Regulation of Insulin Binding to Human Mammary Carcinoma

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ABSTRACT

In vitro binding of labeled insulin was measured in crude membrane fractions of tumor and fat obtained at the time of breast cancer surgery in 23 women. There was significant insulin binding in 22 of 23 tumor specimens and in all 23 fat samples. High-affinity binding sites were present in tumors \(K_a = 1.38 \pm 0.88 \text{ (S.E.) m}^{-1}\) and adjacent fat membranes \(K_a = 1.12 \pm 0.15 \text{ (S.E.) m}^{-1}\). The level of insulin binding was not related to either estrogen receptor status or tumor histological grade. There was a significant negative correlation between insulin binding to fat membranes and fasting insulin levels. In contrast, there was no significant relationship between insulin binding to tumor tissue and serum insulin levels, suggesting the loss of the capacity to “down-regulate” insulin-binding sites by breast tumor cells. The absence of this ability to regulate insulin-binding capacity could result in an increased sensitivity of breast tumor cells to the stimulatory effects of insulin.

INTRODUCTION

Cell surface receptors for insulin have been identified in 2 experimental models of mammary carcinoma in rodents, the DMBA\(^3\)-induced mammary tumor (34) and the R3230AC transplantable mammary adenocarcinoma (11). That this receptor is involved in the biological action of insulin is suggested by the observation that insulin stimulates DNA synthesis (12) and the hexose monophosphate shunt (5) in the DMBA-induced tumor. In addition, insulin has effects on tumor growth. Thus, insulin generally stimulates growth of the DMBA-induced tumor (13, 14), whereas the R3430AC tumor is inhibited by insulin (6, 14). Human breast cancer cell lines cultured in vitro also contain insulin receptors (27), and in some of these lines, insulin stimulated synthesis of macromolecules and fatty acids (27).

Membrane fractions of human breast tumors obtained fresh at the time of surgery also contain specific, high-affinity binding sites for insulin (15). In contrast to experimental tumors, little is known about the functional characteristics of insulin binding in human breast cancer. For example, it has not been determined whether the number of binding sites is fixed for each cell or changes in response to alterations in the extracellular environment. Insulin receptors in normal tissues are subject to regulation by a number of factors including exercise (29), corticosteroids (1), and hypoglycemics (2, 26). Under normal physiological conditions, however, the most important determinant of insulin receptor concentration is the fasting blood insulin level. A reduction in the number of receptors in response to raised levels of insulin (“down-regulation”) has been demonstrated in monocytes and adipocytes (24) in humans and a variety of tissues in rats (32).

In this investigation, we have considered the relationship between fasting insulin levels and insulin binding to human breast tumors. Histological grading of the tumor and ER status were also determined in an attempt to identify other factors which might influence insulin binding.

MATERIALS AND METHODS

Collection and Preparation of Tissues. All tissues were obtained fresh at the time of surgery for breast cancer. Fasting blood for insulin determination was obtained on the morning of surgery prior to the administration of i.v. fluids. Plasma insulin concentration was determined by radioimmunoassay. The tumor specimen was transported in a dry container, on ice, to the pathology department where samples of the tumor and adjacent uninfiltrated fat were removed for analysis. Tumor samples were carefully trimmed free of nonmalignant tissue and placed in storage at \(-70^\circ\) within 2 hr of removal. All samples were assayed within 20 weeks of collection. Plasma membranes were prepared as follows: samples were snap frozen in liquid nitrogen and pulverized with mortar and pestle. The resulting powder was weighed and homogenized in 5 volumes of cold buffer (0.01 M Tris-HCl, 0.0015 M EDTA-0.0025 M dithiothreitol, pH 7.4) using two 15-sec bursts with a Polytron homogenizer set at 3.5. The crude homogenate was then centrifuged at 15,000 \(\times g\) for 20 min at \(4^\circ\), and the resulting supernatant was centrifuged at 100,000 \(\times g\) for 60 min. The supernatant from the 100,000 \(\times g\) centrifugation, containing the cytosol proteins, was assayed for ER. The 100,000 \(\times g\) pellet, containing the cell membranes (35), was resuspended in a volume of assay buffer (0.025 M Tris-HCl, 10 mM MgCl\(_2\), pH 7.6) 0.1% bovine serum albumin) estimated to give a final membrane protein concentration of 3 mg/ml. Protein concentration of the cytosol and membrane preparations was estimated by the method of Lowry et al. (20).

Labeled Hormones. Labeled estradiol (\(^{17}F\)-\(^{6,7-3H}\)estradiol) was purchased from New England Nuclear (Boston, Mass.). Porcine insulin (24 units/mg) was kindly provided by Novo Laboratories, Inc. (Copenhagen). Insulin was labeled with \(^{125}I\) using chloramine-T (9) and purified on Sephadex G-100. Specific activity ranged between 150 and 190 \(\mu\)Ci/\(\mu\)g.

Insulin-binding assay. Membrane preparations (0.1 ml containing approximately 0.3 mg protein) were incubated in duplicate with labeled insulin (0.2 ng) and increasing amounts of unlabeled insulin (0.5 to 5 \(\times 10^5\) ng) in a total volume made up to 0.5 ml with assay buffer. Samples were incubated at \(4^\circ\) for 24 hr. After incubation, the tubes were centrifuged at 3000 \(\times g\) for 40 min, the supernatant was decanted, and the pellet containing membrane-bound labeled insulin was counted in a Packard 1285 gamma counter. Specific binding at each concentration of unlabeled insulin was calculated as the difference in the percentage of added counts bound (total binding) minus the percentage of counts bound in the presence of 5000 ng unlabeled insulin (nonspecific binding). The ranges of insulin concentrations were chosen deliberately so that affinity measurements could be performed with the initial linear high-affinity portion of the Scatchard (33) plot (Chart 2). Data were analyzed using a linear least-squares fit to determine binding site concentration and affinity. Results were expressed as...
either binding site concentration or "specific binding," expressed as the difference in counts bound in tubes containing label only and those containing label plus 5000 ng unlabeled hormone, expressed as a percentage of total counts added. Considering the range of specific binding seen in the absence of membranes (0 to 0.3%) and in the presence of 0.3 mg of denatured (boiled for 30 min) placental membranes (0 to 1.2%), a specific binding greater than 2% was considered to be significant. All specific binding results were adjusted to a membrane protein concentration of 3.0 mg/ml, as specific binding was found to be a linear function of protein concentration in the range 0.5 to 5.0 mg/ml in both tumor and fat.

Degradation of labeled hormone by membranes was tested following incubation of 125I-labeled insulin with membrane preparations under standard conditions. The supernatant following centrifugation was rein- cubated with a fresh sample of placental membranes prepared by the same procedure described above. Binding was compared with the binding seen with fresh labeled hormone.

**Estrogen-binding Assay and Histological Grading.** The supernatant of the 100,000 x g centrifugation was assayed for estradiol binding using a charcoal-dextran assay (16). The concentration of binding sites was determined by Scatchard analysis (33) and expressed as receptor concentration per mg of cytosol protein. Estradiol binding of less than 5 fmol/mg was considered "receptor negative," since such tumors have shown a very low rate of response to endocrine therapy (17).

Tumors were classified according to the grading procedure of Bloom and Richardson (4). Tumor grade was from I to III with Grade III tumors being the most undifferentiated.

**RESULTS**

**Interassay Variation.** Because of the irregular times of availability of tumor samples, individual tissue specimens were processed and assayed on separate occasions. In order to assess interassay variation, a sample of human placental membranes was included in each assay as a control. Fresh human placenta was processed as outlined in "Materials and Methods," and individual aliquots were stored at —70°C. There was no significant decrease in insulin binding to membranes after 4 months of storage at —70°C. The average specific binding to placental membranes was 27.7 ± 4.0% (S.E.) with a between-assay coefficient of variation of 14.4% (n = 10).

Since, in most instances, only a portion of the tumor specimen was available for analysis, the possibility that nonhomo-

geneity of the tumor specimen might introduce sampling error was also considered. One large tumor was divided into 3 equal pieces, and each piece was assayed on a different occasion. Scatchard analysis of binding to the 3 samples is shown in Chart 1. There was no significant difference in affinity or total binding in the 3 fragments. On 2 occasions, a large sample of fat was divided into equal parts and assayed separately with no significant differences in specific binding or affinity among the samples.

**Insulin-binding Studies.** All except one of the 23 tumors assayed demonstrated significant insulin binding (greater than 2%/0.3 mg membrane protein). Specific binding of 125I-labeled insulin ranged from 1.8 to 10.1%, mean 4.8 ± 5.3% (S.E.) per 0.3 mg membrane protein. Two patients had received local radiotherapy preoperatively, and binding to these tumors was close to the average for the group (3.8 and 4.7%). One patient was a non-insulin-dependent diabetic receiving treatment with diet and sulfonylurea and biguanide preparations. Tumor insulin binding in this patient was 5.5%. All fat samples demonstrated significant insulin binding, ranging from 2.2 to 16.7%, mean 8.1 ± 0.85% (S.E.) per 0.3 mg membrane protein.

Chart 2 shows typical Scatchard plots of insulin binding to tumor and fat membranes. Average binding site concentration was 33.1 ± 6.6 fmol/0.3 mg for tumor and 57.4 ± 5.7 fmol/0.3 mg for the fat membranes. Binding affinity was 1.38 ± 0.21 x 10^6 M^-1 in the tumors and 1.12 ± 0.15 x 10^6 M^-1 in the fat specimens.

**Degradation of Labeled Hormone by Membranes.** Labeled insulin was degraded at similar mean rates by fat and tumor membranes [mean rate and range per 24 hr: fat = 21% (18 to 24%); tumor = 22% (14 to 30%), n = 6]. The range of degradation rate was slightly wider in tumor compared with fat membranes.

**Effect of Fasting Insulin Concentration on Insulin Binding to Membranes.** The effect of fasting insulin concentration on insulin binding to tumor and fat membranes is shown in Chart 3. Fasting insulin was not available in one patient. There is a significant inverse relationship between insulin binding to fat and serum insulin concentration (r = 0.55, p < 0.05). By contrast, no significant correlation between serum insulin levels and tumor insulin binding was seen (r = -0.12). A similar result was obtained using insulin-binding site concentrations calculated from Scatchard plots rather than specific binding (fat, r = -0.77, p < 0.01; tumor, r = 0.32, p, not significant). Insulin binding to tumor and fat membranes from individual patients showed no significant correlation (Chart 4).
TUMOR MEMBRANES

Insulin Binding in Breast Cancer

Relationship of Tumor Insulin Binding to ER Status and Histological Classification. Ten tumors were ER positive, and 10 were ER negative. ER status was not measured in 3 tumors. There was no significant difference between insulin binding in ER-positive tumors [4.67 ± 0.95% (S.E.)] versus ER-negative tumors [4.87 ± 0.60]. Tumor insulin binding was not significantly different among the 3 histological grades. Insulin binding to Grade I tumors was 3.8% (n = 1); Grade II tumors, 4.74 ± 0.61% (S.E.) (n = 9); and Grade III tumors, 5.00 ± 0.50% (n = 11).

DISCUSSION

It has been shown previously that human breast cancer cells bind insulin with a specificity and affinity similar to that of nonneoplastic insulin-responsive cells (15). In this investigation, we have considered factors which might influence the concentration of insulin-binding sites in breast tumors, in particular the relationship of binding to the level of circulating insulin. Normal insulin-responsive cells respond to increased ambient insulin levels by reducing the concentration of membrane insulin receptors in a process described as “down-regulation” (19). Direct measurement of this effect in vivo is not feasible in human breast cancer since serial tissue sampling would be necessary. Regulation of insulin binding by circulating insulin in normal tissue is suggested, however, by finding reduced receptor concentrations in monocytes and adipocytes of patients with hyperinsulinemia (24). With the use of a crude preparation of fat cell membranes, we have also observed reduced insulin binding in patients with increased insulin levels (Chart 3). By contrast, no significant relationship between tumor insulin binding and serum insulin was found in the present study. Whether this indicates a fundamental difference between insulin receptors in normal and tumor cells or whether this is due to methodological or sampling problems cannot be entirely resolved.

It is clear that tumors vary in their proportion of tumor, fat, and connective tissue. Thus, insulin binding in a small sample of tumor may not be representative of the overall tumor binding capacity. Autoradiographic studies show that insulin is bound predominantly to tumor cells with considerably less bound to surrounding normal tissue (15). In the present study, all visible fat and connective tissue were trimmed from the tumor specimen. In addition, all of the tumor remaining after histological sectioning was used in the preparation of membranes, thus minimizing the chance of sampling error. For the above reasons, as well as the uniformity of binding seen in the one tumor examined (Chart 1), it seems unlikely that sampling error from tumor specimens is the explanation for the lack of relationship between tumor receptor levels and serum insulin levels.

Studies of membrane insulin receptors most often use either highly purified cell membrane preparations (3) or dispersed cell systems (7). The use of a relatively crude membrane fraction in this study was necessitated by the small size of many tumor specimens and the length of time from surgical removal to laboratory processing. The preparation of highly purified membranes was considered, but since this requires a relatively large amount of homogeneous tissue (23), the method was not feasible for processing small tumor specimens, as in this study. Similarly, the use of a dispersed cell system was not favored because of difficulties in obtaining a suitable prepara-
chronic corticosteroid treatment in which there is diminished somatomedin receptors (30). Insulin may bind to cells such as assessed in parallel with binding studies in the tissue concerned. Our results, which show down-regulation of insulin receptors in fat (Chart 3), suggest that the use of partially purified membranes is adequate for the purpose of detecting physiological differences in insulin binding. Thus, the observation that the insulin-binding capacity of breast tumors is unaffected by insulin levels seems likely to be genuine and not due to assay insensitivity or experimental error, and it thus implies a fundamental difference in receptor regulation between tumor and normal cells. The degree of histological differentiation of breast tumors did not appear to influence insulin binding. Using the grading method of Bloom and Richardson (4), the most undifferentiated tumors (Grade III) bound insulin to the same extent as did the more differentiated tumors (Grades I and II). ER status also appeared to be independent of insulin binding. ER-positive tumors bound insulin to a similar degree as ER-negative tumors. More poorly differentiated tumors and tumors with ER-negative status are associated with an unfavorable prognosis (4) and poor response to endocrine therapy, respectively (18). It thus seems improbable that the extent of insulin binding by breast tumors is reflected in the clinical course of the patient, although this remains to be determined by prospective analysis.

It should be pointed out that the binding of insulin to cells need not equate with insulin responsiveness. For example, although insulin receptors can be detected in human breast cancer cell lines (27), only some of these lines demonstrate a biological response to physiological levels of insulin. In the insulin-responsive lines, however, there is a good correlation between the concentrations of insulin causing one-half maximal displacement of binding and biological response (27). Insulin cannot, however, replace fetal calf serum in maintenance of normal cells. The degree of histological differentiation of breast tumors did not appear to influence insulin binding. Using the grading method of Bloom and Richardson (4), the most undifferentiated tumors (Grade III) bound insulin to the same extent as did the more differentiated tumors (Grades I and II). ER status also appeared to be independent of insulin binding. ER-positive tumors bound insulin to a similar degree as ER-negative tumors. More poorly differentiated tumors and tumors with ER-negative status are associated with an unfavorable prognosis (4) and poor response to endocrine therapy, respectively (18). It thus seems improbable that the extent of insulin binding by breast tumors is reflected in the clinical course of the patient, although this remains to be determined by prospective analysis.

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