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Original Articles

Anti-metastatic outcome of isoform-specific prolactin receptor targeting in breast cancer

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ABSTRACT

Controversy exists concerning the role of the long prolactin receptor (PRLR) in the progression of breast cancer. By targeting pre-mRNA splicing, we succeeded in knocking down only the long PRLR *in vivo*, leaving the short forms unaffected. Using two orthotopic and highly-metastatic models of breast cancer, one of which was syngeneic (mouse 4T1) to allow assessment of tumor-immune interactions and one of which was endocrinologically humanized (human BT-474) to activate human PRLRs, we examined the effect of long PRLR knockdown on disease progression. In both models, knockdown dramatically inhibited metastatic spread to the lungs and liver and resulted in increased central death in the primary tumor. In the syngeneic model, immune infiltrates in metastatic sites were changed from innate inflammatory cells to lymphocytes, with an increase in the incidence of tumor-specific cytotoxic T cells. Long PRLR knockdown in three-dimensional culture induced apoptosis of tumor-initiating/cancer stem cells (death of 95% of cells displaying stem cell markers in 15 days). We conclude that the long PRLR plays an important role in breast cancer metastasis.

Introduction

Prolactin and breast cancer

The PRLR is a compelling target in several cancers [1–3]. For breast cancer, a substantial body of evidence supports an important role in the progression of disease. For example, serum PRL levels in the top quartile of the range considered normal are associated with an increased incidence of breast cancer equivalent to that seen with estrogen [4]; PRLRs are expressed at higher levels in cancerous lesions versus normal tissue [5]; high circulating PRL is correlated with high breast density, itself associated with a higher incidence of breast cancer [6,7]; PRL is an autocrine survival and growth-promoting factor in breast cancer cells and a paracrine factor produced by mammary stromal cells [8,9]; increased autocrine PRL expression in ductal epithelium leads to development of both estrogen receptor positive and estrogen receptor negative cancers in experimental animals [10]; and knockout of the PRLR markedly slows the development of tumors induced by viral oncogene overexpression [11]. Furthermore, normal mammary stem cells have been reported to express the PRLR [12], suggesting the possibility that PRLRs may be present on cancer stem cells and that targeting the PRLR therefore has the potential to be curative. Also of great significance is that up to 95% of primary tumors express the PRLR [13] while approximately 75% express the estrogen receptor [14]. Thus, therapies targeting the PRLR could have greater utility than those targeting the estrogen receptor, including use in patients with estrogen receptor negative tumors and/or metastases.

Prolactin receptor forms and breast cancer

The most abundant PRLR forms in the human and mouse include a long and three short forms (SF1a-c in human [15,16] and S1-3 in mouse [17]). Most work with most cancer types supports the contention that the long PRLR promotes cell proliferation and survival, while one of the short forms (SF1b in human) is anti-proliferative and pro-apoptotic [1–3,18–22], a dominant negative. In breast tumors, the ratio of long to short PRLRs is positively correlated with progression of disease [22]. However, increased nuclear amounts of Stat5, which is a downstream signaling molecule associated with activation of the long PRLR, are associated with a better prognosis in breast cancer [23], and *in vitro* studies have demonstrated that activation of Stat5 enhances the epithelial versus mesenchymal phenotype of breast cancer cells [24]. Thus, there is some controversy about the exact role of the long PRLR in breast cancer. The dominant negative short PRLR inhibits function of the tumor-promoting

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form by hetero-dimerization [25]. In addition, the dominant negative down-regulates expression of the tumor-promoting receptor [18] and, when homodimerized, signals to promote differentiation and apoptosis [1,2,26]. Thus, preservation of the dominant negative PRLR would be predicted to have significant therapeutic advantages.

Materials and methods

Study design

Following pilot experiments to separately assess variation in receptor knockdown and tumor growth and metastatic spread as a function of the number of tumor cells implanted, a power analysis was performed to determine sample size. With longer-term trials where there was a greater chance that some control animals would succumb to their disease, the sample size was increased to ensure appropriate sample size should some animals be lost to analysis. All treatment durations were predetermined and unaltered during the course of the study. Data from animals that died before the tissue collection date were excluded from analysis. This was decided prospectively. No outliers were removed from analyses. A total of 9 trials with, and 3 without, tumors were conducted. Replications are reported in the figure legends.

Breast cancer cell lines

Tumor cell lines were obtained from American Type Culture Collection (Manassas, VA), which repository authenticates via short tandem repeat DNA profiling. Mouse 4T1 and human BT-474 were used for the *in vivo* analyses. Cells were routinely cultured in RPMI 1640 with 10% fetal bovine serum (FBS) (Invitrogen/Life technologies, Grand Island, NY). On the day of harvest, cells were removed from the culture plates by incubation in 0.25% trypsin/2.21 mM EDTA for 5 min at 37 °C, pelleted, washed in Dulbecco's phosphate buffered saline (DPBS) and then suspended in serum-free RPMI 1640 containing matrigel (BD Biosciences, San Jose, CA) such as to produce a 10 mg/ml suspension used for orthotopic injection. Continuous culture *in vitro* results in downregulation of the PRLR and hence only cultures up to 15 generations were used.

Animals

Two strains of mice were used in the study, BALB/cJ and NOD SCID (NOD.CB17-*Prkdc*^{scid}/SzJ) (Jax mice, Bar Harbor, ME). Females aged 8–9 weeks at the time of orthotopic cell placement were used. BALB/cJ mice are syngeneic with the 4T1 mouse breast cancer cell line and NOD SCID mice were used for human BT-474 tumors. In both instances, tumor cells were placed within the mammary fat pad. The number of implanted cells depended on the duration of desired exposure to treatment (see figure legends) since, in these highly metastatic models, death can occur in the control animals before the desired duration of treatment in the experimental group is complete. So as to preserve implanted cell and recipient tissue integrity, cell placement in the mammary fat pad was achieved using a ½ inch 26 gauge needle and gentle pressure during delivery of 50 µl cells. Animals were randomly assigned to experimental groups. Animals from each experimental group were injected in turn to randomize effects due to time since cell harvesting. In order to replicate normal exposure to human PRL, and because mouse PRL does not interact with the human PRLR [27], mice receiving BT-474 cells (both control SMO- and PRLR SMO-treated) also received recombinant human PRL sufficient to result in a circulating level of 10–15 ng/ml (measured in trunk blood as described below). Recombinant human PRL, prepared as previously described [28], was administered via inter-scapular subcutaneous osmotic minipump (Alza Corporation, Cupertino, CA). At the end of the treatment period, the wet weight of the tumor was determined, the tumor was divided radially such that each piece was representative of the whole, and the pieces were processed for histology, gene expression, or for extraction of immune cells. All animal procedures were approved by the University of California, Riverside, Institutional Animal Care and Use Committee and were in accordance with guidelines from the American Association for Laboratory Animal Care. Animals were housed under specific pathogen-free conditions with 12 hour light–dark cycles and ad libitum access to food. Animals were checked daily for external indicators of advanced disease.

Splice-modulating oligomers

The splice-modulating oligomers (SMOs) (Table 1) were custom synthesized as Vivo-Morpholinos by Genetools LLC (Philomath, OR). A scrambled, non-functional oligomer with the same modifications was used as the control. All primers used for RT-PCR are also provided in Table 1. Since focus was on the ability to inhibit metastasis rather than to shrink a pre-existing tumor, treatment with the control or PRLR SMO was begun 3 days prior to tumor cell implantation so that delivery of the SMOs from an Alzet minipump was accurate at the time of tumor cell implantation. For longer treatments, a fresh pump was inserted at day 28.

RT-PCR and qPCR

Tissues were snap frozen and 50–100 mg extracted in RiboZol (Amresco, Solon, OH). Cultured cells were washed once with DPBS before RiboZol was added to culture wells. mRNA was reverse-transcribed with oligo-dT (first-strand cDNA synthesis using M-MLVRT kit, Invitrogen). Analysis used a CFX96 qPCR Detection System (BioRad, Hercules, CA) and SYBR green master mix reagent (iQ SYBR Green, BioRad). All samples for qPCR were confirmed to have only a single peak in the melting curve. All values were normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH) or β-actin mRNA.

General histology/histopathology

Tissues were fixed in 10% formaldehyde in DPBS, pH 7.4, dehydrated and embedded in paraplast. Sections were stained with hematoxylin and eosin and assigned a coded group number. Sections were examined by another individual without knowledge of treatment group identity. Blood smears were produced at the time of euthanasia, stained with Wright's stain and subjected to a white cell count.

EdU and TUNEL staining

The nucleoside analog, 5-ethynyl-2′-deoxyuridine (EdU), was injected intraperitoneally (160 µg/g body weight) 2 hours before tissue harvesting. On tissue sections, incorporated EdU was detected using the Click-IT™ system (Invitrogen/Life technologies). TUNEL staining was accomplished using a DeadEnd kit from Promega (Madison, WI). EdU and TUNEL staining was performed either on sections midway through the primary tumor if the whole tumor was used or on radial sections from the middle to the outer edge if only a portion of the tumor was used for histological analysis. Four sections per tumor were examined and photographed by confocal microscopy. The areas of the sections staining positively for EdU or TUNEL were calculated. Only similarly-sized sections were used for quantitative analysis in order to distinguish effects of tumor size from effects of the PRLR SMO on the area of dead cells. Negative controls included staining of tumors from non-EdU-injected animals, and processing without use of Terminal Deoxynucleotidyl Transferase. Positive controls included section treatment with DNAse 1. Negative control values were subtracted.

Table 1
SMO sequences and Primers used.

SMO sequence		
Human PRLRSMO	GCCCTTCTATTAAAACACAGACACA	
Mouse PRLRSMO	GCCCTTCTATTGAAACACAGATACA	
Human primer sequence for q-PCR		
	Forward primers (5′–3′)	Reverse primers (5′–3′)
LF-PRLR	CCTTGTCCAGGTTCGCTGCAAA	AGATGAGCATCAAATCCTTTTA
SF1b PRLR	TAAATGGTCTCCACCTACCCTGAT	CACCTCCAACAGATGAGCATCAAATCC
Beta-actin	AAAGACCTGTACGCCAACAC	GTCATACTCCTGCTTGCTGAT
Mouse – primer Sequence for q-PCR		
LFPRLR	ATAAAAGGATTTGATACTCATCTGCTAGAG	TGTCATCCACTTCCAAGAACTCC
S1-PRLR	AAGCCAGACCATGGATACTGGAG	AACTGGAGAATAGAACACCAGAG
S2-PRLR	TGCATCTTTCCACCAGTTCCGGGGC	TCAAGTTGCTCTTTGTTGTCAAC
S3-PRLR	TGCATCTTTCCACCAGTTCCGGGGC	TTGTATTTGCTTGGAGAGCCAGT
GHR	TCTCAAGGAAGGGAAGTTGGAG	AGCTCAATGAACTCGACCCA
GAPDH	TGCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC