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Advances in Brief

Correlation between Apoptosis, Tumorigenesis, and Levels of Insulin-like Growth Factor I Receptors[1]

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Abstract

We have investigated whether there is a quantitative relationship between the insulin-like growth factor I receptor (IGF-IR), the extent of apoptosis *in vivo*, and tumorigenesis. C6 rat glioblastoma cells were treated with increasing concentrations of antisense oligodeoxynucleotides to the IGF-IR RNA. The extent of apoptosis *in vivo* is correlated to the decrease in IGF-IR levels and, in turn, tumorigenesis in nude mice is correlated to the fraction of surviving cells. In syngeneic rats, a host response leads to complete inhibition of tumorigenesis. These findings establish, for the first time on a quantitative basis, the relationship between IGF-IR levels and the extent of apoptosis, as well as the relationship between the initial apoptotic event and the time of appearance of transplantable tumors.

Introduction

Evidence is rapidly accumulating that the IGF-IR[3] activated by its ligands plays a crucial role in cell proliferation in at least three different ways: (*a*) it is mitogenic; (*b*) it is required in several types of cells for the establishment and maintenance of the transformed phenotype and for tumorigenesis; and (*c*) it protects tumor cells from apoptosis, both *in vitro* and *in vivo*. These three aspects of IGF-IR function and the appropriate references have been discussed at length in a recent review (1). It is also reasonable to assume that the inhibition of tumorigenesis caused by a loss of function of the IGF-IR (2–6) is, at least in part, related to the ability of the IGF-IR and its ligands to protect cells (especially transformed cells) from apoptosis (7–9).

In a previous paper, we reported a novel way to quantitate the extent of apoptosis *in vivo* by the use of a diffusion chamber that allows the flow in and out of nutrients and proteins but not of cells (9). In this and previous papers, we established that: (*a*) C6 rat glioblastoma cells (and other tumor cells) expressing an antisense RNA to the IGF-IR RNA or cells treated with antisense ODN to the same RNA do not grow in syngeneic animals, grow poorly in nude mice, and undergo massive apoptosis *in vivo* (3, 4, 9); (*b*) the effect is much more pronounced *in vivo* than *in vitro* (8, 9); and (*c*) the antisense strategy specifically targets the IGF-IR (3, 4, 9, 10).

In this article, we investigated whether tumorigenesis was quantitatively related to the extent of induced apoptosis and whether both of them were related to the decrease in the levels of IGF-IR caused by antisense strategies against the receptors. For such quantitation, we can only use antisense ODN to the IGF-IR RNA because expression plasmids are not suitable for this approach. We would like to emphasize, however, that the effects on transformation and apoptosis caused by a loss of function of the IGF-IR have been demonstrated in several different ways, including nonantisense strategies (see above), and that none of the effects observed with antisense ODN can be obtained with sense or randomly synthesized ODN (9).

Materials and Methods

The C6 rat glioblastoma cells used in these experiments, the diffusion chamber used for a quantitative determination of apoptosis, and the ODN sequences have been given in detail in the previous paper (9). The ODN were synthesized by LYNX Therapeutics, Inc. (Hayward, CA), and they do not contain a G-quartet or palindromic sequences. The evidence that the cells thus treated die by apoptosis in the diffusion chambers has also been given in the previous paper (9).

Cell Survival *in Vivo*. Cells (5×10^5) were placed in the diffusion chambers, which were then inserted into the s.c. tissue of 7-week old male BD IX rats (Charles River Breeders) under anesthesia with Halothane (inhalant). After 24 h *in vivo*, the cells were recovered from the chambers and counted using a hemocytometer. Viability was determined by trypan blue exclusion, but it has been omitted from the tables because most of the surviving cells (>95%) did not stain with trypan blue.

Growth Curves. Cells (5×10^4) were plated in 35-mm dishes in 10% serum for 4 h. They were then carefully washed and shifted to serum-free medium (9) supplemented with 10 ng/ml of insulin like growth factor I (Bachem, CA), with or without the ODN. The cells were counted 24 h after shifting to serum-free medium.

Tumorigenesis. C6 wild-type cells or C6 cells treated with ODN at the indicated concentrations for 24 h were trypsinized, washed, and resuspended in PBS. For tumorigenesis in nude mice, 10^5 cells (resuspended in 0.1 ml) were injected s.c. above the hind leg of 7-week old male Balb/c nude mice (Charles River Breeders). For tumorigenesis in syngeneic rats, 10^7 cells (resuspended in 0.2 ml) were injected s.c. above the hind leg of 7-week old male BD IX rats (Charles River Breeders). All animals were observed daily for tumor appearance, and they were sacrificed upon the development of bulky tumors.

Scatchard Analysis. This was performed by using standard methods with the modifications described previously (11).

Results

The purpose of these experiments was to determine whether there is a quantitative relationship among the following parameters: concentration of antisense ODN to the IGF-IR RNA, levels of IGF-IR, induction of apoptosis, tumorigenesis in nude mice, and tumorigenesis in syngeneic rats. We used C6 cells, which are syngeneic in BD IX rats. In all experiments, the cells were incubated for 24 h with the desired concentrations of ODN (the no-treatment cells were also incubated for 24 h but with no ODN), and the number of IGF-IRs was determined at this point. Under these conditions, the cells remain viable *in vitro* for at least 4 days; if the medium is replaced with growth medium, the cells resume growth.

The preincubated cells were tested in 3 different ways: (*a*) they were placed in diffusion chambers that were then implanted into the s.c. tissue of BD IX rats for 24 h to determine the extent of apoptosis

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[3] The abbreviations used are: IGF-IR, insulin-like growth factor I receptor; ODN, oligodeoxynucleotides.

(9); or (*b*) they were injected s.c. into nude mice; or (*c*) they were injected s.c. into syngeneic BD IX rats. We also determined the effect of antisense ODN to the IGF-IR RNA on the growth of cells *in vitro*.

Table 1 shows that there is a correlation between IGF-IR levels and survival of C6 cells *in vivo*. As the concentration of antisense ODN is increased, the number of receptors decreases and the extent of apoptosis increases. The data at the higher concentrations confirm the results reported previously (9). A sizable fraction of cells survives (at least for 24 h) up to a concentration of 2.5 μM of antisense ODN and then survival decreases very rapidly. Untreated cells or cells treated with a random ODN up to 19 μM doubled in number in the diffusion chambers, again confirming that the environment in the diffusion chamber is suitable for their optimal growth (9).

These results are in striking contrast to the effect of the same concentrations of antisense ODN on the growth of C6 cells *in vitro* (Fig. 1). Even at the highest concentration (19 μM), growth inhibition is modest, about 25%, whereas *in vivo*, >99% of cells have died (Table 1). We have omitted the lower concentrations in Fig. 1 because they did not produce any significant inhibition *in vitro*. These experiments confirm our previous report (9) that, with antisense strategies to the IGF-IR RNA, cells are much more sensitive *in vivo* than they are *in vitro*; *in vivo*, they undergo massive apoptosis, whereas *in vitro* one can detect only a modest inhibition of IGF-I-mediated growth.

If the extent of apoptosis *in vivo* is real, then increasing concentrations of antisense ODN should produce a proportional delay in the appearance of palpable tumors after s.c. injection. Because there is a host response to C6 cells treated with antisense strategies to the IGF-IR (Refs. 3, 4 and see below), we first determined tumorigenesis in nude mice (Table 2). There is a clear correlation between extent of antisense ODN-induced apoptosis (as determined in the diffusion chambers) and the time of appearance of palpable tumors in nude mice (Table 2, last column). In the third column, we have given the expected time of appearance of palpable tumors, based on the percentage of surviving cells. Actually, the tumors appeared somewhat later than the expected time of appearance. We can think of two possible explanations: (*a*) the extent of apoptosis is underestimated (we checked it at 24 h only); or (*b*) when only a few cells survive, they may grow more slowly in the first few days after injection. At any rate, tumorigenesis in nude mice is clearly correlated to the extent of *in vivo* apoptosis and not to the extent of *in vitro* inhibition of growth.

Finally, we tested the same cells in syngeneic BD IX rats. The results are summarized in Table 3. At the intermediate or higher concentrations of antisense oligos (from 3 to 19 μM), no tumors appeared after s.c. injection, confirming previous results (3, 9). At the lower concentrations (0.15–2.5 μM) tumors appeared with only 1 day of delay; surprisingly, however, in all these animals, the tumors began

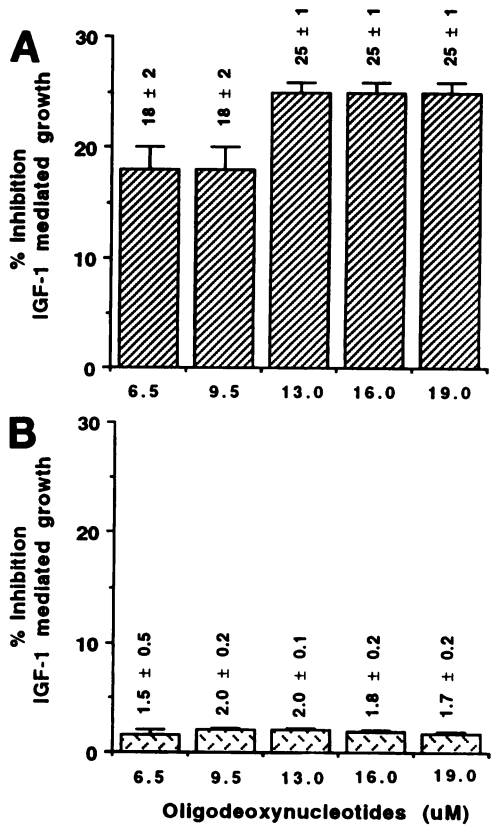

Fig. 1. Effect of antisense oligodeoxynucleotides to the IGF-I receptor on growth of C6 cells *in vitro*. C6 cells were treated *in vitro* with the desired concentrations of ODN, either antisense to the IGF-IR RNA (A) or random (B). The cells were grown in serum-free medium supplemented with insulin-like growth factor type I (IGF-1) (10 ng/ml); the percentage inhibition of growth is expressed in comparison to control cells (no oligos) and was determined 24 h after the beginning of treatment. *Colums*, mean; *bars*, SD.

Table 1 *Survival in vivo correlates with IGF-IR levels*

C6 cells were incubated *in vitro* for 24 h with the oligodeoxynucleotides at the indicated concentrations; they were then transferred to diffusion chambers, which were then implanted into the s.c. tissue of rats. The number of receptors was determined at the end of the *in vitro* incubation; the percentage of cells recovered was determined after 24 h *in vivo*.

Oligo concentration in μM	% of wild-type receptors[a]	% of recovery[b]
Control or random oligo[c]	100	>200
Antisense oligodeoxynucleotides		
0.15	77	54
0.80	85	48
1.50	80	35
2.50	82	35
3.0	56	1.3
6.5	56	0.4
9.5	56	0.4
13.0	42	0.010
16.0	43	0.008
19.0	36	0.001

[a] The number of IGF-IR receptors is expressed as the percentage of receptor levels, compared to untreated C6 cells, as determined by Scatchard analysis.

[b] The percentage of recovery is the fraction of cells recovered from the diffusion chambers after 24 h in the rat.

[c] The random ODN was assayed at different concentrations, the numbers shown here were obtained with 19 μM; *i.e.*, at the same concentration as the maximum antisense ODN concentration. Recovery, with untreated cells or cells treated with a random ODN varied between 200 and 225%.

Table 2 *Tumorigenesis in nude mice*

C6 cells were incubated *in vitro* for 24 h with the indicated ODN before injection into the s.c. tissue of nude mice. The concentration of AS ODN is in μM. Recovery expressed as in Table 1. Expected delay is the number of days after injection before the tumors become palpable, which is 4 days with untreated cells. The other expected delays are based on percentage of cells recovered in Table 1. The last column gives the actual number of days after injection, when tumors became palpable. There were two mice/point.

Treatment	% recovery	Expected delay (days)	Palpable tumors (days)
None or random ODN	>200	4	4
AS[a] 0.15 μM	54	6	6
AS 1.50 μM	35	7	11
AS 3.00 μM	1.3	11–12	17
AS 6.50 μM	0.5	13	17
AS 9.50 μM	0.4	13	17
AS 16.00 μM	0.010	19	24

[a] AS, antisense.

Table 3 *Tumorigenesis in syngeneic rats*

C6 cells were preincubated with ODN as in Tables 1 and 2, then injected s.c. into rats. The animals have been pooled into groups because there was little variation within groups. In the control, the tumors kept growing until they killed the animals. All the other animals are free of tumor, 2 months after the beginning of the experiment.

Treatment	% of IGF-IR	Tumorigenesis
None or random oligo	100	pos.[a] at day 4, kept growing
AS 0.15–2.5 μM	80	pos. at day 5, became smaller at day 19; complete regression by day 25
AS 3.0–9.5 μM	55–60	No tumors
AS 13.0–19.0 μM	30–45	No tumors

[a] pos., time of appearance of palpable tumors.

to get smaller at day 19, and by day 25, they had all regressed. No recurrence has been observed as of day 75. This last experiment confirms that a strong host response is elicited in syngeneic rats by C6 cells treated with antisense strategies to the IGF-IR RNA (3); this host response completely suppresses tumorigenesis when the fraction of surviving cells is about 1%; *i.e.*, 100,000 cells. When 30% or more cells survive, the tumors appear, but the host response eventually causes these tumors to regress.

Discussion

The novel findings in this communication are all based on their quantitative nature: (*a*) the extent of apoptosis *in vivo* is related to the decrease in the number of IGF-IRs; (*b*) tumorigenesis in nude mice is strictly dependent on the fraction of cells escaping apoptosis; (*c*) *in vitro*, the same decrease in the number of IGF-IRs causes minimum or no cell death and only a partial inhibition of growth; and (*d*) apoptosis induced by a decrease in the number of IGF-IRs also induces in syngeneic animals a host response that can eliminate the fraction of surviving cells.

The specificity of the antisense ODN used in these experiments has been repeatedly documented in previous papers (3, 9, 10, 12); they induce a concentration-dependent decrease in the number of IGF-IRs, whereas mismatched or random ODN have no effect at even very high concentrations. In addition, the inhibitory effect on tumorigenesis caused by interference with IGF-IR levels or function has been demonstrated by a variety of approaches [see "Introduction" and review by Baserga (1)]. In all our experiments, we have never been able to decrease the number of IGF-IRs below a 30% value of wild-type levels; we still have no explanation for it, although one could argue that, below that level, the cells would die even *in vitro*.

The extent of apoptosis *in vivo* (9) is not linearly correlated to the decrease in the percentage of IGF-IRs. Although the trend is obvious, there seems to be a sort of threshold, with apoptosis increasing sharply when the percentage of receptors falls to about 50% of untreated cells. This suggests a mechanism, production of apoptosis-inducing molecules, for which, however, we have yet no evidence.

Although it has been known for many years that cell death plays an important role in the growth of tumor cells (13–15), our present findings clearly show the strict correlation between the extent of induced apoptosis and the latent period before the tumors become palpable in nude mice. Even more important, tumorigenesis *in vivo* is dependent on induced apoptosis *in vivo* and not on growth inhibition *in vitro*, which raises some questions on the predictive accuracy of *in vitro* studies. Perhaps, by using *in vitro* assays we are missing agents or mechanisms that are very effective *in vivo*.

Finally, there is the host response elicited in the syngeneic rats, a response that can hardly be neglected because it leads to complete inhibition of tumorigenesis and even tumor regression. Because we have no compelling evidence on the nature of this host response, we simply call it host response, without prejudice as to its mechanism. Rats that have been given injections with C6 cells expressing an antisense RNA to the IGF-IR RNA not only did not develop tumors (3) but they were protected from a subsequent challenge with wild-type C6 cells for at least 3 months. It is hard to imagine cytokine-like molecules circulating in the animals for such long period of time, especially because the antisense cells are essentially all dead within 24 h. Two recent reports in the literature (16, 17) reveal cases in which synthetic ODN can induce a B-cell-mediated immune response. However, we can rule out this artifactual effect because the host response we observe can also be triggered with C6 cells transfected with an antisense expression plasmid. It should also be emphasized that all injections are carried out in the absence of serum. This host response, whatever is its mechanism, is a valuable addition to the apoptotic effect that is induced by a decrease in the number or function of the IGF-IR.

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