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Antisense inhibition of the p65 subunit of NF-κB blocks tumorigenicity and causes tumor regression

(adhesion/transcription factor/extracellular matrix/invasion/solid tumors)

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ABSTRACT **The NF-κB transcription factor, composed of two proteins, p50 and p65, is a pleiotropic activator that participates in the induction of a wide variety of cellular genes. Various cell adhesion molecules have NF-κB binding sites and may play an important role in inflammatory response, tumorigenicity, and metastasis. In an earlier study, we demonstrated that adhesion of diverse transformed cells was blocked by antisense inhibition of the p65 subunit of NF-κB. Since cell–substratum interactions play an important role in tumorigenicity, we reasoned that antisense p65 could inhibit tumorigenicity. In diverse transformed cell lines, phosphorothioate antisense oligonucleotides to p65 inhibited *in vitro* growth, reduced soft-agar colony formation, and eliminated the ability of cells to adhere to an extracellular matrix. Stable transfectants of a fibrosarcoma cell line expressing dexamethasone-inducible antisense RNA to p65 showed inhibition of *in vitro* growth and *in vivo* tumor development. In response to inducible expression of antisense RNA, a pronounced tumor regression was seen in nude mice. The administration of antisense but not sense p65 oligonucleotides caused a pronounced inhibition of tumorigenicity in nude mice injected with diverse tumor-derived cell lines. Inhibitors of NF-κB function may thus be useful in the treatment of cancer.**

Cell–cell and cell–substratum adhesion plays an important role in the regulation of normal and neoplastic cell growth (1). These adhesion events are mediated by diverse cell adhesion molecules (CAMs) and integrins (1, 2). Cell transformation is often associated with qualitative alteration in the integrin repertoire (3). The process of tumor progression is complex and requires malignant cells to modulate their adhesion properties at various points of tumor development (4). Inhibition of NF-κB function by antisense technology elicits a strong block in the adhesion of diverse cell types; if the p65 subunit is inhibited this effect can be observed in most cell types, but if the p50 subunit is inhibited the effect is dependent on the differentiative status of the cells (5). The cellular adhesion of differentiated HL-60 cells stimulated by phorbol 12-myristate 13-acetate is significantly altered, an effect associated with a marked reduction in CD 11b integrin expression (6). These results suggested to us that antisense inhibition of p65 function could have profound effects on cellular adhesion.

Utilizing diverse tumor-derived cell lines, we demonstrate a pronounced inhibition of adhesion and *in vitro* growth after treatment with p65 antisense oligonucleotides. Expression of dexamethasone (Dex)-inducible antisense RNA to p65 in a fibrosarcoma cell line inhibited tumorigenicity and caused regression of tumors in nude mice in a Dex-dependent manner. Based on these results, we have attempted to establish the *in vivo* therapeutic efficacy of antisense p65 oligonucleotides utilizing nude mouse tumor models.

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MATERIALS AND METHODS

Antisense Oligonucleotides. The antisense and sense phosphorothioate analogs of oligonucleotides to the 5′ end of the different subunits of NF-κB, including the ATG initiation codon (18- to 24-mer), were synthesized using an automated synthesizer (model 394, Applied Biosystems) as described (5, 6) following the procedure of Matsukura *et al.* (7).

Cell Lines and Growth Assay. The K-BALB, B-16, SW-480, HT-29, and T-47D cell lines were obtained from the American Type Culture Collection; Rat-1 *ras* (8), HOS-MNNG (9), and Rat-1 p65Δ (10) have been described. In four independent experiments, cells (3×10^6) were trypsinized and plated in the presence of 20 μM of species-specific sense or antisense p65 oligonucleotide in growth medium, and trypan blue-viable cells were counted on triplicate dishes 72 hr later. Soft-agar colony formation was measured by pretreating the cells with the oligonucleotide for 72 hr prior to seeding in 0.33% soft agar (10^3 cells per well for the murine cell lines and 10^5 cells per well for human cell lines) in quadruplicate wells in the presence of 20 μM oligonucleotide. Colonies were scored at the end of 7–14 days.

Adhesion Assays. Diverse transformed cell lines were tested for their ability to adhere to an extracellular matrix (ECM), fibronectin or laminin, in the presence of 20 μM of sense or antisense p65 oligonucleotide as described (5).

Tumorigenicity Assays. The parental K-BALB cells, two independent p65 sense clones, and six independent p65 antisense transfectants of K-BALB cells were tested in two independent nude mouse (BALB/c) assays as described (8) by injecting 5×10^6 cells per mouse into triplicate mice. The mice were fed with or without Dex (0.28 mg/liter) in drinking water (8), and tumor formation was monitored over 2–8 weeks. For tumor regression experiments, Dex was withheld from triplicate mice for 2–3 weeks and then supplied in drinking water over the next 3–6 weeks. Representative mice are shown. A total of 54 nude mice was used in this assay.

For the *in vivo* oligomer experiments, five nude mice were treated with sense or antisense p65 oligonucleotides in phosphate-buffered saline, either twice weekly by subcutaneous administration (1.4 mg) or continuously by means of a time-release, 14-day Alza pump containing 2.8 mg of oligo-

Abbreviations: Dex, dexamethasone; ECM, extracellular matrix; CAM, cell adhesion molecule; RT-PCR, reverse transcriptase polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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nucleotide. The tumor-derived cells (K-BALB or B-16) were injected (5×10^6 cells per mouse) on the contralateral side 72 hr later and the mice were observed over a 2- to 5-week period for tumors. In some experiments, the tumor cell lines were injected 72 hr before treatment was started. Tumors were surgically excised and tumor volume was measured. A total of 160 nude mice was used in this experiment.

Other Methods. RNA isolation, reverse transcriptase polymerase chain reaction (RT-PCR), and construction of Dex-inducible antisense RNA to p65 have been described (5, 8, 11). PCR primers for p65, p50, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been described (5, 6, 8). Gel mobility shift assays to resolve NF-κB complexes have been described (5).

RESULTS

Growth Inhibition of Tumor Cell Lines by p65 Antisense Oligonucleotides. We first investigated the effect of antisense p65 oligonucleotides on cellular growth (Table 1). A 40–60% inhibition of cell numbers as well as complete inhibition of soft-agar colony formation were observed in diverse adherent cell lines exposed to p65 antisense oligonucleotides. Similar inhibition of growth was seen in immortalized murine fibroblasts (Rat-1 and BALB/c 3T3 cells) and in primary cells (human umbilical vein endothelial cells) exposed to these oligonucleotides. In contrast, in suspension cells such as HL-60 (6), WEHI-3B, and Jurkat (J. A. Sokoloski, personal communication), growth was not inhibited. No effect on soft agar colony formation was seen in cells treated with sense p65, sense or antisense p50, or with control *jun-D* antisense oligonucleotides.

Block in Cell Adhesion to ECM by p65 Antisense Oligonucleotides. The mechanism of tumor cell growth, invasion, and metastasis involves complex changes in cell–cell and cell–substratum interactions (4, 12). Since the primary step of tumor cell invasion involves adhesion of tumor cells to the basement membrane substratum, we tested the ability of diverse tumor cell lines to adhere to a fibronectin matrix in the presence of 20 μM of phosphorothioate antisense oligonucleotide to p65. Adhesion to fibronectin (Fig. 1) was completely blocked by antisense oligonucleotides to p65, whereas the control sense p65 oligonucleotides had no effect. In the same experiments, we tested p50, c-*rel*, and several irrelevant oligonucleotides [including *jun-D*, IL-7R, IGF-I, *kit* ligand (KL), and GAPDH] in sense and antisense orientation; all of them failed to alter cell adhesion. Identical results were obtained using laminin as the ECM.

Inducible Antisense RNA to p65 and Tumorigenicity. We hypothesized that the block in adhesion of tumor cells by antisense to p65 would inhibit tumorigenicity *in vivo*. To test this, we first generated cell lines expressing Dex-inducible

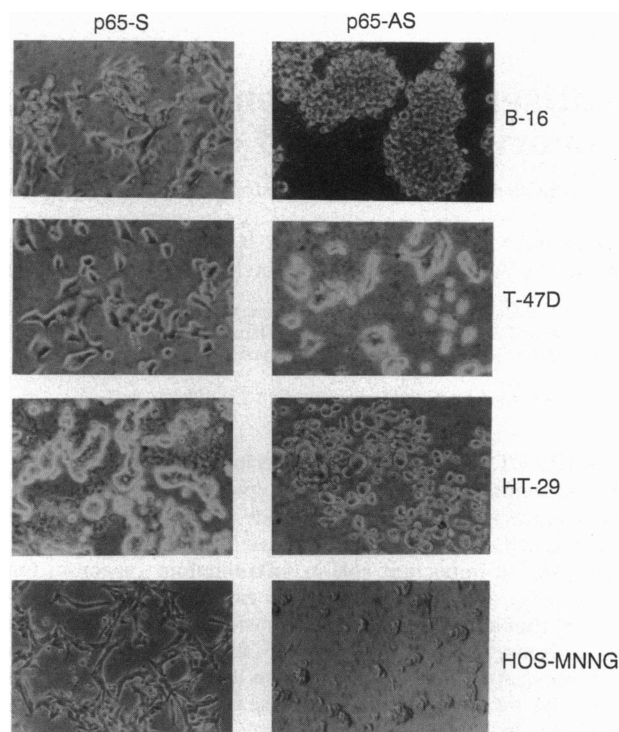

FIG. 1. Adhesion of transformed cells to ECM is blocked by antisense p65 oligonucleotides. Transformed cell lines, B-16 (melanoma), T-47D (breast carcinoma), HT-29 (colon carcinoma), and HOS-MNNG (a chemically transformed osteosarcoma), were tested for their ability to adhere to an ECM (fibronectin) in the presence of sense (S) or antisense (AS) p65 oligonucleotide. Representative results from four independent experiments are shown. (×37.5.)

antisense RNA to p65 (5) from a *ras*-transformed 3T3 cell line (K-BALB). Independent antisense clones but not control sense clones showed a Dex-dependent inhibition of p65 mRNA expression, as monitored by RT-PCR, and failed to form colonies in soft agar. Expression of k-*ras* mRNA was not inhibited in these antisense clones.

The control sense clones formed rapidly growing tumors at the injection site within 7–10 days and large tumors (Fig. 2*A*) developed within 2–4 weeks in the absence and presence of Dex. Dex treatment did not inhibit tumor formation in the control sense clone-injected mice but significantly inhibited tumor growth in the p65 antisense clones. The injection of several independent antisense clones into nude mice resulted in uniformly smaller tumor size, even in the absence of Dex, presumably due to low basal transcription from the mouse mammary tumor virus long terminal repeat (8).

Table 1. Inhibition of *in vitro* growth by antisense oligonucleotides to p65

Cell line	Cell source	Cell no. × 10^6		% inhibition	% soft-agar colony-forming units	
		p65-S	p65-AS		p65-S	p65-AS
K-BALB	Murine fibroblast	9.6	4.5	46.8	3.12	NS
Rat-1 *ras*	Murine fibroblast	8.8	5.0	56.8	ND	ND
Rat-1 p65Δ	Murine fibroblast	9.2	4.2	45.6	ND	ND
B-16	Murine melanoma	7.2	4.8	66.0	3.21	NS
SW-480	Human colon carcinoma	14.0	9.4	67.0	0.08	NS
HOS-MNNG	Human osteosarcoma	9.6	4.8	50.0	0.09	NS
MCF-7	Human breast carcinoma	13.0	7.0	53.8	ND	ND
T-47D	Human breast carcinoma	ND	ND	ND	0.06	NS

Growth of diverse transformed cell lines *in vitro* was measured in the presence of sense (S) or antisense (AS) oligonucleotides to p65. Representative results from one of three independent experiments are shown. ND, not done; NS, not significant (one to three cell clusters compared to 300–500 cell colonies in sense oligomer-treated cultures).

The ability to cause tumor regression by injecting antisense p65 into tumor-bearing nude mice was next investigated. Once palpable tumors had been established in the absence of Dex, the mice were fed with water containing Dex. Mice injected with the antisense p65 clone (Fig. 2*B*) showed a dramatic tumor regression within 1–2 weeks of Dex treatment. These antisense p65-injected mice were alive up to 4 months, whereas the sense p65-injected mice had tumors that continued to grow until the hosts' death in 4–6 weeks. In the same experiment neither the sense clones nor the parental K-BALB cell-injected mice showed any regression of tumor. As an additional control, transfectants of K-BALB cells expressing Dex-inducible antisense RNA to a constitutively expressed gene, *jun-D*, were tested in parallel tumorigenicity assays and did not show tumor inhibition or tumor regression.

Antisense Oligonucleotides to p65 Inhibit Tumorigenicity. The tumorigenicity results with antisense RNA to p65 encouraged us to test whether antisense p65 oligonucleotides could be used to treat tumors in nude mice. We used two modes of oligonucleotide delivery: a subcutaneous, twice-weekly regimen of 1.4 mg of oligonucleotide per injection and a time-release (14-day) Alza pump containing 2.8 mg of oligonucleotide. We chose two murine tumor cell lines: K-BALB (fibrosarcoma) and B-16 (melanoma) cells, both of which form aggressive tumors at the injection site. These tumors grew rapidly in mice treated with sense oligonucleotides by either delivery mode (Fig. 3*A*). In contrast, >70% of the antisense p65-treated mice showed a clear reduction in tumor size. Regardless of whether or not the mice were pretreated with the oligonucleotides prior to the injection of cells, similar inhibition of *in vivo* tumor growth was seen in antisense oligomer-treated mice. Irrelevant antisense oligonucleotides (GAPDH and *jun-D*) were used as control in some experiments; no effect on tumor size was observed. Identical results were obtained with a chemically transformed HOS-MNNG cell line using human p65 antisense oligonucleotides (data not shown). The expression of p65 mRNA was seen in B-16 melanomas excised from nude mice that had been treated with sense oligonucleotides but not in those from mice treated with antisense oligonucleotides (Fig. 3*B*). NF-κB-like binding activity was detected in the nuclear extracts of K-BALB and B-16 cells treated with the control p65 sense oligonucleotides, and this activity was competitively inhibited by a 30-fold molar excess of unlabeled double-stranded NF-κB consensus oligomer. In contrast, the complex was significantly inhibited in the p65 antisense oligomer-treated cells (Fig. 3*C*).

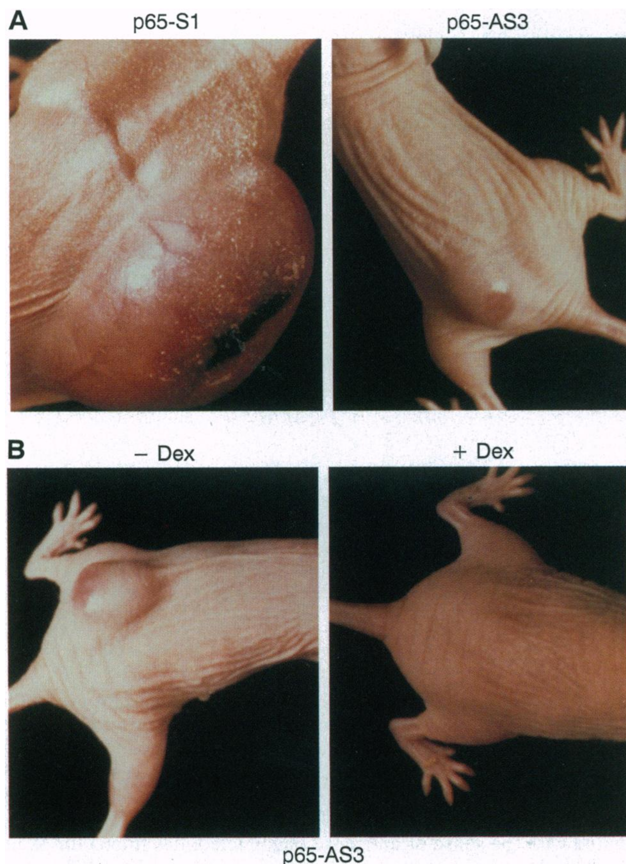

FIG. 2. Inducible expression of antisense RNA to NF-κB-p65 causes tumor inhibition and regression. (*A*) Tumors in nude mice injected with a sense (S1) or an antisense (AS3) clone in the presence of Dex after 6 weeks. (*B*) Regression of tumors by inducible antisense RNA to p65. A representative antisense p65 tumor-bearing mouse (clone 3) was observed in the absence of Dex (– Dex) for 2 weeks (left) and subsequently treated with Dex (+ Dex) for 2 weeks (right). (×0.15.)

DISCUSSION

The studies described herein were undertaken to explore the usefulness of antisense techniques in cancer therapeutics. The antisense approach involves the introduction into cells of polynucleotides complementary to mRNA (13–15). Diverse ECM molecules play a significant role in important cellular events such as adhesion, morphology, spreading, migration, differentiation, tumor cell metastasis, inflammation, and neurite outgrowth (1, 2, 16–19). Various cell surface receptors or CAMs are involved in these complex processes (16, 18, 20).

A variety of adhesion molecules such as ICAM-1 (21), ELAM-1 (22), and VCAM-1 (23) have been shown to have NF-κB binding sites, suggesting that NF-κB may be involved in the regulation of cell adhesion. Since adhesion plays an important role in tumor cell growth and tumor cell metastasis (1, 2, 12), we reasoned that inhibition of NF-κB p65 function should result in antitumor activity.

In diverse adherent transformed cell lines, adhesion to an ECM and cell growth were inhibited by treatment with p65 antisense oligonucleotides *in vitro*. Both forms of inhibition were also seen in similarly treated untransformed cells, suggesting that inhibition of adhesion and cell growth by antisense p65 is not specific to tumor cell lines. Interestingly, in diverse cultured suspension cells, growth was not inhibited by the p65 antisense oligonucleotides, implying that the differences between the growth requirement of adherent cells and that of nonadherent cells could be exploited for therapeutic intervention of solid tumors.

Generation of Dex-inducible antisense RNA to p65 in a *ras*-transformed fibrosarcoma cell line resulted in inhibition of *in vitro* growth and *in vivo* nude mouse tumorigenicity as well as in complete regression of established tumors. In contrast, antisense RNA to *jun-D*, a gene that is constitutively expressed in diverse cells (24), did not result in tumor inhibition or tumor regression, suggesting that the effects of antisense RNA to p65 on inhibition of tumor growth are highly specific.

Based on these results, we investigated the *in vivo* efficacy of phosphorothioate p65 antisense oligonucleotides in tumor models. Using a systemic route of administration (subcutaneous or a sustained time-release method), a significant reduction of tumor size was seen in fibrosarcoma- and melanoma-bearing nude mice. A similar reduction in tumor size was seen after treatment of nonimmune-compromised syngeneic mice with B-16 melanomas (data not shown). Significantly, there was no bone marrow suppression in these antisense oligomer-treated mice.

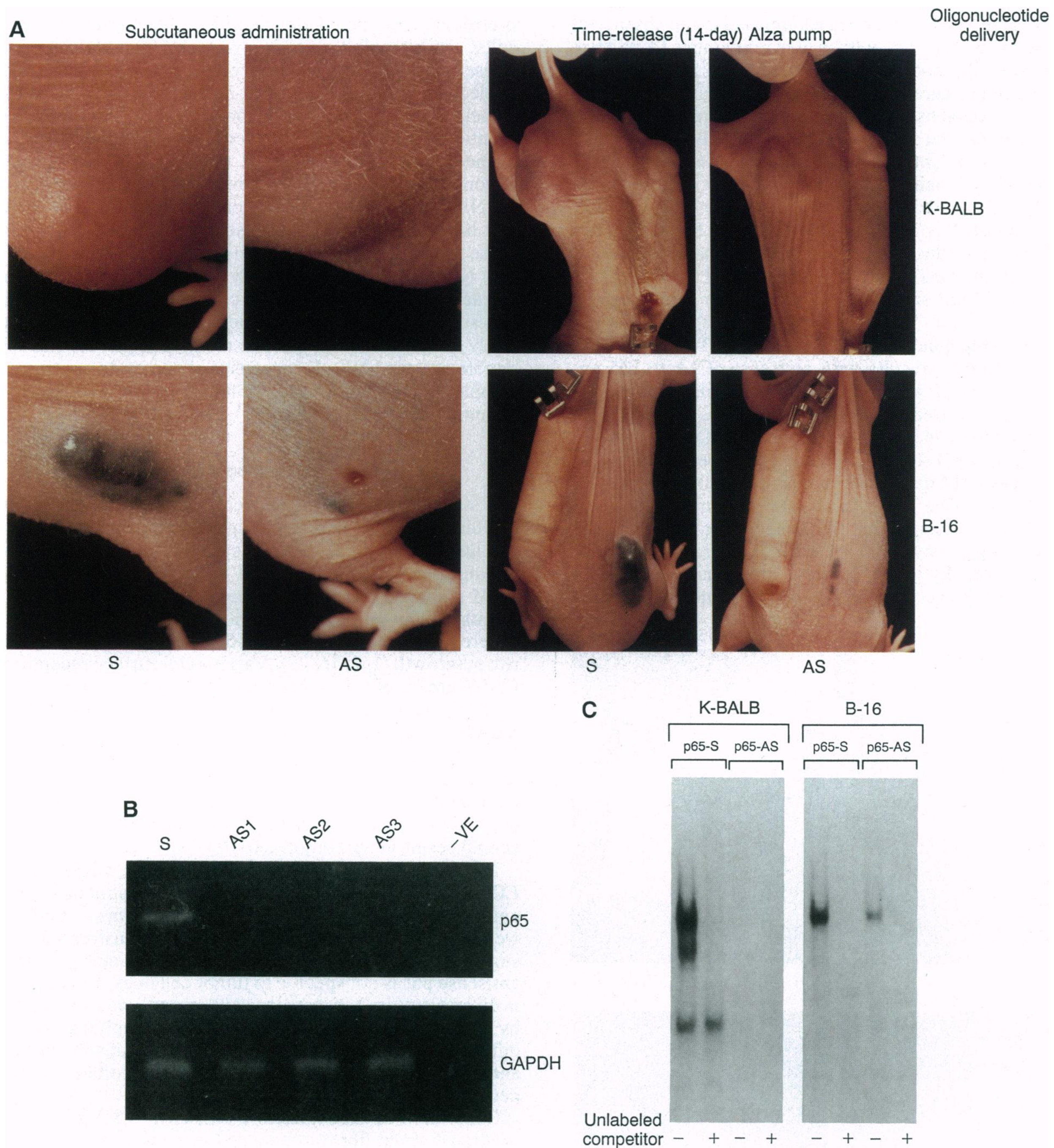

FIG. 3. Inhibition of tumorigenicity in diverse tumor models by antisense oligonucleotides to NF-κB-p65. Five nude mice were treated with sense (S) or antisense (AS) p65 oligonucleotides and injected with tumor-derived cells and observed for tumor formation. (*A*) Representative mice are shown from one of four independent experiments performed with separate preparations of oligonucleotides. Due to the preliminary nature of these series of experiments, all mice were sacrificed at 4–5 weeks. [×0.56 (subcutaneous) and ×0.14 (pump).] (*B*) RNA was isolated from nude mouse tumors obtained by injection with B-16 melanoma cells, following infusion with sense (n = 1) or antisense (n = 3) p65 oligonucleotides for 16 days. Expression of p65 or GAPDH mRNA in these tumors was measured by RT-PCR as described (5). -VE, negative, template minus control. (*C*) Nuclear extracts from K-BALB or B-16 cells treated with the sense or antisense oligonucleotides to p65 for 48 hr were prepared. A ^{32}P-labeled oligonucleotide corresponding to a consensus κB binding site was used as a probe in a gel mobility shift assay in the presence (+) or absence (−) of a 30-fold molar excess of the unlabeled κB oligonucleotide.

How does antisense p65 contribute to inhibition of tumorigenicity and regression of established tumors? We have no *in vivo* evidence that a block of adhesion is responsible for this effect, but in view of our *in vitro* results, it is tempting to speculate that the block of tumor cell growth could result from interference with adhesion mechanisms. The specific inhibition of CD 11b integrin expression in HL-60 cells by p65 antisense oligonucleotides (6) supports this. By the same token, tumor cell invasion and metastasis, events that require cell adhesion, may also be blocked by antisense p65. It is not clear why p50 antisense had no effect on cell adhesion or growth. In the murine embryonic stem cell (ES) system (5)

and in HL-60 cells (6), p50 antisense oligonucleotides did inhibit expression of p50 mRNA, but phenotypic changes were observed only in differentiating ES cells among the many cell types studied (5). These results suggest that the NF-κB-p65 subunit exerts differential effects on cell growth by regulating a distinct set of genes.

Recently, Kitajima *et al.* (25) demonstrated the inhibition of transplanted human T-cell leukemia virus I (HTLV-I) Tax-transformed tumors in mice, utilizing similar p65 antisense oligonucleotides. Interestingly, Tax antisense oligonucleotides did not inhibit the growth of the tumor, suggesting that the p65 antisense effect may not be related to Tax. Indeed, in the present study we found that p65 antisense oligonucleotides did inhibit the growth of tumors that were free of HTLV-I infection and thus lacked the Tax gene. Our findings suggest a general role for the p65 subunit of NF-κB in the maintenance of cellular adhesion. The block of tumor cell adhesion by antisense p65 oligonucleotides that we demonstrate implies that CAMs that are regulated by the p65 subunit of NF-κB are likely to be involved in the ability of transformed cells to establish solid tumors. However, additional effects on growth factors or their receptors, or on other proteins involved in the expression of the tumor cell phenotype, cannot be excluded. For example, in K-BALB and B-16 cells, p65 antisense oligonucleotides inhibited expression of cytokines such as granulocyte/macrophage colony-stimulating factor and transforming growth factor β (data not shown). Our findings open a new approach to the study of tumor cell phenotypes and convey important clinical implications. They demonstrate that antisense oligonucleotides are potential therapeutic agents and that NF-κB antisense, in particular, may be a new drug for the treatment of solid tumors and perhaps other diseases.

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