[CANCER RESEARCH 56, 3898–3901, September 1, 1996]

Advances in Brief

Antisense Epidermal Growth Factor Receptor Transfection Impairs the Proliferative Ability of Human Rhabdomyosarcoma Cells[1]

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Abstract

Human rhabdomyosarcoma cells express membrane epidermal growth factor receptor (EGF-R), which could confer responsiveness to EGF and transforming growth factor-α (TGF-α) of autocrine or paracrine origin. To study the role played by this growth factor circuit in the proliferation and differentiation of myogenic neoplastic cells, human rhabdomyosarcoma EGF-R-expressing cells (RD/18 clone) have been transfected with a plasmid containing a fragment of the EGF-R cDNA in the antisense orientation. *In vitro* growth and differentiative ability were studied on six antisense-transfected clones (AS) in comparison to parental RD/18 cells and to cells transfected with the plasmid containing only the neomycin resistance gene (NEO). A reduced EGF-R membrane expression was found in AS clones by decreased immunofluorescence with an anti-EGF-R monoclonal antibody. All AS transfectants had a greatly impaired proliferative ability, even when cultured in fetal bovine serum-containing medium. Proliferation of AS clones was completely blocked in medium supplemented with 2% horse serum. The differentiation ability of AS clones was heterogeneous, ranging from clones with a percentage of myosin-positive cells higher than controls to clones with a negligible myosin expression. Therefore, the growth impairment determined by the loop interruption is not sufficient to switch on the differentiation program. The role played by EGF-R in the proliferation of human rhabdomyosarcoma cells suggests that this receptor could constitute a target for a therapeutic approach.

Introduction

Rhabdomyosarcoma is a solid tumor arising from skeletal muscle, which generally retains some ability to differentiate along the myogenic pathway (1). It can show a complex machinery of response to growth factors; receptors for IGF[3]-I and IGF-II (2–4), basic fibroblast growth factor (4, 5), and EGF (4) are found expressed by rhabdomyosarcoma. Moreover, all of these growth factors can be provided by the rhabdomyosarcoma itself in an autocrine way (2–6). Some growth factors and receptors expressed in rhabdomyosarcoma cells could sustain proliferation and inhibit differentiation, as suggested by the knowledge of their effect on the normal myogenic counterpart (7, 8). Understanding the actual balance between opposing effects in neoplastic myogenic cells could be useful to put forward new anti-growth factor therapeutic approaches in which the antiproliferative action can be coupled by an induction of differentiation. Strategies to interfere with single autocrine loops in human rhabdomyosarcoma have been mostly applied to the circuit based on IGF-I receptor and comprise blockade with antibodies (2, 4, 9) as well as antisense IGF-I receptor gene transfection (10). In all of these studies, an antiproliferative effect was achieved, but no induction of differentiation was observed. The loop based on EGF and TGF-α, both interacting with EGF-R, has been found in human rhabdomyosarcoma cells, but their role on proliferation and differentiation is not clear (4). In this study, blockade of the EGF-based loop in rhabdomyosarcoma cells by means of antisense gene transfection was studied, along with its effect on differentiation.

Materials and Methods

Cells. The RD/18 clone, derived from the human rhabdomyosarcoma cell line RD (11), was used as the recipient for antisense EGF-R transfection. In RD/18 cells, membrane receptors for EGF and EGF mRNA are expressed as shown previously (4). Cells were cultured in DMEM supplemented with 10% FBS at 37°C in a humidified 7% CO_2 atmosphere. All medium constituents were purchased from Life Technologies, Inc. (Paisley, Scotland).

Antisense Transfection. Antisense pactAS-5′ plasmid contained the 0.89-kb 5′ fragment (*Xba*I-*Sma*I) of the human EGF-R in the antisense orientation downstream from the chicken β-actin promoter (12). For transfection, 4×10^5 RD/18 cells were seeded in 60-mm Petri dishes; 24 h later, cells were transfected by the Lipofectin reagent (Life Technologies, Inc.) with 10 μg pactAS-5′ plasmid and 1 μg pSV$_2$*neo*. Cells were incubated 24 h with plasmid DNA. An additional 48-h incubation with standard medium was performed before splitting the cultures and starting the selection of transfectant cells with the neomycin analogue geneticin (G418) at the concentration of 500 μg/ml in DMEM + 20% FBS. Transfection controls were performed with the pSV$_2$*neo* plasmid alone. Single transfectant colonies were isolated using sterile glass cloning cylinders and expanded for continuous growth in DMEM + 20% FBS with G418.

PCR. PCR was performed on genomic DNA extracted by the QIAamp tissue kit (Qiagen, Chatsworth, CA), DNA concentration was determined by means of A_{260}, and 0.5 μg was used for each PCR reaction in 20 μl final volume. The following primers were used to detect EGF-R cDNA fragment of the antisense pactAS5′ plasmid: direct 5′-GAAAAAGAAAGTTTGC-CAAG-3′ (position 264–283, exon 1–2) and reverse 5′-CACAGATGATTT-TGGTCAG-3′ (position 832–814, exon 6) (13, 14). PCR reaction was performed with a 60°C annealing temperature and 1.2 mM $MgCl_2$ for 30 amplification cycles. A spliced 569-bp product was amplified from plasmid DNA as well as from antisense-containing genomic DNA but not from DNA of parental nontransfected cells. Primers for glyceraldehyde-3-phosphate dehydrogenase (Clontech, Palo Alto, CA) were used as positive controls.

Cytofluorimetric Studies. Cells from subconfluent cultures were resuspended, washed with PBS supplemented with 1% BSA (PBS-BSA), and incubated for 30 min on ice with a 1:40 dilution of monoclonal antibody clone 528 (Oncogene Science, Uniondale, NY) in PBS-BSA. After washing with cold PBS-BSA, cells were incubated for 30 min on ice with a 1:50 dilution of fluoresceinated goat antimouse immunoglobulin antiserum (Technogenetics, Milan, Italy). Cells were again washed and resuspended in PBS containing 1 μg/ml ethidium bromide. Fluorescence was analyzed with a FACScan flow

Received 6/10/96; accepted 7/16/96.

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[1] This work was supported by grants from TELETHON, National Research Council, National Research Council Special Project ACRO, Associazione Italiana per la Ricerca sul Cancro, and Ministero dell'Università e della Ricerca Scientifica e Tecnologica, Italy. I. R. is the recipient of a Fellowship from the Associazione Italiana per la Ricerca sul Cancro.

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[3] The abbreviations used are: IGF, insulin-like growth factor; EGF, epidermal growth factor; EGF-R, EGF receptor; AS, antisense-transfected; FBS, fetal bovine serum; HS, horse serum; NEO, neomycin-transfected; TGF-α, transforming growth factor α.

cytometer (Becton Dickinson, Mountain View, CA). The results shown are from an experiment representative of three.

Proliferation and Differentiation Studies. The proliferative ability of transfectants and controls was evaluated in DMEM supplemented with either 10% FBS or 2% HS as follows. Cells were seeded in DMEM + 10% FBS at the concentration of 10^4 cells/cm^2; 24 h later, one-half of the cultures were shifted to DMEM + 2% HS. Medium renewal was performed every 2–3 days. A flask from each combination was harvested periodically, and cell yield was determined.

For cloning efficiency, cells were seeded in 60-mm Petri dishes at concentrations from 200 to 6400 cells/Petri in DMEM + 10% FBS and incubated 11 days. Plates were then fixed with methanol, and colonies were stained with Giemsa.

Differentiation ability was studied on cultures seeded and cultured as above. DMEM supplemented with 2% HS (also referred to as differentiation medium) is used in rhabdomyosarcoma cell cultures as a differentiation-inducing medium (15) due to the low content of exogenous growth factors. At 3–4-day intervals, cells were harvested and counted; then cytocentrifuge slides were prepared. The percentage of myosin-positive cells was determined after staining with monoclonal BF-G6 antibody (kindly provided by S. Schiaffino, University of Padova, Padova, Italy), reacting with the embryonic myosin heavy chain (16).

Results

To study the role played by the EGF-based loop in proliferation and differentiation of myogenic neoplastic cells, human rhabdomyosarcoma cells (RD/18 clone) were cotransfected with pSV$_2$*neo* and with pact-AS-5′. This plasmid contains an EGF-R cDNA fragment in the antisense orientation and can block translation of the EGF-R mRNA, leading to a decreased EGF-R expression (12). Cotransfectants were selected with the neomycin analogue G418 in 20% FBS-containing medium. Antisense-transfected clones (AS clones) soon showed a growth rate lower than that of clones transfected with the neomycin resistance gene alone (referred to as NEO cells); colonies reached the size to be collected by cloning cylinders 30–40 days after transfection for AS clones and 15–25 days for NEO transfectants. The slow growth rate of AS clones often made difficult their expansion for experimental study.

Six AS clones were studied in comparison to parental RD/18 cells and NEO control transfectant cells. The presence of antisense plasmid in genomic DNA of AS transfectants was shown by PCR (Fig. 1); primers located in different exons of the 5′ antisense EGF-R fragment were able to amplify the spliced 569-bp product from AS genomic DNA and not from genomic DNA of either parental RD/18 or NEO cells. Cytofluorimetric analysis of membrane EGF-R (Fig. 2) showed a decreased expression in AS transfectants when compared to RD/18 or NEO cells.

The proliferative ability of AS transfectants (Fig. 3) was severely impaired in 10% FBS-containing medium and almost completely blocked when the 2% HS medium was used. In either medium, NEO transfection control cells had a growth rate comparable to that of the parental RD/18 clone. The growth impairment of AS transfectants was also shown by a 50 to >90% decrease in cloning efficiency with respect to controls (data not shown).

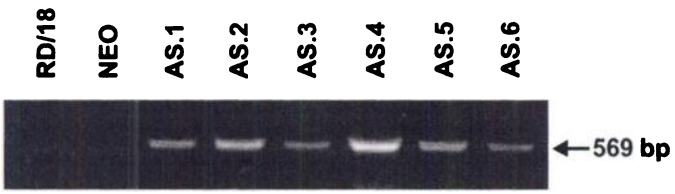

Fig. 1. PCR analysis of genomic DNA extracted from AS transfectants, NEO control, and RD/18 parental cells for the presence of a plasmid expressing an antisense EGF-R 5′ cDNA fragment.

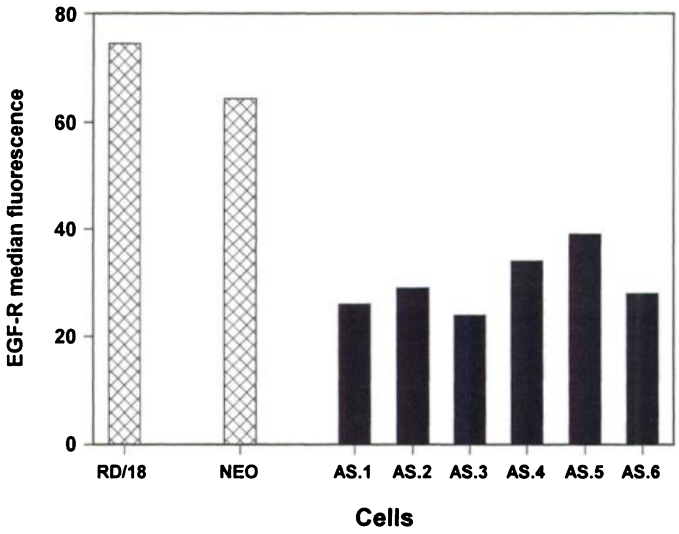

Fig. 2. Cytofluorimetric analysis of membrane EGF-R expression by AS transfectants, NEO control, and RD/18 parental cells.

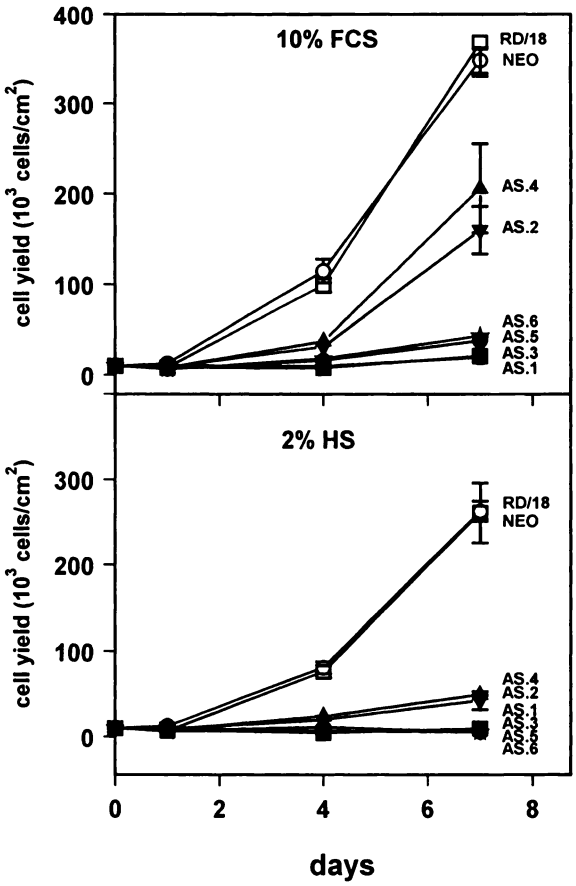

Fig. 3. Proliferative rate of AS transfectants, NEO control, and RD/18 parental cells cultured in DMEM supplemented with either 10% FBS (*upper panel*) or 2% HS (*lower panel*).

Differentiative ability was studied on cells cultured as above, either in 10% FBS- and in 2% HS-containing medium (Fig. 4). Both parental RD/18 and NEO cells showed a negligible percentage of myosin-positive cells in the presence of FBS, whereas the HS-containing differentiation medium allowed the occurrence of about 20% differentiated elements. In either medium, AS transfectants showed a heterogeneous behavior, ranging from clones with a percentage of my-

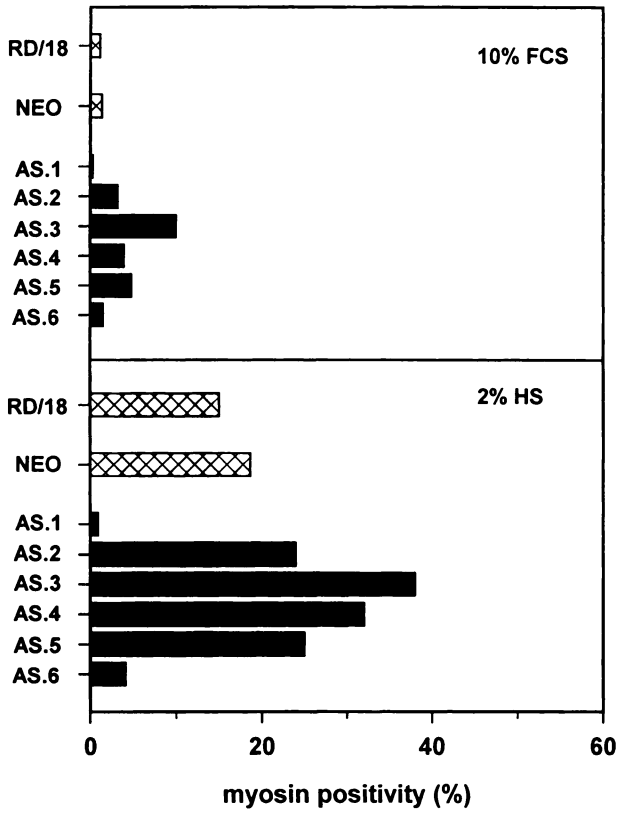

Fig. 4. Differentiative ability of AS transfectants, NEO control, and RD/18 parental cells cultured in DMEM supplemented with either 10% FBS (*upper panel*) or 2% HS (*lower panel*). The percentage of myosin-positive cells was evaluated on cytocentrifuge samples performed after a 7-day culture.

osin-positive cells higher than controls to clones with a lower myosin expression: in particular, myosin positivity in AS.1 and AS.6 clones in 2% HS medium was about 10- and 5-fold lower than control cells. Therefore, the almost complete block of proliferation of AS clones when cultured in 2% HS-containing medium was not sufficient *per se* to induce myogenic differentiation in these rhabdomyosarcoma cells.

Discussion

For this report, we studied, by means of cDNA antisense transfection, the role played by the EGF-R in proliferation and differentiation of the human rhabdomyosarcoma cell clone RD/18. Human rhabdomyosarcoma has a complex machinery of growth factor production and receptor expression that make possible both autocrine effects and susceptibility to exogenous growth factors provided by the microenvironment (2–6).

Autocrine loop based on the interaction between IGF-II and IGF-I receptor has been mainly studied and found to be relevant for proliferation of human rhabdomyosarcoma cells; the reduction of IGF-I receptor available for IGF-II binding achieved either by a specific antibody (2, 4, 9) or by antisense transfection (10) leads to growth impairment and suppression of the malignant phenotype. This autocrine loop is likely to play a central role in the etiopathogenesis of several tumors (17), including rhabdomyosarcomas (18).

The multiplicity of growth factors and receptors observed in rhabdomyosarcoma, however, lends support to the study of other growth factor loops that could be exploited in therapeutic approaches. The role played by EGF and related factors in supporting human rhabdomyosarcoma cell growth has not been extensively studied, even if some interesting evidence has been reported.

We previously found, on two of three human rhabdomyosarcoma cell lines, the expression of membrane EGF-R that could confer responsiveness to growth induction by EGF and TGF-α of either autocrine or exogenous origin (4). It should be noted that the rhabdomyosarcoma RC2 cell line that lacked EGF-R membrane expression has a lower *in vitro* growth rate and a higher serum requirement in comparison to EGF-R-positive cell lines (4, 16). Moreover, EGF was reported to increase rhabdomyosarcoma proliferation and metastasis in a rat model (19). Data on EGF effect on normal myogenic cell proliferation and differentiation have also been reported (7, 20). Here, we found that the decrease in EGF-R membrane expression obtained by transfection of an antisense EGF-R cDNA fragment leads to a severe growth impairment of human rhabdomyosarcoma cells.

The transfection approach with antisense sequence for growth factor receptors was effective in decreasing receptor expression and cell proliferation of neoplastic cells. Several models have been reported for IGF-I-R (21, 22), including rhabdomyosarcoma (10). Antisense EGF-R was found to affect proliferation of carcinoma cells (12, 23, 24), but no data were insofar reported on mesenchymal tumor cells.

Antisense EGF-R transfection gave rise to clones with a very slow growth rate and a high serum requirement that showed a decreased receptor expression, rather than a fully negative phenotype. These observations, true for our present data as well as for literature reports (10, 12, 23), are likely to be due to the relevance of the growth factor studied and could have a functional explanation; different levels of EGF-R can drive neoplastic cells toward distinct phenotypes (25) and can activate different signaling pathways (26). In other models, transformation or maintenance of the transformed phenotype was proportional to the level of EGF-R (12, 23); similarly, a rate-limiting feature for the *in vitro* and *in vivo* growth of rhabdomyosarcoma has been proposed for the IGF-I receptor (10). Therefore, the decrease in EGF-R level obtained here, ranging from 45 to 65% of control cells, could determine a receptor density below the threshold required for efficient growth-stimulating signal transduction.

Normal myogenic differentiation results from a balance of proliferating and differentiating stimuli (8). Among these stimuli, EGF is likely to induce proliferation, although data on its final effect on differentiation are contradictory (7, 20). Therefore, we investigated whether subtracting the proliferative stimulus conferred by EGF allowed rhabdomyosarcoma cells to reach a more differentiated phenotype. Antisense-transfected clones were almost completely blocked when cultured with a low exogenous growth factor content, but differentiation was not always increased. Therefore, this rhabdomyosarcoma model appears to have a differentiation defect that does not depend only on the proliferative activity (4). Clones AS.1 and AS.6, which showed the lowest expression of EGF-R and the lowest cell growth rate, also showed the major decrease in differentiation markers.

The redundancy of growth factor machinery in rhabdomyosarcoma tumors could constitute an additional advantage since interactions between different growth factor responses have been documented in different models. As an example, a functional IGF-I receptor is required for the mitogenic activity of EGF-R (27); it has been demonstrated that the effect of targeting the EGF-R is to affect the activation of the IGF-I receptor (28). EGF can synergize with basic fibroblast growth factor in the induction of myogenic proliferation (29). Human rhabdomyosarcoma tumors can show all of these growth factor response apparatuses, along with the autocrine expression of the relative factors. A combined therapeutic approach directed against multiple growth factor circuits could be the subject of additional experimental studies.

Acknowledgments

We would like to thank Dr. Laura Beguinot (DIBIT, H.S. Raffaele) for providing antisense cDNA plasmid and for continuous discussions and support during the work.

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