Overexpression of Insulin Receptors in Fibroblast and Ovary Cells Induces a Ligand-Mediated Transformed Phenotype

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To investigate whether overexpression of the insulin receptor results in altered cell growth we used NIH 3T3 cells transfected with a bovine papilloma virus/insulin receptor cDNA construct (3T3/HIR). These cells expressed high numbers of insulin receptors (mean ± sd, 631.0 ± 16.7 ng receptors/10^6 cells). Insulin significantly stimulated the growth of 3T3/HIR cells maintained in serum-free medium. Moreover, in these cells, insulin induced marked phenotypic changes, including alterations in cell shape, loss of contact inhibition, and focal growth. In contrast to 3T3/HIR cells, insulin was without effect in either wild-type 3T3 cells (3T3/wt), 3T3 cells transfected with the neomycin resistance gene (3T3/NEO), or the bovine papilloma virus (3T3/BPV).

To assess the presence of anchorage-independent growth, cells were seeded in soft agar and inspected for colony formation. 3T3/HIR cells showed absent or minimal colony growth in the absence of insulin. However, there was a dose-dependent insulin-stimulated increase in both colony size and number. Insulin-stimulated colony formation was specifically inhibited by an insulin antagonist, monoclonal antibody MA-10. In the presence of 100 nM insulin, about 3% of cells formed large colonies. Insulin neither stimulated growth nor induced colony formation in 3T3/wt cells or 3T3/NEO cells. Insulin also stimulated colony formation in CHO cells transfected with an insulin receptor cDNA construct. In conclusion, overexpression of normal insulin receptors induces a ligand-dependent transformed phenotype. This phenomenon may have clinical relevance by conferring a selective growth advantage to tumor cells with high numbers of insulin receptors. (Molecular Endocrinology 5: 452–459, 1991)

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INTRODUCTION

Growth factor receptors of the tyrosine kinase family play a key role in both normal and neoplastic cell growth (1). Overexpression of structurally normal receptor molecules with tyrosine kinase activity in cells is directly involved in inducing a transformed phenotype (2). In addition, structural alterations of some growth factor receptors, by changing their tyrosine kinase domains, may confer an oncogenic potential to these molecules and, thus, induce neoplastic transformation (3–10).

The insulin receptor belongs to the tyrosine kinase growth factor receptor family (11, 12), and insulin mediates proliferative responses in a large variety of both normal and transformed cells (13, 14). However, the role of the insulin receptor molecule in human neoplasia has not yet been established. Recently, we reported that overexpression of the insulin receptor is a characteristic feature of most human breast cancer specimens (15). By using a specific insulin receptor RIA, we found that the average insulin receptor content of human breast cancer specimens was 5- to 10-fold higher than

that of normal breast tissue; employing immunohistochemical analysis, we localized the increased expression of the insulin receptor to the malignant epithelial cells. Moreover, the insulin receptor content of the breast cancer specimens was positively correlated with tumor grade and tumor size. These observations suggested, therefore, a possible role for insulin receptor overexpression in human breast cancer initiation and/or progression.

In the present study, in order to examine the role of insulin receptor overexpression on cell growth, we investigated the growth characteristics of mouse NIH-3T3 fibroblasts transfected with and expressing high levels of insulin receptors (3T3/HIR). We have previously reported that insulin stimulates mitogenesis via its own receptor in these cells, but was without effect in control 3T3 cells (16). We now report that overexpression of the insulin receptor also confers a ligand-dependent transformed phenotype to 3T3/HIR cells.

RESULTS

Insulin Receptor Content

The measurement of insulin receptors in 3T3/HIR cells by RIA confirmed their high level of expression (16). The content of insulin receptors in 3T3/HIR cells was 631.0 ± 16.7 ng/10^6 cells (mean ± SD) or 297.6 ± 11.8 ng/mg protein (21), which is over 100-fold higher than that in 3T3/NEO, 3T3/BPV, or 3T3/wt cells (4.9 ± 0.4, 4.7 ± 0.3, and 5.2 ± 1.4 ng/10^6 cells, respectively).

Effect of Insulin on Cell Growth

In 3T3/HIR cells cultured 4 days in serum-free medium, increasing insulin concentrations stimulated cell proliferation in a dose-dependent manner. This increase in growth was reflected by an increase in both cell number and DNA content (Fig. 1). A small insulin effect was detected at 0.1 nM. At 100 nM insulin, the effect on growth was 268.0 ± 75% of the control value as evaluated by cell number, and 242.2 ± 63% as evaluated by DNA content (mean ± SD; n = 5). We next employed monoclonal antibody MA-10, a specific insulin receptor α-subunit antibody that does not react with the insulin-like growth factor-I (IGF-I) receptor (22). MA-10 almost completely blocked the mitogenic effect of insulin, demonstrating that this effect of insulin on 3T3/HIR cell proliferation was due to the specific action of insulin directly on its own receptor and not on the IGF-I receptor (data not shown). In 3T3/wt cells (Fig. 1) and in 3T3/NEO and 3T3/BPV cells (not shown) there was no significant proliferative response to insulin.

Effect of Insulin on Cell Morphology

When grown in monolayers, 3T3/HIR cell morphology was markedly changed after treatment with insulin (Figs. 2 and 3). Cells grown in the absence of insulin

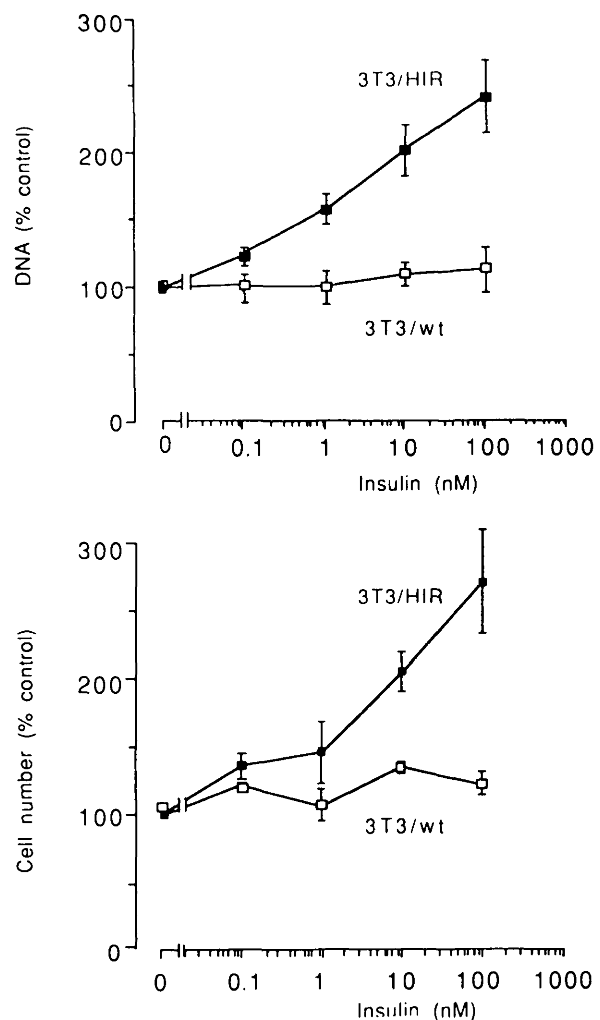

Fig. 1. Proliferation of 3T3/wt and 3T3/HIR Cells in Response to Insulin

Cells were cultured in the absence or presence of increasing concentrations of insulin for 4 days in serum-free medium containing 0.1% BSA. Each value is the mean ± SD for five separate experiments.

had a normal phenotypic appearance; chains of elongated cells were observed, monolayer growth was maintained, and contact inhibition was present. In contrast, addition of insulin to 3T3/HIR cells caused them to lose contact inhibition and form large multilayered focal aggregates. In addition, the cells rounded up, became loosely adherent, and had more intensely staining nuclei. Both the insulin-induced morphological changes and the formation of cell foci were fully prevented by the concomitant addition of monoclonal antibody MA-10 (Fig. 3C). In contrast to 3T3/HIR cells, no significant difference in either cell morphology or cell density was observed in monolayers of 3T3/BPV (Fig. 4) and 3T3/wt cells (not shown) when cultured in either the presence or absence of insulin.

Effect of Insulin on Cell Growth in Soft Agar

Colony formation in soft agar was assessed 7 and 12 days after seeding cells in 0.33% agar in the absence

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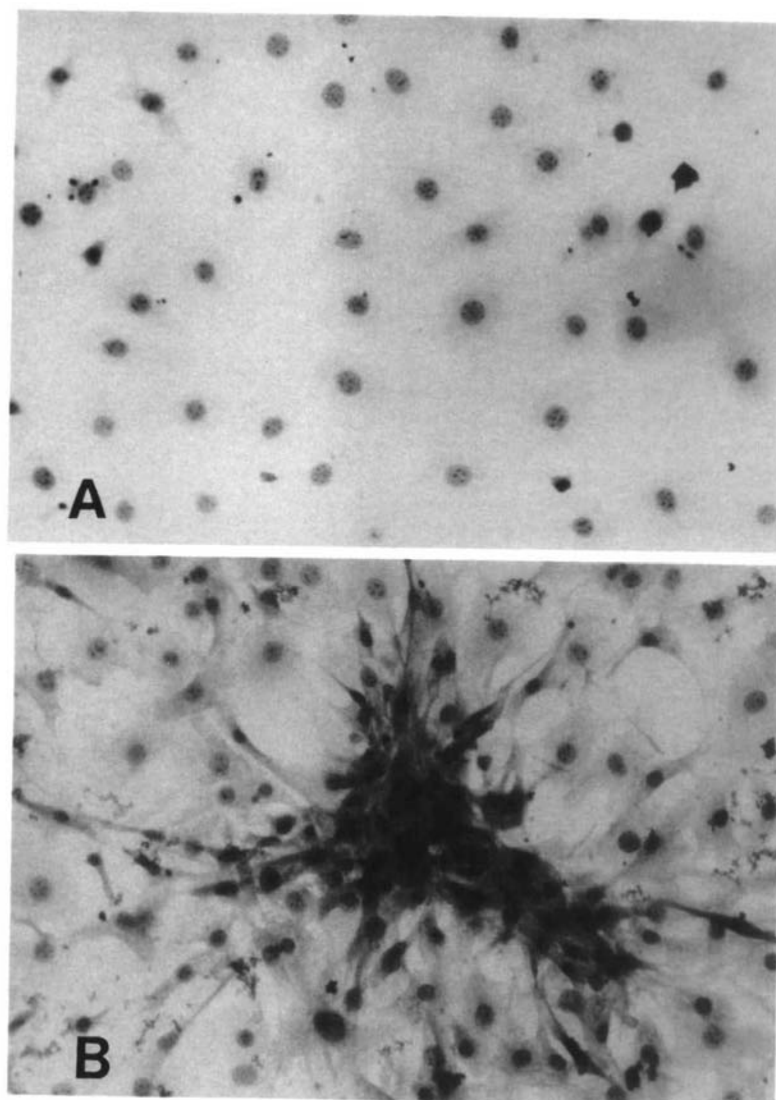

Fig. 2. Light Microscopic Pictures of 3T3/HIR Cells Grown in Medium Containing 0.1% BSA in the Absence (A) and Presence (B) of 100 nM Insulin

Cells were staining with the Papanicolau stain. Magnification, ×100.

or presence of insulin or IGF-I (Table 1 and Fig. 5). When 3T3/HIR cells were seeded and maintained in the absence of insulin, a very small proportion of cells (ranging from 0–0.1%) gave rise to small-sized (~30 cells) colonies. In contrast, when insulin was present, over 3% of the cells seeded formed large-sized colonies (>1000 cells). Both the number and the size of colonies were insulin dose dependent (Fig. 5). Colony formation was observed at insulin concentrations as low as 0.1 nM (Fig. 5). To further demonstrate that insulin-dependent colony formation was stimulated via the insulin receptor, cells were seeded in the presence of monoclonal antibody MA-10. The concomitant addition of insulin and MA-10 blocked the insulin-induced colony formation (Fig. 5).

The insulin-dependent growth of cells with overexpression of the insulin receptor (3T3/HIR) was a genetically stable property. Several colonies were isolated, cloned, and passaged two or three times in monolayer culture and then reanalyzed for their ability to form colonies in soft agar. The ability to exibit colony formation after exposure to insulin was not affected by this procedure, indicating that it was a stable genetic characteristic.[1] In contrast to 3T3/HIR cells, colony formation was not observed when parallel experiments were carried out with either 3T3/wt and 3T3/BPV (not shown) or 3T3/NEO cells (Fig. 5).

IGF-I was also able to induce 3T3/HIR cell growth in soft agar. This ligand was only effective when added at high concentrations (10–100 nM). Moreover, when IGF-

[1] Cells transformed with BPV are known to have variable expression of surface proteins within cloned populations. Therefore, the data with expression of the insulin receptor and colony formation in cloned 3T3/HIR cells are compatible with this type of variable expression (34).

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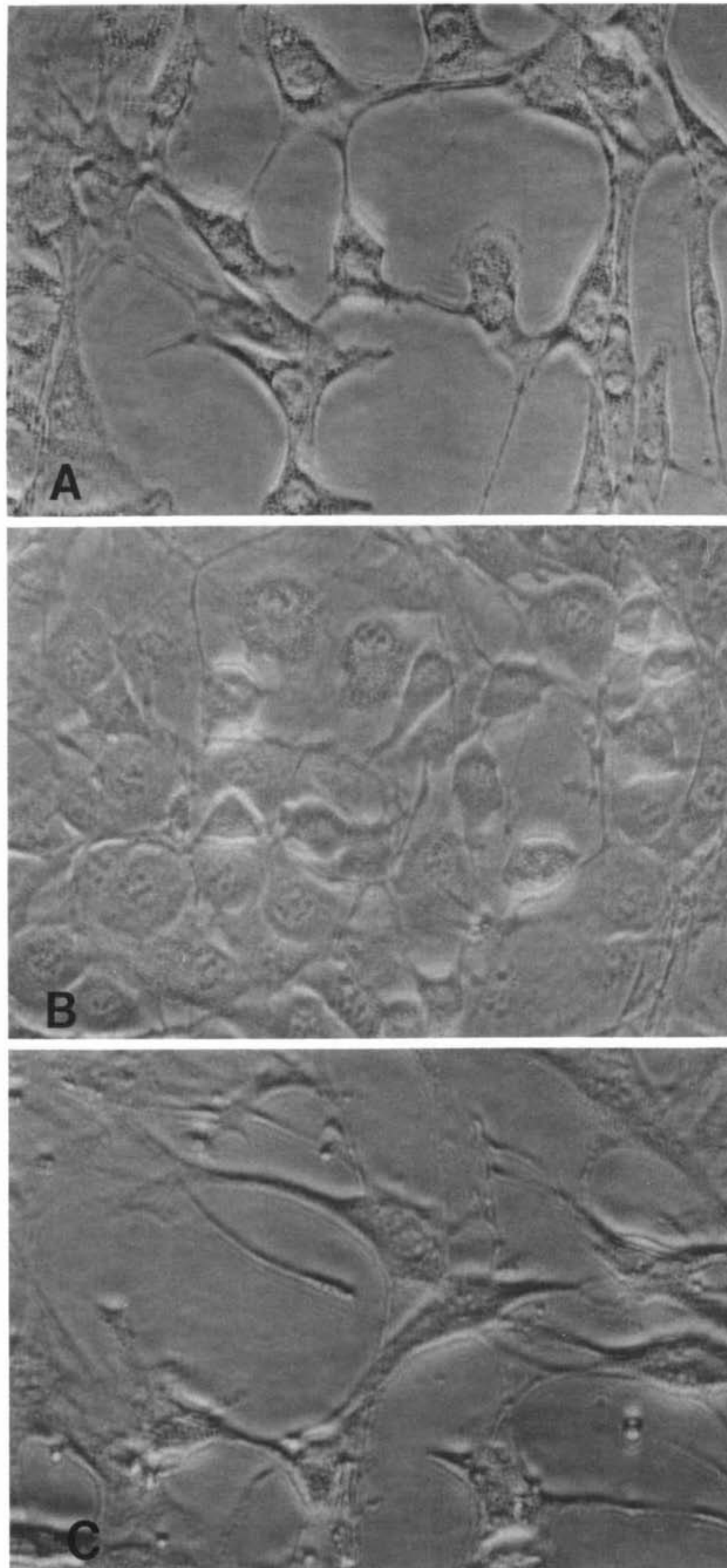

Fig. 3. Phase Contrast Microscopic Pictures of 3T3/HIR Cell Monolayers Grown in DMEM Containing 0.1% BSA without Addition of Insulin (A), in the Presence of 100 nM Insulin (B), and in the Presence of 100 nM Insulin plus 100 nM MA-10 (C)
Magnification, ×400.

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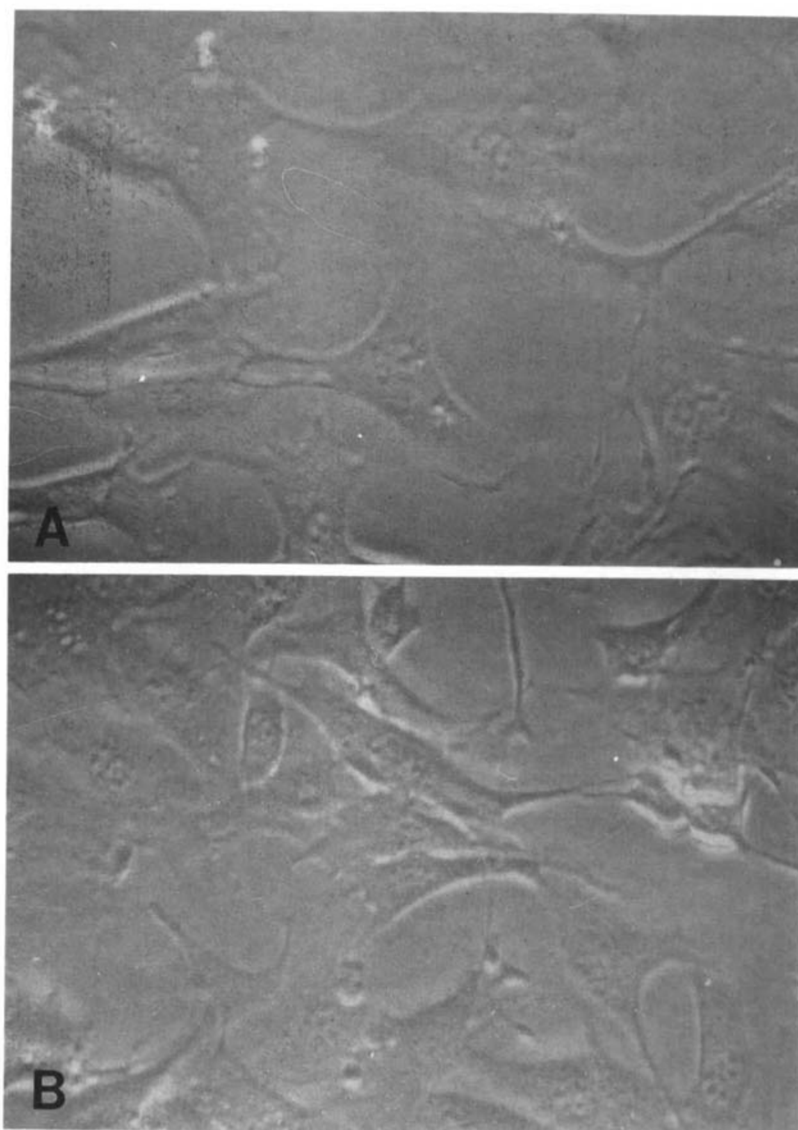

Fig. 4. Phase Contrast Microscopic Pictures of 3T3/BPV Cell Monolayers in Medium Containing 0.1% BSA Grown in the Absence (A) or Presence (B) of 100 nM Insulin
Magnification, ×400.

Table 1. Comparison of 3T3/HIR Cell Colony Formation in Soft Agar in Response to IGF-I and Insulin

Addition	Colonies	
	No.	Size
None	85	Small
IGF-I (nM)		
0.1	83	Small/medium
1.0	112	Small/medium
10.0	115	Small/medium
Insulin (nM) 10.0	480	Large

I was compared to insulin, the percentage of plated cells that formed colonies was much lower (0.4–0.8%), and the colonies formed were smaller.

Studies in Nude Mice

Groups of six mice were injected with either 10^5 or 10^6 3T3/HIR and 3T3/NEO cells. After 8 weeks there was no specific tumor formation.

Studies in CHO Cells

To determine whether insulin could induce colony formation in soft agar in another cell type and in the absence of bovine papilloma genes, we studied wild-type CHO cells (CHO/wt) and CHO cells transfected

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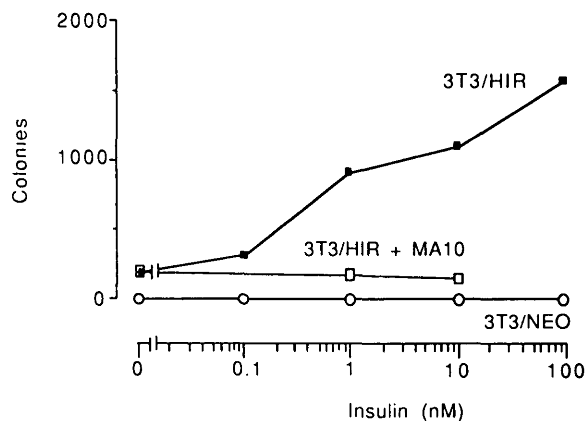

Fig. 5. 3T3/HIR and 3T3/NEO Cell Soft Agar Colony Formation in Response to Insulin

Cells were seeded in 0.33% soft agar in the absence or presence of increasing concentrations of insulin. The figure shows a representative of five experiments.

Table 2. Comparison of CHO/wt and CHO/IR Cell Colony Formation in Soft Agar in Response to Insulin

Addition	Colonies			
	CHO/wt		CHO/IR	
	No.	Size	No.	Size
None	0	0	114	Small
Insulin (100 nM)	0	0	220	Large

with the human insulin receptor (CHO/IR) (Table 2). No colonies were observed in CHO/wt cells grown in either the absence or presence of 100 nM insulin. In CHO/IR cells, only small colonies (<50 cells) were seen in the absence of insulin. In contrast, in CHO/IR cells, insulin induced the formation of large colonies (>200 cells).

DISCUSSION

Overexpression of oncogenes and growth factor receptors of the tyrosine kinase family is often associated with a transformed phenotype (23–25). The insulin receptor belongs to the tyrosine kinase receptor family (11, 12, 26), and insulin exerts its mitogenic effects on a large variety of mammalian cells (14) via this receptor. However, the role of the insulin receptor in the induction of a transformed phenotype has not been extensively studied.

In the present study, in order to investigate the role of insulin receptor overexpression in cell transformation, we employed NIH-3T3 cells transfected with the human insulin receptor gene (3T3/HIR). When 3T3/HIR cells were radioimmunoassayed for insulin receptor content, a value of approximately 300 ng receptor/mg protein was obtained. This value is in the same range as that observed in over 10% of human breast cancer specimens (15) and is much higher than that found in any noncancerous human tissue that has been studied (19). In contrast, nontransfected 3T3/wt cells and 3T3/NEO cells had 100-fold fewer insulin receptors than 3T3/HIR cells. In 3T3/HIR cells, insulin, in a dose-dependent manner, induced a variety of morphological changes and growth responses that can be summarized as follows: 1) a significantly higher growth rate, 2) abnormal morphological characteristics and loss of contact inhibition, and 3) insulin-dependent growth in soft agar with large colony formation. These 3T3/HIR cell responses were specifically blocked by monoclonal antibody MA-10, an insulin receptor antagonist (22). These effects of insulin were absent in three control cell lines, 3T3/wt, 3T3/NEO, and 3T3/BPV. In addition to its effect on 3T3 HIR cells, insulin also induces colony formation in soft agar in transfected CHO/IR cells.

In 3T3/HIR cells, but not in control cells, IGF-I also had a small effect on colony formation in soft agar. IGF-I, however, was effective only at high concentrations. Previous studies in 3T3/HIR cells demonstrated that IGF-I regulated thymidine uptake and other functions via spillover of IGF-I into the highly expressed human insulin receptor (16). Most likely, therefore, in the present study, the small effect of IGF-I on colony formation was also due to spillover into the insulin receptor. In addition to insulin and IGF-I receptors, it has previously been reported that insulin receptor/IGF-I receptor hybrids may exist in 3T3/HIR cells (33). It is unlikely, however, that transformation was mediated by these hybrids, since only insulin and not IGF-I induced this effect.

The present studies indicate, therefore, that 3T3/HIR cells, expressing an increased number of insulin receptors, acquire a transformed phenotype, which is insulin dependent and mediated via the insulin receptor. However, in these cells the expression of the insulin receptor did not induce tumor formation in nude mice. This observation suggests that, in addition to the insulin receptor, other factors may be necessary for full *in vivo* transformation of 3T3 cells into neoplastic cells.

Wang and colleagues (27) reported on a human insulin receptor v-*ros* chimera that was expressed in chicken embryo fibroblasts. As in the present study, insulin induced a transformed phenotype *in vitro*, but not *in vivo*. Also, Kaleko and colleagues (28) overexpressed the related IGF-I receptor in 3T3 cells and obtained a transformed phenotype both *in vitro* and *in vivo*. The appearance of an insulin-dependent transformed phenotype in 3T3 cells transfected with the insulin receptor gene resembles a similar phenomenon described in NIH-3T3 cells transfected with the human epidermal growth factor (EGF) receptor gene (29–31). 3T3 cells overexpressing EGF receptors demonstrate anchorage-independent growth when stimulated with either EGF or with transforming growth factor-α. The present and these prior observations suggest, therefore, that overexpression of insulin receptors and related receptors in the tyrosine kinase family may lead to the initiation and/or progression of certain tumors.

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MATERIALS AND METHODS

Materials

Culture media, glutamine, antibiotics, fetal calf serum (FCS), and trypsin/EDTA solution were purchased from either Gibco (Gettysburg, MD) or the Cell Culture Facility of the University of California, San Francisco. Bacto-Agar was obtained from Difco Laboratories (Detroit, MI). Insulin and tissue culture grade BSA were obtained from Sigma (St. Louis, MO), and recombinant IGF-I from Boeringher Biochemia (Indianapolis, IN).

Cells

3T3/HIR fibroblasts, expressing high levels of human insulin receptors (6×10^6/cell), were obtained by transfection with a bovine papilloma virus/insulin receptor cDNA construct, as previously described (17). These cells not only have over 10^6 high affinity insulin-binding sites (17), but also are fully responsive to insulin (17, 18). 3T3/BPV fibroblasts, expressing only the bovine papilloma gene (17) were also studied. CHO cells, expressing human insulin receptors (1×10^5/cell), were obtained by transfection with a Rous sarcoma virus promoter/human insulin receptor cDNA construct, as previously described (32). 3T3/NEO cells were obtained by transfection of NIH-3T3 cells with the plasmid pSV2Neo containing the bacterial gene for neomycin resistance (17, 18). Cell lines were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FCS, 2 mM glutamine, and 40 μg/ml gentamycin.

Insulin Receptor Content Measurement

The insulin receptor content of cells was measured by a specific RIA, as previously described (19). Cells cultured in monolayer were suspended by treatment with Ca^{2+}/Mg^{2+}-free phosphate buffer (CMF-PBS) plus 0.02 M EDTA and 0.1% BSA, and then solubilized with 50 mM HEPES buffer containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride, and 0.5 mg/ml bacitracin for 60 min at 4 C under continuous shaking. The material was centrifuged at $10,000 \times g$ for 10 min, and the supernatant was analyzed. Values were measured on a standard curve in the range of 25–80% inhibition of ligand binding, using a purified human placenta insulin receptor as a standard (19).

Measurement of Cell Growth

3T3/HIR cell proliferation in response to increasing doses of insulin was assessed by evaluating cell number and DNA content. Growth conditions were standardized as follows. Cells (10^5) were seeded in 35-mm petri dishes in DMEM containing 10% FCS. After 24 h this medium was replaced with DMEM containing 0.1% BSA. After a further 24 h period, the medium was removed, and serum-free medium plus 0.1% BSA was added in the absence or presence of insulin at the indicated concentrations. Media were then changed every other day. At the end of the incubations, cell monolayers were rinsed with PBS, suspended in CFM-PBS with 0.02 M EDTA, and counted in a hemocytometer. DNA content was measured by the Labarca and Paigen fluorimetric method (20). For each condition, experiments were carried out in triplicate.

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 CMF-PBS and suspended by a short treatment with CMF-PBS containing 0.02 M EDTA and 2 mg/ml trypsin. Cells were then centrifuged, and a single cell suspension was obtained by repeated pipetting. Cell plating was performed by mixing 1.5 ml cell suspension (3×10^3 cells) with 1.5 ml prewarmed (42 C) 0.66% Bacto-Agar suspension. The resulting final cell-0.33% agar suspension was poured onto a 2.0-ml layer of 0.66% agar in 35-mm petri dishes. Each petri dish was inspected under an inverted microscope on day 1 after plating to exclude the presence of cell clumps.

The effect of insulin and IGF-I on colony formation in soft agar was tested by adding these hormones to the upper layer. Colonies were scored using an inverted microscope on days 1, 7, and 12 after plating.

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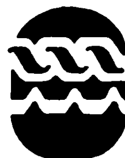

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