Hormone Binding by Human Mammary Carcinoma

Ian M. Holdaway and Henry G. Friesen

Department of Physiology, University of Manitoba, Winnipeg, Canada R3K OW3

SUMMARY

The specific binding of labeled porcine insulin, human prolactin, and human growth hormone was studied in 63 human breast tumors and 15 nonmalignant breast tissues. Most (90%) of the tumors demonstrated significant binding of insulin, as did 80% of nonmalignant tissues. Autoradiographic studies indicated that insulin bound dominantly to tumor cells, rather than to fat and fibrous tissue contained within tumors. Specific binding of prolactin and growth hormone of greater than 1% was seen in 20 and 12% of tumors, respectively, and one tumor studied in detail showed a small amount of saturable, high-affinity prolactin binding. The affinity of binding of insulin and prolactin to tumor was similar to that seen in target tissues in subprimate species (Kd = 4 x 10^-10 M), but the prolactin-binding capacity in the one tumor studied in detail was very low (10 fmoles/mg membrane protein), compared with prolactin-responsive experimental mammary carcinoma.

INTRODUCTION

It has been recognized for some years that many experimental (2, 19) and human (13, 17) mammary tumors possess specific binding sites for estrogens, and retrospective surveys have suggested that human tumors that lack estrogen binding sites frequently fail to respond to endocrine treatment (13). In addition, it has recently become apparent that some mammary cancers of the rat also bind a number of polypeptide hormones, in particular PRL (4, 14, 30), and INS (14). In hormone-responsive rat mammary tumors, the degree of PRL binding appears to correlate with tumor response to endocrine treatment (10), including both administration (14) and withdrawal (11) of PRL. Since PRL has been suggested as a factor possibly involved in the maintenance of some human mammary tumors in vivo (8, 28) and in vitro (23), the possibility exists that the presence of binding sites specific for PRL may have therapeutic implication similar to that for estrogens (see above). However, to date, there has been no report of binding of polypeptide hormones by human mammary tissue, and this study was undertaken to survey a group of human tumors and benign breast lesions for the presence of binding sites for polypeptide hormones and estrogens.

MATERIALS AND METHODS

Standard and Labeled Hormones. Human growth hormone (NIH 1934D; 2.6 units/mg) was kindly supplied by the Endocrine Study Section of the Institute of Arthritis, Metabolic and Digestive Diseases, Bethesda, Md. INS (24.4 units/mg) was supplied by Connaught Laboratories (Toronto, Ontario, Canada), and Eli Lilly Co. Indianapolis, Ind. Kindly supplied porcine proinsulin and insulin from a number of other species. Human prolactin was prepared by the method of Hwang et al. (12); 2 preparations were used in the study, and both were active in receptor-binding systems using plasma membranes from pregnant rabbit mammary glands (27) with potency 90 to 100% in terms of hPRL Friesen No. 1 (28 IU/mg). Labeled estradiol (17β-[6,7-H]estradiol) was purchased from New England Nuclear (Boston, Mass.). Labeling of hPRL and hGH with 125I (New England Nuclear) was performed by a soluble lactoperoxidase method (29), with purification of the labeled hormone by chromatography, with the use of Sephadex G-100. On several occasions, labeled hPRL was further purified by combination with particulate plasma membranes from pregnant rabbit mammary gland (27), followed by elution of bound hormone with 2 M thiocyanate and rechromatography and desalting, with Sephadex G-100. Such "receptor-purified" labeled hormone did not demonstrate significantly superior binding properties, compared with the starting material. Insulin was labeled with 125I, using chloramine-T oxidation (7).

Collection and Processing of Tissues. Sixty-three tumors (50 infiltrating duct carcinomas, 2 medullary carcinomas, 1 comedocarcinoma, 1 cystosarcoma phylloides, 6 lymph node metastases and 3 skin metastases) and 15 nonmalignant mammary samples (6 specimens of mammary fat, 3 fibroadenomas, and 6 samples with cystic hyperplasia) were obtained during mastectomy. Specimens were collected either at the time of histological examination by frozen section in the operating suite or following transfer to the pathology department 30 to 60 min after excision. After collection, samples were kept on ice for approximately 15 min until frozen in n-hexane chilled to −65° in a bath of dry ice and ethanol. More recently, a number of samples have been delivered from peripheral hospitals necessitating a period of up to 2 hr on ice before freezing. Samples were kept at −20° or −70° for up to 2 weeks before processing. Plasma membranes were prepared by differential ultracentrifugation as follows: frozen samples were sliced finely with a scalpel in a Petri dish standing on dry ice, thawed, and further minced with scissors and homogenized for 60 sec in 2 to 4 volumes of cold buffer (0.01 M Tris-HCl-0.0015 M EDTA, pH 7.4, with 0.5 mM dithiothreitol), with a Polytron.
tissue homogenizer. The crude homogenate was centrifuged at 15,000 × g for 20 min, and the resulting supernatant was recentrifuged at 100,000 × g for 60 min. The 100,000 × g pellet, containing the cell membranes (26) and hereafter described as the particulate membrane fraction, was homogenized in assay buffer (0.01 M Tris-HCl, pH 7.6-10 mM MgCl₂-0.1% bovine serum albumin) prior to assay. The supernatant from the 100,000 × g centrifugation was used for estimation of estradiol-binding proteins. Protein concentration was determined by the method of Lowry et al. (15). In some cases, ultrathin (20-μm) slices of tissue were obtained from 1-cm frozen blocks of tumor for assessment of binding in untreated samples (11).

Binding Assay for Peptide Hormones. Particulate membrane preparations (0.1 ml, containing approximately 500 μg protein) were incubated in duplicate with 100,000 cpm (approximately 0.5 ng) of labeled hormone with or without excess unlabeled hormone (10 μg/ml) in a total volume made up to 0.5 ml with assay diluent. Samples containing labeled hPRL or hGH were incubated for 6 hr at room temperature; samples for estimation of insulin binding were incubated for 24 hr at 4°C; the lower temperature and correspondingly longer incubation time to achieve binding equilibrium were found necessary to prevent degradation of labeled insulin at room temperature. At the end of the incubation period, 3 ml cold assay buffer were added, samples were centrifuged at 3000 × g for 20 min, the supernatants were decanted, and the pellets, containing membrane-bound hormone, were counted in an automatic γ-counter. The binding of labeled hormone was also assessed with the use of 20-μm tissue slices (11). In all cases, the uptake of labeled hormone was calculated as specific binding, being the difference between counts bound in the absence and presence of excess unlabeled hormone, expressed as a percentage of total counts added. In some cases where sufficient material was available, Scatchard analysis (24) was used to quantify binding affinity.

Binding of Labeled Estradiol. Estradiol binding was measured by the technique outlined by McGuire and De La Garza (18). Scatchard (24) analysis of binding data was performed to quantitate the number of binding sites per mg cytosol protein. Binding in the presence of excess unlabeled hormone was subtracted from each assay point, and the data were computed with a Hewlett-Packard desk computer.

Autoradiography of Binding of ¹²⁵I-labeled Insulin to Tumor Tissue. Frozen sections of tissue (5 to 8 μm) were obtained in a cryostat and flash-dried onto glass slides at room temperature. Sections were incubated at 37° for 30 min with 0.1 ml assay buffer containing 150,000 cpm ¹²⁵I-labeled INS, washed thoroughly in distilled water, dried, coated with Kodak NTB2 photographic emulsion, and exposed for 10 to 14 days prior to developing. Sections adjacent to those used for autoradiography were stained with hematoxylin and eosin to show histological detail.

RESULTS

Lower Limit of Experimental Significance in Estimates of Specific Binding. Three methods were used to assess the lower limit of significance in estimating specific binding of ¹²⁵I-labeled hPRL to particulate membrane preparations (Table 1). Plastic tubes showed a particular tendency to bind ¹²⁵I-labeled hPRL, and assays were performed in glass tubes in most instances. There was a negligible contribution to observed specific binding from counting and pipetting errors, as shown by the attempt to displace adsorbed ¹²⁵I from tumor membranes with unlabeled hPRL. Hormone from a subprimate species that might not be expected to bind to human tissue, bovine growth hormone, also showed only a small degree of specific binding. On the basis of these criteria, it was considered that specific binding in excess of 1% might be experimentally significant; this figure represents a difference of at least 1000 cpm between the means of duplicate samples measured with or without excess unlabeled hormone.

Influence of Protein Concentration on Specific Binding. Specific binding of 1% or more was never observed in particulate membrane samples with a protein content less than 1.5, 3, or 5 mg/ml for ¹²⁵I-labeled INS, hPRL, and hGH, respectively. Samples with a protein content less than this were thus excluded from this study on the basis that recovery of membranes might have been too low to permit adequate measurement of hormone binding. In 2 tumors tested, specific binding of labeled INS and hPRL was linearly related to membrane protein concentration over the range 400 to 1100 μg/tube, beyond which binding was not tested. The relationship between binding of labeled hGH and protein concentration was not determined.

Binding of Labeled Insulin. The extent of binding of labeled insulin by human mammary tissue is shown in Chart 1. Most (43 of 48; 90%) tumor specimens exhibited more than 1% specific binding of ¹²⁵I-labeled INS, as did 9 of 11

<table>
<thead>
<tr>
<th>Binding agent</th>
<th>Labeled material</th>
<th>No. of observations</th>
<th>% of total counts added apparently displaced by 1 μg unlabeled PRL</th>
<th>MEAN</th>
<th>RANGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test tubes containing assay diluent:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glass</td>
<td>¹²⁵I-labeled hPRL</td>
<td>3</td>
<td>0.2</td>
<td>0-0.4</td>
<td></td>
</tr>
<tr>
<td>Plastic</td>
<td>¹²⁵I-labeled hPRL</td>
<td>3</td>
<td>0.4</td>
<td>0-0.9</td>
<td></td>
</tr>
<tr>
<td>Tumor membranes</td>
<td>¹²⁵I</td>
<td>10</td>
<td>0.1</td>
<td>0-0.1</td>
<td></td>
</tr>
<tr>
<td>Tumor membranes</td>
<td>¹²⁵I-labeled bovine growth hormone</td>
<td>4</td>
<td>0.2</td>
<td>0-0.5</td>
<td></td>
</tr>
</tbody>
</table>


Nonmalignant tissues. The uptake of insulin by a representative tumor plotted according to Scatchard (24) is shown in Chart 2. Also shown for comparison is the uptake of hormone by mammary fat cell membranes obtained from the same patient. The affinity of binding is the same for the 2 tissues (K_a approximately 2.5 x 10^9 liters/mole), but the tumor possesses almost twice the number of binding sites per mg protein as the fat tissue.

In Table 2, data from 5 subjects are shown in which insulin uptake was studied in both neoplastic and mammary fat tissue from the same patient. K_a values are of the same magnitude in the 2 tissues, but in only 2 cases did the concentration of binding sites in fat exceed that in tumor. The overall binding capacity (mean ± S.D.) in 19 tumor samples was 126 ± 85 fmoles/mg protein and, in 5 fat samples, was 144 ± 80 fmoles/mg protein; the mean K_a values were 2.2 and 1.2 x 10^9 liters/mole, respectively. Autoradiographs of insulin uptake by tumor slices in vitro (Figs. 1 and 2) showed binding dominantly over areas of tumor with much lower uptake over regions of fat and fibrous connective tissue.

**Binding of Labeled PRL and Growth Hormone.** Specific binding of [125I]labeled hPRL of 1% or more was observed in 8 of 41 tumors (range, 1.1 to 4.2%/500 ¿g membrane protein), and 2 of 16 tumors demonstrated more than 1% specific binding of [125I]labeled hGH (1.0, 1.2%/500 ¿g membrane protein). Specific binding of both polypeptides was less than 1% in all nonmalignant tissues studied. A comparison of specific and nonspecific binding in the 8 tumors with more than 1% specific binding of [125I]labeled hPRL is shown in Chart 3. Although total binding was as high as 10% of total counts added, specific binding was always less than one-half total binding (range, 14 to 38% total binding). In one case, sufficient membrane was available for Scatchard analysis of binding of [125I]labeled hPRL, and this showed a small component of saturable high-affinity binding (Chart 2). In 10 of 20 Scatchard analyses of insulin binding, and in the single study of PRL binding shown in Chart 2, there was a suggestion of a 2nd set of low-affinity binding sites seen at high concentrations of unlabeled hormone (K_a approximately 4 x 10^8 liters/mole).

**Uptake of Labeled PRL by Various Tumor Fractions.** Since specific binding of PRL by tumor tissue membranes appeared low, compared with that for insulin, various side-fractions prepared during ultracentrifugation, and unprocessed aliquots of tumor were investigated for specific binding of labeled PRL to see whether binding sites might be lost during sample handling. As shown in Chart 4, tumors where particulate plasma membranes (100,000 x g pellet) showed specific binding of [125I]labeled hPRL of greater than 1% also demonstrated significant specific binding when...
unprocessed tumor (ultrathin sections) was tested. However, the precipitates from low-speed centrifugation (15,000 \( \times g \) pellet) of these tumors did not show significant PRL binding. Similarly, in tumors showing insignificant binding of hPRL to particulate membrane fractions, specific binding was correspondingly low in the 15,000 \( \times g \) pellet and also in the 100,000 \( \times g \) supernatant (data not shown). On 3 occasions, specific binding of 1 to 2% was seen in either crude homogenates or tumor slices when values were less than 1% in the membrane fractions.

**Specificity of Binding of Insulin and PRL.** Insulin from a number of species was able to cross-react with \(^{125}\text{I}\)-labeled INS bound to tumor membranes (Chart 5); guinea pig and rat insulin were slightly less effective, and porcine proinsulin was almost 100 times less potent than hormone from other species. The displacement of bound PRL from a particulate membrane preparation of a human mammary tumor by various unlabeled hormones is also shown in Chart 5. Hormones structurally and functionally unrelated to PRL were without effect, whereas bovine and rat PRL, hGH, and human placental lactogen were equipotent with hPRL at displacing labeled hormone from tumor-binding sites. By comparison, ovine PRL appeared more potent than hPRL, although lack of tumor material made definitive assessment of potency impossible.

**Relation of Binding of Estradiol and Polypeptide Hormones.** The relationship between the concentration of estradiol-binding protein and specific hormone binding for all tumors with significant uptake of polypeptide hormones is shown in Chart 6. There was no significant correlation between the absolute levels of estradiol-binding protein and the extent of binding of any of the polypeptide hormones, although, in general, tumors with more than 1% specific binding of a polypeptide hormone also demonstrated significant estrogen binding. All tumors with more than 1% binding of labeled PRL had significant binding of insulin; conversely, 7 of 22 tumors with significant insulin binding showed more than 1% specific binding of PRL. The 2 tumors with more than 1% binding of hGH also bound significant amounts of PRL and insulin.

**DISCUSSION**

It is recognized that experimental animal mammary tumors bind PRL, insulin, and estrogens. Although indirect evidence has suggested that there may be uptake of PRL by human tumors in vitro (23), there have as yet been no studies reporting binding of polypeptide hormones by human breast cancers. The results of this study indicate that many human tumors possess appreciable numbers of binding sites for insulin, and an occasional tumor may contain a small number of high-affinity binding sites for hPRL.

The reaction of labeled insulin with mammary tissues...
I. M. Holdaway and H. G. Friesen

(Chart 1) indicates that most tumors possess insulin-binding sites. The mean binding site concentration is comparable to that seen in human placenta (22) and rat fat cells (5). Since many tumor samples unavoidably contain a proportion of fat and fibrous tissue, it was important to decide whether the observed hormone uptake could be due to these components which are themselves able to bind insulin (5, 9). Estimates of binding site content in tumor and mammary fat tissue obtained from the same patients (Table 2) reveal that even a 50% content of fat in tumor samples could not account for the observed uptake. This finding was confirmed by autoradiography in a number of tumors (Figs. 1 and 2) which indicated that labeled hormone is undoubtedly bound to tumor tissue. It should be noted that, in the autoradiographs, tissues of high membrane content (tumor cells) are contrasted to those of low membrane content (fat cells). When the membranes were concentrated and fat was removed by centrifugation, the capacity to bind labeled insulin was seen to be similar in the 2 tissues (Table 2). The affinity of insulin for binding sites in mammary tissues was approximately the same as in other insulin-binding tissues (5). Insulin-binding sites have been found in the dimethylbenz(a)anthracene-induced rat mammary tumor (14), since this tumor frequently regresses following destruction of the pancreatic islets (2). It is interesting to speculate whether there is any connection between insulin-binding sites in human breast tumors and the role of obesity and high fat intake, both potential causes of insulin hypersecretion, as possible risk factors in the etiology of human breast cancer (16, 25). Similarly, if insulin is important in tumor maintenance, decreased plasma insulin levels following hypophysectomy may provide one possible mechanism for tumor regression sometimes seen with this procedure. Interaction of factors such as nonsuppressible insulin-like material (20) and epidermal growth factor (3) with insulin-binding sites might provide an additional mechanism for tumor maintenance.

Compared with the findings for insulin, only a few tumors demonstrated experimentally significant binding of labeled PRL and hGH. In these tumors, background binding, either nonspecific to binding to mammary membranes or to test tubes, was high, constituting up to 8% of total counts added. Since a small amount of apparently specific binding of labeled polypeptides can occur to inert surfaces (6), it could be argued that the apparently specific binding of $^{125}$I-labeled hPRL observed in Chart 3 might be "nonspecific." However, in the 1 tumor in which there was sufficient membrane for further analysis, there was a small amount of specific (Chart 5), high-affinity, saturable (Chart 2) binding present, having the characteristics of PRL binding observed in target tissues in subprimate species (26, 27). The content of binding sites in this tumor was only 1% of that observed in PRL-sensitive rat mammary tumors (14), although the affinity constant for PRL binding to both human and murine tissues is such that plasma hormone at physiological concentrations could interact with these sites.

The finding that only a minority of tumors possess binding sites for PRL is possible evidence against an important role for PRL in the development and maintenance of human breast cancer. It is, however, obviously important to ensure that the degree of binding observed here genuinely reflects the binding capacity of human mammary tissues. The possibility that the low order of binding of hPRL might be due to loss or destruction of binding sites during tumor processing is unlikely in view of the similarity of binding measurements made on unprocessed slices of tumor and partially purified membranes (Chart 4). Specific binding of 1 to 2% was observed in slices from several tumors that showed insigificant binding to membranes, possibly because the lower limit of significance is greater with the less precise slice technique. Alternatively, these may have been tumors that lost a small number of binding sites during processing. Further evidence that processing does not cause loss of binding sites is the finding that, where particulate membrane preparations were negative for specific binding, crude tissue homogenates, with one exception, were also negative. Rapid degradation of binding sites during collection and storage before tumor processing may also have caused loss of binding activity; the presence of significant insulin binding in most of the specimens argues against this possibility, although the rate of degradation might vary between different classes of binding site.

A further possible reason for low tumor uptake of PRL and hGH might be reduced biological and receptor activity of the labeled hormones used in this study. Formal biosays of the labeled materials were not performed to answer this point; however, all labeled preparations were tested for ability to bind to standard receptor preparations (rabbit mammary tissue membranes for hPRL and rabbit liver membranes for hGh) and showed full binding activity in this regard. Although possible, it seems unlikely that the PRL and hGH receptors in these tissues would be widely different from their human counterparts. For instance, it has been demonstrated that the physicochemical reactions of $^{125}$I-labeled hGh with human liver membranes are similar to those of $^{125}$I-labeled hGh with rabbit liver preparations (1).

When the binding of steroid and polypeptide hormones to human breast tumors is compared, it appears that most tumors with significant binding of polypeptide hormones also contain significant amounts of estradiol-binding protein (Chart 6). This is particularly evident for tumors with more than 1% binding of hPRL, all of those measured for estradiol uptake having binding protein contents of greater than 8 fmoles/mg cytosol protein. This relationship was less noticeable for tumors with significant insulin binding, 20% of which did not have significant estradiol binding. It is possible that tumors with significant polypeptide hormone uptake represent a group with a generally high content of hormone binding sites. This "differentiation" with respect to binding sites could permit speculation that they may be particularly responsive to endocrine therapy. Careful study of the behavior of these tumors following hormone manipulation may allow this hypothesis to be tested.

ACKNOWLEDGMENTS

We are grateful to Dr. T. Hyodo for supplying iodinated insulin and to D. Maughn, L. Bloom, and Dr. R. Cooke for help with specimen collection. We thank D. Bazak and E. Zernickel for help in preparation of the manuscript and J. Harris for preparing the illustrations. We particularly acknowledge the help of Dr. I. Worsley who prepared human prolactin used in this study.

1950
REFERENCES

Fig. 1. Autoradiograph of in vitro $^{125}$I-labeled insulin uptake (left) and hematoxylin and eosin stain (right) of adjacent sections of human breast cancer 22.
Fig. 2. Autoradiograph of in vitro $^{125}$I-labeled insulin uptake (top) and hematoxylin and eosin stain (bottom) of adjacent sections of human breast cancer 17.
Hormone Binding by Human Mammary Carcinoma
Ian M. Holdaway and Henry G. Friesen