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Insulin Receptor Overexpression in 184B5 Human Mammary Epithelial Cells Induces a Ligand-Dependent Transformed Phenotype

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Abstract To determine the role of the insulin receptor overexpression in breast epithelial cell transformation, the 184B5 human breast epithelial cell line was transfected with human insulin receptor cDNA. In two cell lines transfected with and overexpressing human insulin receptors (IR) (223.8 and 184.5 ng IR/10^6 cells), but not in untransfected cells, insulin binding and tyrosine kinase activity were elevated, and insulin induced a dose-dependent increase in colony formation in soft agar. © 1995 Wiley-Liss, Inc.

Key words: breast cancer, insulin, malignant transformation, tyrosine kinase, insulin receptor

Recently, we have reported that overexpression of the insulin receptor is a characteristic feature of many human breast cancer specimens [1] and that in human breast cancer cells in tissue culture insulin promotes growth via its own receptor [2]. Moreover, we have recently demonstrated that in fibroblasts and ovary cells transfected with and overexpressing insulin receptors, the addition of insulin induces a ligand-dependent transformed phenotype [3]. A similar observation has been reported for the overexpression of the closely related IGF-1 receptor [4]. These observations suggested, therefore, a possible role for insulin receptor overexpression in the initiation and/or progression of human cancers. In the present study we have employed an immortally transformed human breast epithelial cell line, 184B5, which has a low insulin receptor content [5]. The insulin receptor has been overexpressed in these cells to determine whether insulin could induce a transformed phenotype.

MATERIALS AND METHODS

Cells and Transfection

The immortally transformed 184B5 human mammary epithelial cell line was obtained as described following carcinogen exposure of a reduction mammoplasty derived cell strain, 184 [5]. The cells were grown in mammary epithelial growth medium (MEGM) (Clonetech, San Diego, CA) as previously described [5–7]. Electroporation was used to cotransfect the cells with two expression plasmids containing human insulin receptor cDNA (pCMV-IR) and pSV2-NEO (both under the control of the cytomegalovirus promoter) at a 10:1 ratio. 184B5-NEO cells were obtained by transfection of 184B5 cells with the plasmid pSV2-NEO only. After 3 weeks the neomycin-resistant colonies were isolated and grown in 12 multiwell plates.

Cell Growth in Soft Agar

To test the ability of cells to form colonies in soft agar, a double layer culture technique was used. Subconfluent cell monolayers were rinsed with Ca^{++}/Mg^{++}-free PBS and suspended by a short treatment with trypsin [3]. Cells were then centrifuged and resuspended in MEGM medium without insulin containing 0.1% fetal calf serum. Cell plating was performed by mixing a 2.5 ml prewarmed suspension (10^5 cells) with a 0.5 ml prewarmed 2% Bacto-Agar suspension. The resulting final suspension cell in 0.33% agar was poured onto a 2.0 ml layer of 0.66% agar in 35 mm petri dishes. Each petri dish was inspected under microscope on day 1 after plat-

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ing to exclude the presence of cell clumps. The effect of insulin on colony formation in soft agar was tested by adding hormones to the upper layer every other day. Colonies were scored after 3 weeks and classified according to their size as small (with a diameter < 100 μm), medium (100–150 μm), or large (> 150 μm).

RESULTS AND DISCUSSION

Isolation of Cell Lines Overexpressing Insulin Receptors

Thirty-two individual neomycin (G-418) resistant cell lines were isolated after cotransfection of 184B5 cells with pCMV-IR and pSV2-NEO. Of these cell lines, seven demonstrated elevated ^{125}I-Insulin binding (range 30–161% ^{125}I-Insulin bound per 0.5×10^6 cells) that was at least threefold greater than that of cells transfected with pSv2-NEO alone (5–10% bound). Two cell clones, 184B5-IR14 and 184B5-IR12, having the highest binding (161 and 107% bound), were selected for further studies.

Insulin Receptor Content

Insulin receptor content, measured by radioimmunoassay [8], was 223.8 ng receptor per 10^6 cells in 184B5-IR12 cells, 184.5 ng receptor per 10^6 cells in 184B5-IR14 cells, and 24 ng receptor per 10^6 cells in 184B5-NEO cells.

These receptors were functional as increased insulin binding was observed in transfected 184B5 cells (Fig. 1a). Scatchard analysis [9] revealed that high affinity insulin receptor binding was increased. 184B5-NEO cells had a binding capacity of 0.6 femtomoles/cell, while the transfected cells had increased binding capacities of 31.2 and 25 femtomoles/cell.

Insulin Receptor Tyrosine Kinase

Cells were stimulated with 100 nM insulin for 10 min at 37°C and the cell lysate subjected to Western blotting analysis utilizing an antiphosphotyrosine antibody [10]. In all the three cell lines, insulin increased the phosphotyrosine content of insulin receptor beta subunit, thus demonstrating that the transfected insulin receptor tyrosine kinase activity was functional (Fig. 1b). Moreover, in these experiments both 184B5-IR14 and 184B5-IR12 cells showed a higher content of phosphotyrosine residues in the beta subunit when compared to 184B5-NEO cells. These findings were consistent with the observations from binding and radioimmunoassay data that transfected cells expressed a higher level of the insulin receptor protein.

Effect of Insulin on Cell Growth in Soft Agar

Colony formation in soft agar was assessed 21 days after seeding cells in 0.33% agar in the absence or presence of insulin (Table I). No colonies were observed in the 184B5-NEO cells either in the absence or presence of insulin (data not shown). The plating efficiencies observed were low (0.001–0.01%), as has been reported in 184B5 cells [11]. When 184B5-IR14 cells were seeded and maintained in the absence of insulin, a very small proportion of cells gave rise to small-sized colonies (< 100 μm). In contrast, when insulin was present the cells formed more and larger colonies (> 100 μm). Even at 0.1 nM, the lowest concentration of insulin tested, the increase in small colonies was maximal. The number of the medium and large colonies, however, was insulin dose-dependent. To demonstrate that insulin-dependent colony formation was stimulated via the insulin receptor, cells were seeded in the presence of the specific anti-insulin receptor monoclonal antibody MA-10 [12]. The concomitant addition of insulin and MA-10 blocked insulin-induced colony formation. Similar results were obtained with 184B5-IR12 cells with maximum effect at 100 nM insulin (% of medium/large colony formation = $10.7 \pm 3.2 \times 10^3$; n = 6).

DISCUSSION

We have reported that overexpression of insulin receptor content is a characteristic feature of many human breast cancer specimens [1]. Elevated insulin receptor content correlates with both tumor size and tumor grading, suggesting that the receptor is associated with larger and more aggressive tumors. In addition to data with the insulin receptor, there are data suggesting that insulin plays a role in breast cancer. Obesity causes insulin resistance and hyperinsulinemia [14], and it is well documented that obese women, especially those with android obesity, are at a significantly higher risk for breast cancer [15]. In rats there is direct evidence that insulin plays a role in breast cancer biology. Alloxan diabetes completely blocks the formation of mammary tumors induced by 7,12-dimethylbenz(a)anthracene [16]. This effect of diabetes can be mimicked by food deprivation with subsequent hypoinsulinemia. Moreover, the induction of alloxan diabetes in rats with estab-

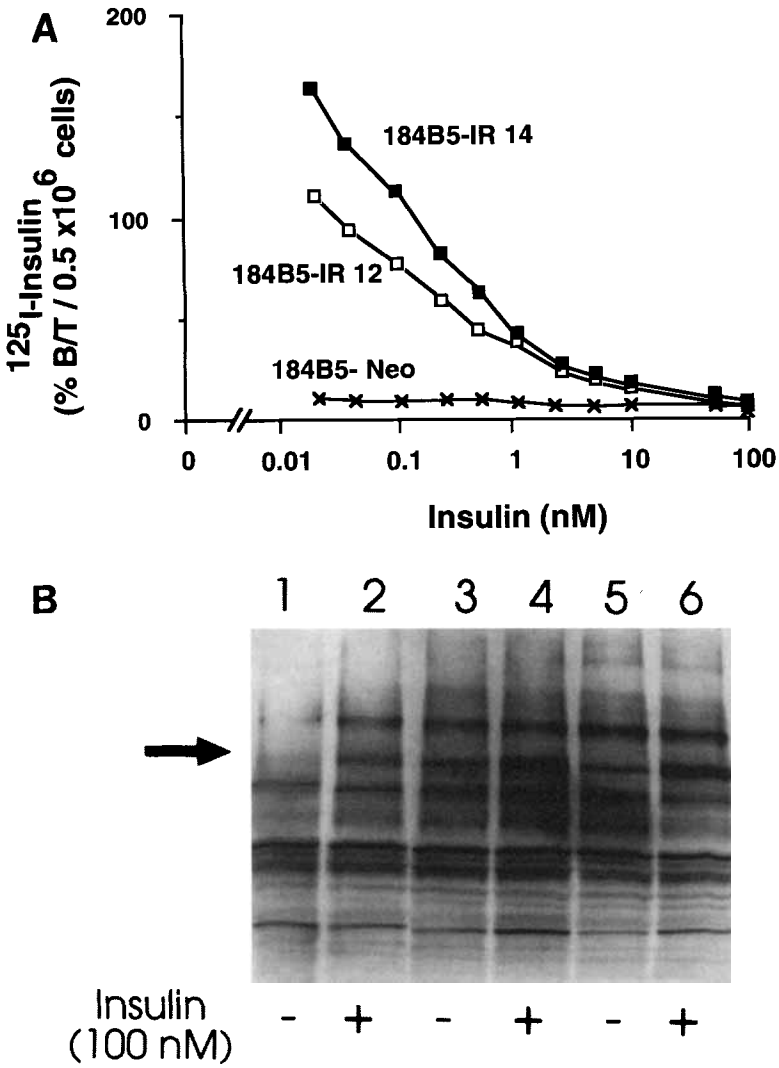

Fig. 1. **A:** Inhibition-competition studies of ^{125}I-Insulin (18 pM) and unlabelled insulin in 184B5-NEO cells (x) and in two lines transfected with and overexpressing insulin receptors (1845-IR14 (■) and 184B5-IR12 [□]). **B:** Western blot analysis of tyrosine phosphorylated proteins in 184B5-NEO cells and in two lines transfected with and overexpressing insulin receptors (1845-IR14 and 184B5-IR12). In these kinase experiments, cells were incubated for 10 min without or with 100 nM insulin and then solubilized. Cell lysates were resolved by SDS-Page, transferred to PVDF membranes, and then immunoblotted with anti-PY antibody [18]. The 95 KDa band, corresponding to the insulin receptor beta subunit, is indicated by the arrow. Lanes 1,2: 184-B5-NEO. Lanes 3, 4: 185-B5-IR14. Lanes 5, 6: 184-B5-IR12.

lished tumors causes rapid tumor regression [16]. Conversely, insulin administration to rats with mammary tumors enhances tumor growth [17]. Finally, it has been reported that rats injected with the highly potent insulin analogue, B10Asp, develop breast cancer [18].

In the present study we find that an eight- to tenfold overexpression of insulin receptors in nonmalignantly transformed human breast epithelial cells, an increase similar to that found in human breast cancer specimens [1], is associated with ligand-induced colony formation in soft agar, a characteristic associated with malignant transformation. These present data, therefore, are in concert with previous data indicating that overexpression of members of the tyrosine kinase family, including the insulin receptor, can induce malignant transformation in various cell types including breast epithelial cells [13]. While the exact role of insulin and insulin receptor in the biology of breast and other cancers has not yet been established, the present studies in transfected breast epithelial cells support the concept that insulin and its receptor may have a role in breast cancer cell growth. Thus, measurement of insulin receptors in breast cancer speci-

TABLE I. Effect of Insulin on Colony Formation in Soft Agar†

Ligand	% of colony formation (x 10^3) small (< 100 μm)	medium (100–150 μm)	large (> 150 μm)
None	2.0 ± 2.8	0	0
Insulin (nM)			
0.1	48.0 ± 28	0	0
1.0	28.0 ± 19	6.0 ± 4.2	1.0 ± 1.7
10.0	58.0 ± 43	9.0 ± 1.1	4.7 ± 4.0*
100.0	25.0 ± 11	14.0 ± 11.0	13.5 ± 5.1*
Insulin (10 nM) + MA-10 (10 nM)	2.5 ± 0.7	0	0

†Colony formation in soft agar was evaluated 21 days after seeding 10^5 cells in 0.33% agar in the absence or presence of the indicated concentrations of ligands. Hormones were added every other day to the upper layer of the cultures. Each value is the mean ± SE of four separate determinations.

*$P < 0.02$ in respect to values at 1 nM insulin.

mens may provide a new parameter for staging breast cancers.

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