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 predict the relative risk for prostate and breast cancer (7, 8). Despite the growing interest in the tumor biology of IGF-IR signaling, there have been few experimental models that are designed or suitable for analyzing the role of the receptor pathway in oncogenesis. Transgenic models for studying IGF-IR signaling were generated by the overexpression of IGF-I or IGF-II (9–14). In mouse skin, overexpression of IGF-I led to hyperplasia and hyperkeratosis, and papilloma formation after treatment with chemical carcinogens (10, 11). Transgenic expression of IGF-I/IGF-II in the mammary glands led to inhibition of mammary involution and, in some models, the formation of mammary adenocarcinomas (9, 12–14). In one IGF-2 transgenic mouse model, lung tumors were observed after a latency of >6 months but a direct role of IGF-IR signaling in the tumorigenic process has not been shown (15). However, whereas some of these models confirmed a link between tumorigenesis and IGF-I signaling, it is uncertain how the limited half-life of the ligand peptides may have dampened the potential phenotype of receptor hyperactivation. Most importantly, their utility has been restricted by the long latency period of tumor development. To establish an animal model to study the biological function of IGF-IR, we chose to use a chimeric receptor that would form a constitutive homodimer, thus obviating the complexity and uncertainty in ligand dynamics associated with the reliance on the wild-type IGF-IR. The chimeric receptor was constructed by an in-frame fusion of the cytoplasmic sequence of the human IGF-IR to the extracellular and transmembrane sequences of the human T-cell antigen CD8α. Overexpression of the fusion receptor disrupted normal mammary development and resulted in the rapid appearance of mammary and salivary gland tumors. These tumors were transplantable into nude mice and their growth could be inhibited by a novel inhibitor of the IGF-IR kinase.

Materials and Methods

Generation of the mouse mammary tumor virus CD8-IGF1R construct. A HindIII/BamHI fragment encoding amino acid residues 1 to 218 of human CD8α was cloned into the HindIII/BamHI site of pcDNA3.1(+). A BamHI fragment encoding amino acid residues 964 to 1367 of the human IGF-IR (numbering refers to the sequence NP 000865 in the National Center for Biotechnology Information protein database) was then inserted into the BamHI site of the vector containing the CD8 fragment. The transgene was subcloned into the pAA derived mouse mammary tumor virus (MMTV) transgene vector to yield the resulting construct shown in Fig. 1 (16). Generation and identification of CD8-IGF1R transgenic mice. Transgenic mice harboring the MMTV CD8-IGF1R construct (MCI) were generated by microinjection of a PvuII fragment from the above construct into the pronuclei of B6D2 embryos using the techniques described (17). Injected embryos were transferred to pseudopregnant ICR female mice and allowed to develop to term. Mice harboring the transgene were identified by a PCR strategy designed to detect the CD8-IGF1R fusion using the following

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oligonucleotides: 5'-AGGTTGTGGGTAAGAGTA-3' for the CD8 portion of the sequence and 5'-CGTCCAGTAACTGTTGAAGA-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 mm², 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 adeno-carcinoma 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 line was designated IGF1R-Sal and was propagated as a tumor xenograft in nude mice. Tumors were passed every 2 weeks, at which time the tumor reached ~500 to 1,000 mm³ in size. For treatment studies, nude mice bearing IGF1R-Sal tumors of about 100 mm³ in size were sorted into groups of eight for treatment with vehicle (80% polyethylene glycol 400 in water) alone or with BMS-536924. Tumor weight is derived from size measurements using the formula: tumor weight = (length x width²)/2.

A cell line was derived from an IGF1R-Sal tumor xenograft that had been propagated in nude mice for three passages. The tumor was excised and 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 x 10⁶) were seeded initially onto 6-well tissue culture plates coated with poly-L-lysine (Becton Dickinson, Rockford, IL). 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 toluene 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 bichinchoninic acid protein assay kit (Pierce, Rockford, IL). Lysates prepared from tumors/tissues (~2 mg) or cultured cells (0.5 mg) were immunoprecipitated using monoclonal antibody to the human CD8 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 were transfected using siRNAfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. siRNA expression was detected by Western blotting using antibodies specific to the IGF-IR (Santa Cruz Biotechnology, Santa Cruz, CA).

Figure 1. Diagrammatic representation of the transgenic construct, MMTV CD8-IGF1R. A, the fusion cDNA was constructed by ligation of codons 1 to 218 of the human CD8a gene to codons 964 to 1367 of the human IGF-1R gene. The coding sequence is transcribed from the MMTV promoter. B, the resulting fusion protein contains the extracellular and transmembrane sequences of CD8a and the entire cytoplasmic sequence of the IGF-1R. Intermolecular disulfide bonds that result in receptor homodimerization. C, PCR analyses of transgene transmission in founder mice. DNA from mouse tails was amplified with primers specific to the fusion receptor and the reaction products were analyzed by agarose gel electrophoresis. Representative data from the analyses of 32 lines and the positive identification of seven of the eight positive founder mice, including MCI-9, MCI-15, MCI-19, and MCI-21. Lanes N, M, and P contained DNA size markers (M), and PCR products from negative control (N), or positive control (P) using plasmid DNA as template.
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'-UGGAUGGU-AGCGAGAAGU-3' and antisense 5'-AUCCUUGGCUACAGGGATT-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 (Packard, Meriden, CT). Results are expressed as an IC50, which is the drug concentration required to inhibit cell proliferation by 50% to that of untreated control cells Data shown are averages of triplicate wells with SEs indicated.

**Results**

**Expression of CD8-IGF1R perturbs mammary 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 offspring 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 the ductal tree never filled the fat pad and often did not extend past the lymph node. Pregnancy-mediated lobuloalveolar development was also aberrant in the transgenic mice (Fig. 2C) and also failed to stimulate growth of the ductal system. The failure of the ductal cells to undergo proper lobuloalveolar development (data not shown) resulted in a complete absence of milk production.

Histologic analysis at 6 weeks of age revealed marked hyperplasia in the mammary glands of the transgenic mice (Fig. 2D), which was associated with hyperproliferation as assessed by bromodeoxyuridine incorporation (Fig. 2E). Consistent with the

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**Figure 2.** Whole-mount analyses of mammary gland development from wild-type and CD8-IGF1R transgenic mice. Inguinal mammary gland whole mounts were prepared from nontransgenic (left) and transgenic MCI-15 littermates at 4 weeks (A), and 6 weeks (B) of age, and also at 16 days of pregnancy (C). Thin arrows point to terminal end buds. Thick arrow points to the beginning of the duct at the nipple. ○, lymph nodes that serve as a point of reference in the mammary gland. D, histopathology of nontransgenic and transgenic MCI-15 mammary glands at 6 weeks of age. E, BrdUrd staining of serial sections similar to those shown in (D). Brown color results from positive staining against a blue background from the counterstain. Bar, 50 μm (D and E).
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. 3). 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, expansile 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 expansile 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.](image-url)
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\textsubscript{50} of 100 nmol/L for the recombinant enzyme.\textsuperscript{6} Treatment of the IGF1R-Sal cells with BMS-536924 resulted in a dose-dependent inhibition of proliferation, with an IC\textsubscript{50} of 0.11 \mu mol/L (Fig. 5f). MCF7 cells, which were previously shown to be sensitive to IGF-IR inhibition, were also significantly inhibited (IC\textsubscript{50} = 0.46 \mu mol/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\textsubscript{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. 5j), 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.\textsuperscript{1} 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 development of other organ systems in a transgenic mouse model. J Clin Invest 1996;98:507–17.

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