

Insulin-like Growth Factor I Receptor-mediated Circuit in Ewing's Sarcoma/Peripheral Neuroectodermal Tumor: A Possible Therapeutic Target¹

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Abstract

The disappointingly low survival rate observed in Ewing's sarcoma (ES)/peripheral neuroectodermal tumor (PNET) despite the adoption of aggressive multimodal treatments prompted us to study the existence of autocrine circuits to be used as innovative therapeutic targets. Of the several circuits analyzed, only the insulin-like growth factor receptor (IGF-IR)-mediated loop was found to be constantly present both in cell lines and clinical samples, suggesting a role for this autocrine circuit in the pathogenesis of ES/PNET. The *in vitro* inhibition of the IGF-IR-mediated circuit by the specific IGF-IR binding antibody α IR3 suppressed the growth of ES/PNET cells by decreasing the proliferative rate and increasing apoptosis. α IR3 also significantly inhibited the ability of ES/PNET cells to grow in soft agar and to migrate following a chemotactic stimulus. Inactivation of the IGF-IR signaling pathway may therefore be considered as an effective therapeutic modality for patients with ES/PNET.

Introduction

ES³ is a malignant small round cell tumor of bone and soft tissues, arising in children and young adults, and showing an extremely aggressive clinical course. It constitutes a single pathological entity with Askin's tumor of the thoracic wall and PNET. These lesions share a common origin and feature a spectrum of phenotypic patterns, with the poorly differentiated ES at one end and PNET at the other end, showing evidence of neuroectodermal differentiation (1). Although the survival of ES/PNET patients has been improved significantly by the addition of multidrug chemotherapy to the surgical or radiotherapeutic control of the primary lesion (2), the rate of systemic relapse is still disappointingly high, especially in high-risk groups (3). With the aim of identifying innovative, more effective therapeutic strategies for this tumor, we analyzed the production of growth factors and the expression of their corresponding receptors in cell lines and clinical samples of ES/PNET to define the presence of autocrine circuits. Cells that synthesize a growth factor and display its receptor have the potential for self-stimulatory or autocrine growth. In particular, malignant cells can produce growth factors that bind to their own cell surface receptors, ensuring the continuous growth of neoplastic cells, and participate in neoplastic transformation. The possibility of

blocking these autocrine loops has been proposed recently as a novel, nonconventional therapeutic tool for human cancers (4). In this paper, we report on the presence in ES/PNET of a unique, specific autocrine loop that is mediated by the IGF-IR. We demonstrate that the blockage of the IGF-IR-mediated circuit by a monoclonal antibody that specifically blocks the IGF-IR (5) can effectively inhibit the *in vitro* growth and the motility of ES/PNET cells.

Materials and Methods

Cell Lines. ES cell lines SK-ES-1 and RD-ES, and Askin's tumor cell line SK-N-MC were obtained from the American Type Culture Collection (Rockville, MD). ES cell line TC-71 was a generous gift from T. J. Triche (Childrens Hospital, Los Angeles, CA). PNET cell lines LAP-35 and IOR/EW4 were established previously at the Istituti Ortopedici Rizzoli. Cell were cultured routinely in IMDM supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin (Life Technologies, Inc., Paisley, Scotland), and 10% inactivated FCS (Biological Industries, Kibbutz Beth Haemek, Israel). Cells were maintained at 37°C in a humidified 5% CO₂ atmosphere.

Tissue Samples. We studied surgical specimens from patients operated on at the Istituti Ortopedici Rizzoli, including five cases of ES, two cases of PNET, and one case of Askin's tumor. Each specimen was partly processed for histopathological evaluation, and partly snap frozen in liquid nitrogen and stored at -80°C for molecular analysis. By RT-PCR, in all of the cases we found the fusion products of either the t(11;22) or the t(21;22), two specific chromosomal aberrations, the presence of which is diagnostic for ES/PNET (6, 7).

Growth Factor Analysis. The mRNAs for growth factors expressed by ES/PNET cells were evaluated by RT-PCR. Total RNA was extracted from frozen pelleted cells or from 5 μ m-thick cryogenic tissue sections using the TRIzol extraction kit (Life Technologies, Inc.). cDNA was obtained by using Moloney-Murine Leukemia Virus (M-MLV) reverse transcriptase (Life Technologies, Inc.) in the presence of oligodeoxythymidylate and deoxynucleotide triphosphate. The RT-PCR exponential phase was determined on 15-40 cycles to allow a semiquantitative comparison among the cDNAs developed from identical reactions. Specific primer pairs for growth factors were obtained, respectively, from Clontech (Palo Alto, CA; IGF-I, 514-bp product; IGF-II, 538-bp product; TGF β 1, TGF β 1, 161-bp product; TGF α , TGF α , 297-bp product; EGF, 527-bp product) and Stratagene (La Jolla, CA; FGF- β , 366-bp product). For NGF, the 397-bp amplicon sequence was designed as reported previously (8). β -Actin primer pairs were obtained from Clontech (479-bp product). Amplified products were identified by gel electrophoresis (2% agarose gels) and ethidium bromide staining. Gel images were digitized using the Gel Doc 1000-PC and the Molecular Analyst/PC software (Bio-Rad, Hercules, CA).

Growth Factor Receptor Expression. In cell lines, expression of specific membrane receptors was determined by flow cytometry (FACSscan, Becton Dickinson, Mountain View, CA) after indirect immunofluorescence with the following primary antibodies: clone α IR3 (antihuman IGF-IR, Oncogene Science, Cambridge, MA), diluted 1:10; clone 528 (antihuman EGF receptor, Oncogene Science), diluted 1:40; clone 8211 (antihuman NGF receptor, Boehringer Mannheim, Mannheim, Germany), diluted 1:20; clone C-16 (antihuman TGF β receptor, Santa Cruz Biotechnology, Santa Cruz, CA), diluted 1:5; and antihuman FGF receptor (Upstate Biotechnology, Lake Placid, NY), diluted 1:40. In clinical samples, the expression of membrane receptors was deter-

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³ The abbreviations used are: ES, Ewing's sarcoma; PNET, primitive neuroectodermal tumor; IMDM, Iscove's modified Dulbecco's medium; RT-PCR, reverse transcription-PCR; IGF, insulin-like growth factor; IGF-IR, IGF-I receptor; TGF, transforming growth factor; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor; BrdUrd, bromodeoxyuridine.

mined by the avidin-biotin complex immunoperoxidase method on cryostatic sections using the above-mentioned antibodies. The final reaction product was visualized with 1% diaminobenzidine plus H_2O_2 . Sections were counterstained with Gill's hematoxylin.

In Vitro Cell Growth. To study the effects of the blockage of IGF-IR-mediated autocrine circuit, cells were seeded in 24-well plates (cells/well: 20,000 for TC-71, SK-N-MC, SK-ES-1, and RD-ES, and 100,000 for LAP-35 and IOREW4) in IMDM plus 10% FCS. After 24 h, medium was changed with IMDM plus 1% FCS, with or without (control) the blocking antibody α IR3 (10 ng/ml–5 μ g/ml). As an additional control, a class-matched mouse IgG (MOPC-21) (Sigma Chemical Co., St. Louis, MO) was also used (1 μ g/ml). Medium renewal (with or without the antibodies) was made every 3 days. Cell growth was evaluated on harvested cultures by trypan blue vital cell count.

BrdUrd Labeling Index. Cells (5,000–10,000/cm²) were seeded in IMDM plus 10% FCS. After 24 h, medium was changed with IMDM plus 1% FCS either without (control) or with 1 μ g/ml α IR3 antibody (blocking antibody) or 1 μ g/ml MOPC-21 antibody (additional control), respectively. At 48–96 h after seeding, cell cultures were incubated with 10 μ M BrdUrd (Sigma Chemical Co.) for 1 h in a CO₂ atmosphere at 37°C. Harvested cells were fixed in 70% ethanol for 30 min. After DNA denaturation with 2 N HCl for 30 min at room temperature, cells were washed with 0.1 M Na₂B₄O₇ (pH 8.5). Cells (10⁶) were then processed for indirect immunofluorescence staining, using α -BrdUrd (Euro-Diagnostics, Milan, Italy) diluted 1:4 as a primary antibody and analyzed by flow cytometry.

Morphological Assessment of Apoptotic Nuclei. Cells were seeded and treated as reported above. After a 24–72 h *in vitro* treatment, cells were fixed in methanol/acetic acid (3:1) for 15 min and stained with 50 ng/ml Hoechst 33258 (Sigma Chemical Co.). Cells with three or more chromatin fragments were considered apoptotic. The percentage of apoptotic nuclei was evaluated out of 1000–2000 nuclei.

Motility Assay. The motility assay was performed using Transwell chambers (Costar, Cambridge, MA) with 8- μ m pore size, polyvinylpyrrolidone-free, polycarbonate filters (Nucleopore, Pleasanton, CA). IMDM plus 10% FCS alone, IMDM plus 10% FCS with IGF-I (10 ng/ml), or IMDM plus 10% FCS with IGF-II (10 ng/ml) were placed in the lower compartment of the chamber. Cells (10⁵) in IMDM plus 10% FCS with or without α IR3 (1 μ g/ml)

were then seeded in the upper compartment and incubated for 18 h at 37°C. Cells migrated toward the filter to reach the lower chamber base were counted after Giemsa staining. All of the experiments were performed in triplicate.

Soft-Agar Assay. Anchorage-independent growth was determined in 0.33% agarose (SeaPlaque; FMC BioProducts, Rockland, ME) with a 0.5% agarose underlay. Cell suspensions were plated in semisolid medium (IMDM plus 10% FCS containing 0.33% agarose) with or without α IR3 (1 μ g/ml; cells/dish: TC-71, 1,000–3,300; SK-N-MC, 3,300–10,000; and LAP-35, 33,000–100,000). Dishes were incubated at 37°C in a humidified atmosphere containing 5% CO₂, and colonies were counted after 14 days.

Results

Presence of Autocrine Circuits. The production of several growth factors in ES/PNET was analyzed both in cell lines and tissue samples by RT-PCR (Fig. 1). Only TGF β and IGF-I mRNA were present in all of the cell lines and in all of the clinical samples, the remaining growth factors being barely or modestly expressed only in a minority of cases. The expression of the corresponding growth factor receptors was analyzed by immunofluorescence in ES/PNET cell lines (Fig. 2) and by avidin-biotin complex immunoperoxidase on cryostatic sections of tissue samples. In clinical specimens, EGF receptor and FGF receptor were expressed only in one of eight cases, whereas NGF receptor was expressed in seven of eight cases, and IGF-IR was expressed in all of the cases, in substantial agreement with data obtained in cell lines. On the contrary, TGF β receptor was not expressed in ES/PNET cell lines but appeared to be present in four of eight clinical samples, possibly reflecting actual differences between *in vitro* conditions and clinical settings, or simply methodological variability. In summary, among all of the growth factors and corresponding receptors considered, only IGF-I and IGF-IR were found to be constantly present in all of the cell lines and tissue samples of ES/PNET, indicating that the IGF-IR-mediated circuit is specific and possibly relevant for the pathogenesis of these tumors.

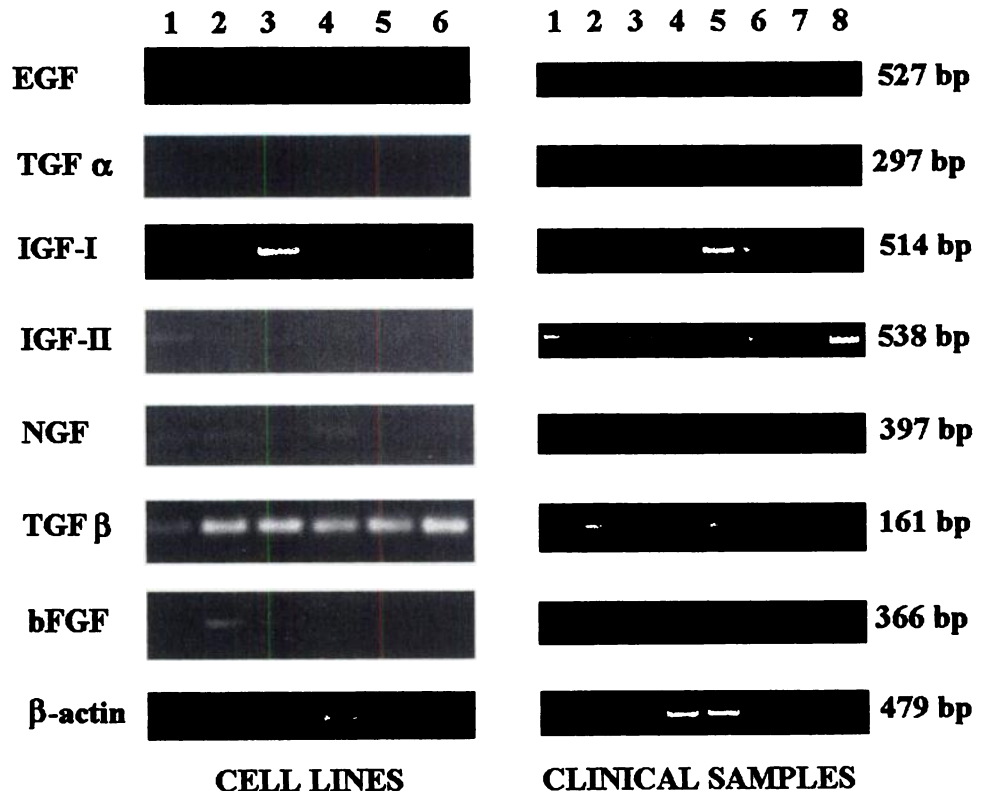


Fig. 1. Expression of growth factors in cell lines and tissue samples of ES/PNET. Methods for reverse transcription of mRNA and PCR amplification of cDNA fragments were described in "Materials and Methods." Amplification products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Cell lines: Lane 1, TC-71; Lane 2, SK-ES-1; Lane 3, SK-M-NC; Lane 4, RD-ES; Lane 5, LAP-35; and Lane 6, IOR-EW4. Clinical samples: Lanes 1 and 6 are PNETs; Lanes 2, 3, 4, 5, and 7 are ESs; Lane 8 is an Askin's tumor.

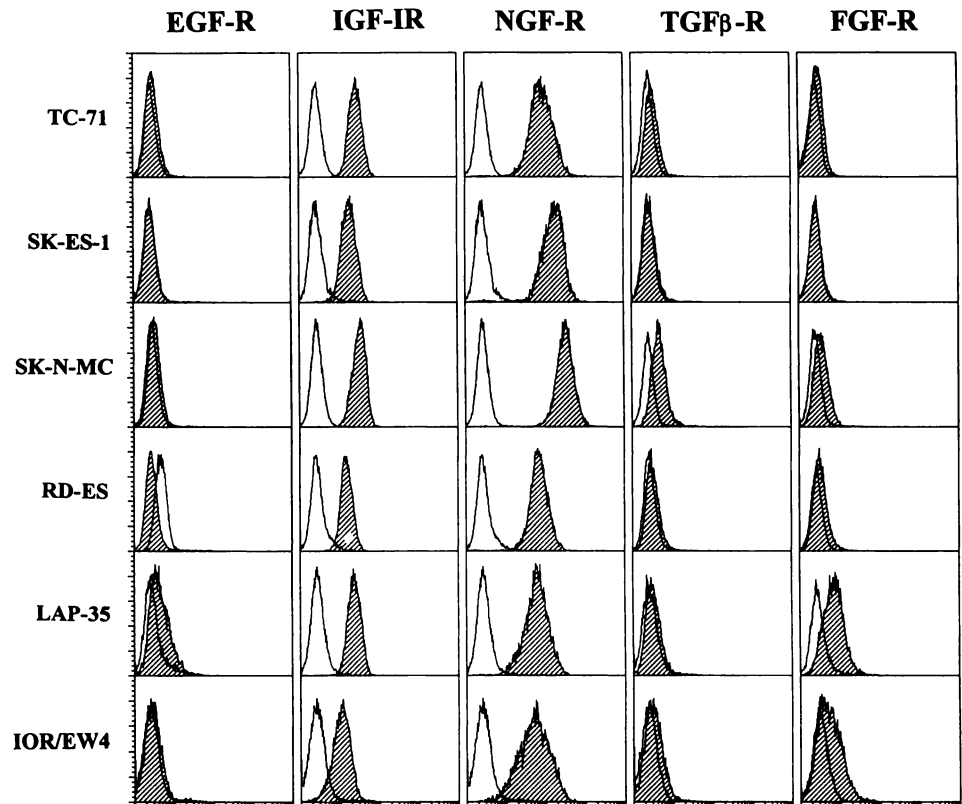


Fig. 2. Expression of different growth factor receptors in ES/PNET cell lines as determined by flow cytometry. Results of individual experiments, representative of at least two different similar experiments, are shown.

Inhibition of the IGF-IR-mediated Circuit. The neutralizing antibody α IR3 was used to block the IGF-IR-mediated loop and test the role of this circuit for the *in vitro* growth of ES/PNET cells. A relevant dose-dependent growth inhibition was induced in all of the ES/PNET cell lines by the continuous *in vitro* treatment with different doses of α IR3 (10 ng/ml–5 μ g/ml) for 6 days (Fig. 3a). The growth inhibition induced by α IR3 was due to a cytostatic rather than a cytotoxic effect, as indicated by the growth curves of three cell lines that represent the spectrum of the ES/PNET group of tumors, including conventional ES (TC-71), PNET (LAP-35), and Askin's tumor (SK-N-MC; Fig. 3b). To ascertain whether this effect was due to a reduction in the proliferative rate and/or an induction of apoptosis, the incorporation of BrdUrd and the percentage of apoptotic nuclei were analyzed. α IR3 treatment induced both a consistent reduction in the S-phase rate and a significant induction of apoptosis in all of the cell lines (Table 1).

Effects on Colony Formation in Soft Agar. Treatment with α IR3 antibody induced a significant inhibition, ranging from 66 to 99%, in the number of colonies of all of the cell lines (SK-N-MC, 645 ± 28 versus 1912 ± 66 , $P = 0.003$; TC-71, 22 ± 7 versus 1082 ± 133 , $P = 0.01$; and LAP-35, 210 ± 12 versus 2174 ± 91 , $P = 0.002$, Student's *t* test).

Effects on Migratory Ability. Migratory ability of TC-71 and SK-N-MC cells was significantly stimulated both by IGF-I and IGF-II (Fig. 4), indicating that these peptides act also as motility factors for ES/PNET cells. Treatment with α IR3 (1 μ g/ml) significantly inhibited the migratory ability of ES/PNET cells either when complete medium alone was used as a chemoattractant or when IGF-I or IGF-II was added.

Discussion

Expression of growth factors and/or their receptors may be an important mechanism by which tumors develop abnormal growth. In this report, we documented the presence of an IGF-IR-mediated

circuit both in cell lines and clinical samples of ES/PNET and demonstrated the functional role of IGF-IR for the growth of ES/PNET cell lines. The analysis of six cell lines and eight clinical samples of ES/PNET, all defined cytogenetically either by the t(11;22) or the t(21;22), revealed a high expression of IGF-I and IGF-IR in all of the cases, whereas IGF-II was only partly present in tissue samples and barely evident in cell lines. The production of IGF-I has already been reported in several cell lines showing the t(11;22) (9). In this paper, we identified the specificity of the IGF-IR-mediated autocrine loop in ES/PNET and ruled out the existence of circuits mediated by other growth factors, including EGF-TGF α , TGF β , and NGF. In addition, we also demonstrated the clinical relevance of this phenomenon, based on the finding that IGF-I and its receptor are simultaneously present in tissue samples of a representative series of ES/PNET.

The occurrence of IGF-I expression in ES/PNET is not completely unexpected in ES/PNET. This is a group of poorly differentiated tumors of bone and soft tissues of uncertain histogenesis, which may express a spectrum of differentiative features, including mesenchymal and neuroectodermal markers (10). IGF-I is a widely expressed growth factor that is important for the physiological growth and development of many tissues (11). Different cell types that are present in the skeleton require IGF-I for growth, including fibroblasts, epithelial cells, bone marrow stem cells, and osteoblasts (12), and IGF-I plays an important role in normal bone growth, cell turnover, and metabolism (13). Besides, IGF-I is an important survival factor for neurons, oligodendrocytes, and their precursor (14), and IGF-I mRNA expression is present in proliferating embryonic neurons (15). Therefore, it is very likely that IGF-I may be involved in the up-regulated proliferation of musculoskeletal stem cells, possibly of neuroectodermal derivation, with residual potential for multilineage differentiation. Because of the absence of a normal counterpart of ES/PNET, we could not compare the level of expression of IGF-I/IGF-IR in normal and neoplastic cells. We could only define the existence of an auto-

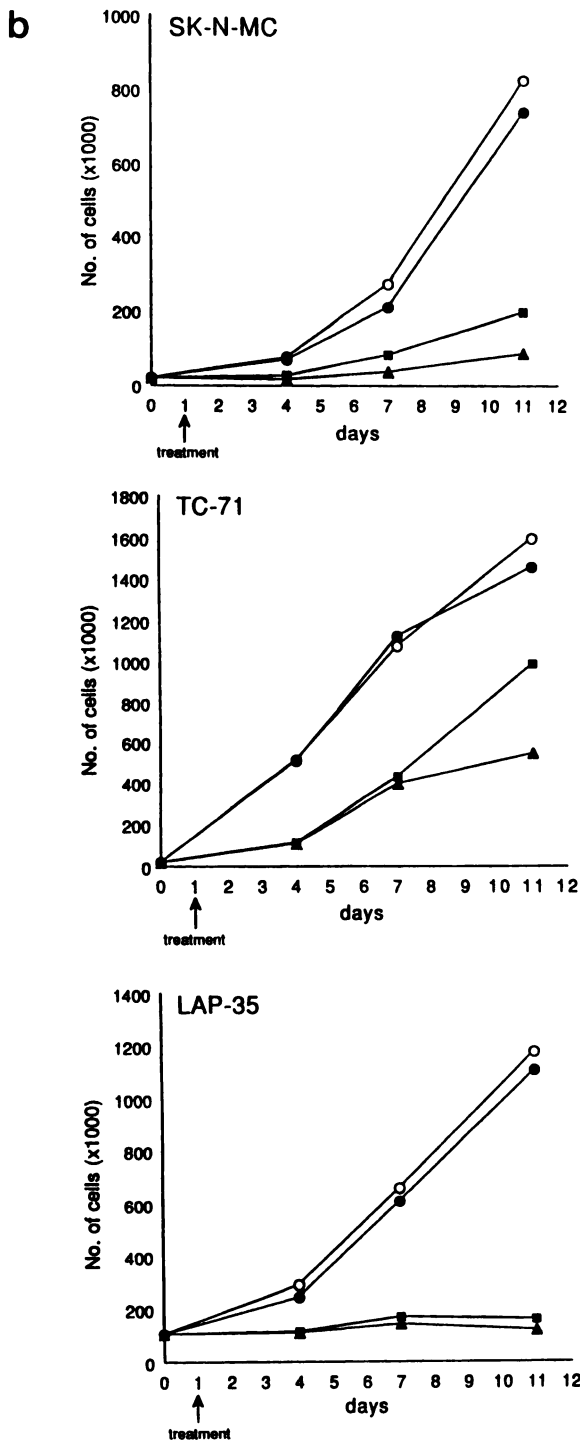
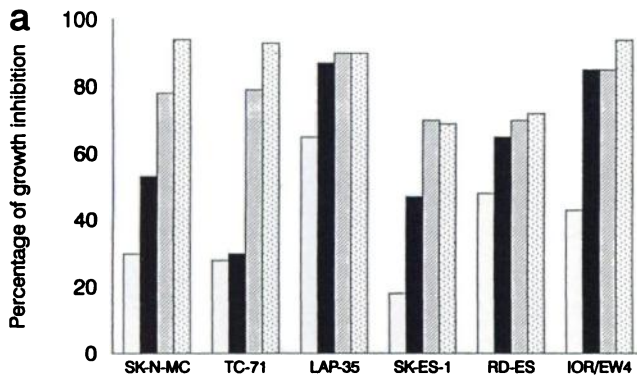


Table 1 Effects on proliferative rate and apoptosis of ES/PNET cell lines by blocking the IGF-IR-mediated circuit

Cell line	Treatment ^a	BrdUrd labeling index ^b	Apoptosis ^c
TC-71	None	46.7 ± 4.5	0.8 ± 0.2
	MOPC-21 (1 µg/ml)	40.2 ± 3.0	0.7 ± 0.1
	αIR3 (1 µg/ml)	31.0 ± 2.2 ^d	4.4 ± 0.1 ^e
SK-N-MC	None	53.7 ± 1.8	0.7 ± 0.1
	MOPC-21 (1 µg/ml)	50.9 ± 3.6	0.6 ± 0.2
	αIR3 (1 µg/ml)	46.1 ± 1.9 ^d	2.2 ± 0.1 ^e
LAP-35	None	17.1 ± 1.0	2.6 ± 0.3
	MOPC-21 (1 µg/ml)	18.1 ± 1.5	2.5 ± 0.2
	αIR3 (1 µg/ml)	7.4 ± 0.8 ^e	20.0 ± 3.0 ^d

^a Cells were treated for 72 h before analysis.

^b Average ± SD of three independent experiments.

^c Percentage of apoptotic nuclei. Average ± SD of three independent experiments.

^d Significantly different by the Student's *t* test; *P* < 0.05.

^e Significantly different by the Student's *t* test; *P* < 0.001.

ocrine loop, the blockage of which greatly inhibits the proliferation and the motility ability of tumor cells and significantly decreases their survival.

IGF-IR is a transmembrane heterotetramer that exhibits tyrosine kinase activity after binding of its ligands to the extracellular domain (11). Previous studies have demonstrated that ES/PNET cells exhibit specific high-affinity IGF-I and IGF-II receptors, and that the metabolic action of both IGF-I and IGF-II appears to be mediated through the type I receptor (16). We found that the blockage of IGF-IR with αIR3 antibody significantly inhibits the *in vitro* growth of ES/PNET cell lines in a dose-dependent way, indicating that, in this group of tumors, IGF-I plays a role as an autocrine growth factor, and that the presence of an intact, activated IGF-IR is important for cell growth. We also observed that inactivation of IGF-IR decreases the growth rate of ES/PNET cells, partly because of a reduction of the proliferative rate, and partly because of an induction of apoptosis. The role of the IGF-IR-mediated autocrine circuit as a survival mechanism has already been established for different cell types, suggesting that the inactivation of IGF-IR may cause oncogene-driven tumor cells, particularly those overexpressing *c-myc*, to undergo apoptosis (17, 18). This might be the case with ES/PNET, which are known to overexpress *c-myc* (19).

In addition to the effects on cell growth, we found that inactivation of IGF-IR by αIR3 strongly impairs the ability of ES/PNET cells to form colonies in soft agar, suggesting a role for IGF-IR in the malignant behavior of this tumor. We also found that IGF-IR ligands can act as motility factors for ES/PNET cells, and that the blockage of IGF-IR by αIR3 significantly reduces their ability to migrate after a chemotactic stimulus, suggesting a possible role for IGF-IR in the modulation of the metastatic ability of ES/PNET cells. It is well known that IGF-IR plays a central role in the mechanisms of transformation of different tumors, including glioblastoma, small cell lung carcinoma, melanoma (18), rhabdomyosarcoma (20), and osteosarcoma (21). In these tumors, treatment with αIR3 antibody (22) or with antisense expression plasmids either to IGF-I or IGF-IR (18, 20) effectively reversed the transformed phenotype and/or inhibited tumorigenesis.

In conclusion, our results suggest that inhibition of the IGF-IR signaling pathway may be used as an effective therapeutic target for ES/PNET. Interestingly, the observed effects on cell growth induced

Fig. 3. Inhibition of ES/PNET cell growth after 6 days of continuous *in vitro* treatment with different doses of anti-IGF-IR neutralizing antibody αIR3 (a). Results are expressed as percentage of growth inhibition compared to controls. □, 10 ng/ml; ■, 100 ng/ml; ▤, 1 µg/ml; ▥, 5 µg/ml. b. *In vitro* growth curves of three different ES/PNET cell lines after treatment with αIR3 or MOPC-21 antibodies. Data are from one experiment, representative of two different experiments. ○, control; ■, 100 ng/ml αIR3; ▤, 1 µg/ml αIR3; ●, 1 µg/ml MOPC-21.

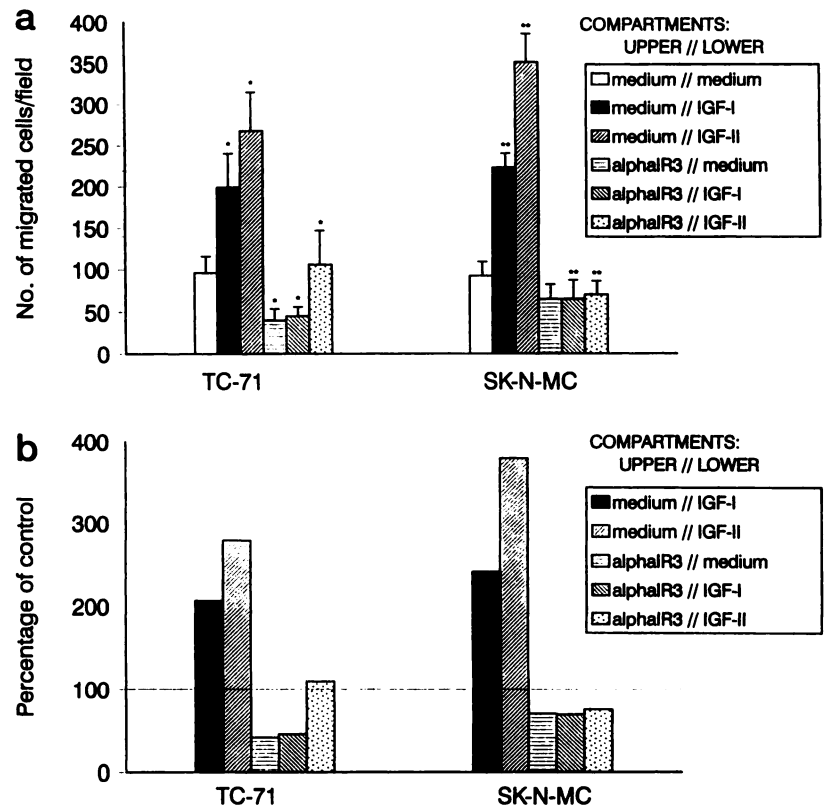


Fig. 4. Motility response of ES/PNET cells after treatment with α IR3 antibody. Cells (20,000) were incubated in the upper compartment of a Transwell chamber with or without α IR3 (1 μ g/ml). In the lower compartment, IMDM plus 10% FCS, IMDM plus 10% FCS and IGF I (10 ng/ml), or IMDM plus 10% FCS and IGF-II (10 ng/ml) was used as the source of chemoattractant. *a*, each column represents the mean of five independent experiments. (*, $P < 0.05$; **, $P < 0.001$; Student's *t* test). *b*, columns represent the percentage of migrated cells compared to control.

in vitro by blocking the IGF-IR-mediated circuit by α IR3 antibody in ES/PNET cells might even be more relevant *in vivo* (23). A number of different approaches could also be considered, including the use of peptide analogues or antisense strategies to block the autocrine loop. This wide range of therapeutic possibilities offers us a fascinating scenario to be investigated further in an effort to provide ES/PNET patients with a better outcome.

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