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Gene therapy of murine teratocarcinoma: Separate functions for insulin-like growth factors I and II in immunogenicity and differentiation

(episome/antisense/immunotherapy/tumor regression)

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ABSTRACT **Teratocarcinoma is a germ-line carcinoma giving rise to an embryoid tumor with structures derived from the three embryonic layers: mesoderm, endoderm, and ectoderm. Teratocarcinoma is widely used as an *in vitro* model system to study regulation of cell determination and differentiation during mammalian embryogenesis. Murine embryonic carcinoma (EC) PCC3 cells express insulin-like growth factor I (IGF-I) and its receptor, while all derivative tumor structures express IGF-I and IGF-II and their receptors. Therefore the system lends itself to dissect the role of these two growth factors during EC differentiation. With an episomal antisense strategy, we define a role for IGF-I in tumorigenicity and evasion of immune surveillance. Antisense IGF-I EC transfectants are shown to elicit a curative anti-tumor immune response with tumor regression at distal sites. In contrast, IGF-II is shown to drive determination and differentiation in EC cells. Since IGF-I and IGF-II bind to type I receptor and antisense sequence used for IGF-II cannot form duplex with endogenous IGF-I transcripts, it follows that this receptor is not involved in determination and differentiation.**

Recently we observed that C6 rat glioblastoma cells transfected with an episomal expression vector producing antisense insulin-like growth factor I (IGF-I) RNA lost tumorigenicity and induced a T-cell mediated immune reaction both against themselves and against their nontransfected tumorigenic progenitor cells in syngeneic animals (1–3). C6 cells (4) as well as many primary human malignant tumors and derived cultured cells have been reported to express high levels of IGF-I or IGF-II (5–17). It is thus important to assess the generality of the antisense IGF effect on tumor immunogenicity in order to help evaluate its potential clinical significance. In addition, IGF-II is considered to be an important growth and/or differentiation factor during normal fetal development. However, the roles of IGF-I or IGF-II in influencing cell determination and differentiation in embryonic and fetal development remain undetermined.

Teratocarcinoma is a well-characterized tumor type that lends itself well to the analysis of the role of IGFs in tumor development and tumor immunity as well as their role in directing differentiation. Human and mouse teratocarcinoma cell lines produce IGF-I and the type I IGF receptor (15, 18–20). Teratocarcinoma consists of malignant undifferentiated embryonic carcinoma (EC) cells and various somatic tissue intermingled (21, 22). This is thought to reflect disturbed development. In most instances, these tumors originate from ectopic germ cells outside of the gonads or from embryonic inclusions (23), with the pluripotent cells escaping the influence of embryonic organizers. The EC stem cell resembles a normal embryonic cell (24, 25) and thus may be of blastodermic origin.

The histopathology of murine teratocarcinoma resembles human teratocarcinoma. Both comprise tissues derived from the three primary germ cell layers: mesoderm, endoderm, and ectoderm. Teratocarcinoma cell lines have been used to study regulation of cell determination and differentiation in mammalian embryogenesis (26). EC cells have the potential to develop malignant tissues derived from the three primary germ cell layers. During embryoid development, the morphologic changes resemble those of the inner cell mass in the mammalian blastocyst stage of normal embryos (27). In addition, the EC cells may revert from their malignant phenotype and participate in normal development (28). Due to the pluripotent nature of EC cells and the availability of a syngeneic mouse strain (129SV), and because they express IGF-I, IGF-II, and both type I and type II IGF receptors (15), mouse PCC3 EC cells seemed a uniquely informative system with which to explore the effects of these two closely related IGFs on tumorigenicity and immunogenicity as well as their involvement in determination and differentiation.

MATERIALS AND METHODS

To inhibit IGF-I expression, we used the episome-based antisense IGF-I vector (pAnti-IGF-I) we described previously (1). For IGF-II inhibition, we assembled an episome-based vector (pMT/EP) for producing antisense IGF-II RNA (Fig. 1). A synthetic double-stranded oligonucleotide of 99 nucleotides in length, spanning the 1st through the 33rd amino acid of the processed mouse IGF-II peptide, was inserted into pMT/EP in the antisense orientation (5′-GCTTACGGCCCCGGAGAGACTCTGTGCGGAGGG-GAGCTTGTTGACACGCTTCAGTTTGTCTGTTCG-GACCGCGGCTTCTACTTCAGCAGGCCTTCAAGC-3′). This sequence is unique in that it bears little homology to the murine IGF-I transcript. Expression of this antisense RNA by Zn^{2+} activation of the MT-1 promoter in stably transfected murine myoblasts blocked the accumulation of endogenous IGF-II transcripts completely (D. Montarras, C. Pinset, F. Gros, and Jo.I., unpublished observations). Transfection of mouse PCC3 cells was performed with Lipofectin reagent (Bethesda Research Laboratories). Immunoperoxidase and

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Abbreviations: EC, embryonic carcinoma; IGF, insulin-like growth factor; H&E, hematoxylin/eosin.

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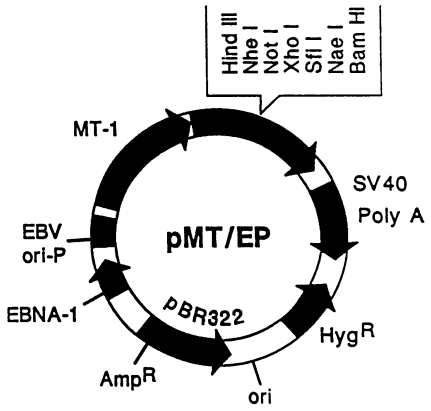

FIG. 1. Structure of expression vector pMT/EP. EBV, Epstein–Barr virus; EBNA-1, EBV-encoded nuclear antigen 1; SV40, simian virus 40; Hyg^R, hygromycin resistance; Amp^R, ampicillin resistance.

fluorescent antibody methods were performed as we described (1, 2). Polyclonal antibody against IGF-I was kindly provided by the National Hormone and Pituitary Program (National Institute of Diabetes and Digestive and Kidney Diseases). Anti-IGF-II antibodies were obtained from Calbiochem; anti-T-cell receptor was kindly provided by S. Emancipator (Institute of Pathology, Case Western Reserve University). RNase protection assays were performed essentially according to published methodologies.

RESULTS

The PCC3 murine teratocarcinoma used in this study is a pluripotent line generated by the laboratory of François Jacob (29). When 5×10^6 PCC3 cells are injected subcutaneously into 129 SV mice, a tumor develops in 6–10 days. This tumor includes many poorly differentiated malignant tissues, representing all three germ layers—e.g., neuroblastic cells, hepatocytes, and adipocytes. Tissues of mesodermal origin—e.g., muscle and cartilage—stained intensely for both IGF I and IGF-II (Fig. 2). Tissues of endodermal origin immunostained more weakly for IGF-I and IGF-II than those from mesodermal origin. The hepatocytic cells, which are poorly differentiated, were apparently negative for both IGF-I (Fig. 2) and IGF-II (not shown).

Cultured PCC3 cells express IGF-I transcripts as shown by an RNase protection assay (Fig. 3). Moreover, when PCC3 cells stably transfected with vector for antisense IGF-I were grown in the presence of Zn^{2+}, in order to activate the MT-1 promoter and produce antisense RNA, the endogenous IGF-I transcript disappeared, as shown by RNase protection assay (Fig. 3, lane 3).

When 5×10^6 PCC3 cells stably transfected with vector producing antisense IGF-I RNA were injected subcutaneously into 129 SV syngeneic mice the cells lost their tumorigenicity. The MT-1 promoter is known to be active *in vivo* (30). Instead of forming a tumor, the antisense IGF-I PCC3 transfectants evoked a strong immune response in five of five animals, with an extensive mononuclear cell infiltrate evident 4–5 days after injection. The majority of these mononuclear cells are CD8 positive as shown by immunocytochemistry and fluorescence microscopy (Fig. 4 *Left, b*). All of the five injected mice have been tumor free for 10 months. When 5×10^6 PCC3 cells stably transfected with vector alone devoid of antisense sequences or with a vector with antisense to IGF-II were injected, an initial infiltration of mononuclear cells is apparent due to inflammation. However, these infiltrates are CD8 negative (Fig. 4 *Left, c–e*), and all developed tumors. When PCC3 cells stably transfected with the pMT/EP vector, devoid of antisense IGF-I, were injected, teratocarcinoma tumors developed in five of five mice (Table 1).

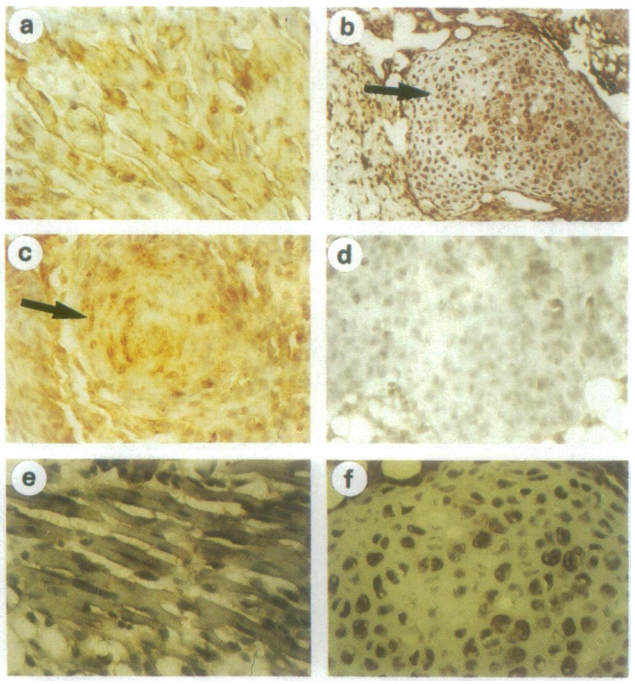

FIG. 2. Immunocytochemical labeling of mouse teratocarcinoma using anti-IGF-I (*a–d*) and anti-IGF-II (*e* and *f*) antibodies, detected by immunoperoxidase. (*a*) Muscle. (*b*) Cartilage (single arrow). (*c*) Epidermoid. (*d*) Liver. (*e*) Muscle. (*f*) Cartilage. (*a*, *c*, and *d*, ×80; *b*, ×50; *e* and *f*, ×200.)

Table 1 summarizes experiments aimed at gene therapy of teratocarcinoma. The initial subcutaneous injection of 5×10^6 EC parental cells (nontransfected) was above the left hind leg. A second injection of transfected or nontransfected cells was administered above the right hind leg either simultaneously or 6–8 days later, when a tumor of 0.5–1.0 cm in diameter was apparent at the site of the first injection. As shown in Table 1, antisense IGF-I transfected cells protect the animal from tumor formation. Moreover, when transfected antisense IGF-I cells are injected into animals with established teratocarcinoma tumors, this treatment results in complete regression of the tumors. These animals have been tumor free for 10 months. Furthermore, when transfected cells were irradiated

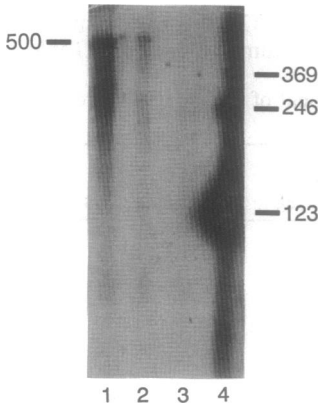

FIG. 3. Suppression of endogenous IGF-I RNA transcripts in PCC3 cells transfected with pAntiIGF-I. Total RNA was isolated from untransfected C6 glioma cells (lane 1), transfected PCC3 cells (lane 2), and transfected PCC3 cells exposed to 50 μM Zn^{2+} for 6 hr prior to RNA isolation (lane 3). Cellular RNA was hybridized to a 500-bp rat antisense cRNA probe transcribed *in vitro* in the presence of [^{32}P]UTP. Following hybridization the samples were digested with RNases A and T1 and analyzed on a denaturing acrylamide gel. Lane 4, size markers (123-bp ladder, Bethesda Research Laboratories).

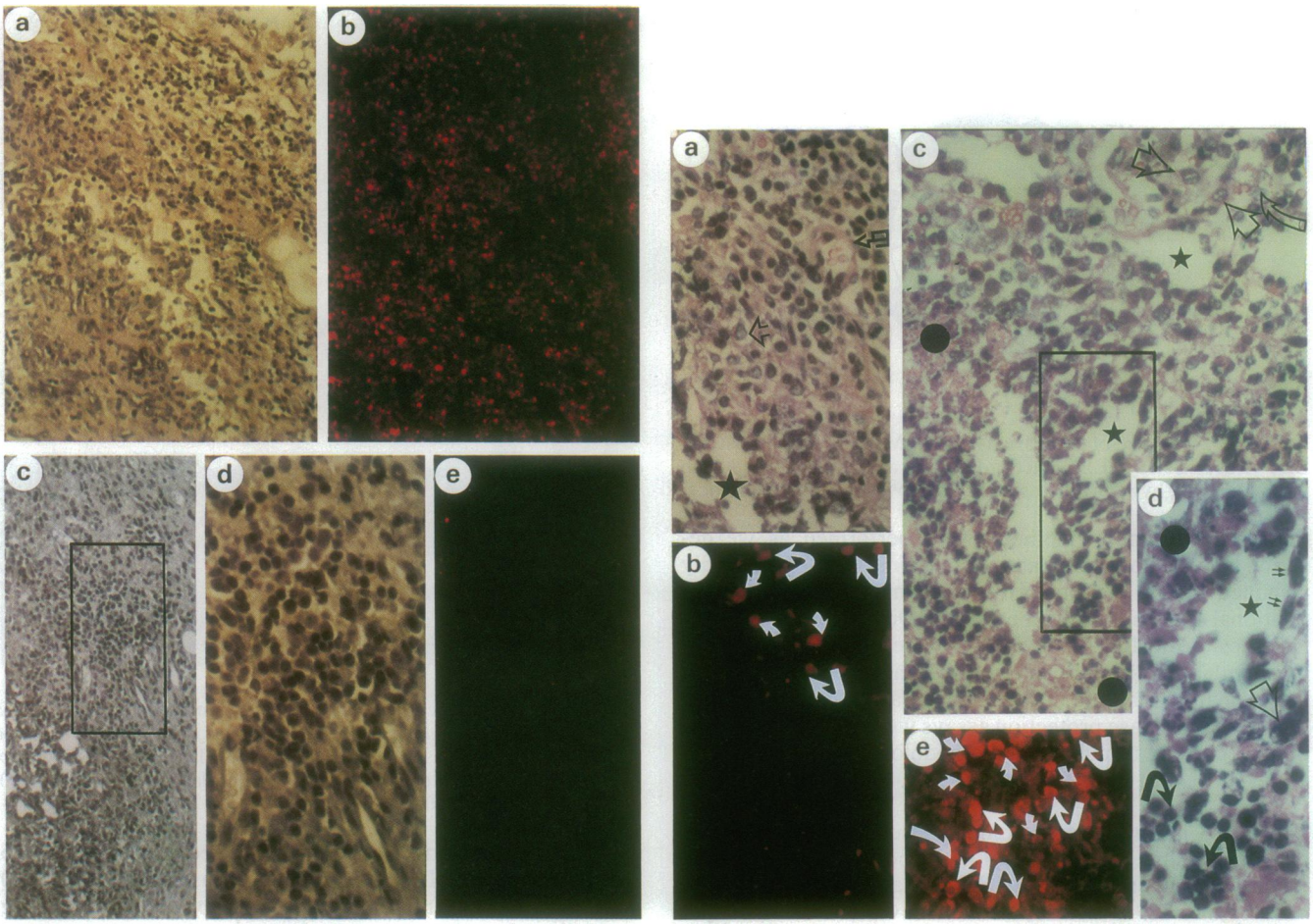

FIG. 4. (*Left*) Lesions resulting from injection of PCC3 cells expressing antisense IGF-I RNA (*a* and *b*) and antisense IGF-II (*c–e*) into 129 SV mice. (*a*) Lymphocyte infiltration. [Hematoxylin/eosin (H&E), ×80.] (*b*) Serial section to *a* stained with anti-CD8 antibodies. (×80.) (*c*) Mouse teratocarcinoma presenting poorly differentiated neuroepithelial clusters (at bottom) accompanied by lymphocytic cells (boxed). (×55.) (*d* and *e*) Higher magnification of boxed region in *c* showing lymphocytes (*d*) and stained with anti-CD8 antibodies (*e*). (×220.) (*Right*) PCC3 tumor regression induced by injection of antisense IGF-I expressing transfected cells. The experiment was that described in Table 1, line 4. (*a*) Teratocarcinoma 4 days following transfected cell injection, showing EC cells (open arrow), neuroblastic cells arranged in pseudorosettes (star), and nervous system-derived cells scattered about a blood vessel (broken arrow). (H&E, ×110.) (*b*) Serial section to *a* stained with monoclonal rat anti-mouse antibody to CD8 conjugated to rhodamine. (×110.) (*c*) Teratocarcinoma 9 days following transfected cell injection, showing EC cells and pseudorosettes of neuroblastic cells (open arrows and stars, respectively) and disintegrating and necrotic tissue (black circle). (H&E, ×110.) (*d*) Boxed area of *c* shown at higher magnification. Double arrows, neuroblastic cells of pseudorosette (star); solid arrows, lymphocyte clusters; open arrow, EC cells. (H&E, ×220.) (*e*) Section adjacent to *d* depicting lymphocytes (solid arrows) stained for CD8 as in *b*. (×135.)

with 5000 rad of ^{60}Co before injection, they retained the capacity to evoke tumor regression (Table 1).

Table 1. Treatment of murine teratocarcinoma with IGF-I antisense

Injection protocol		Time between injections, days	Tumor development	
Left leg	Right leg		Left leg	Right leg
EC	EC	0	5/5	5 /5
EC	EC	6	5/5	5 /5
EC	EC T	0	0/6	0 /6
EC	EC T	6–8*	0/5	0 /5
—	EC IR	—	—	0 /5
EC	EC IR	0	5/5	0 /5
EC	EC T IR	0	0/4	0 /4
EC	EC T IR	6–8*	0/4	0 /4
EC	EC V	0	4/4	4 /4
EC	EC V	6–8*	6/6	6 /6

Cells were injected into mice subcutaneously above the left hind leg or the right hind leg. T, transfected; IR, irradiated cells with 5000 rad (1 rad = 0.1 Gy) from ^{60}Co source; V, vector only.
*Second injection was administered after a solid tumor of 0.5–1 cm in diameter was formed. Two to 3 weeks after injection tumor regression was apparent.

The experiments described in Table 1, line 4, were repeated. After 8 days, when solid tumors of about 0.5 cm in diameter were apparent, antisense IGF-I stably transfected PCC3 cells were injected above the right hind leg as described (Table 1, line 4). At intervals, the solid tumors above the left leg were removed, fixed in formaldehyde, and serially sectioned. Fig. 4 *Right, a–e* depicts the results of this experiment. The tumors show progressive necrosis and infiltration with CD8-positive lymphocytes.

To investigate the role of IGF-II in EC differentiation, PCC3 cells were stably transfected with an episomal vector producing IGF-II antisense RNA. It seems reasonable to state that the sequence chosen for antisense IGF-II cannot form a duplex with endogenous IGF-I transcripts (*Discussion*). When these transfectants were injected subcutaneously into syngeneic mice, there was initial infiltration of mononuclear cells 4–5 days after injection. However, none of the infiltrated cells stained positively for CD8 (Fig. 4 *Left, c, d,* and *e*). The fate of PCC3 cells stably transfected with episome-based antisense for IGF-II was different from those expressing antisense IGF-I RNA. These cells remained subcutaneous for 2.5–3 months without forming any pathological lesion or apparent tumor. However, after 2.5–3 months the

injected cells spontaneously differentiated into structures that resemble mature differentiated murine tissues. Such differentiation into mature structures is often seen in human teratocarcinoma (27) but rarely is observed in murine teratocarcinoma. Fig. 5 depicts tissues developed from PCC3 cells stably transfected with vector producing IGF-II antisense RNA. A squamous epidermoid is shown in Fig. 5 *Upper, a* and a stratified squamous keratinized epithelium as well as bone are evident in Fig. 5 *Upper, b*. The organized liver structure (Fig. 5 *Lower, a*) stains intensively with fluorescent antibodies to α-fetoprotein (Fig. 5 *Lower, b*). This liver structure also stains intensely with fluoresceinated antibody to IGF-II (data not shown), as does rodent fetal liver (31).

These results support the notion that after 2.5–3 months without antibiotic selective pressure, the transfected PCC3 cells either lost the episome or the episome underwent rearrangement rendering it incapable of suppressing IGF-II production. We favor the first hypothesis since we previously observed that human cultured lymphocytes transfected with an episomal expression construct lost the episome after 70 days when kept without the selection pressure of hygromycin B (32). Similar results were obtained with rat C6 glioblastoma cells (T.R.J. and Jo.I., unpublished results). Once the episome is lost, IGF-II is expressed and cells differentiate.

DISCUSSION

Our previous work demonstrated that transfection of rat C6 glioblastoma cells with an episome-based vector expressing antisense IGF-I RNA abolished the tumorigenicity of these cells and, in addition, elicited an immune response that could eradicate preexisting tumors derived from nontransfected C6 cells (1, 2). Many human primary malignant tumors and derived cultured cells have been reported to express high levels of IGF-I or IGF-II (5–17). As one approach for assessing whether the antisense IGF-I effect extends to tumor types other than rat C6 glioblastoma, we performed experiments similar to those reported earlier using mouse PCC3 teratocarcinoma cells.

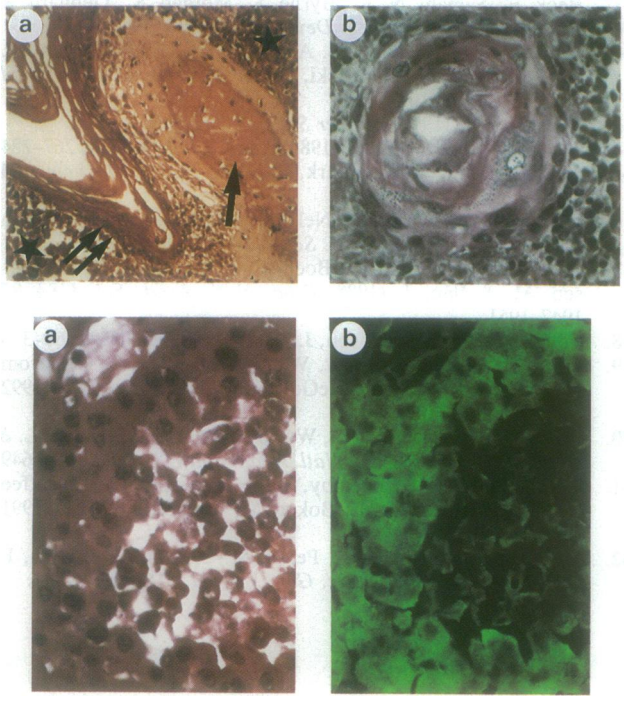

FIG. 5. (*Upper*) Mouse teratocarcinoma induced by injection of PCC3 cells transfected with vector expressing antisense IGF-II RNA. (*a*) Mature cartilage showing ossification (single arrow) and well-differentiated epidermoid epithelium (double arrow). Star, neuroepithelial sheets of individual cells. (H&E, ×75.) (*b*) Mature epidermoid structure. A sheet of neuroepithelial dark cells is visible (right). (H&E, ×125.) (*Lower*) Differentiated hepatic tissue (fetal–newborn type) from mouse teratocarcinoma derived from PCC3 cells transfected with vector expressing antisense IGF-II RNA. The same section is stained with H&E (*a*) or fluorescent anti-α-fetoprotein antibodies (*b*). (×200.)

The neoplastic tissues apparent in PCC3-derived tumors develop from the three embryonic germ layers, with many different lineages represented. Our finding that no tumor elements grow from antisense IGF-I transfectants is consistent with the possibility that malignant PCC3 cells starting down different lineages share at least one common, tumor-specific antigen serving as a T-cell receptor target. Moreover, it supports the general principle that in all tumors expressing high levels of IGF-I, a complete suppression of the growth factor by genetic manipulation may evoke a therapeutic immune response. The murine teratocarcinoma system will likely provide further insights into the relationship between differentiation state and immunogenicity, particularly since differentiation of these cells can be regulated by retinoic acid in culture (33).

IGF-I and IGF-II can both mediate signal transduction via type I receptor. IGF-I is expressed in PCC3 cells but not IGF-II. The latter is expressed during cell and tissue differentiation. IGF-I expression is maintained in antisense IGF-II transfectants. It seems reasonable to state that the antisense sequence for IGF-II cannot form a hybrid with endogenous IGF-I transcripts. The 99 nucleotides used as antisense IGF-II have 65 nucleotides interspersed in positions identical to mouse IGF-I. However, there is only one region of 8 sequential nucleotides with an identical sequence position. Taking into account 59% G + C, 35% mismatch, as well as intracellular 0.15 M KCl, the apparent melting temperature (t_m) is 41.2°C, assuming that 1% mismatch decreases the t_m by 1.5°C. Under these conditions practically no duplex formation is expected (34). This supports the idea that the type I receptor is not involved in controlling the tumorigenicity and immunogenicity of PCC3 cells. It is possible that IGF-I may affect tumorigenic and immunogenic capacity by intracellular processes. Such an intracellular mechanism bypassing the conventional receptor was proposed by Heldin and Westermark (35).

Alternatively, our observations may also be explained by assuming that the differentiation role of IGF-II in EC cells is mediated by type II IGF receptor. In the presence of IGF-I, IGF-II is incapable of directing differentiation to fully differentiated tissues and structures. In normal rodent early embryonic and fetal development, IGF-II is expressed in all tissues, while IGF-I cannot be detected (33). It is generally believed that IGF-II is the major growth factor during mammalian embryonic and fetal development and acts through autocrine and/or paracrine mechanisms (36, 37). Our experiments defined the role of IGF-II as a differentiation factor, while IGF-I expression is involved in the mechanism responsible for the cell to evade the immune surveillance. Alternatively, it is also possible that both IGF-I and IGF-II must be present to stimulate the pathway leading to tumor formation.

Our gene therapy approach is based upon antisense RNA complementary to transcripts for regulatory molecules expressed by the neoplastic cells. Other investigators have devised alternate genetic routes for modulating tumor cell immunogenicity. Thus, it was recently shown that transfecting cultured murine melanoma cells with the costimulator B7 evokes an effective immune response that brings about regression of existing tumors in syngeneic animals (38, 39). Likewise, the introduction of other exogenous genes, such as foreign recombinant major histocompatibility complex (40) or cytokine genes (41), has led to effective tumor cell immu-

nogenicity. Our approach is distinct from these in that it entails inhibition of an endogenous gene, rather than expression of an exogenous gene, in tumor cells. Though the precise molecular mechanism whereby IGF-I inhibition affects immunogenicity remains to be elucidated, the present findings with teratocarcinoma, taken together with earlier glioblastoma findings, support a critical regulatory role for IGF-I in tumor immunosurveillance. While the findings with IGF-II support the role of this growth factor in directing tissue differentiation in teratocarcinoma, this role of IGF-II in differentiation is not unique to teratocarcinoma. It was recently shown that when cultured myoblasts are induced to differentiate to myotubes, they express IGF-II as well as the known regulatory factors involved in myogenesis, MyoD, myogenin, and MlCla. Transfection of these cells with antisense episomal vectors and blocking endogenous IGF-II expression prevent myogenic differentiation as well as the expression of all the above-mentioned myogenic regulatory factors (42). These findings indicate that IGF-II plays a role of a master gene overlooking myogenesis. Thus, not only cancer cell differentiation but also normal differentiation pathways may be regulated by IGF-II.

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