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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

Insulin receptor substrate 1 (IRS-1) was constitutively activated in the majority of breast cancers. Blocking IRS-1 signaling with a dominant-negative IRS-1 mutant F18 dramatically reduced the cancer cell growth. These studies suggest that constitutive IRS-1 activation plays a central role in breast cancer cell growth and that IRS-1 could be an attractive therapeutic target. IRS-1 has complicated downstream signaling pathways that play important roles in multiple cellular functions. An ideal IRS-1 inhibitor for cancer therapy would block only the IRS-1 growth-related signaling, with minimal interruption of other IRS-1 signaling. To determine the IRS-1 downstream signaling pathway that is critical for growth of breast cancer cells, we expressed six IRS-1 mutants, each with the mutation of critical tyrosine residue(s) that initiates a particular IRS-1 downstream pathway, in breast cancer cells and determined the dominant-negative effects of each IRS-1 mutant on tumor cell growth. Our results showed that IRS-1 defected in SHP-2 binding had similar tumor suppressor function as F18, suggesting IRS-1 promote cancer cell growth by activating SHP-2 signaling. A synthetic peptide representing SHP-2 binding site was then used to screen a compound library. Preliminary analysis showed that 3 small molecular compounds were capable of inhibiting SHP-2 binding in vitro.

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Introduction

Insulin receptor substrate 1 (IRS-1) is a major substrate of insulin, insulin-like growth factors (IGF-1 and IGF-2), estrogen, prolactin, epidermal growth factor, platelet derived growth factor and growth hormone and is at the intersection of several signaling pathways known to be involved in breast cancer. [1-4] Our previous studies showed that IRS-1 is constitutively activated in the majority of breast cancers, and that 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 the cancer cell growth.[5] These studies suggest that constitutive IRS-1 activation plays a central role in breast cancer cell growth and that IRS-1 could be an attractive therapeutic target. We have two main goals in this project: (1) To determine the IRS-1 downstream signaling pathway that is critical for breast cancer growth. IRS-1 signaling plays important roles in numerous cell functions, such as carbohydrate metabolism and cell adhesion, in addition to mediating cell growth. An ideal IRS-1 inhibitor for cancer therapy would block only the IRS-1 growth-related signaling, with minimal interruption of other IRS-1 signaling. (2) To identify small molecule compounds that specifically block the IRS-1 growth-related signaling pathway.

Body

(1) To determine the IRS-1 downstream signaling pathway that is critical for breast cancer growth

Our previous studies showed that a IRS-1 mutant construct F18 with all 18 potential tyrosine-phosphorylation sites replaced by phenylalanines was able to block IRS-1 signaling and inhibit tumor cell growth. To determine the specific tyrosine-phosphorylation site(s) that are critical to tumor cell growth, we established a series of IRS-1 mutants with individual tyrosine residues replaced by phenylalanines and studied their capabilities of inhibiting tumor cell growth.

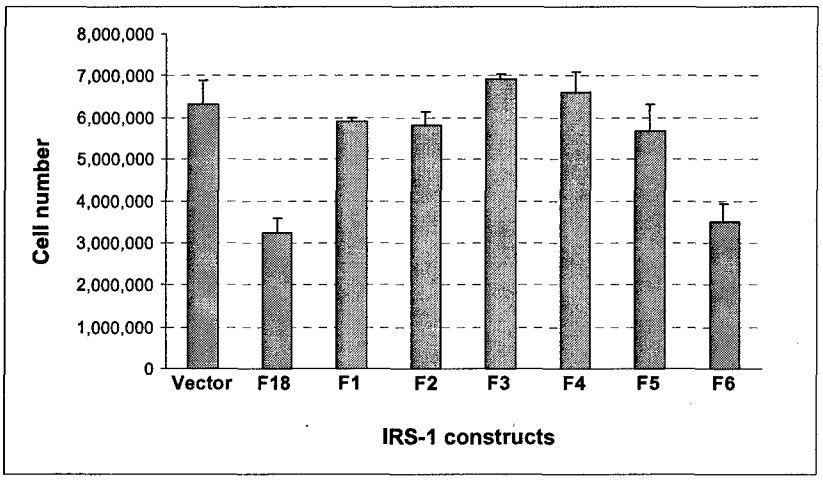
IRS-1 mutant constructs. We assembled a wild-type IRS-1 construct that has a MYC tag and a HIS tag at the C-terminus (pcDNA4/IRS-1). Six IRS-1 mutant constructs were created by site-directed mutagenesis on the wild-type pcDNA4/IRS-1, each with the critical tyrosine residue(s) that serves as the binding sites of p85, Fyn, Grb2, Nck, Csk, or SHP-2 replaced by phenylalanines (Table 1)

Table 1 *Six IRS-1 constructs with the mutations of different tyrosine residues*

IRS-1 Constructs	Mutations	Defect of binding sites
F1	Y151F	Nck
F2	Y662F	P85
F3	Y896F	Grb2
F4	Y941F	Csk
F5	Y1179F	Fyn
F6	Y1229F	SHP-2

Cell proliferation and transformation assay. Each of the six IRS-1 mutants (F1-6), F18 (positive control) and empty vector (negative control) were transfected into breast cancer cells (HTB22) with LipofectAmine Plus Reagent (Invitrogene). Forty-eight hours after transfection, viable cells were counted directly by a hemocytometer in the presence of 0.4% (v/v) trypan blue. Similar expression of each IRS-1 mutant was ensured by performing western blots with an antibody to MYC tag. Our results showed that F18, similar to our previous studies, inhibited about 40-50% tumor cell growth relative to the control cells. One of the IRS-1 mutants defected in SHP-2 binding (F6) showed tumor cell growth inhibition with an efficacy similar to F18, while the remaining five IRS-1 mutants (F1-5) had little effects on tumor cell growth, suggesting that the IRS-1 promote tumor cell growth through activating SHP-2 signaling pathway (Figure 1a and 1b).

a.



b.

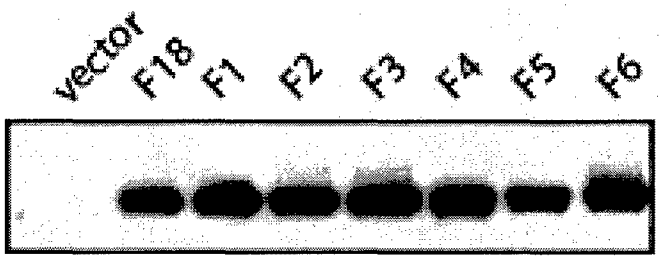
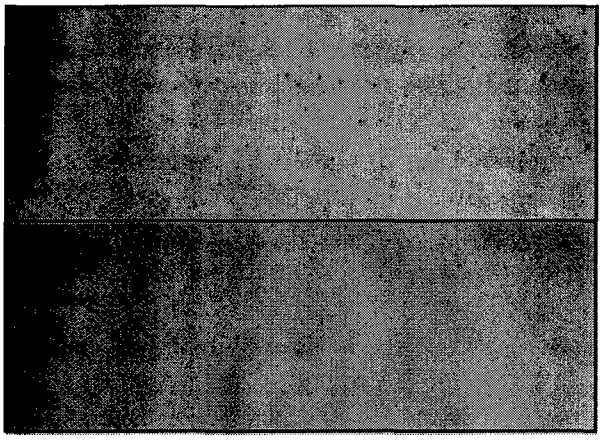


Figure 1 *Inhibition of tumor cell growth by expressing dominant negative IRS-1 mutants. (a). Breast cancer cell line HTB22 was transfected with F1-6, F18 and an empty vector. Living cells were determined by trypan blue exclusion 48 hours after transfection. IRS-1 F6 with the defect of SHP-2 binding inhibited tumor cell growth with an efficacy similar to F18. The remaining five IRS-1 mutants had little effects on tumor cell growth. The number of cells is the average value based on three independent experiments. (b). Similar expression of IRS-1 mutants in tumor cells. Western blot analysis of 50 μg of cell lysis was performed with an antibody specific to MYC tag.*

The critical role of SHP-2 signaling on tumor cell growth was confirmed in a soft agar assay. Tumor cells (HTB22 and HTB27) expressing IRS-1 mutants and vector were selected in the presence of Zeocin (500 μg/ml) for 2 weeks before subjected to soft agar assay. While the IRS-1 mutant F6, similar to F18, inhibited colony formation in soft agar plates, the remaining IRS-1 mutants did not inhibit the anchorage independence growth of tumor cells (Figure 2)(manuscript in preparation).

a.



b.

Cases	Number of colonies in soft agar plates	
	Cells expressing IRS-1 F6	Control cells
HTB22	13	217
HTB27	49	287

Figure 2 *Inhibition of anchorage independent growth of tumor cells by expressing dominant negative IRS-1 mutants. IRS F6 and F18 significantly inhibited tumor cell growth in soft agar cultures while IRS-1 F1-5 showed no such an effect. (a). Breast cancer cell line HTB22 was transfected with an empty vector (top) or IRS-1 F6 expression construct (bottom), selected in the presence of Zeocin for two weeks, and planted in 35-mm plates containing 0.3% agar and cultured for two weeks. Colonies were fixed with methanol and stained with Giemsa. (b) Colonies that exceeded 120 μm in diameter in soft agar plates for breast cancer cell line HTB22 and HTB27 expressing IRS-1 F6 or vector only (control) were counted. The number of colonies is the average value based on six dishes in two independent experiments*

(2). Screening a compound library.

A biotin-labeled IRS-1 peptide containing 20 amino acid residues surrounding the critical phosphotyrosine residue that binds to SHP-2 was synthesized (Biosynthesis) and immobilized to microtiter plates coated with streptavidin. The plates were washed and blocked. Purified recombinant SHP-2 was added to the plates and incubated with or without the test compounds (ChemBridge Corporation, San Diego, CA) at room temperature for 2 hours. The plates were washed and incubated with an antibody to SHP-2 for 1 hour at room temperature and incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The peroxidase substrate was added and incubated for 15 minutes. The absorbance was measured at 450 nm with a SpectraMax 340 plate reader. Our initial screening identified 3 compounds that inhibited SHP-2 binding. We are further analyzing the specificity of the compounds for blocking the binding of SHP-2 to tyrosine-phosphorylated peptide. We are challenging the binding of tyrosine-phosphorylated peptide, in the presence of the test compounds, with either a phosphotyrosine peptide or a non-tyrosine-phosphorylated peptide. Phosphotyrosine peptide but not the non-tyrosine-phosphorylated peptide will compete with a specific compound.

Future studies will focus on evaluating the specificity of the compound for blocking SHP-2 growth-related pathway at cell level. Breast cancer cells with constitutive IRS-1 activation will be cultured in the presence of variable concentrations of the compounds. The IRS-1 substrates, including p85, Fyn, Grb2, Nck, Csk, and SHP-2, will be immunoprecipitated with antibodies to specific substrates and immunoblotted with an antibody to phosphotyrosine-containing proteins (PY99). A specific compound will block only the tyrosine phosphorylation of SHP-2. Finally, we will test the inhibition effect of a compound on proliferation and transformation of breast cancer cells.

Key Research Accomplishments

In the one year period supported by the concept award, we have determined that the critical IRS-1 signaling pathway for tumor cell growth is mediated by the SHP-2 signaling. This finding is important because it allowed us to design a short peptide representing the binding site for SHP-2, which was used for screening a compound library. Our initial screening resulted in the identification of 3 small molecular compounds that inhibited the binding of the SHP-2 to the synthetic peptide in vitro.

Reportable outcomes

Our work on the characterization of IRS-1 growth-related signaling pathway is being prepared for publication.

Conclusions

We found that SHP-2, by binding to IRS-1, initiated the signaling pathway for breast cancer cell growth. Blocking SHP-2 signaling was able to inhibit breast cancer cell growth. We found 3

compounds that were able to inhibit the binding of SHP-2 in an *in vitro* system.

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