Overexpression of Insulin-like Growth Factor-binding Protein-2 Results in Increased Tumorigenic Potential in Y-1 Adrenocortical Tumor Cells

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Abstract

Increased concentrations of insulin-like growth factor-binding protein-2 (IGFBP-2) have been observed in human malignancies including adrenocortical carcinomas. To elucidate the functional consequences of IGFBP-2 overexpression, we have stably transfected the cDNA of murine IGFBP-2 in mouse adrenocortical tumor cells (Y-1). Long-term overexpression of IGFBP-2 was associated with significant morphological alterations, enhanced cell proliferation, and increased cloning efficiency as compared with mock transfected control cells. The enhanced proliferation of IGFBP-2 secreting clones was independent of exogenous insulin-like growth factors (IGFs). These data suggest that elevated levels of IGFBP-2 may contribute to the highly malignant phenotype of adrenocortical cancer by a thus far unknown, presumably IGF-independent, mechanism.

Introduction

Recently, it has become apparent that the IGF3 system plays a central role in the mechanism of transformation and tumorigenesis (1). IGFs inhibit apoptosis, promote tumor growth, and induce transformation and metastasis in many types of malignancies. These mitogenic effects of IGF-I and IGF-II are mediated through interaction with the IGF-I receptor. In addition, the biological actions of IGFs are modulated by a family of at least six IGFBPs that are synthesized locally by most tissues, including cancer cells. Depending on the cellular context, IGFBPs are capable of inhibiting or enhancing IGF actions and even have ligand-independent effects (2). Accumulating data point to an important role of IGFBP-2 in human malignancies. Increased levels of IGFBP-2 have been demonstrated in serum from patients with ovarian cancer (3), colon cancer (4), and prostate carcinoma (5) and in cerebrospinal fluid of patients with central nervous system malignancies (6). Elevated serum levels of IGFBP-2 have been positively correlated with the tumor stage of the patients and are assumed to originate from the tumor cells (5). High concentrations of IGFBP-2 have been found in a variety of carcinomas, including colon tumors (7), prostate carcinoma (5), and adrenocortical carcinomas (8) and in leukemias (9). There is increasing evidence, that the IGF system plays a crucial role in adrenocortical tumorigenesis. IGFs are mitogenic for adrenocortical cells in vitro and in vivo, and a strong overexpression of IGF-II and IGF-I receptors, which might contribute to the neoplastic cell proliferation, has been found in adrenocortical carcinomas (8, 10–12). However, although IGFBP-2 overexpression was positively correlated with the malignant phenotype of adrenal tumors (8), the functional significance of elevated IGFBP-2 levels in carcinomatous adrenocortical tissue remains unclear. In vivo, IGFBP-2 has been identified as a negative regulator of growth because transgenic mice overexpressing IGFBP-2 display reduced body weight gain (13). To clarify the significance of IGFBP-2 in the tumorigenesis of the adrenal gland, we have transfected the murine IGFBP-2 cDNA into the Y-1 mouse adrenocortical tumor cell line and investigated long-term effects of IGFBP-2 overexpression on adrenocortical tumor cell growth.

Materials and Methods

Cell Culture and Stable Transfection of Y-1 Adrenocortical Tumor Cells. Murine adrenocortical tumor cells (Y-1 cells; American Type Culture Collection, Rockville, MD) were grown in Ham's F10 cell culture medium with 2.5% FCS and 15% horse serum. Y-1 cells were transfected using the DMRIE-C reagent according to the manufacturer's instructions (Life Technologies, Inc., Eggenstein, Germany). In brief, Y-1 cells were cultured in Petri dishes (10 cm in diameter) to 60% confluence and incubated for 12 h in 3.5 ml of transfection solution containing serum-free Ham's F10 medium, 10 µg pCMV-int-mIGFBP-2 (14) and 1 µg of EcoRI linearized the neomycin-resistance plasmid pSV2neo (Clontech, Heidelberg, Germany). Mock transfections were performed using the resistance plasmid alone. Monolayers were washed once in serum-free medium and kept in culture medium for 24 h. Positive clones were selected by G418 (Life Technologies, Inc.; 250 µg/ml) in the culture medium for 2 weeks. G418 was replaced every 2 days. Colonies were isolated from the Petri dish and subcultured more than 12 times before their characterization.

RT-PCR. Total RNA was isolated using the Trizol reagent (Boehringer Mannheim, Mannheim, Germany). Two µg of total RNA were DNaseI-digested for 1 h at 37°C, and cDNA was transcribed according to standard protocols from 1 µg of total RNA. Primers used in the PCR analysis were as follows: (a) mIGFBP-2 sense TGG CCA AAG TGT GTG CA; (b) mIGFBP-2 antisense CTC TCT AAT GAA GAG G; (c) IGF-I sense AAA ATC AGC AGT CTT CCA AC; (d) IGF-I antisense AGA ATC ACA GCT CCG GAA GCA; (e) IGF-II sense GCC CCG GAG AGA CTG TGG G; (f) IGF-II antisense GCC CAC GGG GTA TCT GGG GAA; (g) mIGF-I receptor sense ATG CTT TGT GAA CTG CGC ATG TGG; (h) mIGF-I receptor antisense CGG CTT GCT CTT GCT CCC GCA TAT; (i) β-actin antisense ACC ATG TCG CCA TCG ATG AC; (j) β-actin antisense GTG TCC AAA GTA TCA ATG AC; (k) GH receptor sense TGC CTT GAT TAT GCT TCT GCT GGA AAA; and (l) GH receptor antisense TAA GAA CCA TGG AAA CTG GAT. PCR conditions for all of the primer pairs with the exception of the β-actin primers were as follows: 94°C for 4 min and 35 cycles at 45°C for 30 s, 72°C for 30 s, and 94°C for 30 s. For β-actin PCR we used the following program: 94°C for 4 min and 35 cycles at 60°C for 40 s, 72°C for 20 s, and 94°C for 60 s. No band was visible from DNaseI-digested non-reverse transcribed total RNA demonstrating the absence of DNA in the RNA samples.
Characterization of the Y-1 Cell Clones. To demonstrate the secretion of functional IGFBP-2, conditioned medium from the selected cell clones was analyzed by Western ligand blot analysis as described previously (14). Briefly, after incubation of 2 × 10^6 cells for 72 h in 5 ml of serum-free cell culture medium, the medium was aspirated, diluted 1:5 with sample buffer [50 mM Na_2HPO_4 (pH 7.0), 1% (w/v) SDS, and 50% (w/v) glycerin], boiled (5 min), electrophoresed on a 5% stacking/12% separating SDS-polyacrylamide gel, and transferred to a nitrocellulose membrane (Millipore, Eschborn, Germany). The blots were blocked with 1% fish gelatin and incubated with 125I-IGF-II (10^6 cpm per blot). Binding proteins were visualized on Phospho-Imager Storm (Molecular Dynamics, Krefeld, Germany). All of the hybridization and washing steps were performed at 4°C. Furthermore, IGFBP-2 in the conditioned media was identified by immunoblot analysis. Membranes were prepared as described above, with the only exception that proteins were separated under reducing conditions and incubated with a specific peptide-induced antiseraum against a partial sequence from murine IGFBP-2 as described previously (13).

To demonstrate the presence of intact IGF-I receptors, binding studies of 125I-IGF-I to Y-1 adenocortical cells were performed and IGF-I receptor number and affinity were determined by Scatchard analysis as published previously (12). In addition, the concentration of IGFBP-2, IGF-I, and IGF-II in conditioned media from the different cell clones was measured by RIA as described previously (10, 13).

Cell Proliferation and Colony Formation Assay. For the assays, only cells with passage numbers higher than 12 were used. Proliferation was assessed in Ham’s F10 medium containing 2% serum as described previously (14). In brief, 2 × 10^3 cells were suspended in medium containing 2.5% FCS, 15% horse serum, and 0.9% methylocellulose (Fluka, Deisenhofen, Germany) and plated into 35-mm bacteriological Petri dishes. Cells were incubated at 37°C and 5% CO_2 for 2 weeks. Colonies of more than 50 cells were counted under an inverted microscope. All of the measurements were carried out in triplicate, and each experiment was repeated independently at least three times. Data are depicted as means ± SE, and statistical analysis was performed using Student’s t test.

**Results**

**Overexpression of IGFBP-2.** Although no expression of murine IGFBP-2 could be detected by RT-PCR in wild-type or in mock-transfected Y-1 cells, IGFBP-2-specific bands of correct size (199 bp) were found in all of the selected IGFBP-2 overexpressing cell clones (Fig. 1A). Furthermore, the secretion of functional IGFBP-2 by the IGFBP-2-transfected cell clones could be demonstrated by Western ligand blotting, immunoblotting, and RIA, whereas no IGFBP-2 protein was found in the control cells (Fig. 1, B and C). After the incubation of 2 × 10^6 cells for 72 h, the concentration of IGFBP-2 was 104 ng/ml and 143 ng/ml in conditioned medium from cell clones Y11 and Y15, respectively but was below the detection limit (1.25 ng/ml) in control cells as assayed by RIA. In addition to IGFBP-2, a faint band at Mr 24,000—presumably representing nonglycosylated IGFBP-4—was detectable by Western ligand blotting of conditioned medium from stable transfectants and controls (data not shown).

**Cellular Morphology.** Although the morphology of control transfected cells was unaltered as compared with wild-type Y-1 cells (Fig. 2A), IGFBP-2 overexpressing cell clones displayed significant changes in their morphological appearance with a spherical cell shape and a disturbed growth in monolayer (Fig. 2B).

Cell Proliferation. When cell proliferation was assayed in serum-reduced medium (2% serum), IGFBP-2-overexpressing cell clones displayed a significantly enhanced proliferation (186 ± 12% of control; P < 0.001) versus control transfected cells (Fig. 3A). This increased mitogenic activity of IGFBP-2 secreting cell clones was retained even in the presence of the highest serum concentration (17.5%). When IGF-I in a concentration of 11 ng/ml was added to the proliferation experiments, only a small and nonsignificant mitogenic effect was observed in both control and IGFBP-2-transfected cell clones (106 ± 6%). At high concentrations (300 ng/ml), IGF-I stimulated cell proliferation in mock transfecants and in IGFBP-2-secreting Y-1 cells to a similar extent (maximally 144 ± 7% in controls and 147 ± 8% in IGFBP-2-secreting cells; P < 0.01). However, the mitogenic effect was similar in both groups and the lower proliferation rate of the mock-transfected cells could not be compensated for even by very high concentrations of IGF-I. A similar but slightly weaker mitogenic effect was observed when IGF-II was used instead of IGF-I (in mock-transfected cells: IGF-I, 133 ± 13%; IGF-II, 117 ± 2%; in IGFBP-2-transfected cells: IGF-I 127 ± 16%; IGF-II 109 ± 19%), which suggests that the IGF-induced cell proliferation was mediated through interaction with the IGF-I receptor in these cells. Furthermore, we have evaluated the effect of Long R3 IGF-I, an IGF analogue that does not bind to IGFBPs. As compared with equal concentrations of native IGF-I (200 ng/ml), a similar mitogenic effect was found for IGF-I and Long R3 IGF-I in both control and transfected clones (mock transfected cells: IGF-I 114 ± 5%; Long R3 IGF-I 114 ± 11%; IGFBP-2 transfected cells: IGF-I 114 ± 2%; Long R3 IGF-I 118 ± 2%). Cell proliferation of Y-1 cells was unaffected by exogenous rhGH (data not shown).

**Cloning Efficiency.** In addition, we have measured cell growth under anchorage-independent conditions. In comparison to mock-transfected Y-1 cells, IGFBP-2 secreting clones formed significantly more colonies in methylocellulose (P < 0.001; Fig. 3B). In mock transfecants, X2 and X4 cloning efficiency was 7.5 ± 3.05% and 8.8 ± 2.1%, respectively, whereas in IGFBP-2 secreting clones, Y11 and Y15 cloning efficiency was 17.5 ± 2.2% and 19.8 ± 3.1%, respectively.

**Characterization of Y-1 Cell Clones.** The expression and secretion of IGF-I and IGF-II was analyzed by RT-PCR and RIA. When the secretion of IGFs into conditioned medium was measured after incubation of confluent Y-1 cells in serum-free medium for 72 h, control
clones and wild-type cells secreted 6.5 ± 3.7 ng of IGF-I per million cells, whereas IGF-I secretion of IGFBP-2 expressing cell clones was significantly reduced (0.7 ± 0.6 ng of IGF-I per million cells; \( P < 0.05 \)). Accordingly, IGF-I mRNA levels (Fig. 4) were significantly lower in IGFBP-2 overexpressing clones as compared with controls. In contrast to IGF-I, IGF-II concentrations were below the detection limit (1 ng/ml) in all of the cell clones tested, and no mRNA transcripts of murine IGF-II could be detected by RT-PCR (Fig. 4).

\( ^{125} \text{I}-\text{IGF-I} \) binding to Y-1 adrenocortical cells was characteristic of the IGF-I receptor. Unlabeled IGF-I was potently displaced by the radioligand, whereas IGF-II was less potent, and insulin competed only at high concentrations. Scatchard analysis revealed a single class of high-affinity binding sites for IGF-I with a low number of approximately 4000 IGF-I receptors/cell and a dissociation constant of 1.6 ± 0.4 nM both in transfected cells and in controls. Furthermore, Y-1 cells express mRNA transcripts specific for IGF-I receptor as analyzed by RT-PCR and semiquantified relative to \( \beta \)-actin mRNA expression (Fig. 4). However, no alteration of relative mRNA expression was found in IGFBP-2 secreting Y-1 cells if compared with mock controls or nontransfected Y-1 cells. In addition, we have detected small amounts of GH receptor mRNA expression in all of the Y-1 clones examined (Fig. 4).

**Discussion**

In this paper, we describe how long-term overexpression of IGFBP-2 in Y-1 mouse adrenocortical cells is associated with enhanced tumorigenic potential and altered cellular morphology. This observation is in accordance with clinical findings of increased IGFBP-2 levels in human adrenocortical tumors, and adds further
IGFBP-2 has been demonstrated to represent an inhibitor of IGF actions both in vivo and in vitro, in which IGFBP-2 overexpression results in reduced body weight of IGFBP-2-transgenic mice or reduced cell proliferation, respectively (13, 14). However, the observation that many human malignancies are associated with elevated IGFBP-2 serum or tumor tissue levels has shifted the focus of interest to possible tumor growth-promoting effects of IGFBP-2 (3–9). Similar to our findings in adrenocortical tumor cells, a stimulatory effect of IGFBP-2 on DNA synthesis has been reported in human MCF-7 and porcine aortic smooth muscle cells (15, 16). In accordance with our data, Menouny et al. (17) recently reported that the expression of IGFBP-2 in human epidermoid carcinoma cells (KB 3.1) is associated with an increased tumorigenicity of the cells. However, the mechanism of the IGFBP-2-associated increase in tumorigenesis remains unclear. Whereas in KB 3.1 cells it has been postulated that increased IGFBP-3 proteolysis might be responsible for the IGFBP-2-associated tumorigenicity (17), no change in IGFBP secretion or proteolysis could be detected in the described IGFBP-2-overexpressing Y-1 cell clones. Similarly, in adrenocortical cancer tissue no changes in IGFBP-3 concentrations or IGFBP proteolysis were detected (8). Furthermore, IGFBP-2 overexpression in Y-1 cells was not associated with a different expression of IGF-I receptors or IGF-II ligand. In contrast, IGF-I mRNA expression and secretion were significantly lower in IGFBP-2-transfected cell clones as compared with wild-type and control transfected cells, possibly due to a negative feedback regulation of IGFBP-2 on IGF-I expression. This makes an indirect IGF-dependent mitogenic effect of IGFBP-2 through up-regulation of IGF-I receptors or IGF ligands or through down-regulation of inhibiting IGFBPs in our cell culture system very unlikely. In addition, proliferation of Y-1 cells is only weakly stimulated by exogenous IGF ligands, and the IGFBP-2-associated increase in cell proliferation was independent of levels of endogenous or exogenous IGF-I, even in the presence of supraphysiological concentrations of exogenous IGF-I. This makes an IGF-ligand-induced IGFBP-2-specific blockade of action unlikely. Taken together, these data strongly suggest that IGF-independent mechanisms are responsible for the IGFBP-2-associated increase in tumorigenicity of Y-1 cells. Whereas most effects of IGFBPs involve interactions with IGFs and IGF receptors, accumulating data indicate that IGFBPs are capable of exerting biological actions in an IGF-independent manner (18). Thus far, IGF-independent effects of IGFBP-2 have been reported in porcine aortic smooth-muscle cells and in rat osteosarcoma cells, in which IGFBP-2 increases DNA synthesis and GH binding to the GH receptor, respectively (16, 19). Although we were able to demonstrate the presence of GH receptor mRNA in Y-1 cells, our data did not allow us to determine the molecular mechanism through which IGFBP-2 enhances adrenocortical tumor cell growth because exogenous GH had no effect on cell proliferation of Y-1 cells. However, the tissue culture model described in this paper provides a useful tool to further delineate the mechanisms involved in IGF-independent growth/tumor promoting-effects of IGFBP-2. In conclusion, we show for the first time that long-term overexpression of IGFBP-2 in adrenocortical tumor cells is associated with an enhanced tumorigenic potential of these cells. Our data support the hypothesis that IGFBP-2 contributes to the highly malignant phenotype of adrenocortical cancer. Additional studies investigating the cellular mechanism of this presumably IGF-independent tumor growth-promoting action of IGFBP-2 will improve our understanding of the role of IGFBP-2 in human malignancies and may contribute to the development of future diagnostic and therapeutic approaches in the management of adrenocortical tumors.

References


Fig. 3. Proliferative activity under anchorage-dependent and anchorage-independent conditions in selected Y-1 clones. In A, cells were grown in monolayer under serum-reduced conditions in the presence of increasing concentrations of IGF-I for 5 days, and their anchorage-dependent proliferative activity was analyzed by MTT assay as described. The data are representative for six independently performed experiments. In B, anchorage-independent growth was assessed by colony formation assay in 0.9% methylcellulose as described. Data represent the means ± SE from three independently performed experiments.

Fig. 4. mRNA expression of growth-relevant genes in Y-1 cell clones as analyzed by RT-PCR analysis. One μg of total RNA was reverse transcribed into cDNA, and PCR was performed as described in “Materials and Methods.” cDNA from adult murine liver was used as the positive control for IGF-I, IGF-I receptor (IGF-I-R), GH receptor (GH-R), and β-actin. A plasmid containing the IGF-II cDNA was used as the positive control for the IGF-II RT-PCR analysis; +, positive control; −, double distilled H2O was used as the negative control; X2 and X4, mock controls; Y11 and Y15, IGFBP-2-transfected cells.


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