binding method (2, 3), both in the presence and absence of molybdate (3–7), and the results compared with the receptor values for specimens not embedded in O.C.T. compound. Addition of sodium molybdate, 5–10 mmol/L, to the homogenization buffer of breast cancer specimens inhibits a time- and temperature-dependent loss of ER- and PR-binding capacity in the cytosol (2–7). Currently, laboratories participating in the quality-assurance programs established by our laboratory for various cooperative trial groups and the College of American Pathologists (8) are divided in their use of molybdate, so we evaluated both types of assays.

Materials and Methods

Tissue Collection and Cytosol Preparation

Biopsies of human breast cancer were submitted by the surgeons and pathologists at hospitals utilizing the clinical laboratory services of the Hormone Receptor Laboratory. All analyses were first conducted to meet the primary clinical need of our patients; only unneeded residual tissue was used for additional assays. Tissue specimens embedded in O.C.T. compound ("Tissue-Tek"; Miles Laboratories Inc.) before being submitted to the laboratory were noted in the record. Tissues were brought to the laboratory on solid CO₂ and stored at −86°C for one or two days until analyzed.

While frozen, samples embedded in O.C.T. compound were trimmed to remove the bulk of the resin without sacrificing the tissue sample. Biopsies were promptly sliced with a scalpel and homogenized with a "Polytron" (Brinkmann Instruments, Inc., Westbury, NY) in the buffer provided in the DuPont/NEN Products ER and PR kits, both in the presence and absence of sodium molybdate, 10 mmol/L.

Fourteen specimens that were submitted in the frozen state were divided into an untreated control and a portion embedded in O.C.T. Both specimens were examined for ER and PR identically with radioligand-binding assays (2, 3).

Steroid Receptor Assay

Cytosols were prepared and assayed by radioligand-binding procedures, with dextran-charcoal used to remove unbound steroid (2, 3). The kits (cat. no. NEA-087 for ER, and NEA-091 for PR) supplied by DuPont/NEN Products for analyses of ER and PR were used with both daily laboratory controls and occasional interlaboratory comparisons as part of our responsibility as the national reference facility for these procedures (2, 8). Protein concentrations in cytosol were determined by the Bradford method (9) and adjusted to 2 g of protein per liter. Cytosol (0.1 mL per
assay) was incubated with either \(^{3}H\)estradiol-17\(\beta\) or \(^{3}H\)promegestone \((^{3}H)R5020\), at six standard concentrations, at 4 °C for 16 to 24 h. Non-specific binding was assessed in the presence of a 200-fold excess of either unlabeled diethylstilbestrol or R5020 for ER or PR, respectively. Unbound hormone was separated from bound \(^{3}H\)steroid by incubation with 0.3 mL of dextran-coated charcoal for 15 min. All assays were performed in duplicate, and specific binding capacities and \(K_d\) values were calculated according to the Scatchard method \((10)\) with a computer program designed by the Hormone Receptor Laboratory for the Apple IIe computer. This program is available for a marginal sum by writing to our laboratory.

Statistical Results

The Kolmogorov–Smirnov test was used to determine the nature of the distribution of the specific binding capacities and \(K_d\) values of each receptor. The distribution of each of the parameters significantly deviated from the gaussian pattern. Therefore, we performed nonparametric, distribution-free Wilcoxon matched-pairs test \((11)\) to analyze the effect of O.C.T. on steroid receptor values in matched samples from the same tumor biopsies. The Kruskal–Wallis test was used to evaluate the O.C.T. effect on ER and PR values for historical control specimens. Data distribution was, thus, expressed in terms of minimum, median, and maximum value, instead of mean ± standard deviation. All calculations were performed by using the SPSS statistical package \((11)\).

Results

We compared ER and PR values derived from the same tumor biopsies that were either embedded in O.C.T. compound or remained untreated (Table 1). There was no statistically significant difference in values for either the ER \((P = 0.695)\) or the PR \((P = 0.879)\) by the Wilcoxon matched-paired test. Application of the O.C.T. compound did not alter the receptor status of any of the 14 specimens we examined, using the cutoff values of 10 fmol per milligram of cytosol protein suggested by the National Surgical Adjuvant Breast Project \((12)\). Protein concentrations in the two experimental groups were not statistically different.

A retrospective study was conducted on a much larger patient population to compare the influence of O.C.T. compound on the specific binding capacities and affinities of ER and PR in biopsies of breast cancer. We compared the influence of O.C.T. under conditions in which sodium molybdate was included in the homogenization buffer \((3–7)\) with determinations in the absence of molybdate, because the original data indicating the clinical utility of ER and PR as predictive markers of endocrine-responsive cancer were derived from receptor assays performed without the stabilizing agent \((cf. 2, 3)\).

We compared results from ER and PR determinations of 306 historical controls with those of 80 biopsies embedded in O.C.T. compound, using the ligand titration assay in the absence of molybdate (Table 2). There was no statistically significant difference in either ER \((P = 0.679)\) or PR \((P = 0.132)\). Nor was any significant difference in either affinities of ER \((P = 0.236)\) or PR \((P = 0.421)\) found by the same (Kruskal–Wallis) statistical test.

Because O.C.T. compound reportedly \((1)\) may influence the association of ligand with receptor, we compared ER and PR binding values from 288 historical controls with those of 39 biopsies embedded in the compound obtained in the presence of 10 mmol of sodium molybdate per liter \((e.g., 7)\) in the ligand titration assay (Table 2). No statistically significant difference was observed in either ER \((P = 0.421)\) or PR values \((P = 0.840)\) between the two groups by the Kruskal–Wallis test. Furthermore, no statistically significant difference was observed in the \(K_d\) values for either ER \((P = 0.362)\) or PR \((P = 0.621)\) by the same test.

Discussion

Embedding breast tumors with O.C.T. compound at frozen-section diagnosis evidently has little influence on the specific binding capacities and affinities of either ER or PR, contrary to the results published by Muench and Maslow \((1)\). Also, there was no statistically significant difference in these biochemical parameters in the group of tissues treated with O.C.T. compound as compared with untreated biopsies when assays were performed in either the presence or the absence of sodium molybdate.

The data in Table 2 indicate that sodium molybdate did not increase ER and PR binding, in contrast to reports by other workers \((e.g., 3–7)\). However, the comparison in our study was conducted on historical controls in which the effects of molybdate were not evaluated on the same cytosolic preparations. If, in fact, ER and PR are determined simultaneously in the presence and absence of sodium molybdate \((5–10 \text{ mmol/L})\) in the same cytosol, most studies report a modest increase \((5–20\%)\) in ER values and a 40–75% increase in PR \((e.g., 3–7)\), with little influence on receptor status \((7)\). Our findings on using O.C.T. compound in conditions with and without molybdate are significant, because many laboratories continue to determine ER and PR without sodium molybdate in homogenization buffers \((12)\).

An apparently critical difference between our study and that of Muench and Maslow \((1)\) is that they added O.C.T. compound directly to cytosols at concentrations that would be unlikely to be encountered in the clinical labo-

| Table 1. Influence of O.C.T. Compound on Sex-Steroid Binding Capacities (fmol/mg Cytosol Protein) in Split Tumor Biopsies from 14 Breast-Cancer Patients |
|---|---|---|---|---|---|---|---|
| Estrogen receptors | | | Progestin receptors | | | |
| 295 | 630 | 39 | 56 | 117 | 53 | 132 | 150 |
| 49 | 173 | 30 | 101 | 0 | 4 | 0 | 0 |
| 154 | 192 | 208 | 112 | 28 | 25 | 62 | 106 |
| 150 | 74 | 35 | 35 | 61 | 72 | 234 | 218 |
| 17 | 23 | 0 | 0 | 27 | 18 | 26 | 25 |
| 11 | 11 | 15 | 22 | 173 | 142 | 42 | 13 |
| 25 | 22 | 94 | 76 | Median | 39 | 37 | 45 |

Wilcoxon matched-pairs test
ER \(Z = -3.3922\) \(P = 0.6949\)
PR \(Z = -1.1529\) \(P = 0.8785\)

* Biopsies exhibiting a numerical decrease in concentrations of ER and (or) PR.
Table 2. Influence of O.C.T. Compound on Sex-Steroid Binding Capacities (fmol/mg Cytosol Protein) Determined in
the Presence and Absence of Sodium Molybdate

<table>
<thead>
<tr>
<th>Age, y</th>
<th>Molybdate</th>
<th>O.C.T. comp.</th>
<th>No. of specimens</th>
<th>Estrogen receptor</th>
<th>Progesterin receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mean</td>
<td>Median</td>
</tr>
<tr>
<td>i1 ± 14</td>
<td>−</td>
<td>−</td>
<td>306</td>
<td>84 ± 10</td>
<td>20*</td>
</tr>
<tr>
<td>i9 ± 14</td>
<td>−</td>
<td>+</td>
<td>80</td>
<td>116 ± 41</td>
<td>41*</td>
</tr>
<tr>
<td>i1 ± 14</td>
<td>+</td>
<td>−</td>
<td>288</td>
<td>90 ± 98</td>
<td>26*</td>
</tr>
<tr>
<td>i8 ± 15</td>
<td>+</td>
<td>+</td>
<td>39</td>
<td>85 ± 26</td>
<td>47*</td>
</tr>
</tbody>
</table>

* P value insignificant in all four pairs. ER,Mo−,±:O.C.T.; *P = 0.679. ER,Mo+,±:O.C.T.; *P = 0.421. PR,Mo−,±:O.C.T.; *P = 0.132. PR,Mo+,±:O.C.T.; *P = 0.840.

*P value insignificant in all four pairs. ER,Mo−,±:O.C.T.; *P = 0.679. ER,Mo+,±:O.C.T.; *P = 0.421. PR,Mo−,±:O.C.T.; *P = 0.132. PR,Mo+,±:O.C.T.; *P = 0.840.

Patient support of this research was provided by a grant from the Make a Grant to the University of Sarajevo, Yugoslavia.

References

CLIN. CHEM. 35/12, 2319–2321 (1989)

Chemiluminescent Enzyme Immunoassay of α-Fetoprotein Based on an Adamantyl Dioxetane Phenyl Phosphate Substrate

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We have evaluated a new chemiluminescent substrate for the alkaline phosphatase (EC 3.1.3.1) label used in a Hybtech Tandem™-E immunoassay of α-fetoprotein (AFP). The new substrate, adamantyl 1,2-dioxetane phenyl phosphate (AMPFP™), emits light at 477 nm when acted upon by the enzyme. Detection limits for AFP with this method were 33 ng/L (mean of 20 replicates of the zero standard + 2 SD) and 470 ng/L (two background). Between-batch CVs ranged from 43.1% to 9.60% for AFP in the range 29.1–132.0 μg/L. Comparison of results for 49 specimens assayed with use of the chemiluminescent kit and a colorimetric version of the AFP assay gave statistical values as follows: slope = 0.88, intercept = 4.19, and r = 0.94.

Additional Keyphrases: cancer · neural tube defects · Down's syndrome · prenatal diagnosis

α-Fetoprotein (AFP) is widely measured to screen for fetal neural tube defects (1) and Down's syndrome (2), and to detect hepatoma (3) and teratomas of the ovary and testis (4). Several nonisotopic immunoassays of AFP have been developed based on enzyme labels such as horseradish peroxidase (5), alkaline phosphatase (6), and β-galactosidase (7); chemiluminescent labels (8); and europium chelate labels (9, 10). Recently we have described the application of adamantyl dioxetane phenyl phosphates such as

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Received July 19, 1989; accepted August 15, 1989.

CLINICAL CHEMISTRY, Vol. 35, No. 12, 1989 2319