Advances in Brief

Growth Inhibition of Human Melanoma Cells in Nude Mice by Antisense Strategies to the Type 1 Insulin-like Growth Factor Receptor[1]

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Abstract

The growth of human melanoma cells FO-1 in nude mice is strongly inhibited or even abrogated when the cells are stably transfected with a plasmid expressing an antisense RNA to the insulin-like growth factor 1 receptor (IGF-1R) RNA, which causes a marked reduction in the number of IGF-1 receptors. When a tumor arises after a long delay in nude mice, it can be shown that the tumor cells have lost the expression plasmid and that the number of IGF-1 receptors has returned to wild-type levels. The antisense effect is even more remarkable, since the growth of FO-1 melanoma cells in monolayers is not affected by the expression of the antisense RNA. Inhibition of tumorigenesis was also evident when FO-1 melanoma cells were treated with antisense oligodeoxynucleotides to the IGF-1R RNA prior to injection into nude mice. These results confirm in human cells that the IGF-1R plays a dominant role in transformation and tumorigenesis and that its effect on tumorigenesis is more profound than its effect on mitogenesis.

Introduction

The generation of mice in which the *IGF-1R* genes have been disrupted by targeted homologous recombination has demonstrated the important role that the IGF-1R[3] and its ligands play in embryonal development (1, 2). The establishment of cell lines, R⁻ cells, from these knockout mouse embryos has been extremely helpful in defining the role of the IGF-1R in the growth and transformation of cells. Briefly, it was shown that: (*a*) the IGF-1R is not necessary for the growth of cells in culture, although it is necessary for optimal growth, even in 10% serum. In its absence, all cell cycle phases are elongated (3); (*b*) a functional IGF-1R is necessary for the growth-promoting action of other growth factors like epidermal growth factor (4); and (*c*) the IGF-1R is obligatory for the establishment and maintenance of the transformed phenotype (3–6). The last finding has suggested the hypothesis that interference with the expression and/or activation of the IGF-1R could conceivably lead to the reversal of the transformed phenotype in other types of cells. This was shown to be true. C6 rat glioblastoma cells expressing an antisense RNA to the IGF-1R RNA do not grow at all in syngeneic rats, and in fact cause complete regression of established contralateral wild-type tumors (7). These findings are supported by other reports, indicating that antibodies to the IGF-1R (8, 9); antisense expression plasmids to either IGF-2 (10), IGF-1 (11, 12) or their receptor (13); and a dominant negative mutant of the IGF-1R (14) can all reverse the transformed phenotype and/or inhibit tumorigenesis. The present investigation was undertaken with

two purposes: (*a*) to determine the effect of a reduction in the number of IGF-1Rs (by antisense strategies) on the growth of human melanoma cells in nude mice; and (*b*) to determine the mechanism(s) allowing, in some cases, the outgrowth of tumors from cells expressing the antisense RNA to the IGF-1R RNA.

Materials and Methods

Cells Lines and Cultures. FO-1 human melanoma cells, originated from a metastatic melanoma lesion and previously characterized in detail (15, 16), were transfected with the plasmids HSP/IGF-1RS and HSP/IGF-1R AS described previously (7, 17). These plasmids express sense and antisense transcripts (309 base pairs), respectively to the IGF-1R RNA, under the control of a Drosophila HSP70 promoter, and also contain the gene for resistance to G418. Transfected cells were selected in 0.5 mg/ml of G418 and characterized as described below.

Cells were passaged in RPMI 1640 supplemented with 5% calf serum and 5% fetal bovine serum. For growth experiments, 8×10^4 cells were plated in 35-mm dishes in 10% serum; after 12 h, the growth medium was removed and replaced with serum-free medium supplemented with 0.1% bovine serum albumin (fraction V) and 1.0 μM ferrous sulfate, with or without IGF-1 (10 ng/ml).

IGF-1R Number. IGF-1R number was determined by Scatchard plot analysis as described previously (18) on 8×10^4 cells that had been incubated for 72 h in serum-free medium at 39°C.

Soft Agar Assay. Growth in soft agar was carried out as described in detail by Sell *et al.* (5). The number of colonies >125 μm in diameter was determined at the times indicated.

Tumorigenicity in Nude Mice. Twenty-four h prior to injection, cells were incubated in serum-free medium at 39°C. After gentle trypsinization, 10^7 cells were injected s.c. (suspended in 0.2 ml of phosphate-buffered saline) above the hind leg of 7-week-old BALB/c nude mice (Charles River Breeders). When cells were preincubated with either sense or antisense oligodeoxynucleotides, we used a concentration of 80 μg/ml for 24 h prior to injection; 1 h prior to injection, additional oligos (40 μg/ml) were added.

Polymerase Chain Reaction. PCR was carried out by standard methods (19), omitting the reverse transcriptase step, and using the following amplimers: 5′ AAG GAA TGA AGT CTG GCT CC (exon 1) and 3′ AGA ACT GCA CGG TGA TCG A (exon 2). The expected size of the PCR product is 172 base pairs; because the amplimers are in different exons separated by a large intron, only the plasmid DNA is detected by this method. Our procedure (18) includes a standard to monitor the amount of DNA.

Materials. Human recombinant IGF-1 was obtained from Bachem, Inc. (Torrance, CA). Sense and antisense oligodeoxynucleotides to the IGF-1R RNA were synthesized as described previously (20). The sense sequence was 5′ AAG TCT GGC TCC GGA GGA and the antisense sequence was 5′ TCC TCC GGA GCC AGA CTT; they represent codons 2–7 of the prepropeptide (21), and they have been shown previously to effectively decrease the number of IGF-1 receptors (20, 22).

Statistics. All data represent the mean ± SE of duplicate determinations. Statistical analysis was performed by Student's *t* test.

Results

FO-1 human melanoma cells stably transfected with a plasmid expressing an antisense RNA to the IGF-1R RNA displayed a 70%

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[3] The abbreviations used are: IGF-1R, insulin-like growth factor 1 receptor; PCR, polymerase chain reaction.

decrease in the number of IGF-1Rs, as measured by Scatchard plot analysis, confirming previous results from our laboratory that this antisense plasmid does indeed decrease the number of IGF-1Rs (7). The number of receptors in wild-type FO-1 cells was 49×10^4 receptors/cell, while in FO-1 cells expressing the antisense plasmid, there were 14.7×10^4 receptors/cell. At variance with previous results, however (7, 20), this decrease in the number of receptors had no effect on the growth of FO-1 melanoma cells in monolayers (Table 1). Notice that these cells grow well even in serum-free medium, although their growth is enhanced by the addition of IGF-1 (10 ng/ml). Even at 39°C at which temperature there is a maximum expression of the antisense RNA, the effect of the antisense plasmid is extremely modest, if present at all.

The reduction in the number of IGF-1Rs was more critical for anchorage-independent growth, i.e., colony formation in soft agar, which is an accepted criterion for transformation (23). The cells expressing the antisense to the IGF-1R RNA showed a marked inhibition of colony formation in soft agar, compared to the clonogenic efficiency of wild-type cells or of cells expressing the sense RNA (Fig. 1). This inhibition was more prominent when the assays were carried out at 39°C (Fig. 1) than when incubated at 37°C (not shown), confirming that the expression of the antisense RNA in this plasmid is temperature dependent (7, 17).

An adequate number of IGF-1Rs was even more important for tumorigenesis in nude mice. FO-1 melanoma cells were not tumorigenic (or weakly so) in BALB/c nude mice up to a concentration of 10^6 cells/mouse, s.c. However, at 10^7 cells, both wild-type cells and sense cells (FO-1 cells expressing the sense RNA) gave tumors in all mice with a latent period of only 4 days (Table 2). Cells expressing the

Table 2 *Growth of human melanoma cells in nude mice*

Human melanoma cells (10^7) were injected s.c. in 0.2 ml of phosphate-buffered saline above the right hind leg of 7-week-old BALB/c nude mice. T_1, T_2, and T_3 are the tumors that grew out of the 3 mice in which the antisense cell lines gave tumors (see text). The latent period is the time between injection and the appearance of a palpable tumor.

Cell type	Animals with tumors	
	No. of animals	Latent period (days)
Expression plasmids		
FO-1 wild type	3/3	4
FO-1 sense	6/6	4
FO-1 antisense	3/6	28
T_1, T_2, T_3	9/9	4
Oligodeoxynucleotides		
FO-1 wt[a] plus antisense	0/3	NA
FO-1 wt plus sense	3/3	4

[a] wt, wild-type; NA, not applicable.
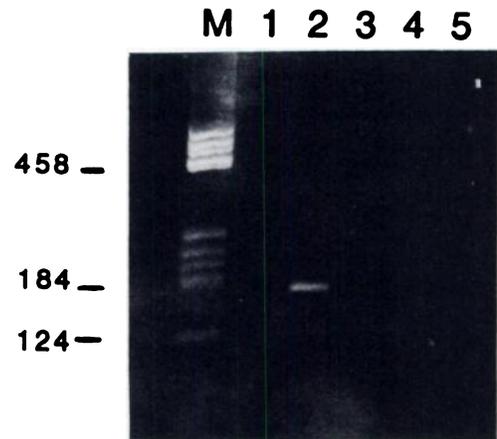
Fig. 2. PCR analysis of FO-1 melanoma cells for the presence of a plasmid expressing an antisense RNA to the IGF-1R RNA. The technique used is described in "Materials and Methods" (30 cycles of amplification). *M*, markers; *Lane 1*, wild-type FO-1 cells; *Lane 2*, FO-1 cells stably transfected with the plasmid expressing the antisense RNA to the IGF-1R RNA; *Lanes 3–5*, tumors T_1, T_2, and T_3 (see text).

Table 1 *Growth of human melanoma cells in monolayers*

Cells (8×10^4) were plated in 35-mm dishes in 10% serum for 12 h. The growth medium was then replaced with serum-free medium (SFM) with or without the addition of IGF-1 (10 ng/ml). Plates were incubated at either 34°C or 39°C, and the cells were counted after an additional 24-h (not shown) or 48-h incubation.

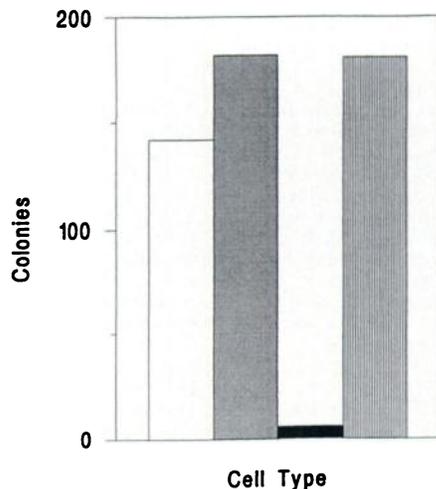| Cell type	No. of cells $\times 10^4$			
	34°C		39°C	
	SFM	IGF-1	SFM	IGF-1
Wild type	28.8 ± 0.5	37.2 ± 0.6	35.8 ± 0.9	42.5 ± 0.6
Sense	24.9 ± 0.7	44.3 ± 0.4	31.1 ± 0.5	45.9 ± 0.3
Antisense	24.7 ± 0.4	41.0 ± 0.4	29.5 ± 0.4	39.2 ± 0.3

Fig. 1. Growth in soft agar of FO-1 melanoma cells and derivatives. Cells were seeded at a concentration of 3×10^3 cells and the number of colonies in duplicate plates was determined after 3 weeks of growth in 10% serum at 39°C. □, wild type cells; ▧, sense cells; ■, antisense cells; ▥, T_1, T_2, and T_3 tumor cells (see text).

antisense RNA gave tumors in 3 of 6 nude mice; the latent period in the three mice that developed tumors was 28 days (no range; all 3 tumors became palpable at 28 days).

The delayed growth of these 3 tumors, referred to as T_1, T_2, and T_3, could be due to either a very slow growth of the originally injected cells, or, alternatively, clonal selection. To distinguish between these two alternatives, we examined these 3 tumors for the presence of the original antisense plasmid for the levels of IGF-1Rs and for their ability to grow when reinjected into nude mice. By PCR, it was shown that the antisense plasmid, clearly detectable in the original antisense cells (Fig. 2, *Lane 2*), was no longer detectable in the 3 tumors that grew out in nude mice (Fig. 2, *Lanes 3–5*). Because of the large number of amplifications and the intensity of the positive signal, it is reasonable to conclude that the plasmid is essentially absent from the T_1 to T_3 tumors. This conclusion was confirmed by the determination of the number of IGF-1Rs using Scatchard analysis; T_1 cells had the same number of IGF-1Rs ($\sim 50 \times 10^4$ receptors/cell) as the wild-type FO-1 cells. The cells isolated from this tumor also formed colonies in soft agar as efficiently as wild-type cells (Fig. 1). We give only one bar because all 3 tumors gave a similar number of colonies, indistinguishable from wild-type FO-1 cells. Finally, all 3 tumors, T_1, T_2, and T_3, when reinjected into nude mice, grew very rapidly, with a latent period of only 4 days, like the original wild-type and sense cells (Table 2).

Because these experiments indicate that FO-1 melanoma cells stably transfected with a plasmid expressing the antisense to the IGF-1R can escape inhibition by losing the plasmid, we investigated the possibility that tumorigenesis by FO-1 melanoma cells could also

be inhibited by the use of antisense oligodeoxynucleotides to the IGF-1R RNA. Wild-type FO-1 cells preincubated with antisense oligodeoxynucleotides to the IGF-1R RNA did not produce tumors when injected s.c. into nude mice, while the same cells preincubated with sense oligodeoxynucleotides gave rise to tumors with the usual latent period of 4 days (Table 2).

Discussion

In this paper, we have shown that the important role of the IGF-1R in tumorigenesis extends also to a human tumor cell line, growing in nude mice, which is of primary interest in itself. However, other novel findings are: (a) the same antisense plasmid that inhibits growth in soft agar and in nude mice has very little effect on monolayer growth, indicating a dissociation between effect on cell proliferation and on transformation, a dissociation that could predict a differential effect of the antisense strategies on normal and tumor cells (see below); (b) when tumors arise in nude mice from cells expressing the antisense plasmid, it can be shown that the tumor cells have escaped inhibition because they have lost the antisense plasmid, and the number of receptors has now returned to normal. Incidentally, similar results have been obtained with C6 rat glioblastoma cells injected into nude mice;[4] and (c) inhibition of tumorigenesis in nude mice is also obtained with antisense oligodeoxynucleotides, whose delivery into animals, at present, offers fewer problems than the stable transfection in vivo of expression plasmids.

It should be emphasized that in every case, wild-type, sense, and antisense cells were treated exactly the same, and that although the number of nude mice tested may be small, the differences were dramatic. Combining the various groups, wild-type and sense cells gave tumors in 4 days in 12 of 12 mice, while the antisense cells gave tumors in 3 of 9 mice, with a 28-day latent period.

We have discussed above the evidence indicating that a decrease in the number of IGF-1Rs reproducibly causes an inhibition or even an abrogation of tumorigenicity. The reverse is also true; overexpression of the IGF-1R by itself can cause ligand-dependent transformation (4, 24-26). While there is no question that the IGF-1R plays an important role in transformation and tumorigenesis, very little is known about the mechanism(s). We would like to propose a hypothesis that takes into consideration our previous paper (7), the startling finding by Harrington et al. (27) that IGF-1 protects cells from c-myc-induced apoptosis, and the present data. These 3 sets of data are compatible with the following explanation: when tumor cells expressing an antisense RNA to the IGF-1R RNA are injected into syngeneic rats or nude mice, they undergo apoptosis in large numbers. In syngeneic rats, the few cells that survive are destroyed by immune mechanisms (7, 11, 12), while in nude mice, some of them may escape inhibition by eliminating the plasmid. Repeated injections of antisense oligodeoxynucleotides could obviate the problem of escaping cells, as suggested by our results. However, the most important question is whether the induction of apoptosis is limited to tumor cells or, at least, more extended in tumor cells than in normal cells. The fact that in the experiments of Harrington et al. (27) the protection from apoptosis by IGF-1 is exerted on cells induced to die by an overexpressed c-myc suggests that the effect of our antisense strategies may be more accentuated in oncogene-driven cells than in normal cells. However, only further experimentation in vivo will answer this question.

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