Treatment of murine breast cancer cells with antisense RNA to the type I insulin-like growth factor receptor decreases the level of plasminogen activator transcripts, inhibits cell growth in vitro, and reduces tumorigenesis in vivo

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Aims: To establish that cells from the murine mammary carcinoma cell line, EMT6, express type I insulin-like growth factor receptor (IGF-IR), tissue-type plasminogen activator (tPA), and urokinase-type plasminogen activator (uPA). To investigate the role of IGF-IR in growth, transformation, and tumorigenesis in addition to its relation to tPA and uPA in EMT6 cells. To assess the suitability of the EMT6/syngeneic mouse model for studying the role of IGF-IR in tumorigenesis.

Methods: The presence of transcripts for IGF-IR, tPA, and uPA was determined by northern blot analysis using poly (A) RNA derived from EMT6 cells transfected with an antisense IGF-IR construct or a construct lacking the antisense IGF-IR insert. Flow cytometry was used to measure IGF-IR protein. Assays were performed to determine cell proliferation, transformation, and the tumorigenicity of antisense IGF-IR transfected EMT6 cells and control transfected EMT6 cells.

Results: There was strong expression of IGF-IR, tPA, and uPA in EMT6 cells. EMT6 cells from clones carrying antisense IGF-IR displayed a significant decrease in cell proliferation and lost the ability to form colonies in soft agar. A decrease in tumour size occurred when cells carrying the antisense IGF-IR were injected into syngeneic mice. Reduced expression of tPA and uPA was seen in EMT6 cells carrying the antisense IGF-IR construct.

Conclusions: The IGF-IR plays a role in the progression, transformation, and tumorigenesis of EMT6 murine mammary carcinoma cells. The suppression of IGF-IR mRNA in EMT6 cells decreases tPA and uPA expression. EMT6 cells and the syngeneic mouse provide a suitable model for studying the role of IGF-IR in breast tumour progression.

“Tumour recurrence and the development of metastases are common problems in the treatment of breast cancer. Many transformed or malignant tumour cells including cells from breast cancers are known to produce plasminogen activator (PA) which is a serine protease existing in two forms known as tissue-type (tPA) and urokinase-type (uPA). Plasminogen activators contribute to the lysis of tumour associated fibrin, suppression of tumorigenesis, a reduction in the metastatic potential, and prolonged survival after the injection of MDA-MB-435s cancer cells expressing antisense IGF-IR into the mammary fat pads of immune compromised mice. Alternatively, it has been shown that overexpression of the IGF-IR promotes aggregation, growth, and cell survival in MCF-7 human breast cancer cells in vitro.”
which is a crucial step in the generation of metastases. Indeed, both tPA and uPA play crucial roles in tumour growth and metastasis for several types of cancer including breast cancer. A previous study from our laboratory showed that tPA and uPA were decreased in rat prostate cancer cells transfected with an antisense IGF-IR construct. Moreover, a recent report has demonstrated that the disruption of IGF-IR in human breast cancer cell lines suppressed invasion through Matrigel and decreased the expression of uPA mRNA.

To investigate the role of IGF-IR in the pathogenesis of breast cancer, we have characterised the murine mammary carcinoma cell line, EMT6, for the expression of IGF-IR gene transcripts and for protein expression. In addition, we determined whether the EMT6 cells expressed tPA and uPA at the mRNA level. We then applied an antisense strategy that targeted the IGF-IR to EMT6 cells to test the hypothesis that IGF-IR plays a role in cell growth and tumorigenesis in this cell line. In our study, we provide data to show that targeting the IGF-IR in EMT6 murine breast cancer cells reversed the malignant phenotype in vitro and reduced tumour growth in vivo. Moreover, the data revealed that the treatment of EMT6 cells with an antisense IGF-IR construct resulted in decreased expression of tPA and uPA. These studies provide a basis for future experiments that will allow us to elucidate the function of the IGF-IR in breast cancer in an immune competent syngeneic animal model system.

METHODS

Cell line and cell culture

The EMT6 murine mammary carcinoma cells that were used in our study were kindly provided by Dr Emmanuel Akporiaraye (University of Arizona, Tucson, Arizona, USA). The EMT6 cell line is a subline of an early passage of the KJHJ line that was derived from a mammary carcinoma arising in a BALB/c mouse. EMT6 cells form progressive tumours in syngeneic mice and immunisation of mice with irradiated EMT6 cells does not generate significant protective immunity against this tumour. The cells were maintained in minimum essential medium (αMEM) (Life Technologies, Gaithersburg, Maryland, USA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, Utah, USA). Culture medium for transfected EMT6 cells was supplemented with genetin at 500 µg/ml (Life Technologies) to maintain selection pressure. All of the experiments were carried out using cells from early passages (P1 or P2). Before injection into 4–6 week old female syngeneic BALB/c mice the cells (1 x 10⁷) were washed once in phosphate buffered saline (PBS), treated with versene (1/5000 dilution; Life Technologies) for three to five minutes and resuspended in PBS.

Construction of vectors

To assemble the pRcII/cytomegalovirus (CMV) vector (fig 1A) used in these experiments a pRS/CMV vector (Invitrogen, Carlsbad, California, USA) was modified by removing DNA sequences from nucleotide position 995 (ApaI site in the multiple cloning site) to nucleotide position 2100 (SmaI site from the human uPA sequence, and a 2.2 kb fragment of chicken β actin—were labelled with [³²P]dCTP (DuPont NEN Research Products, Boston Massachusetts, USA) using the random hexanucleotide primer method. Northern blot hybridisation was carried out in 5x saline sodium citrate (SSC), 5x Denhardt’s solution, 10% (wt/vol) dextran sulfate, 0.1% (wt/vol) sodium dodecyl sulfate (SDS), and 100 µg/ml denatured salmon sperm DNA at 65°C for 18 hours. After hybridisation, the membrane was washed for 15 minutes at room temperature with 2x SSC, followed by a final 15 minute wash in a solution consisting of 0.5x SSC and 0.5% SDS (wt/vol) at 65°C. The membranes were exposed to x ray film for five minutes to 12 hours at room temperature or at ~80°C. x Ray films were analysed with a ScilScan 5000 laser densitometer (United States Biochemical Corp, Cleveland, Ohio, USA) and normalised relative to β actin mRNA.

Transfection

EMT6 murine mammary cancer cells were transfected with the antisense IGF-IR vector or with the pRcII/CMV control vector using Lipofectin (Life Technologies), according to the supplier’s instructions. Geneticin (G-418 sulfate; Life Technologies) at a concentration of 1 mg/ml was used to select for cell clones that were neomycin resistant, indicating that the vector was present in the cells. Several (n = 9) individual cell clones were isolated from the population of cells carrying the antisense IGF-IR vector. All of the transfected cell clones were maintained in αMEM with 10% FBS and G418 (0.5 mg/ml).

Northern blot analysis

Total RNA was isolated from cells with Trizol reagent (Life Technologies). Poly A+ RNA was then selected using the Megasamer reagent assembly (Life Technologies), according to the manufacturer’s instructions. Samples (8 µg poly A+ RNA) were electrophoresed on a 1% denaturing agarose gel followed by transfer to a Hybond-N nylon membrane (Amersham Lifesciences, Arlington Heights, Illinois, USA). The cDNA probes—a 0.7 kb fragment from the human IGF-IR sequence, a 1.7 kb fragment from the mouse tPA sequence, a 1.3 kb fragment from the human uPA sequence, and a 2.2 kb fragment of chicken β actin—were labelled with α [³²P]dCTP (DuPont NEN Research Products, Boston Massachusetts, USA) using the random hexanucleotide primer method.
Flow cytometry

The EMT6 cells (1 × 10^6) were plated into 100 mm Petri dishes and grown in medium containing 10% FBS for 24 hours. The cells were rinsed with PBS and detached with versene, (1/5000 dilution; Life Technologies) at 37°C for 10 minutes, followed by washing in PBS containing 2% FBS. Cells (2 × 10^5) were transferred to Eppendorf tubes, spun at 300 × g for three minutes at 4°C and washed twice in PBS containing 2% FBS. The primary antibody, mouse monoclonal antibody α-IR-3 (Oncogene Research Products, Cambridge, Massachusetts, USA) was used at a 1/30 dilution for 30 minutes at 4°C, followed by two rinses in PBS containing 2% FBS. The secondary antibody, B-phycoerythrin (PE) conjugated goat antimouse IgG Fab (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania, USA) was applied at a 1/60 dilution for 30 minutes at 4°C in the dark. Cells were rinsed as above and suspended in freshly prepared 2% paraformaldehyde at 4°C in the dark.

Controls consisted of incubation with no antibodies or incubation with the secondary antibody only. Data were acquired using an EPICS XL-MCL (Beckman Coulter, Miami, Florida, USA). System II (Version 3) acquisition software was used to acquire and analyse the data. A 488 nm air cooled argon ion laser, operating at 15 mW, was used to excite the PE conjugated antibody, and a 575 nm band pass optical filter was used to collect the emitted light. Fluorescence data were visualised as a histogram. The fluorescent signals for the cells from clones carrying the antisense IGF-IR construct were compared...
numbers were extrapolated from a standard curve generated and a reference wavelength of 630 nm. The reported cell used to measure the absorbance at a wavelength of 570 nm were counted manually after two weeks. Results are expressed as mean (SE) of six wells. All assays were repeated several times.

**Soft agar colony forming assay**

For soft agar assays, a bottom layer of 1 ml of the corresponding culture medium containing 0.6% granulated agar (Difco Laboratories, Detroit, Michigan, USA) and allowed to solidify. Cells (1 × 10⁶) from the H, J, and O clones carrying antisense IGF-IR or cells transfected with the vector lacking the insert were suspended in 50 µl of αMEM containing 0.3% agar, 10% FBS and 0.5 mg/ml G418. Culture medium (1 ml) containing 0.3% agar (Difco Laboratories) was added to the cell suspension before seeding on the dishes. Triplicates were performed for each type of cell. Cells were incubated at 37°C in 5% CO₂ atmosphere. Dishes were examined twice a week and colonies were counted manually after two weeks. Results are expressed as mean (SE).

**Assay of tumour growth in animal models**

Female syngeneic Balb/c mice 4–6 weeks of age were used. Mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and maintained in the animal resource facility at Case Western Reserve University. The EMT6 murine mammary cancer cells from clones H, J, and O carrying the antisense IGF-IR construct or the control cells carrying the construct lacking the antisense IGF-IR insert were injected through a 22 gauge needle over the right scapula of the mice. All cells were injected in a volume of 100 µl at a concentration of 1 × 10⁶ viable cells, as determined by trypan blue exclusion. The tumour take rate when the control cells were injected in the scapular region was 100%. Animals were sacrificed on day 14 and the tumours excised and weighed.

**RESULTS**

The goal of these studies was to characterise the EMT6 murine mammary cancer cell line for IGF-IR expression, in addition to the expression of the plasminogen activators, tPA and uPA. We also wanted to establish whether the EMT6 cell line would provide a suitable syngeneic animal model for examining the role of IGF-IR in tumorigenesis using an antisense gene targeting approach.
The first objective of the series of experiments carried out in our study was to determine whether the EMT6 murine mammary carcinoma cells express IGF-IR. The second aim was to establish that EMT6 cells could be transfected with our antisense IGF-IR expression construct. After transfection of the EMT6 cells with the control vector (construct lacking the IGF-IR inserts) and clonal selection, northern blot analysis was carried out using poly (A+) RNA derived from cells. The EMT6 cells carrying the control construct were found to express both the 7 kb and 11 kb transcripts for IGF-IR (fig 2A). After transfection of the EMT6 murine mammary cancer cells with a vector containing the IGF-IR cDNA in an antisense orientation, several clones (n = 9) were identified and maintained in culture. All of these cell clones were assessed for the presence of the antisense IGF-IR expression vector (data not shown). The three clones, H, J, and O (fig 2A) that displayed the strongest signal for antisense IGF-IR expression by northern blot analysis were used to assess further the role of IGF-IR in cell growth and tumour formation. The 7 kb and 11 kb transcripts for IGF-IR were apparent in cells from the J, H, and O clones carrying the antisense IGF-IR construct (fig 4C). The filter was rehybridised with a cDNA probe for chicken β actin to verify the integrity and amount of poly (A+) RNA in the samples (fig 2B).

We also assessed uPA expression in the EMT6 cells. Northern blot analysis was carried out on poly (A+) RNA derived from cells from the clones expressing antisense IGF-IR and on poly (A+) RNA derived from cells from the clone transfected with the control vector. We found that uPA was expressed by all of the cell samples (fig 4A). The expression of uPA mRNA in the IGF-IR antisense transfected cells was reduced by 25% in clone H, by 65% in clone J, and by 45% in clone O relative to that in the cells from the clone carrying the control CMV construct (fig 4C). The filter was rehybridised with a cDNA probe for chicken β actin to verify the integrity and amount of poly (A+) RNA in the samples (fig 4B).

A series of assays was performed to characterise the proliferative capabilities of the EMT6 cells after treatment with the antisense IGF-IR construct. The proliferation rate of the cells from clone H, clone J, and clone O (fig 5A) carrying the antisense IGF-IR construct was significantly inhibited on day 5 when compared with the proliferation rate of cells from the clone carrying the control CMV construct lacking the IGF-IR insert (fig 5A). In addition to slower growth rates, large numbers of non-adherent cells were present in cultures of the clones carrying the IGF-IR antisense construct compared with the cultures of the clone carrying the control construct.

To determine whether the treatment of EMT6 cells with antisense IGF-IR RNA could affect the expression of the plasminogen activator, tPA, we analysed poly (A+) RNA derived from EMT6 cells from clones, H, J, and O, and compared it with poly (A+) RNA derived from EMT6 cells from the clone with the CMV vector by northern blot hybridisation. We found that tPA was expressed by all of the cell samples (fig 3A). The expression of tPA mRNA in the IGF-IR antisense transfected clones was reduced by 60% in clone H, by 40% in clone J, and by 30% in clone O relative to that in the cells from the control transfected clone (fig 3C). The filter was rehybridised with a cDNA probe for chicken β actin to verify the integrity and amount of poly (A+) RNA in the samples (fig 3B).

Figure 6  Soft agar colonisation of EMT6 murine mammary carcinoma cells. Assays were performed in parallel on all cells under identical culture conditions. (A) Photographs are from a soft agar assay carried out on cells from the H, J, and O clones (panels 1, 2, and 3, respectively), carrying the antisense insulin-like growth factor I receptor (IGF-IR) construct and cells carrying the cytomegalovirus construct lacking the antisense IGF-IR insert (panel 4). Original magnification, ×1. (B) High power photographs of sample fields taken from assays depicted above. Original magnification, ×40.
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Table 1  Anchorage independent colony formation by EMT6 murine mammary carcinoma cells carrying an antisense IGF-IR expression plasmid

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies</th>
</tr>
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<tbody>
<tr>
<td>pRc/IRAS H</td>
<td>660 (53)</td>
</tr>
<tr>
<td>pRc/IRAS J</td>
<td>0</td>
</tr>
<tr>
<td>pRc/IRAS O</td>
<td>0</td>
</tr>
<tr>
<td>pRc/CMV</td>
<td>0</td>
</tr>
</tbody>
</table>

Triplicate plates were seeded with 1 x 10⁵ cells each and colonies were counted 14 days later. Each number represents the average of triplicate determinations expressed as mean (SE).

To assess whether treatment of the EMT6 mouse mammary carcinoma cells with antisense IGF-IR affects the tumorigenic potential of the cells in vivo, assays were carried out in female syngeneic mice. Mice (six/group) were injected with cells from the antisense IGF-IR H, J, or O clones or cells from the control clone carrying the construct lacking the antisense IGF-IR insert. A dramatic inhibition in tumorigenesis was seen in the mice injected with cells from the H, J, and O clones. In contrast, the animals injected with cells from the clone carrying the construct lacking the antisense IGF-IR insert developed large tumours by day 14 post-injection (fig 7B). All of the mice injected with cells from the H, J, and O clones developed tumours; however, the tumours were significantly smaller than those that formed after injection of the control cells (fig 8).

DISCUSSION

We have characterised cells from the EMT6 murine mammary carcinoma cell line for the expression of IGF-IR, tPA, and uPA mRNA and for the production of IGF-IR protein. In addition, we have established that the syngeneic Balb/c mouse provides a suitable in vivo model for studying the role of IGF-IR in tumorigenesis. We then applied an antisense gene therapy strategy that targets the IGF-IR to define mechanisms that are involved in the growth of this cell line. In addition, we have shown that the treatment of EMT6 cells with antisense IGF-IR reduces IGF-1 protein as well as the mRNA expression of the plasminogen activators, tPA and uPA.

“We have established that the syngeneic Balb/c mouse provides a suitable in vivo model for studying the role of type I insulin-like growth factor receptor in tumorigenesis”

IGF-IR is produced by normal and malignant cells and is a crucial coordinator of cell cycle progression, cell survival, and the establishment and maintenance of the transformed phenotype. IGF-IRs are overexpressed in virtually all breast cancer cell lines, in which they are believed to enhance growth and inhibit apoptosis. In our study, we have established that cells from the murine mammary carcinoma cell line, EMT6, express transcripts for IGF-IR in addition to IGF-IR protein. Several reports, including those from our group, have shown that one mechanism by which gene therapy strategies that target IGF-I or IGF-IR in rat glioblastoma cells inhibit tumour growth is by the stimulation of a cellular immune response. The EMT6 mouse mammary cell tumour has been shown to be a useful model system for studying another member of the growth factor family, transforming growth factor β, and has also been useful in studies that investigated the role of the immune system in host–tumour interactions in vivo. Therefore, we plan to use the EMT6 model system to elucidate further the role of antisense IGF-IR in an immune competent animal.

Because EMT6 cells express IGF-IR transcripts we then used this model system to study the role of IGF-IR in murine mammary tumorigenesis. We carried out a series of experiments to determine whether the treatment of EMT6 cells by an antisense IGF-IR gene targeting approach could change the malignant phenotype of the murine breast cancer cells. We extended the findings of others, in addition to studies by our group, which showed that targeting the IGF-IR by both
monoclonal antibodies directed against IGF-IR and antisense strategies inhibits breast cancer cell growth. The monoclonal antibody α-IR-3 has been used to block the IGF-IR and can successfully inhibit the growth of human breast cancer cells, MDA-MB-231. A recent report from our laboratory showed that the treatment of MDA-MB-435 cells with an antisense IGF-IR expression construct suppressed the growth of this metastatic human breast cancer cell line. We were unable to show a quantitative decrease in IGF-IR expression at the mRNA level by northern blot analysis of the antisense IGF-IR EMT6 cell clones; however, small changes in mRNA values may not be detectable using this method. In contrast, we found a decrease in IGF-IR protein concentrations in cells from the antisense IGF-IR clones using flow cytometry, in addition to a significant inhibition in the cell proliferation rate. It has been shown that a small increment in the number of receptors for each cell, well within the physiological range, can modulate the mitogenic and transforming activities of the IGF-IR in mouse embryo fibroblast 3T3-like cells. Although it was not in the scope of our study to assess the number of IGF-IR binding sites on the EMT6 cells, it is likely that the treatment of the cells with our antisense IGF-IR construct resulted in a reduction in the number of binding sites for IGF-IR. Indeed, our results indicate that the reduction in the IGF-IR protein concentration in EMT6 cells treated with an antisense IGF-IR construct has profound effects on the mitogenic and transforming activities of the IGF-IR.

“The use of antisense type I insulin-like growth factor receptor for cancer treatment in humans has far reaching implications”

The abolition of anchorage independent growth in soft agar by cells from the H, J, and O clones suggests that the transformed phenotype has been reversed after treatment with the antisense IGF-IR expression construct. Several laboratories (including our group) have reported that the treatment of cancer cells with antisense IGF-IR results in the reversal of the transformed phenotype. We found that the antisense strategy was more effective in inhibiting the growth of EMT6 cells in soft agar than in the cell proliferation assay. This is in agreement with a report showing that the IGF-IR has a more profound influence on the transformed phenotype than on cellular proliferation for several tumour cell lines, including MCF-7 human breast cancer cells. We showed that there was a dramatic reduction in tumour size when cells from the H, J, and O clones carrying antisense IGF-IR were injected in the region of the mammary fat pad in syngeneic mice. A recent report from our group demonstrated that human breast cancer cells treated with a construct carrying antisense IGF-IR have a delay in the formation of tumours and significant inhibition of tumour growth in immune compromised mice. A previous report from our laboratory showed that the treatment of rat C6 glioblastoma cells with a transfection vector that was constructed to drive transcription of the homopurine (AG) sequence 3′ to the termination codon of the IGF-IR that can form a potential triple helix resulted in dramatic inhibition of tumour growth in vivo. In addition, the downregulation of IGF-IR by either antisense or a potential triple helix approach resulted in upregulation of the cell surface expression of major histocompatibility complex I, suggesting that there may be an immune system component involved in the suppression of tumorigenesis. A study by Rensicoff et al showed that the injection of C6 cells expressing antisense IGF-IR RNA into syngeneic animals provided protection against tumour induction by C6 wild-type cells and, in fact, caused complete regression of established C6 wild-type tumours. Furthermore, the direct injection of an antisense IGF-IR expression vector into established neuroblastoma tumours growing in syngeneic mice resulted in a pronounced inhibition of tumour growth, with complete and durable tumour regression in half of the animals. Therefore, the mechanisms by which gene therapy strategies targeting the IGF-IR inhibit the growth of some tumour cells may be through a decrease in receptor number as well as by yet undefined immune components. Indeed, the use of antisense IGF-IR for cancer treatment in humans has far reaching implications. A recently completed phase I study of 10 terminally ill patients with malignant glioblastoma treated with an 18 mer oligonucleotide that targets the sequence of IGF-I mRNA resulted in a dramatic long term response for two patients, an objective but transient response for four patients, and no response in four others. This study demonstrates the importance of using syngeneic animal models to provide information that can then translate to potential treatments for cancer in humans.

In our present study, we found a reduction in the expression of the plasminogen activators, tPA and uPA, in antisense IGF-IR transfected EMT6 cells. These serine proteases are involved in tumour invasion in vivo. Indeed, both tPA and uPA play crucial roles in tumour growth and metastasis. IGF-I induces tPA activity in developing astrocytes. Breast cancer recurrence is more likely if tumours express high amounts of IGF-IR, whereas early relapse is associated with the overexpression of uPA. A recent report has shown that in serum starved MDA-MB-231 human breast cancer cells uPA mRNA increased in expression over time in the presence of IGF-I. These investigators also showed that the inhibition of IGF-IR by the stable expression of a dominant negative inhibitor of IGF-IR, 486stop, suppressed invasion and decreased the expression of uPA mRNA in MDA-MB-231 cells. Moreover, uPA overproduction results in increased skeletal metastasis by human prostate cancer cells. uPA expressed by PA-III prostate cancer cells has been implicated in the osteoblastic process through its protease action by hydrolysing IGF binding proteins, which results in increased bioavailability of IGFs. A report from our group showed that PA-III rat prostate cancer cells transfected with an antisense IGF-IR construct have a decreased expression of tPA and uPA.

In conclusion, our data have shown that the IGF-IR plays a role in proliferation, transformation, tumorigenesis, and growth progression in a murine mammary carcinoma cell line. In addition, we have established a correlation between IGF-IR and plasminogen activators in the murine EMT6 cells. The EMT6 murine mammary carcinoma cell line will provide an in vitro/in vivo model for studying the role of the IGF/plasminogen system in invasion and metastasis. Moreover, we have provided data showing that the EMT6 cell line and its syngeneic mouse can be useful for studying the role of the IGF-IR in tumorigenesis and the host immune response.

**Take home messages**

- The type I insulin-like growth factor receptor (IGF-IR) plays an important role in the progression, transformation, and tumorigenesis of EMT6 murine mammary carcinoma cells.
- The suppression of IGF-IR mRNA in EMT6 cells decreases the expression of tissue-type and urokinase-type plasminogen activator.
- EMT6 cells and the syngeneic mouse provide a suitable model for studying the role of IGF-IR in breast tumour progression.

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