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14. ABSTRACT:
Prolactin receptor (PRLR) is a single transmembrane receptor that normally requires ligand-binding to trigger intracellular signaling. Several isoforms of the human PRLR have been identified, including a long form (LF) and two short forms (SF1a and SF1b). These isoforms share identical amino acid sequence in the extracellular domain, but altered cytoplasmic domain as a consequence of alternative splicing. The extracellular domain consists of two fibronectin-like subdomains, S1 and S2. Recently we have identified the existence of naturally-occurring S2 deleted (delta S2) variants in several human cancer cell lines. We also showed that these human delta S2 isoforms were constitutively dimerized in the absence of PRL. When overexpressed in breast cancer cells, the delta S2 LF increased cell proliferation. The aim of our proposed training grant was to analyze the effect of delta S2 PRLR in a stable transfection system. We found that one of the S2 deleted short isoforms, delta S2 SF1b, was able to inhibit cell growth and migration.

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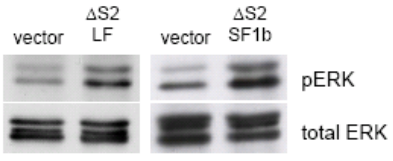
INTRODUCTION

Prolactin (PRL) is a hormone that stimulates mammary gland development and milk protein production. It has also been indicated as a contributing factor to the pathogenesis and progression of several types of cancers, including breast cancer. Recent studies suggest PRL be an autocrine growth factor in normal and cancerous breast tissues (1-4). PRL acts through dimerized single membrane-spanning receptors, PRL receptors (PRLRs). Conformational changes of the dimerized receptors trigger intracellular signals via Jak2-Stat5, ERK, PI3K, and a number of other pathways (5-10). Several PRLR isoforms have been identified, including a long form (LF) and two short forms (SF1a and SF1b) (11, 12). These isoforms are produced by alternative splicing, which generates transcripts different in length of their cytoplasmic termini. The N-terminal extracellular domain of PRLR, which is identical in primary sequence in all three isoforms, consists of two fibronectin-like subdomains, S1 and S2 (13, 14). Preliminary studies from our laboratory have identified the existence of naturally-occurring S2 deleted (ΔS2) isoforms in several human cancer cell lines. We have also shown that the deleted ΔS2 isoforms are constitutively dimerized and activated in the absence of PRL. The ΔS2 PRLR LF has a growth-promoting effect in T-47D human breast cancer cells in a transient transfection experiment. We therefore established stable cell lines expressing ΔS2 PRLR LF, SF1a or SF1b to assess the biological activity of these isoforms. In this report, we show that all three isoforms can produce intracellular signals and one particular isoform, ΔS2 SF1b, can inhibit growth and migration of a human cancer cell line.

BODY

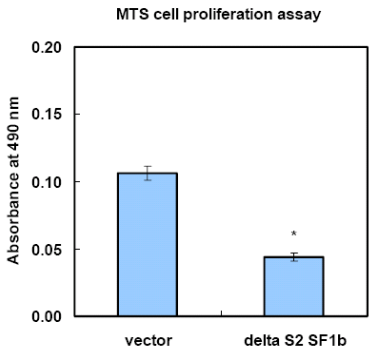
The aim of first year's work was to establish all expression constructs and perform a number of proposed assays so that necessary adjustments could be made. The first step was to subclone the ΔS2 PRLR cDNA (LF, SF1a, or SF1b) to a pcDNA3.1(+) expression vector (Invitrogen, Carlsbad, CA). After the correct DNA sequence was confirmed, these plasmids were transfected individually into several human cancer cell lines, including a well differentiated breast cancer cell line T-47D, poorly differentiated MDA-MB-231, and PC-3, a prostate cancer cell line that was used in one of the PI's major projects. Western blot analysis showed the proteins were well expressed and migrated toward the expected sizes (not shown).

Next we examined if the ΔS2 PRLR isoforms were able to produce intracellular signals. PRLR short forms were considered dominant negatives when first discovered (11, 12). We have previously shown that the molecular mimic of phosphorylated PRL, S179D PRL, can trigger a prolonged signal from the short receptors through the ERK signaling pathway (15). Therefore ERK activation of the ΔS2 PRLR short forms as well as ΔS2 LF was tested by Western blotting. Results indicated that all three S2 deleted isoforms could signal in the absence of PRL. Examples of ΔS2 LF and SF1b are shown here.

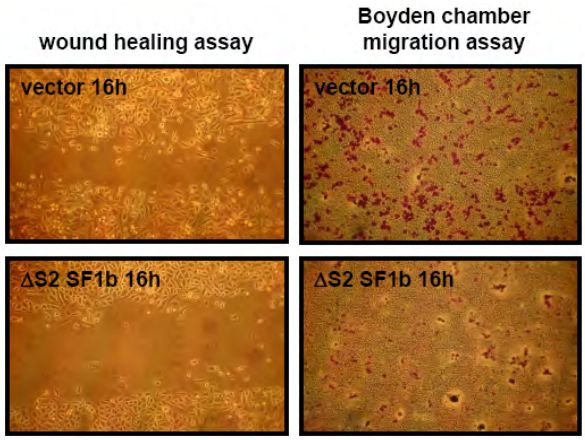


Our preliminary data suggest in a transient transfection system, ΔS2 PRLR LF has a promoting effect on cancer cell growth, whereas ΔS2 SF1b marginally decreases cell number. This is an effect when low transfection efficiency in these cells is expected. We therefore established stable cell lines to improve gene

delivery, and long-term activation of ΔS2 PRLRs could also be monitored. After 2-3 weeks of antibiotic selection, isolated colonies were analyzed for protein expression by Western blotting. Selected ΔS2 PRLR expressing cells were used for proliferation assays including the colorimetric formazan reduction assay (16) and direct cell counting. Taking one of the short forms as an example, the results showed a significant decrease in cell growth in ΔS2 SF1b cells compared to vector controls.



PRL has been shown to be a chemoattractant by activating the Ras and Rac signaling cascade (17). However, little is known how different PRLR isoforms alter cell migration. Migratory capacity was conducted using the wound healing assay and Boyden chamber migration assay. In the wound healing assay, wounds were introduced with a sterile tip to a monolayer of confluent cells. The extent of wound closure was measured after 16 hours. In the Boyden chamber migration assay, single-cell suspension was placed at 10^5 cell per well in the upper permeable support (8.0 μm, Costar, Acton, MA). Medium containing 10% fetal bovine serum was placed in the lower chamber as a chemoattractant. After 16 hours, cells were methanol-fixed, and those which had not migrated through the membrane were wiped off with a cotton swab. Cells that had migrated to the lower surface of the membrane were stained with 1% toluidine blue. As shown in the figure, ΔS2 SF1b cells (lower panels) exhibit decreased motility compared to vector controls (upper panels).



KEY RESEARCH ACCOMPLISHMENTS

- established ΔS2 PRLR expression plasmids
- established ΔS2 PRLR stable cell lines
- conducted a series of *in vitro* assays regarding cell proliferation and migration using the ΔS2 PRLR stable cell lines

REPORTABLE OUTCOMES

Data obtained during this training period was in part presented in a poster session in the American Society for Cell Biology annual meeting. Two manuscripts were prepared for submission.

1. Huang KT and Walker AM. (2006) A naturally occurring, inducible, constitutively active isoform of the human prolactin receptor ΔS2 SF1b, reduces proliferation and migration in human prostate cancer cells. The American Society for Cell Biology 46th Annual Meeting, p66. Manuscript in preparation.
2. Tan D, Huang KT, Ueda E and Walker AM. (2007) S2 deletion variants of human PRL receptors demonstrate that extracellular domain conformation can alter conformation of the intracellular signaling domain, submitted.

CONCLUSION

From our preliminary results described above, one can conclude that the short receptor ΔS2 SF1b, inhibits cancer cell growth and migration. One can also expect a growth promoting effect from the ΔS2 LF. However, the level of endogenous full-length PRLR in the cells should also be considered. Several studies, including some from our lab, have noted heterodimerization of the long and short receptors can occur (18, 19). In addition, Gadd et al. report that artificially truncated PRLRs can also heterodimerize with endogenous receptors and alter PRL signaling (20). Therefore heterodimerization of the ΔS2 and endogenous PRLRs is possible. In cell lines with high PRLR expression, such as T-47D, heterodimerization is more likely to occur than those with very few normal receptors, such as MDA-MB-231. We would like to examine this possibility and have a better picture if interactions of different receptors may lead to different outcomes.

We have shown the inhibition of cell growth and migration in the ΔS2 SF1b stable cell line. However, we did not observe noticeable cell cycle defects or cell death. We suspected this was mainly because during the process of antibiotic selection, cells had been accustomed to the constitutive expression of ΔS2 SF1b. Changes in cell cycle might be compromised. To address this issue, an inducible expression system will be developed and used in future experiments, so that ΔS2 PRLR proteins can be expressed when needed.

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