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Advances in Brief

Constitutive Activation of Insulin Receptor Substrate 1 Is a Frequent Event in Human Tumors: Therapeutic Implications[1]

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Abstract

Insulin receptor substrate 1 (IRS-1) is a major substrate of insulin, insulin-like growth factors, and cytokine signaling and plays an important role in mediating apoptosis, cell differentiation, and cell transformation. We found that IRS-1 is constitutively activated in a variety of solid tumors, including breast cancers, leiomyomas, Wilms' tumors, rhabdomyosarcomas, liposarcomas, leiomyosarcomas, and adrenal cortical carcinomas. Blocking the constitutively activated IRS-1 signaling in breast cancer cells with a dominant-negative IRS-1, an IRS-1 with all 18 potential tyrosine-phosphorylation sites replaced by phenylalanines (F18), dramatically reduced cancer cell growth. Breast cancer cells that expressed F18 also formed smaller and far fewer colonies in soft agar culture than did the cells that did not express F18. These studies suggest that constitutive IRS-1 activation is a common phenomenon in tumors and that activated IRS-1 may present an attractive therapeutic target.

Introduction

IRS-1[3] is an immediate substrate of IGF-IR, IR, RET, and JAKs (1–4). IRS-1 itself does not possess kinase activity but is phosphorylated after binding to activated receptors through its PTB (5). IRS-1 is phosphorylated on multiple tyrosine residues that serve as docking sites for a variety of signaling molecules, including p85, Fyn, Grb2, Nck, Csk, and SH-PTP2 (Syp), each then initiating the distinct signaling pathways that contribute to IRS-1 biological effects (6).

IRS-1 activation is known to be critical for cell mitogenesis. Murine hematopoietic 32D cells that do not express IRS-1 lose the ability to proliferate in response to IL-4 or insulin. Expression of IRS-1 in these cells, however, restores their mitogenic response to insulin and IL-4 (7). In hepatoma cells (8) and Chinese hamster ovary cells (9), the expression of an antisense *IRS-1* RNA greatly reduces cell growth rate and thymidine incorporation in response to insulin. In Rat-1 fibroblasts overexpressing IR, microinjection of affinity-purified IRS-1 antibody completely inhibits DNA synthesis after the cells are stimulated with insulin or IGF-I (10).

Several studies also demonstrate transformation potential for IRS-1. Overexpression of IRS-1 in NIH3T3 cells induces neoplastic transformation by interacting with Grb2 and SH-PTP2 (Syp), which in turn leads to activation of mitogen-activated protein kinase signaling pathways (11, 12). Fibroblasts (R-cells), derived from mouse embryos with a targeted disruption of the IGF-IR gene, are resistant to transformation by oncogenes such as SV40 T antigen. Coexpression of IRS-1 and the SV40 T antigen, however, induces R-cell transformation (13). Furthermore, IRS-1 is associated with SV40 T antigen in transformed cells (14). In murine hematopoietic 32D cells and LNCaP prostate cancer cells, expression of IGF-IR induces cell differentiation and subsequent cell death after stimulation by IGF-I. Coexpression of IRS-1 and IGF-IR in those cells, however, effectively inhibits cell differentiation, induces malignant transformation in 32D cells (15), and restores the full transformed phenotype in LNCaP cells (16).

Because IRS-1 plays an important role in both cell proliferation and cell transformation, we asked the question whether IRS-1 signaling is activated in tumors. We examined 34 tumors of 7 different tissue origins and found constitutive IRS-1 activation in 27 of 34 cases. The oncogenic significance of IRS-1 activation was demonstrated by inhibition of cancer cell growth through the expression of a dominant-negative IRS-1 in breast cancer cells.

Materials and Methods

Antibodies. Affinity-purified polyclonal antibody to IRS-1 was supplied by Upstate Biotechnology (Lake Placid, NY). Monoclonal antibody to phosphotyrosine residues (PY99) and affinity-purified polyclonal antibodies to IR and IGF-IR were supplied by Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies to IR and IGF-IR were supplied by Oncogene Research Products (Cambridge, MA). Monoclonal antibodies to c-myc and polyhistidine were supplied by Invitrogen (Carlsbad, CA).

Immunoprecipitation and Western Blotting. A 1×3×3-mm slice of frozen tissue was lysed in 1% NP40, 50 mM Tris (pH 8.0), 100 mM sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium vanadate, 5 μg/ml aprotinin, 5 μg/ml leupeptin, and 50 μg/ml phenylmethylsulfonyl fluoride. Protein lysate was precleaned with 20 μl of protein A-agarose and incubated with the immunoprecipitating antibodies (10 μg) for 2 h at 4°C. Protein A-agarose (30 μl) was added and rotated overnight at 4°C. After three washes, the immunoprecipitates were denatured at 90°C for 5 min, separated by SDS-PAGE (4–12% gradient gel), and electrophoretically transferred to polyvinylidene difluoride membranes, which were blocked with 5% milk in PBST (or 3% BSA in PBST if antiphosphotyrosine antibody PY99 was used for immunoblotting). Primary antibodies were added and incubated at room temperature for 2 h. Detection was performed with enhanced chemiluminescence Western blotting detection reagents from Amersham Pharmacia Biotech (Piscataway, NJ). For restaining the blot with different antibodies, the blots were stripped in 100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C and reimmunoblotted as described above.

Constructs. An IRS-1 cDNA construct with all 18 potential tyrosine-phosphorylation sites replaced by F18 was created by site-directed mutagenesis on a wild-type IRS-1 cDNA in pBluescript as described previously (17). A c-myc epitope and a polyhistidine epitope were added by amplifying F18 by PCR with primers containing an *Eco*R V (forward) and *Not* I (reverse) sites and digesting and ligating in frame with pcDNA4/TO/myc-His C (Invitrogen).

Transfection. Cells were passed the day before transfection and cultured in medium without antibiotics. DNA was mixed with 8 μl of LipofectAMINE Plus Reagent and 12 μl of LipofectAMINE Reagent from Life Technologies, Inc. (Rockville, MD) and added to each of the T 25 flasks containing fresh serum-free medium and cultured for 3 h. The medium was then replaced with fresh complete culture DMEM/F12. For transfection of cells cultured in

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[3] The abbreviations used are: IRS-1, insulin receptor substrate 1; IR, insulin receptor; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; PTB, phosphotyrosine-binding domain; IL, interleukin; BrdU, bromodeoxyuridine; PTP1B, protein-tyrosine phosphatase 1B.

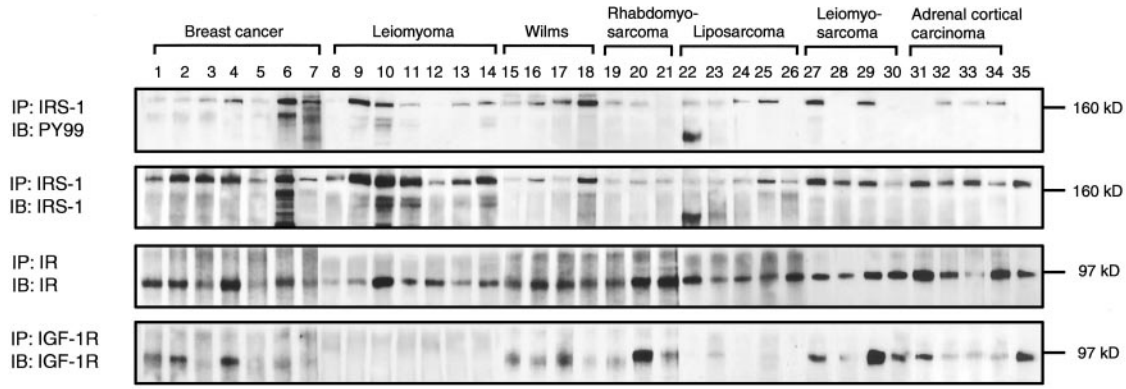

Fig. 1. Expression and/or activation of IRS-1, IR, and IGF-IR in 34 human tumors. Case 35 is a control cell line known to express nontyrosine-phosphorylated IRS-1, IR, and IGF-IR.

96-well plates, DNA was mixed with 1 μl of LipofectAMINE Plus Reagent and 0.5 μl of LipofectAMINE Reagent.

Cell Growth Assay. Cells cultured in 96-well plates were transfected and incubated for 48 h. BrdU (10 μM) was added, and the cells were reincubated for 4 h. The cells were then fixed, incubated with anti-BrdU antibody, and developed using a colorimetric reaction using a cell proliferation ELISA kit from Roche Molecular Biochemicals (Mannheim, Germany). The results were read at 450 nm in an ELISA reader.

Apoptosis Assay. Cells were plated on Lab-Tek Chamber Slide System from Fisher Scientific (Pittsburgh, PA) and transfected. Apoptotic nuclei were evaluated by the end labeling of DNA 3′-OH ends with FITC-dUTP using an *in situ* death detection kit from Roche Molecular Biochemicals according to the manufacturer's instructions. For the DNA laddering assay, 10 μg of DNA isolated from transfected cells were separated on a 1% agarose gel.

Soft Agar Assay. Transfected cells were selected in 0.75 mg/ml Zeocin for 4 weeks. Cells (1×10^4) were suspended in 2 ml of soft agar (0.35% Bactoagar in DMEM/F12 with 20% FCS), plated onto 5 ml of solidified agar (0.75% Bactoagar in DMEM/F12), and cultured at 37°C in 5% CO_2 for 3 weeks. Colonies were fixed with methanol and stained with Giemsa.

Results

Constitutive Activation of IRS-1 in Tumors. Of the 34 tumors studied, all but 2 breast cancer cell lines were primary tumors. Cell lines were cultured in serum-free medium for 24 h before being processed for IRS-1 evaluation. For primary tumors, frozen tumor tissues were used directly for IRS-1 evaluation. IRS-1 was immunoprecipitated with antibody to IRS-1 and immunoblotted with antibody to phosphorylated tyrosine residues. The blots were then stripped of bound antibodies and reprobed with antibody to IRS-1. IRS-1 was found to be tyrosine phosphorylated in 7 of 7 breast cancers, 5 of 7 leiomyomas, 4 of 4 Wilms' tumors, 2 of 3 rhabdomyosarcomas, 4 of 5 liposarcomas, 2 of 4 leiomyosarcomas, and 3 of 4 adrenal cortical carcinomas (Fig. 1). The degree of IRS-1 tyrosine phosphorylation varied from case to case and did not necessarily correlate with the total IRS-1 expression level (Fig. 1). One liposarcoma (case 22 in Fig. 1) expressed an activated IRS-1 of aberrant size, which potentially representing a rearranged IRS-1 or an isoform arising from alternative splicing. In 3 Wilms' tumors and 1 breast cancer, a phosphotyrosine-containing protein(s) of M_r ~190,000 was coimmunoprecipitated with IRS-1 (Fig. 1).

Because IRS-1 is the major substrate for both IR and IGF-IR, we evaluated the possibility that IR and IGF-IR activation in these tumors might lead to IRS-1 activation. However, no apparent tyrosine phosphorylation of IR and IGF-IR was observed (data not shown). All of the tumors expressed IR, whereas expression of IGF-IR was less common (Fig. 1), particularly in leiomyomas, with none of the 8 expressing detectable levels of IGF-IR.

Inhibition of Tumor Cell Growth with a Dominant-negative IRS-1. The impact of IRS-1 constitutive activation on tumor cell growth was studied by introducing a dominant-negative IRS-1 with all 18 potential tyrosine-phosphorylation sites replaced by phenylalanines (F18) into breast cancer cells that expressed an activated IRS-1 (case 6 in Fig. 1). As a control, breast cancer cells were transfected with an empty vector expressing the marker only. Cell growth was significantly inhibited in cells expressing F18 compared with cells expressing vector only. The BrdU incorporation in cells expressing F18 was 32–60% lower than that of control cells expressing vector 48 h after transfection (without selection; Fig. 2). Growth inhibition was more marked when more F18 DNA was used for the transfection (Fig. 2). Similar studies were performed in cells selected for stable transfection for 4 weeks. However, no obvious difference in cell growth was observed between breast cancer cells expressing F18 *versus* those with vector, presumably because of the low level of sustained expression of F18, as indicated by Western blot analysis (data not shown). These findings suggest that only cells expressing a low level of F18 can survive and/or that cells expressing a lower level of F18 had a growth advantage over those cells expressing a higher level of F18 during prolonged cell culture.

Because activation of IRS-1 signaling is known to protect cells from apoptosis, (18) we studied whether expression of a dominant-negative IRS-1 in breast cancer cells induces apoptosis. However, no significant apoptosis was noted in F18-transfected cells in both a DNA ladder assay and a terminal deoxynucleotidyl transferase-

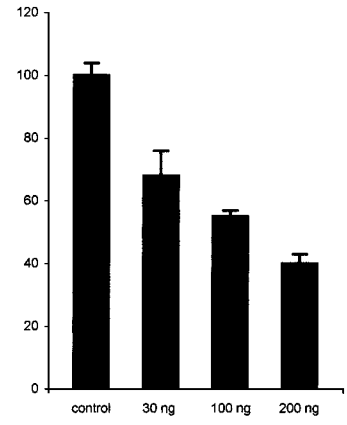

Fig. 2. Growth inhibition in breast cancer cells expressing a dominant-negative IRS-1 (F18). Cells seeded in 96-well plates were transfected with empty vector (pcDNA4/TO/myc-His C) or F18 at different concentrations (30, 100, or 200 ng/well), and the BrdU incorporation was evaluated 48 h after transfection. Results were expressed as percentage of the control cells expressing empty vector.

mediated nick end labeling assay. These findings suggest that other mechanisms such as inhibition of the IRS-1 mitogenic signaling pathway could be responsible for the suppression of cell growth in breast cancer cells expressing F18.

To determine whether the expression of a dominant-negative IRS-1 in breast cancer cells reduced tumor cell transforming capability, we studied anchorage-independent cell growth of breast cancer cells expressing F18, *versus* cells expressing vector only, *versus* untransfected parental cells by colony formation in soft agar. Transfected cells were selected for 4 weeks before the soft agar assay was performed. All of the cells were seeded at 10^3 cells/35-mm plate and cultured for 3 weeks. Parental breast cancer cells, as expected, formed many colonies in soft agar. Breast cancer cells expressing F18, however, formed significantly smaller and fewer colonies. Breast cancer cells expressing the vector only behaved like untransfected parental cells (Fig. 3). Similar results were obtained from two independent experiments performed by different personnel. The dramatic difference in cell growth in soft agar between cells expressing F18 and those with vector was somewhat unexpected because the same group of stably transfected cells (selected for 4 weeks), as described before, showed no significant differences in cell proliferation. A possible explanation for this apparent discrepancy is that only very low levels of F18 expression are needed to reduce cell transformation, whereas similar levels do not substantially inhibit proliferation. Previous studies have shown similar findings in lung carcinoma cells expressing a dominant-negative IGF-IR. Whereas the expression of the dominant-negative IGF-IR dramatically reduced colony formation in the soft agar assay, cell proliferation was not inhibited in stably transfected cells (19).

Discussion

We have found constitutive activation of IRS-1 in a broad range of human tumors, including breast cancer, leiomyoma, Wilms' tumor, rhabdomyosarcoma, liposarcoma, leiomyosarcoma, and adrenal cortical carcinoma. We chose to evaluate these particular tumor types, in part, because they are known to occasionally overexpress IGF-II (20). IGF-II binds to both IGF-IR and one of the IR isoforms (IR-A; Ref. 21). Therefore, one might wonder whether IGF-II/IGF-IR and/or IGF-II/IR signaling was responsible for the constitutive activation of IRS-1, which we found in most of the study tumors. This did not appear to be the case: IR was expressed on all of the tumors we studied, and IGF-IR was expressed in about half of the tumors, but we did not detect IR or IGF-IR tyrosine phosphorylation in any of the tumors. Although we cannot rule out that IR or IGF-IR phosphorylation was present at low levels and went undetected [IRS-1 contains 18 potential tyrosine-phosphorylation sites, (2) whereas IR/IGF-IR have only 7 such sites] (22), it is also possible that IRS-1 is activated by other mechanisms.

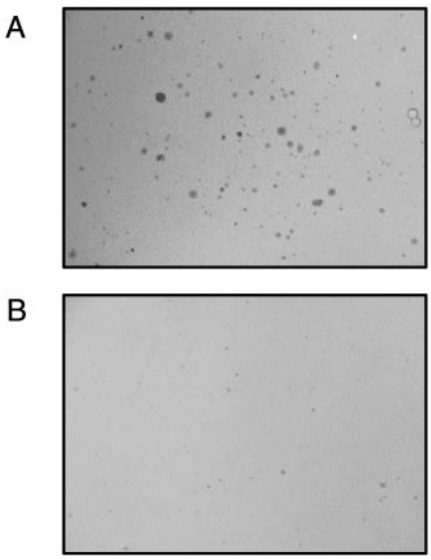

Fig. 3. Inhibition of anchorage-independent growth in breast cancer cells expressing F18. Cells were transfected with empty vector (pcDNA4/TO/myc-His C; *A*) or F18 (*B*), selected in the presence of 0.75 mg/ml Zeocin for 4 weeks, and plated in 35-mm plates containing 0.35% agar and cultured for 3 weeks. Colonies were fixed with methanol and stained with Giemsa.

IRS-1 activation is negatively regulated by PTP1B, which dephosphorylates IRS-1 (23). Protein-tyrosine phosphatases have long been speculated to play a role in tumor suppression because of their ability to inactivate protein tyrosine kinases and inhibit cell growth. One such gene, *PTEN*, has been confirmed as a tumor suppressor gene (24, 25). To study the possibility that constitutive IRS-1 activation in tumors is associated with inactivation of PTP1B, we performed Western blot analysis of 5 breast cancers and their matched nonneoplastic breast cells. None of the tumors expressed detectable PTP1B, whereas the matched nonneoplastic breast cells expressed PTP1B. However, no genomic mutation of *PTP1B* gene was found in those tumors.[4]

Our studies reported herein provide a rationale for the use of IRS-1 as a potential drug target. For example, small molecule inhibitors could be screened for their ability to disrupt IRS-1 signaling. One attractive targeting site is the IRS-1 PTB domain. The IRS-1 PTB domain binds to activated receptors leading to IRS-1 tyrosine phosphorylation (5). Inhibitors that block the PTB domain would likely disrupt IRS-1 signaling and thus inhibit cell growth. Such inhibitors could be pharmacologically pleiotropic, however, because IRS-1 signaling plays a critical role in normal cell functions such as carbohydrate metabolism and cell adhesion in addition to mediating cell growth. Alternatively, an IRS-1 downstream signal pathway that is responsible for cell growth could be targeted. For example, inhibitors could be developed that block the binding of the growth-related IRS-1 substrate to specific IRS-1 phosphorylated tyrosine residue(s). Our studies suggest that such approaches could be relevant therapeutically to a wide variety of tumor types. In summary, we find that constitutive IRS-1 activation is a common phenomenon in human tumors, and we provide evidence that IRS-1 signaling plays a crucial role in tumor cell proliferation and transformation.

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[4] S. Xiao, unpublished data.

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