Decreased Insulin-like Growth Factor-II/Mannose 6-Phosphate Receptor Expression Enhances Tumorigenicity in JEG-3 Cells

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Abstract

The insulin-like growth factor-II/mannose-6-phosphate receptor (IGF-II/M6PR) is believed to bind and degrade the potent mitogen IGF-II, a growth factor for many tumors. This receptor has been shown to be mutated and/or lost in a significant percentage of a variety of tumors, implying that it may act as a negative regulator of cell growth. In this study, we demonstrate that down-regulation of this receptor, mediated by antisense IGF-II/M6PR cDNA transfection into JEG-3 choriocarcinoma cells, results in increased growth rate in vitro and increased tumor growth rate in vivo. These findings demonstrate that a decrease in IGF-II/M6PR expression results in a growth advantage in JEG-3 cells and are consistent with the hypothesis that the IGF-II/M6PR is an inhibitor of tumor growth.

Introduction

The IGF-II/M6PR is a monomeric glycoprotein found in two forms: a membrane-associated molecule of M_1 ~ 270,000 and a soluble receptor of M_1 ~ 220,000 as assessed by SDS-PAGE. This receptor interacts with at least two distinct classes of ligand. One is the mitogen IGF-II, and the IGF-II/M6PR is believed to compete with the type-I IGF receptor for this peptide by sequestering excess mitogen and transporting it to the lysosomes for degradation. It has been proposed that the IGF-II/M6PR may prevent tissue overgrowth and perinatal mortality, which can be prevented by concurrent disruption of either the IGF-II gene or the IGF-I receptor gene. The second class of ligand interacting with the IGF-II/M6PR is glycoproteins bearing M6P moieties on their carbohydrate side chains. Several of these are important regulators of tumor cell growth and metastasis, including lysosomal proteases and precursor TGF-β1. It has been proposed that the IGF-II/M6PR may prevent tissue overgrowth by mediating the degradation of IGF-II and the activation of TGF-β1. A wide variety of tumor types have been shown to display loss of heterozygosity and mutation of IGF-II/M6PR, leading to the hypothesis that the IGF-II/M6PR may be a negative regulator of tumor growth. That increased IGF-II levels and decreased active TGF-β1 levels have been noted in tumors featuring IGF-II/M6PR inactivating mutations support this hypothesis. To further test this hypothesis, we have transfected JEG-3, a choriocarcinoma cell line that secretes both IGF-II and TGF-β1 with an antisense IGF-II/M6PR cDNA construct to determine whether reduced IGF-II/M6PR levels lead to a growth advantage for tumor cells and thus enhance their tumorigenicity.

Materials and Methods

Human IGF-II/M6PR cDNA Constructs. Human IGF-II/M6PR cDNA (originally cloned by Dr. W. Sly, GenBank accession no. J03528) was a kind gift from Dr. Richard McDonald (University of Nebraska, Omaha, NE). Sense and antisense constructs, consisting of the nucleotides 1 to 718 of the IGF-II/M6PR cDNA, were subcloned into pBlUESCRIPT SK + (Stratagene) as a HindIII fragment in both orientations. The sense and antisense IGF-II/M6PR cDNA constructs were then cloned into the KpnI and XbaI sites of pOP13 MCS2, which allows transcription from the Rous sarcoma virus promoter. The vector pOP13 MCS2, a derivative of the LacSwitch vector (Stratagene) pOP13CAT, was generated by replacing the chloramphenicol acetyltransferase gene with the multiple cloning sites XbaI, BglII, Smal, ClaI, KpnI, and XhoI. Stable transfection of JEG-3 cells (American Type Culture Collection, Rockville, MD), cultured in αMEM with 10% FCS, was performed using Fugene 6 transfection reagent (Boehringer Mannheim), and transfectedants were selected for Geneticin resistance (G418) at 400 μg/ml. Clonal foci were isolated, grown under selection, screened for soluble IGF-II/M6PR expression using a specific ELISA, and were infected with recombinant lentivirus. Tumor growth was rapid in this protocol, and each experiment was performed in quadruplicate and repeated at least three times. Tumor Growth in Vivo. JEG-3 clones were harvested by trypsinization, washed, and resuspended at 1 × 10⁶ viable cells/ml in αMEM medium with 0.2% BSA. Viability was assessed by trypan blue exclusion and was routinely >90%. Cells (1 × 10⁶ cells in 100 μl) were injected s.c. at the dorsal neck into four groups (two sense and two antisense IGF-II/M6PR cDNA transfected cell lines) of eight athymic nu/nu female mice. Results were pooled from four separate experiments. Tumor growth was rapid in this protocol, and each experiment was terminated at a single time point when the largest tumors were ~1 cm in diameter (10–12 days after injection). Postmortem blood samples were taken from all test animals. Tumors were paraffin embedded and stained with H&E for histology. The experiments were repeated four times and carried out with approval of the Institutional Animal Care and Ethics Committee.

Results

JEG-3 choriocarcinoma cells stably transfected with either an antisense cDNA construct or a sense cDNA construct were assessed for IGF-II/M6PR mRNA and protein expression by Northern blot analysis and receptor ELISA, respectively. Although no significant difference in mRNA levels were detected between antisense and sense IGF-II/M6PR cDNA transfecants (data not shown), soluble IGF-II/M6PR protein levels in conditioned medium were decreased in the majority of antisense cDNA transfecants (Fig. 1). Antisense IGF-II/M6PR cDNA transfected clones showed displayed a marked decrease in intracellular IGF-II/M6PR levels when assessed by immunohistochemistry (data not shown). Clones AS/8, AS/10, AS/12, and AS/13, which exhibited >50% decrease in soluble IGF-II/M6PR expression (Fig. 1), were chosen for further study.

Effect of Antisense-mediated Reduction of IGF-II/M6PR Expression on Cell Proliferation. To determine whether antisense IGF-II/M6PR cDNA transfection increased the rate of cell proliferation, cell growth rates were assessed in antisense and sense IGF-II/M6PR cDNA transfected cells over 4 days. As seen in Fig. 2, all of the selected antisense IGF-II/M6PR cDNA transfected cells appeared to grow at a faster rate compared with sense IGF-II/M6PR cDNA.

Received 8/2/99; accepted 10/5/99.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: IGF-II/M6PR, insulin-like growth factor-II/mannose 6-phosphate receptor; TGF, transforming growth factor; uPAR, urokinase plasminogen activator receptor.

References
highly vascular and exhibited a large percentage of mitotic cells. No significant morphological differences were observed between the antisense IGF-II/M6PR cDNA-derived and the sense IGF-II/M6PR cDNA-derived tumors.

To confirm that the transfected cells were maintaining a decreased IGF-II/M6PR expression in vivo, soluble human IGF-II/M6PR was quantitated in a representative subset of the mouse sera (Table 1). Sera from mice injected with antisense IGF-II/M6PR cDNA clone AS/13 displayed significantly decreased serum human IGF-II/M6PR levels/mg of tumor weight (P = 0.013), whereas the reduction in serum human IGF-II/M6PR levels in Clone AS/10 did not reach significance (P = 0.173 as assessed by one-way ANOVA). When assessed as a group (i.e., AS/10 and AS/13), the serum of animals injected with antisense IGF-II/M6PR cDNA transfected cells contained less human IGF-II/M6PR per mg of tumor than those injected with sense cDNA transfected control cells (P = 0.032).

**Discussion**

Optimal invasion of the uterus by trophoblasts is believed to be regulated by a mixture of autocrine and paracrine systems. A variety of growth factors are produced at the interface between the maternal decidua and the invading trophoblasts, including TGF-β and IGF-II (8). TGF-β is produced in the inactive precursor form by the decidual and has been shown to reduce proliferation and invasion of cytotrophoblasts in vitro when activated (9). IGF-II has been shown to enhance the migratory and invasive potential of first-trimester extravillous trophoblasts. Trophoblasts also express IGF-II/M6PRs and, because the IGF-II/M6PR interacts with both IGF-II and TGF-β, it is likely that this receptor is involved in the invasion process (10).

JEG-3 choriocarcinoma cells, like their trophoblastic precursors, express both IGF receptors, the type 1 IGF receptor (11), and the
IGF-II/M6PR (12). Because JEG-3 cells express IGF-II but not IGF-I mRNA (13, 14), it is probable that these cells undergo autocrine growth stimulation from endogenous IGF-II via their type 1 IGF receptors, a feature of several tumor cell lines (15). JEG-3 cells also express TGF-β1 but are insensitive to the growth-inhibitory effects of this cytokine (9). These characteristics make the JEG-3 cell line useful for examining the effects of altered IGF-II/M6PR expression in the absence of confounding effects of TGF-β1 on growth rate.

In this study, we demonstrate that antisense cDNA-mediated decrease in endogenous IGF-II/M6PR results in an increase in the growth rate of JEG-3 choriocarcinoma cells in vitro. Because this receptor is believed to sequester and degrade IGF-II (2), a decrease in IGF-II/M6PR could potentially result in decreased degradation and hence increased bioavailability of IGF-II to the type 1 IGF receptor. Because the IGF-II/M6PR has been shown to be an integral part of the activation of precursor TGF-β1 to TGF-β1 (16), a reduction in IGF-II/M6PR availability could also result in a decrease in TGF-β1 activation. However, because JEG-3 cells are insensitive to the growth-inhibitory effects of TGF-β1 (9), any alteration in TGF-β1 processing would not affect proliferation in this system.

Antisense IGF-II/M6PR cDNA transfected JEG-3 clones form significantly larger tumors when injected into nude mice. The percentage of animals that developed tumors after injection with the antisense IGF-II/M6PR cDNA transfected cells was also significantly higher than those injected with sense IGF-II/M6PR cDNA transfected cells, suggesting that not only the growth rate but also the tumorigenicity of the antisense IGF-II/M6PR cDNA transfected cells is increased. These tumors retain decreased IGF-II/M6PR expression in vivo, as shown by an overall decrease in soluble human IGF-II/M6PR levels in the mouse sera compared with sera taken from animals with sense IGF-II/M6PR cDNA-derived tumors. Although the mechanisms involved in the increased tumor growth rate are not characterized yet, there are several likely candidates. An increase in IGF-II bioavailability to type 1 IGF receptors is the most obvious. The IGF-II/M6PR is also known to be involved in the transport of lysosomal proteases, and mouse lines deficient in IGF-II/M6PR display decreased lysosomal storage and a corresponding increase in lysosomal enzyme secretion (17). The antisense cDNA transfected clones may therefore secrete more lysosomal proteases than the controls cells, and this could result in increased extracellular matrix degradation and subsequently increase the invasion rate of the tumor cells. The IGF-II/M6PR has been implicated in the specific degradation of the uPAR; therefore, a decrease in IGF-II/M6PR expression may result in an increase in cell surface concentrations of this receptor (18). Increased uPAR expression has been shown to be associated with increased cellular adhesion and tumor cell migration both in vivo and in vitro (19); therefore, this decrease in uPAR degradation may lead to increased cellular adhesion and invasion. A combination of all of these mechanisms may be responsible for the marked increase in tumor growth demonstrated in this study, and future work will focus on examination of the JEG-3 sense and antisense IGF-II/M6PR cDNA transfected cell lines and tumors to identify genes whose expression has been regulated by the altered IGF-II/M6PR levels. The results reported here support the hypothesis that the IGF-II/M6PR may act as a negative regulator of cell growth and that reduction of its expression leads to enhanced tumorigenicity.

Acknowledgments

We thank the Department of Anatomical Pathology, specifically Dr. Jeanette Phillips, for assessing the histopathology of the tumors, Sue Smith for embedding and sectioning of the tumor tissues, and Stella Panaretos for technical assistance.

References

16. Dennis, P. A., and Rifkin, D. B. Cellular activation of latent transforming growth factor-β1 but are insensitive to the growth-inhibitory effects of TGF-β1 (9); therefore, this study, and future work will focus on examination of the JEG-3 sense and antisense IGF-II/M6PR cDNA transfected cell lines and tumors to identify genes whose expression has been regulated by the altered IGF-II/M6PR levels. The results reported here support the hypothesis that the IGF-II/M6PR may act as a negative regulator of cell growth and that reduction of its expression leads to enhanced tumorigenicity.
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