Tumor Development by Transgenic Expression of a Constitutively Active Insulin-Like Growth Factor I Receptor

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Abstract

The insulin-like growth factor I receptor (IGF-IR) is a transmembrane tyrosine kinase that is essential to growth and development and also thought to provide a survival signal for the maintenance of the transformed phenotype. There has been increasing interest in further understanding the role of IGF-I signaling in cancer and in developing receptor antagonists for therapeutic application. We describe herein a novel animal model that involves transgenic expression of a fusion receptor that is constitutively activated by homodimerization. Transgenic mice that expressed the activated receptor showed aberrant development of the mammary glands and developed salivary and mammary adenocarcinomas as early as 8 weeks of age. Xenograft tumors and a cell line were derived from the transgenic animals and are sensitive to inhibition by a novel small-molecule inhibitor of the IGF-IR kinase. This new model should provide new opportunities for further understanding how aberrant IGF-IR signaling leads to tumorigenesis and for optimizing novel antagonists of the receptor kinase. (Cancer Res 2005; 65(9): 3781-7)

Introduction

The importance of the insulin-like growth factor I (IGF-I) signaling pathway to growth and development has been well established (1, 2). Early demonstration that the IGF-I receptor (IGF-IR) was required for oncogenic transformation suggested that receptor antagonists may have utility as anticancer agents (3). Activation of the receptor is initiated with binding of IGF-I or IGF-II to the α-subunits of the IGF-IR, resulting in autophosphorylation of tyrosine residues in the β-subunit. The ligand dependence of receptor activation increases the complexity with which the pathway may regulate normal growth and development, as well as the oncogenic process. Overexpression of IGF-I was previously reported in tumor tissues of colon, breast, and lung (4–6). In addition, it has been suggested that circulating IGF-I levels may have been increasing interest in further understanding the role of IGF-IR in cancer and in developing receptor antagonists for therapeutic application. We describe herein a novel animal model that involves transgenic expression of a fusion receptor that is constitutively activated by homodimerization. Transgenic mice that expressed the activated receptor showed aberrant development of the mammary glands and developed salivary and mammary adenocarcinomas as early as 8 weeks of age. Xenograft tumors and a cell line were derived from the transgenic animals and are sensitive to inhibition by a novel small-molecule inhibitor of the IGF-IR kinase. This new model should provide new opportunities for further understanding how aberrant IGF-IR signaling leads to tumorigenesis and for optimizing novel antagonists of the receptor kinase. (Cancer Res 2005; 65(9): 3781-7)

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oligonucleotides: 5'-AGGGTTGGTGGGAAAGTGAC-3' for the CD8 portion of the sequence and 5'-CGTCCGATGATGTTGAAGA-3' for the IGF-IR portion of the fusion. Founder mice (F0) shown to harbor the transgene were then outbred to the ICR background, and progeny (F1) were again tested for transmission of the transgene. Mice were observed twice daily for the presence of tumors, and the date of first observation of lesions was noted. Animals were euthanized when tumors grew to >1,000 mm³, or if lesions impaired movement or feeding. Animal handling was done under the guidelines of the institutional Animal care and Use Committee in an Association for Assessment and Accreditation of Laboratory Animal–accredited facility.

Establishment of xenografts and cell line. A salivary gland adenocarcinoma that developed spontaneously in a transgenic mouse (MCI-19) was excised and cut into fragments of about 20 mg. Tumor fragments were implanted s.c. into the ventral thoracic region of a group of six female, athymic BALB/c nu/nu mice (Harley Sprague-Dawley, Indianapolis, IN), using a 13-gauge trocar. Once established, the salivary gland–derived tumor was minced with scissors and was subjected to enzyme dissociation using a cocktail consisting of 0.025% collagenase (Sigma Chemical Co., St. Louis, MO), 0.05% Pronase (Calbiochem, La Jolla, CA) and 0.04% DNase (Sigma Chemical) for 1 hour at 37°C. The cell suspension was passed through a 70-μm nylon screen, and the cells were washed in PBS, counted and resuspended in complete RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. Cells (3 × 10⁶) were seeded initially onto 6-well tissue culture plates coated with poly-δ-Lysine (Biocohat Cellware, Becton Dickinson, Mountain View, CA) The cultured cells were passaged by trypsinization once every 3 to 4 days. After an additional six to seven passages, the cultured cells were adapted to growth on noncoated tissue culture flasks (Falcon, Lincoln Park, NJ).

Mammary gland whole mount analysis. Whole mount analysis was done on female wild-type and transgenic littermates. Inguinal mammary glands were dissected, placed on a glass slide, and fixed overnight in ethanol/acetic acid. Glands were washed in 70% ethanol for 30 minutes, rinsed in water for 10 minutes (twice), and stained overnight with carmine alum. Glands were then destained in 70%, 95%, and 100% ethanol each for 30 minutes and soaked in tolune until the fat cleared. Glands were then placed in methylsalicylate and photos taken using a Nikon dissecting microscope.

Tissue preparation and histology. Complete necropsies were done on transgenic mice to identify gross evidence of tumor masses. Samples of all masses observed at necropsy, along with lung, liver, kidney, heart, and spleen were evaluated histologically. These tissues were fixed in 10% neutral-buffered formalin, sectioned and stained with H&E. For bromodeoxyuridine (BrdUrd) labeling of cells, mice were injected with BrdUrd (100 mg/kg) 2 hours before sacrifice. BrdUrd was detected by immunohistochemistry as described previously (18).

Western blot analyses. Lysates were prepared from tumors and tissues by homogenization in lysis buffer [1% Triton X-100, 5% glycerol, 0.15 mol/L NaCl, 20 mmol/L Tris-HCl (pH 7.6), 1 mmol/L EDTA, 1 mmol/L, sodium vanadate, 40 μmol/L ammonium molybdate, and 1% Complete protease inhibitors (Roche Biochemicals, Nutley, NJ)] at 4°C. Insoluble materials were removed by centrifugation, and protein concentration was determined using a Bio-Rad Micro biinchoninic acid protein assay kit (Pierce, Rockford, IL). Lysates prepared from tumors/tissues (~2 mg) or cultured cells (0.5 mg) were immunoprecipitated using a monoclonal antibody to the human CD8α chain (Biosource, Camarillo, CA). The immunoprecipitates were fractionated on 10% polyacrylamide gels (NuPage, Invitrogen, San Diego, CA) and were analyzed by Western blotting with antibodies to phospho-tyrosine (BD Biosciences, San Jose, CA) and with antibodies specific to the IGF-IR (Santa Cruz Biotechnology, Santa Cruz, CA). Analyses of IGF-IR signaling were also done by Western blots using unfractionated cell lysates (20 μg each). Proteins were separated on 4% to 12% polyacrylamide gels (NuPage, Invitrogen) and subjected to Western blot analysis using antibodies to Akt, phospho-Akt, mitogen-activated protein kinase (MAPK), and phospho-MAPK (all from Cell Signaling Technology, Beverly, MA). Antibody binding was detected using chemiluminescence substrate (Pierce).

Synthetic small interfering RNAs and transfection. IGF1R-Sal cells were grown in RPMI 1640 containing 10% FCS. Cells were seeded into 96-well plates at a density of 1,500 cells per well 1 day before transfection. On the following day, the culture medium was replaced with 89 μL of antibiotic-free medium immediately before transfection. Small interfering RNAs (siRNAs) that target the cytoplasmic portion of the CD8-IGF1R fusion protein.
mRNA were designed using a proprietary sequence selection algorithm and were custom synthesized by Qiagen, Inc. (Chatsworth, CA). Of three siRNA oligonucleotides synthesized, one siRNA (IGFR2: sense 5'-UUGCAUGGU-AGGCGAGAAGU'T-3' and antisense 5'-ACUUUCGCUACAAUGCAATT-3') was found to silence the expression of the CD8-IGF1R transgene, as measured by quantitative reverse transcription-PCR (data not shown). siRNA specific for Eg5 was prepared as described (19). Oligonucleotides were annealed and transfected into cells using LipofectAMINE 2000 (Invitrogen) with OptiMEM media (Life Technologies, Gaithersburg, MD). A 10× mixture of duplex siRNA was prepared by diluting in OptiMEM (serum-free transfection media). A 1:1 mixture of duplex siRNA oligonucleotides and LipofectAMINE 2000 was allowed to incubate for 20 minutes at room temperature and 20 μL was added to each well. Following the addition of the transfection mixtures, cells were allowed to incubate at 37°C for 72 hours before cell growth was measured by incorporation of 3H-thymidine as described below. Cell lysates were also prepared from separate transfected cultures for Western blot analyses as described above.

Cell proliferation assays. Cells were plated at 1,500, 7,500, and 3,000 cells per well for IGF1R-Sal, BT474, and MCF-7, respectively, in 96-well microtiter plates. Compounds dissolved in dimethyl sulfoxide were added 24 hours later and the cultures were incubated for an additional 72 hours. Cells were pulsed with 4 μCi/μL [3H] thymidine (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom) for 3 hours, trypsinized, and harvested onto UniFilter-96, GF/B plates (Perkin-Elmer, Boston, MA). Incorporation into DNA was measured by scintillation counting on a TopCount NXT (Perkin-Elmer). Data shown are averages of triplicate wells with SEs.

Results

Expression of CD8-IGF1R perturbs mammmary development and drives tumorigenesis. The CD8α extracellular sequence was chosen as the fusion partner because of its shown ability to promote intermolecular disulfide bond formation, which was previously exploited to generate a chimeric receptor kinase that is constitutively activated in the absence of ligand stimulation (20). The CD8-IGF1R fusion gene was placed under the control of the mouse mammary tumor virus promoter, which drives transcription in the mammary and salivary glands of transgenic mice (Fig. 1; ref. 21). The DNA construct was microinjected into the pronuclei of fertilized oocytes and resulted in the generation of eight founder mice carrying the transgene DNA. In subsequent breeding, the transgene was found transmitted to offsprings in a Mendelian fashion. There was no apparent clinical abnormality in the fertility or development of the transgenic mice, except that females were unable to lactate and the transgene had to be transmitted through male mice. The latter observation suggested that transgene expression may have perturbed mammary development, and this was further investigated by whole-mount analyses of mammary glands. At 4 weeks of age, when puberty growth starts, mammary glands from CD8-IGF1R mice were highly abnormal, showing a reduction in terminal end buds (TEB, the proliferating end of the ductal structure). There was also reduced ductal bifurcation, dilated ducts, and aberrant side branching (Fig. 2A). In particular, it was apparent that the main duct leading directly from the nipple (Fig. 2A, thick arrow) was often highly dilated. There was a dramatic reduction in mammary ductal growth and development, such that at 6 weeks of age the ductal structure in wild-type mice had virtually filled the fat pad, whereas in transgenic mice the ductal growth was completely retarded (note the ductal structure is still behind the lymph node; Fig. 2B). The blockade of mammary gland development in transgenic mice was persistent through 15 weeks of age such that...
early hyperplasia, two of the transgenic lines, MCI-15 and MCI-19, that had particularly high levels of transgene expression developed tumors of the salivary and mammary glands (Fig. 3A). Salivary gland tumors emerged as early as 8 weeks in the MCI-15 line and by week 12 in the MCI-19 line. Enlargement of the salivary gland first became evident clinically at weaning and rapidly progressed as the animals aged. All salivary masses were lobular adenocarcinomas with occasional squamous differentiation. Tumors were multilobular, circumscribed, expansive masses made up of dense sheets of cells with scant cytoplasm (Fig. 3B). In some areas of the tumors, there was differentiation into serous-type cells with highly eosinophilic granular cytoplasm, with and without acinar organization (suggestive of Parotid gland origin). Scattered lymphatic vessels contained neoplastic nodules and there was invasion into adjacent striated muscle.

Mammary tumors were first palpable also at 8 weeks of age in virgin female mice. Multifocal masses in the mammary tissue were often randomly distributed in various glands. At sites of mammary masses, adenocarcinomas and adenomas were multifocal within adjacent lobules. The adenocarcinomas were multinodular expansive masses with variable encapsulation. The nodules were made up of dense sheets of a fairly monomorphic population of neoplastic cells with large areas of central necrosis and cyst formation (Fig. 3C). Some nodules had extensive squamous differentiation and cystic degeneration.

Establishment of xenograft animal model. The aggressive growth rate of the transgenic tumors and their relatively homogeneous histology suggested that they may be transplantable as xenografts, which would provide a tool for pharmacologic evaluation of IGF-IR inhibitors. Salivary gland tumors were excised from the MCI mice and fragments were implanted s.c. on the ventral side of athymic nu/nu mice. Within a few weeks of implantation, the tumors grew at a rapid rate and could be passaged serially as xenografts. One of the low-passage xenografts was explanted in culture, and from that, a cell line (IGF1R-Sal) was derived. Transgenic mammary tumors also grew as xenografts in nude mice, but numerous attempts to isolate a stable cell line were unsuccessful.

Expression of the CD8-IGF1R fusion protein was assessed in tissues and cell line by immunoprecipitation and Western blot analyses (Fig. 4A). A monoclonal antibody specific to the extracellular sequence of human CD8α was used to immunoprecipitate the chimeric protein from tissue and cell lysates. The immunoprecipitates were analyzed by Western blotting with antibodies to phospho-tyrosine and to the cytoplasmic sequence of the IGF-IR. A polypeptide of ~75 to 80 kDa in size was detected in the transgenic and xenograft tumors but not in tissue from wild-type mice, thus verifying the expression of the CD8-IGF1R fusion protein (Fig. 4A). The fusion protein was phosphorylated on tyrosines, consistent with activation of the receptor kinase as a result of constitutive dimerization. A similar analysis verified the expression of the fusion receptor in the IGF1R-Sal cells and revealed extensive tyrosine phosphorylation of the receptor in the cultured cells. Dependence of the cell line on receptor expression or function was assessed by transfection with siRNA specific to the receptor sequence. In cells transfected with the silencing siRNA, the fusion protein level was reduced by ~50% relative to cells transfected with a nonsilencing siRNA (Luc4, designed against the firefly luciferase mRNA; Fig. 4B). Treatment of the IGF1R-Sal cells with transgene-targeted siRNAs resulted also in a 50% reduction in thymidine incorporation relative to cells treated with a nonsilencing control siRNA (Fig. 4C). The partial inhibition

Figure 3. Salivary and mammary gland tumors in CD8-IGF1R transgenic mice. A, transgenic mice with representative salivary and mammary tumors from line MCI-19. B, histopathology of a salivary tumor that developed in the CD8-IGF1R transgenic mice. Salivary adenocarcinoma characterized by neoplastic cells forming dense sheets (X) and more differentiated serous-type cells forming acinar structures (>). C, histopathology of a mammary gland tumor. Arrows, areas of squamous differentiation and cystic degeneration. Arrowheads point to mitotic figures (A and B). Bar, 20 μm.
of proliferation following siRNA treatment is consistent with the incomplete suppression of protein accumulation for the fusion receptor. By contrast, cells transfected with an siRNA previously reported to silence expression of the kinesin motor protein Eg5 resulted in close to 80% inhibition of cell proliferation (Fig. 4C; ref. 19).

The utility of the IGF1R-Sal transgenic tumor and cell line was further evaluated using a small-molecule antagonist of the IGF-IR kinase. BMS-536924 is a novel synthetic molecule that has been
optimized for potent inhibition of the IGF-IR kinase, with an IC_{50} of 100 nmol/L for the recombinant enzyme. Treatment of the IGF1R-Sal cells with BMS-536924 resulted in a dose-dependent inhibition of proliferation, with an IC_{50} of 0.11 μmol/L (Fig. 5d). MCF7 cells, which were previously shown to be sensitive to IGF-IR inhibition, were also significantly inhibited (IC_{50} = 0.46 μmol/L; ref. 22). A different breast tumor cell line, BT-474, that has been known to depend on HER2 gene amplification and signaling for growth, was relatively insensitive to the compound, with an IC_{50} that is >10 fold higher than that for the IGF1R-Sal cells (Fig. 5d; ref. 23). Because the three cell lines were comparable in their sensitivity to the cytotoxic agent paclitaxel (Fig. 5b), their differential sensitivity to BMS-536924 may be a reflection of the different extent to which their proliferation or survival depends on the IGF-IR pathway. Immunoprecipitation and Western blot analyses were used to verify that inhibition of cell proliferation was accompanied by a modulation of signaling by the fusion receptor. Receptor phosphorylation, as well as the phosphorylation of Akt and MAPK, were inhibited with a dose response that parallels the inhibition of cell proliferation (Fig. 5c). The pharmacokinetic properties of BMS-536924 are such that the compound should be suitable for oral administration for efficacy evaluation in animal models. Nude mice were implanted with the IGF1R-Sal tumors and were treated with a once-daily regimen of BMS-536924. There was a dose-dependent increase in antitumor efficacy when animals were treated for 14 days (Fig. 5d). The tumors grew to a size of >2 g in control mice treated with vehicle alone but were nearly completely inhibited in mice treated with the compound. Tumor growth was delayed for a further 5 to 6 days following the termination of drug treatment.

Discussion

The genetic deletion of IGF-IR in mice led to a fundamental understanding of the role of IGF-IR in growth (3). Characterization of fibroblasts that lack IGF-IR revealed a critical role of IGF-IR in both growth and transformation, with the IGF-IR-null fibroblasts failing to be transformed by a number of oncogenes (3). Using a transgenic mouse model, we have shown that a CD8-IGF1R fusion protein functions as a dominant oncogene. The incidence and kinetics of tumor emergence suggest that the transgene is sufficient to initiate and maintain the transformation process. The data presented here clearly support the IGF-IR as an oncogene and an attractive target for therapeutic intervention. At present, it cannot be excluded that the fusion receptor acquired novel signaling functions that are not associated with the native IGF-IR.

Expression of the fusion receptor in the mammary gland caused severe perturbation of development and actually resulted in a retardation of ductal growth. These transgenic mice, with their robust phenotype, provide a model for further dissecting the role of IGF-IR signaling in mammary gland development, which is not feasible with knockout mutants or with ligand-expressing transgenic mice. IGF-I and its receptor are implicated in ductal branching (18, 24, 25). Mammary glands from CD8-IGF1R transgenic mice showed aberrant and excessive side branching (represented by multiple buds along the duct) and this often occurred on the end of the duct where the TEB should be. It is possible that this aberrant side branching impairs the ability of the TEB to form and grow. A similar pattern of branching was recently noted in mice overexpressing an inducible fibroblast growth factor receptor 1, suggesting that common signaling pathways downstream of both growth factor receptors may be involved (26).

Recent progress in discovering IGF-IR kinase inhibitors has led to the observation that multiple myeloma cells may be exquisitely dependent on IGF-IR signaling for proliferation and survival (27, 28). However, there is still a great need for preclinical models that can be used to assess the therapeutic potential of IGF-IR inhibitors in solid tumors. Although other transgenic mouse tumors had previously been developed for studying IGF-I signaling, the long latency in tumor development in these models makes them impractical for use in the optimization of pharmacologic inhibitors of the receptor kinase. We have shown that the IGF1R-Sal tumors could be transplanted in nude mice and the xenografts were shown responsive to treatment with an inhibitor of the IGF-IR kinase. Furthermore, a cell line was established from one of the xenograft tumors and was shown inhibited in proliferation by a silencing siRNA that targets the transgene. The sensitivity of the cell line to both siRNA and a small-molecule inhibitor provides confidence that the tumor and cell models are indeed dependent on receptor signaling, thus making available an experimental system suitable for in vitro and in vivo evaluation of chemical entities designed to inhibit the IGF-IR kinase activity.

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