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# Proliferation of human malignant melanomas is inhibited by antisense oligodeoxynucleotides targeted against basic fibroblast growth factor

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**Human malignant melanomas, unlike normal melanocytes, can proliferate in the absence of exogenous basic fibroblast growth factor (bFGF). Exposure of primary melanomas in the vertical growth phase and metastatic melanomas to antisense oligodeoxynucleotides targeted against three different sites of human bFGF mRNA inhibited cell proliferation and colony formation in soft-agar. In contrast, exposure of human bFGF sense or antisense oligonucleotides complementary to human β-nerve growth factor or insulin-like growth factor I mRNA had no such effects. These experiments indicate that activation of the bFGF gene may play an important role in the progression from melanocytic precursor lesions to malignant melanoma.**

*Key words:* antisense oligodeoxynucleotides/basic fibroblast growth factor/human malignant melanoma

## Introduction

Approximately 95% of familial malignant melanomas and 40% of sporadic melanomas arise from precursor lesions (Clark *et al.*, 1984, 1986; Clark, 1988). The three types of melanocytic lesions are: congenital, common acquired and dysplastic nevi. Congenital and common acquired nevi represent focal proliferations of normal human melanocytes. In contrast, dysplastic nevi consist of a heterogeneous population of normal melanocytes and melanocytes showing increased pigmentation, nuclear pleomorphism and mitotic atypia (Greene *et al.*, 1985). For this reason, melanocytes of dysplastic nevus are considered to represent precursor lesions of human malignant melanoma. Human melanoma can be classified into three stages: i) melanoma in the radial growth phase, ii) melanoma in the vertical growth phase and iii) metastatic melanoma. Primary melanoma in the radial and vertical growth phase and metastatic melanoma demonstrate significant biological, biochemical and karyotypic differences from normal human melanocytes and from melanocytic precursor lesions. For example, primary melanoma in the vertical growth phase and metastatic melanomas, unlike normal melanocytes, do not require the presence of phorbol esters for proliferation *in vitro*, display anchorage-independent growth *in vitro*, form tumors in nude mice (Herlyn *et al.*, 1985, 1987) and demonstrate severe karyotypic abnormalities (Balaban *et al.*, 1984, 1986). Most importantly, however, metastatic melanoma cells can grow in protein-free medium (Rodeck *et al.*, 1987) whereas normal melanocytes require exogenous basic fibroblast growth factor (bFGF) for proliferation *in vitro* (Halaban *et al.*, 1987, 1988a; Herlyn *et al.*, 1988), suggesting that tumor progression in the melanocytic system is characterized by increasing independence from exogenous bFGF.

Human bFGF cDNA clones were first isolated and characterized by Abraham *et al.* (1986a). The coding region of the human bFGF gene is interrupted by two introns and the estimated size of the gene represents at least 34 kb (Abraham *et al.*, 1986a). In the case of primary human melanomas in the vertical growth phase and metastatic melanomas, three bFGF-specific mRNA transcripts of 7.0, 3.7 and 1.2 kb were detected (H.Menssen, U.Rodeck, L.Otwos, M.L.Mancianti and M.Herlyn, manuscript submitted). bFGF shares 55% homology with acidic FGF (Abrahams *et al.*, 1986a) and between 40 and 50% homology with oncogene *hst(K-fgf)* (Sakamoto *et al.*, 1986; Taira *et al.*, 1987; Delli-Bovi and Basilico, 1987; Delli-Bovi *et al.*, 1987, 1988), the *FGF-5* gene (Zhan *et al.*, 1987, 1988) and the *int-2* gene (Moore *et al.*, 1986). bFGF is synthesized as a 155 amino acid protein (Abraham *et al.*, 1986a), which as recently shown by Feige and Baird (1989), can serve as a substrate for protein phosphorylation.

To determine the role of bFGF in regulating melanoma development, we studied the effect of antisense oligodeoxynucleotides complementary to three different regions of human bFGF mRNA on primary melanomas in the vertical growth phase and on metastatic melanomas. In an attempt to bypass cell surface-mediated events and directly influence the expression of a given gene of interest, application of antisense oligodeoxynucleotides has proved to represent a powerful tool. For example, Zamecnik *et al.* (1986) and Goodchild *et al.* (1988) demonstrated inhibition of HIV replication upon addition of antisense oligomers targeted against viral mRNA. Studies by Wickstrom *et al.* (1988) and Holt *et al.* (1988) demonstrated inhibition of HL60 cell proliferation and c-*myc* expression following addition of antisense oligomers directed against human c-*myc* mRNA. Oligomer complementary to c-*myb*-encoded mRNA was recently shown to inhibit normal human hematopoiesis *in vitro* (Gewirtz and Calabretta, 1988) and proliferation of human myeloid leukemia cell lines (Anfossi *et al.*, 1989).

In the present study, we demonstrate inhibition of proliferation of primary human melanoma in the vertical growth phase and metastatic melanoma upon addition of antisense oligodeoxynucleotides complementary to the translation start site and two different splice donor–acceptor sites of human bFGF.

## Results and Discussion

Unmodified, 15 bp oligodeoxynucleotides complementary to three sites of the sense or antisense strands of human bFGF mRNA were synthesized. The first 15mer antisense

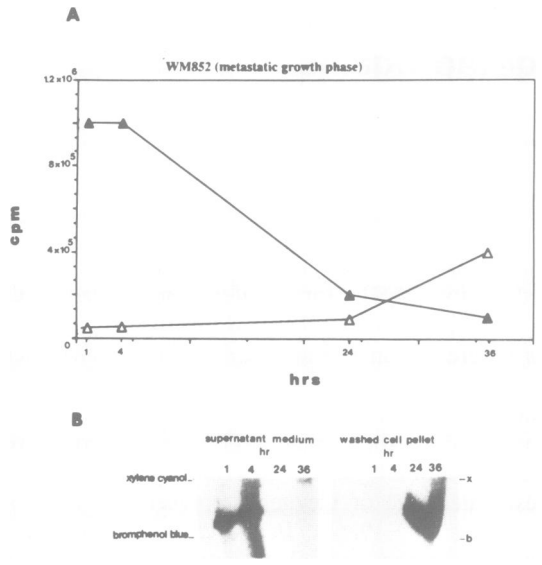


**Fig. 1.** (**A**) Kinetics of bFGF-specific oligomer uptake. $^{32}P$ 5′ end labeled $1^0$splice-junction-specific antisense oligomer (5′-TAG CTT GAT GTG AGG-3′) was added to WM852 metastatic melanoma cells and incubated with the cells for 1, 6, 24 and 36 h. Oligomer uptake was determined by liquid scintillation counting of the supernatant media -▲- and the aqueous phases of the cell extracts -△-. (**B**) Stability of bFGF-specific $1^0$splice-junction oligomer upon incubation with WM852 melanoma cells. Aliquots representing equal numbers of c.p.m. of the supernatant media and the aqueous phases of the cell extracts at 1, 6, 24 and 36 h were lyophilized and electrophoresed in a 20% denaturing polyacrylamide gel. The size of DNA fragments comigrating with xylene cyanol (x) and bromophenol blue (b) in a 20% denaturing polyacrylamide gel are ~28 and 8 nucleotides respectively.

oligomer (5′-GGC TGC CAT GGT CCC-3′) was directed against the translation start site (AUG codon) (Abraham *et al.*, 1986a) and surrounding nucleotides. The second antisense oligomer (5′-TAG CTT GAT GTG AGG-3′) was complementary to codon 60, the first splice donor−acceptor site (Abraham *et al.*, 1986a), and is referred to as $1^0$splice-junction oligomer. The third antisense oligomer (5′-ACA TTT AGA AGC CAG-3′) was targeted against codons 94 and 95, representing the second splice donor−acceptor site (Abraham *et al.*, 1986a) ($2^0$splice-junction oligomer). Although the human bFGF gene shares ~50% homology with acidic FGF (Abraham *et al.*, 1986a) and the genes *hst(K-fgf)* (Sakamoto *et al.*, 1986; Taira *et al.*, 1987; Delli-Bovi and Basilico, 1987; Delli-Bovi *et al.*, 1987, 1988), *FGF-5* (Zhan *et al.*, 1987, 1988) and *int-2* (Moore *et al.*, 1986), the sequence of the three different human bFGF specific antisense oligomers was sufficiently divergent from the sequence of each of these genes to prevent duplex formation with their respective mRNAs.

The four cell lines used in the present study were isolated from melanomas WM75 (primary melanoma in the vertical growth phase) (Herlyn *et al.*, 1985), WM983-A (primary melanoma in the vertical growth phase) (R.Kath, U.Rodeck, A.Parmiter, J.Jambrosie and M.Herlyn, manuscript submitted), WM983-B, a metastatic melanoma removed from the same patient as melanoma WM983-A (Kath *et al.*, manuscript submitted), and a second human metastatic melanoma (WM852) (Herlyn *et al.*, 1985).

Northern blot analysis demonstrated bFGF gene expression in these cell lines, as indicated by the presence

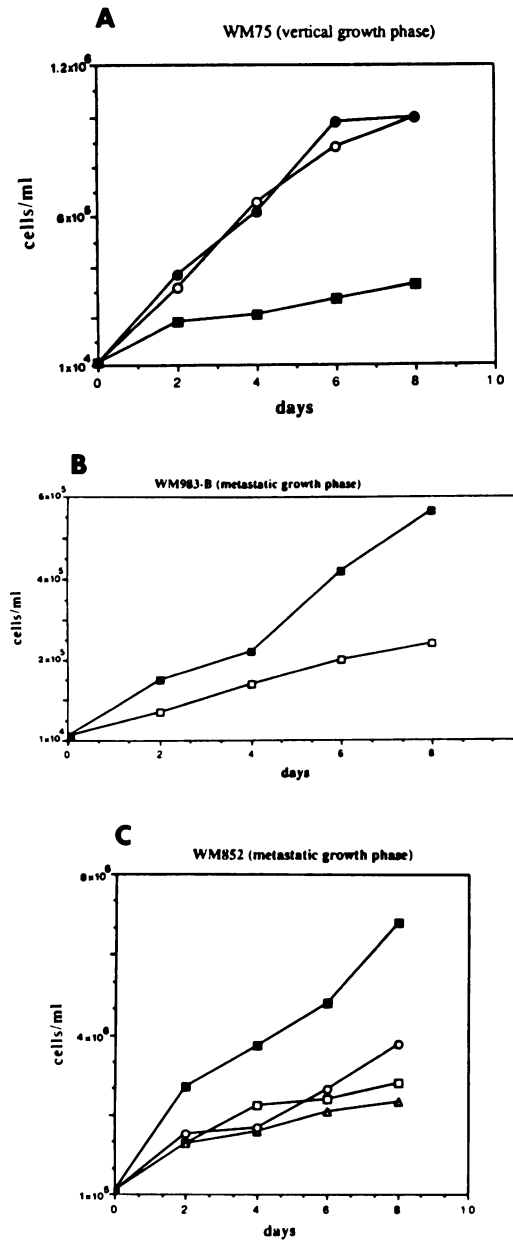


**Fig. 2.** Inhibition of melanoma cell proliferation upon addition of bFGF-specific antisense oligomers. (**A**) WM75 cells were plated at a density of $10^4$ cells/microtiter well in medium containing 5% serum. After 24 h, the medium was changed and sense and antisense oligomers were added at 50 μM/microtiter well. Cell counts were performed over 8 days, with two replicate cultures for each time point. Symbols: -○- no oligomer added; -●- sense oligomer, AUG codon (5′-GGG ACC ATG GCA GCC-3′); -■- antisense oligomer, $1^0$splice-junction (5′-TAG CTT GAT GTG AGG-3′). (**B**) WM983-B cells were plated, and oligomers were added as described in (A). Symbols: -■- sense oligomer, $2^0$splice-junction (5′-CTG GCT TCT AAA TGT-3′); -□- antisense oligomer, $2^0$splice-junction (5′-ACA TTT AGA AGC CAG-3′). (**C**) WM852 cells were plated at a density of $10^5$ cells/microtiter well and incubated in protein-free medium. Oligomers were added at 50 μM/microtiter well 24 h later. Symbols: -■- sense oligomer, $1^0$splice-junction (5′-CGT CAC ATC AAG CTA-3′); -□- antisense oligomer, $1^0$splice-junction; -△- antisense oligomer, AUG codon (5′-GGC TGC CAT GGT CCC-3′).

of 7.0, 3.7 and 1.2 kb mRNA transcripts (Menssen *et al.*, manuscript submitted) detected upon hybridization of the melanoma mRNAs to a 1.4 kb bovine bFGF-specific cDNA probe (Abraham *et al.*, 1986b). In contrast, Halaban *et al.*

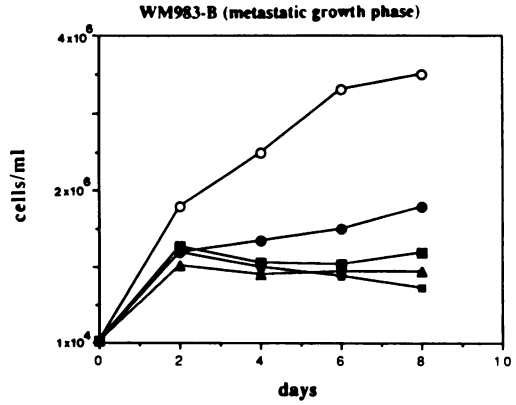


**Fig. 3.** Dose–response curve of bFGF-specific antisense oligomer. WM983-B cells were plated at a density of $10^4$ cells/microtiter plate. After 24 h 10 -●-, 25 -■-, 50 -▲- and 75 -■- μM of $1^0$splice-junction-specific antisense oligomer was added to the cells with two replicate cultures for each time point. Control cultures -○- did not receive oligomer.

(1988b) demonstrated that bFGF mRNA transcripts and bFGF immunoprecipitable protein cannot be detected in normal human melanocytes suggesting that the lack of bFGF production is at the level of transcription.

### *Kinetics of bFGF-specific oligomer uptake by human melanoma cells*

To first determine whether oligomers complementary to bFGF mRNA were efficiently taken up by melanoma cells *in vitro*, purified 5′ end labeled antisense oligomer directed against the $1^0$splice-junction was added to WM983-A and WM852 cells ($1.2 \times 10^6$ c.p.m./$10^6$ cells/60 mm plate) grown in 5% fetal bovine serum, heat inactivated at 65°C to destroy nuclease activity. Oligomer uptake was determined by liquid scintillation counting of the supernatant media and the cell extract aqueous phases over 36 h. Figure 1A shows a representative example of the kinetics of oligomer uptake. Little antisense oligomer was associated with individual extracts of WM852 cells within 24 h of its addition. More than 30% of the initial radioactivity added to the cells was detected between 24 and 36 h in the cell extract aqueous phases suggesting that the uptake of oligomers by human melanoma cells represents a rather slow process. To determine bFGF-specific oligomer stability equal numbers of c.p.m. ($5 \times 10^3$ c.p.m./lane) representing the supernatant media and the aqueous phases representing the cell pellets were lyophilized and electrophoresed in a 20% denaturing gel. As shown in an autoradiograph depicted in Figure 1B, the 5′ end labeled bFGF-specific antisense oligomer was not degraded even after 36 h of incubation with WM852 cells. The finding that oligomers are stable for a significant period of time upon incubation with cells was also reported by Holt *et al.* (1988) who observed the presence of full-length c-*myc* oligomer 5 days after addition to the medium of HL60 cells.

### *Oligomers complementary to human bFGF are growth inhibitory to human malignant melanomas*

To assess the effects of bFGF-specific sense and antisense oligomers, WM75 and WM983-B cells were plated in medium containing 5% fetal bovine serum at a density of $10^4$ cells/microtiter well in 24 well microtiter plates. After 24 h, at which point the cells had reached 30–40% confluency, single doses of 50 μM bFGF-specific sense or antisense oligomers were added. On days 2, 4, 6 and 8 following addition of the oligomers, the cells were trypsinized, resuspended in 1.00 ml of medium, assayed for viability by trypan blue exclusion and counted. Regardless of whether incubation took place in the presence of sense or antisense oligomers, the cells demonstrated 98–100% viability and no signs of morphological changes. Furthermore, in neither of the experiments described here, did the sense, antisense or untreated melanoma cells reach confluency on day 8 of the incubation, thereby ruling out the possibility that the results obtained were due to having measured confluency rather than antisense oligomer induced inhibition of cell proliferation.

As shown in Figure 2A, WM75 cells grown in the presence of sense oligomer specific for the initiation codon (5′-GGG ACC ATG GCA GCC-3′) grew from $10^4$ to $\sim 10^6$ cells/ml which was comparable to the number of cells counted on day 8 which had not received oligomers. In comparison, WM75 cells grown in the presence of bFGF-specific antisense oligomer complementary to the $1^0$splice-junction, revealed ~60% growth inhibition measured on day 8. Similar results (data not shown) were obtained in case of WM983-A, a primary melanoma in the vertical growth phase.

Figure 2B demonstrates the growth inhibitory effects of 50 μM $2^0$splice-junction-specific antisense oligomer on the metastatic melanoma cell line, WM983-B. By days 6 and 8, the extent of growth inhibition in the presence of antisense compared with sense oligomer (5′-CTG GCT TCT AAA TGT-3′) represented 50–70%. Addition of the same $2^0$splice-junction-specific sense and antisense oligomers to WM983-A cells resulted in a similar extent of growth inhibition (data not shown).

Since it is one of the characteristics of metastatic melanoma cells to proliferate in the absence of serum without demonstrating any signs of morphological changes (Rodeck *et al.*, 1987), WM852 and WM983-B cells were adapted to growth in protein-free medium over a period of 5 weeks. At the end of 5 weeks, WM852 and WM983-B were plated at a density of $10^5$ cells/microtiter well followed 24 h later by the addition of 50 μM $1^0$splice-junction-specific sense oligomer (5′-CCT CAC ATC AAG CTA-3′) or 50 μM of each of the three bFGF-specific antisense oligomers. The results demonstrated between 50 and 75% inhibition of proliferation of WM852 (Figure 2C) and WM983-B cells (data not shown) in the presence of each of the three antisense oligomers compared with the $1^0$-splice-junction-specific sense oligomer.

The dose of 50 μM of bFGF-specific antisense oligomers was chosen for the studies presented here based upon the result of a dose–response curve (Figure 3), which revealed that incubation of WM983-B cells in the presence of 10 μM $1^0$splice-junction-specific antisense oligomer compared with 50 μM $1^0$splice-junction-specific antisense oligomer showed ~50% less growth inhibition. On the other hand, addition of 75 μM in comparison with 50 μM bFGF-specific $1^0$splice-junction antisense oligomer did not demonstrate a significantly higher degree of inhibition of proliferation.

The extent of growth inhibition of 50–75% upon addition of bFGF-specific antisense oligomers to human malignant melanomas, compared to untreated or bFGF-specific sense

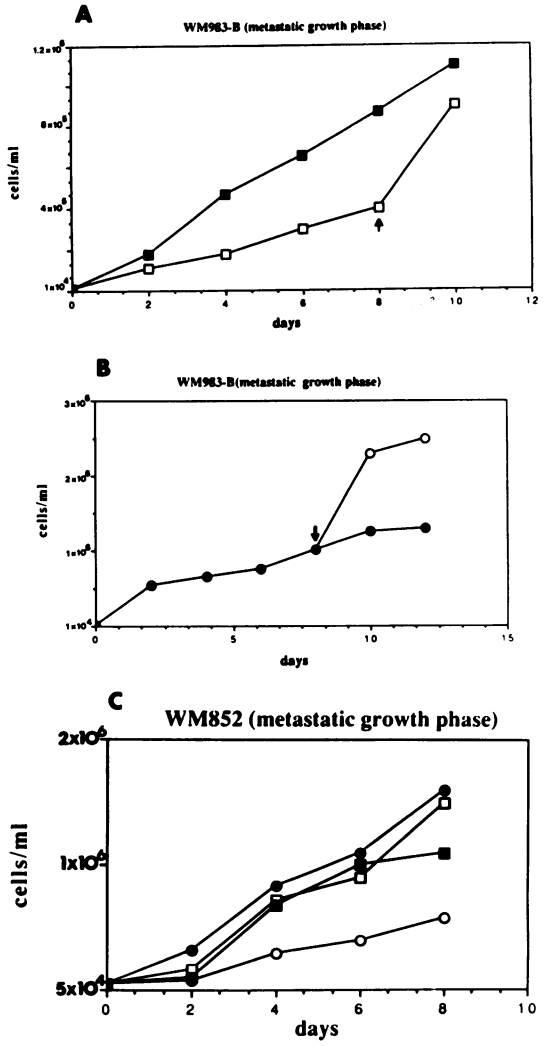


**Fig. 4.** (**A**) Inhibition of proliferation of human melanomas by bFGF-specific antisense oligomers is reversible. Replicate cultures of WM983-B cells ($10^4$ cells/microtiter well) were incubated in the -■- absence of oligomer or in the -□- presence of 50 $\mu$M $1^0$splice-junction-specific oligomer for 8 days. On day 8, the two melanoma cell cultures were rinsed three times with medium and incubated for an additional period of 48 h in the presence of medium containing 5% serum but no oligomer. Growth curves were established as described in Figure 2. (**B**) Two sets of replicate cultures of WM983-B cells ($10^4$ cells/microtiter well) were cultured for 8 days in the presence of 50 $\mu$M bFGF-specific antisense oligomer targeted against the $1^0$splice-junction -●-. On day 8, 3-fold excess (150 $\mu$M) $1^0$splice-junction-specific sense oligomer was added to half of the cell cultures -○-, whereas the other half did not receive additional oligomer -●-. WM983-B cells were cultured for another 2 days and growth curves were established as described. (**C**) Exogenous bovine bFGF functions as a 'competitor' for antisense oligomer-induced inhibition of proliferation. WM852 cells adapted to growth in protein-free medium were plated at a density of 5 × $10^4$ cells/microtiter well and incubated for 8 days in the absence of oligomer -□-, in the presence of -●- 40 ng/ml of bovine bFGF, in the presence of 50 $\mu$M antisense oligomer directed against the $1^0$splice-junction -○-, or in the presence of 50 $\mu$M $1^0$splice-junction-specific antisense oligomer and 40 ng/ml of bovine bFGF -■-.

oligomer treated melanoma cells, was similar to the decrease in the growth rate reported for HL60 cells upon incubation with c-*myc* antisense oligomer (Holt *et al.*, 1988) or upon addition of c-*myb* antisense oligomer (Anfossi *et al.*, 1989).

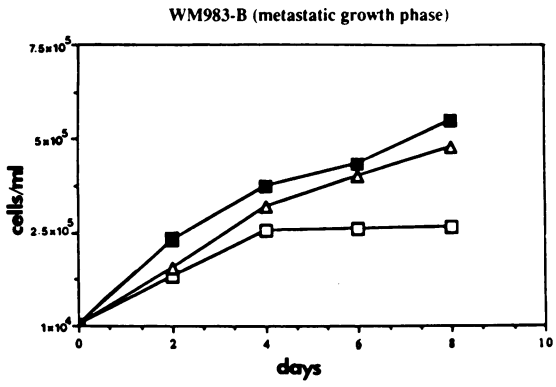


**Fig. 5.** bFGF-specific antisense oligomer induced inhibition of melanoma cell proliferation demonstrates sequence specificity. Oligomers were added at 50 $\mu$M/microtiter well to WM983-B cells grown in medium containing 5% serum. The growth curves were performed as described in Figure 2, with replicate cultures for each time point. Symbols: -■- sense oligomer directed against the $1^0$splice-junction (5′-CCT CAC ATC AAG CTA-3′); -△- 5 bp mismatched antisense oligomer, $1^0$splice-junction (5′-TAG CTT ATG TGG AGG-3′); -□- antisense oligomer, $1^0$splice-junction (5′-TAG CTT GAT GTG AGG-3′).

### *Antisense oligomer effects on melanoma cell proliferation are reversible*

Assuming that the mechanism responsible for the observed inhibition of proliferation of human malignant melanomas is due to duplex formation between intracellular bFGF mRNA and the corresponding antisense oligomers, one would predict that antisense oligomer effects are reversible. Two independent experiments were designed to provide experimental evidence for this hypothesis.

WM983-B cells were incubated in the presence of 50 $\mu$M antisense oligomer targeted against the $1^0$splice-junction for 8 days, which led to 70% growth inhibition compared to untreated WM983-B cells (Figure 4A). On day 8, the medium was either replaced with fresh medium containing no oligomer (Figure 4A) or as a separate experiment (Figure 4B), 3-fold (150 $\mu$M) excess of $1^0$splice-junction-specific sense oligomer was added to the cells without a medium change. Replacement with medium containing no oligomer, as well as hybridization competition with sense oligomer led to complete reversal of growth inhibition within 48 h (Figure 4A and B). Reversible antisense oligomer induced inhibition of proliferation has also been demonstrated for HL60 cells incubated in the presence of c-*myc* specific antisense oligomers (Holt *et al.*, 1988) and for BALB/c3T3 cells incubated in the presence of antisense oligomers complementary to PCNA, a nuclear protein representing a cofactor of DNA polymerase $\delta$ (Jaskulski *et al.*, 1988).

Given the context of the present study, another important aspect to investigate was the possibility that bFGF-specific antisense oligomer induced inhibition of proliferation of human melanomas would be abolished in the presence of exogenous bFGF. To test this hypothesis, WM852 cells adapted to growth in protein-free medium were plated at a density of 5 × $10^4$ cells/microtiter well. After 24 h, the cells received either no oligomer, no oligomer but 40 ng/ml exogenous bovine bFGF, 50 $\mu$M bFGF-specific antisense oligomer directed against the $1^0$splice-junction or the same antisense oligomer together with 40 ng/ml of bovine bFGF.

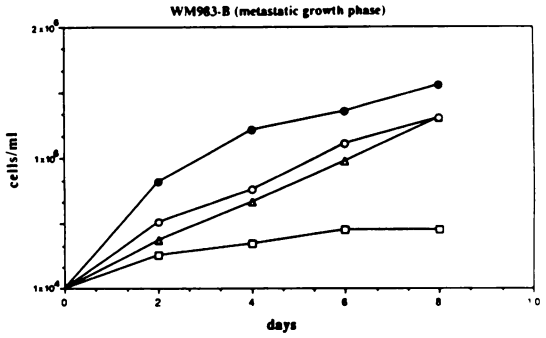


**Fig. 6.** Antisense oligomers targeted against human β-NGF and IGF-I mRNA have little growth inhibitory effect on melanoma cell proliferation. Oligomers were added at 50 μM/microtiter well to WM983-B cells grown in medium containing 5% serum. The growth curves were performed as described in Figure 2. Symbols: -●- no oligomer; -○- antisense oligomer directed against human IGF-I mRNA (5′-CAT CTT CAC CTT CAA-3′); -△- antisense oligomer complementary to human β-NGF mRNA (5′-ATG CAC CTC ACT GCG-3′); -□- bFGF-specific antisense oligomer targeted against the $1^0$splice-junction.

The data presented in Figure 4C revealed two interesting findings. Firstly, the growth rate of WM852 cells incubated in the presence of exogenous bFGF was not significantly elevated compared with the growth rate of WM852 cells cultured in protein-free medium, thus supporting the hypothesis that human malignant melanomas synthesize sufficient quantitities of bFGF required for their proliferation. Secondly, WM852 cells grown in the presence of bFGF-specific antisense oligomer and exogenous bFGF reached similar cell densities on days 2, 4 and 6 as untreated WM852 cells, thus indicating that exogenous bFGF functioned as a 'competitor' for antisense oligomer-inducible inhibition of proliferation.

### *'Mismatched' bFGF antisense oligomer and antisense oligomers unrelated to bFGF have few growth inhibitory effects*

To address the aspect of the specificity of inhibition of the human bFGF gene, a 15mer antisense oligomer complementary to the $1^0$splice-junction with ten identical and five mismatched base compositions (5′-TAG CTT ATG TGG AGG-3′) produced no apparent inhibition of cellular growth when added to WM983-B metastatic melanoma cells at a concentration of 50 μM (Figure 5), thus indicating that the specific hybrid formation between bFGF-specific antisense oligomers and endogenous bFGF mRNA was responsible for the inhibition.

Ross *et al.* (1984) demonstrated the expression of nerve growth factor receptor on melanoma and dysplastic nevus cells, as Peacocke *et al.* (1988) did on normal human melanocytes upon the addition of the tumor promoter 12-*O*-tetradecanoyl phobol-13-acetate (TPA) to the culture medium. Furthermore, recent investigations (Rodeck *et al.*, 1987) indicated that insulin-like growth factor I (IGF-I) and insulin represent major growth factors for melanomas. To determine whether the synthesis of human β-nerve growth factor (β-NGF) and/or IGF-I by human malignant melanomas is as important for their proliferation as the production of bFGF, 15mer antisense oligomers targeted against the splice donor−acceptor site of human β-NGF (residues −166/−125) (5′-ATG CAC CTC ACT GCG-3′) (Ullrich *et al.*, 1983) and human IGF-I (first exon−intron) (5′-CAT CTT CAC CTT CAA-3′) (Bell *et al.*, 1985) were added to WM983-B cells. As compared with untreated cells, no significant growth inhibition was observed in the presence of either of the two bFGF-unrelated antisense oligomers over a period of 8 days (Figure 6). On the other hand, the $1^0$splice-junction bFGF-specific antisense oligomer inhibited the proliferation of WM983-B cells by 70% in comparison with WM983-B cells which did not receive oligomers (Figure 6). These results therefore suggest that the proliferation of human malignant melanomas is particularly dependent upon the autocrine production of bFGF rather than the synthesis of human β-NGF or IGF-I.

**Table I.** Inhibition of anchorage-independent growth by antisense oligomers directed against human bFGF mRNA

| WM983-B (metastatic growth phase) | | | | |
|---|---|---|---|---|
| Cell type ($5 \times 10^3$) | Oligodeoxynucleotide 25 μM | Number of soft agar colonies counted on day 14 | | |
| | | Dish 1 | 2 | mean |
| WM983-B | – | 35 | 27 | 31 |
| WM983-B | sense $1^0$splice-junction | 28 | 13 | 21 |
| WM983-B | antisense AUG codon | 8 | 2 | 5 |
| WM983-B | antisense $1^0$splice-junction | 4 | 5 | 5 |
| WM983-B | antisense $2^0$splice-junction | 4 | 4 | 4 |

Soft agar colony forming efficiency of human melanomas in the presence of bFGF-specific oligomers. $5 \times 10^3$ WM983-B melanoma cells were resuspended in 0.3% soft agar medium containing 5% serum, and either no oligomers or 25 μM of either sense oligomer specific to the $1^0$splice-junction or antisense oligomers complementary to the AUG codon or to the $1^0$ or $2^0$splice-junction were added. The cells were plated into 35 mm Petri dishes and individual soft agar colonies were counted on day 14 following addition of the different oligomers. The data presented here represent one of three soft agar assays performed, all of which led to similar results.

### *Inhibition of anchorage-independent growth of human malignant melanomas upon addition of bFGF-specific antisense oligomers*

Since the initial experiments described here suggested that the synthesis of bFGF was important to the autocrine growth activities of human malignant melanomas, we investigated whether bFGF-specific antisense oligomers also inhibited the ability of melanoma cells to form colonies in soft agar. The results, as shown in Table I, revealed 40% fewer soft agar colonies in the presence of 25 μM of each of the three antisense oligomers compared with AUG codon-specific sense oligomer-treated cells and 75% less colony formation than seen in untreated cells.

The data presented here demonstrate that proliferation of primary human melanomas in the vertical growth phase and metastatic melanomas is particularly dependent upon the autocrine production of bFGF. This finding, together with the fact that normal human melanocytes cannot proliferate in the absence of exogenous bFGF (Halaban *et al.*, 1987, 1988a; Herlyn *et al.*, 1988), suggest that bFGF gene activation may represent an important step in the

development of human melanomas, possibly correlating with the onset of melanoma progression through the epidermis–dermis in a vertical fashion.

The possibility that autocrine production of bFGF represents an event which may contribute to tumor progression was also suggested by Schweigerer *et al.* (1987), who demonstrated that human embryonal rhabdomyosarcomas, unlike normal myoblasts (Gospodarowicz *et al.*, 1976), produce bFGF which stimulates their own growth and that of vascular endothelial cells.

Finally, as shown in this study, application of antisense oligodeoxynucleotides has proven to represent an excellent approach to determine the importance of specific gene(s) involved in the progression of human malignant melanoma and its precursor lesions.

## Materials and methods

### *Synthesis and purification of oligomers*

Unmodified, 15mer oligodeoxynucleotides were synthesized on a 1 μM scale on an Applied Biosystems 380B DNA synthesizer by means of β-cyanoethyl phosphoramidite chemistry. The oligomers were purified by HPLC, lyophilized, resuspended in 0.1 M NaCl and purified further on ion exchange resin columns (NACS Prepac, BRL).

### *Cell lines*

The primary melanoma cell lines in the vertical growth phase WM75 and WM983-A, and the metastatic melanoma cell lines WM983-B and WM852 employed in this study, have been described (Herlyn *et al.*, 1985; Kath *et al.*, manuscript submitted).

The cells were maintained in three parts Temins's modified MEM and one part Leibovitz's L15 medium supplemented with 5% heat inactivated (56°C for 30 min) fetal bovine serum at 35°C in 5% $CO_2$. WM852 and WM983-B melanoma cells adapted to growth in protein-free medium were cultured in three parts Temin's modified MEM and one part Leibovitz's L15 medium over a period of 5 weeks by plating the cells at a minimal density of $3 \times 10^4$ cells/cm$^2$.

Experiments involving oligomers were performed with a single lot of fetal bovine serum which had been heat inactivated at 65°C for 45 min to destroy nuclease activity.

### *Determination of oligomer uptake and stability*

$1^0$splice-junction specific antisense oligomer was 5′ end labeled with [γ-$^{32}$P]ATP (3000 Ci/mmol; NEN) with T4 polynucleotide kinase and purified on a NACS Prepac column or a 20% denaturing polyacrylamide gel. $1.2 \times 10^6$ c.p.m. of 5′ end labeled antisense oligomer was added to WM852 cells which had been plated at a density of $10^6$ cells/60 mm plate in medium containing 5% serum 24 h prior to addition of the oligomer. Following addition of the oligomer samples were incubated for 1, 6, 24 and 36 h. Supernatant media (1.00 ml/plate) were removed at each time point and centrifuged to remove cellular debris (Wickstrom *et al.*, 1988). The cell pellets were rinsed twice with phosphate-buffer saline (PBS), lysed in 1.00 ml of Tris-buffered saline containing 1% SDS (Wickstrom *et al.*, 1988) and extracted with phenol–SSC. Uptake of antisense oligomer at each time point was determined by liquid scintillation counting of aliquots of the supernatant media, the PBS washes and the aqueous phases of the cell pellets. Oligomer stability was determined by analysis of lyophilized aliquots ($5 \times 10^3$ c.p.m./sample) of the supernatant media and the aqueous phases of the cell pellets in a 20% denaturing polyacrylamide gel. The lyophilized aliquots were resuspended in formamide buffer containing bromophenol blue and xylene cyanol which served as size markers.

### *Determination of growth rates*

Melanoma cells were plated at a density of $10^4$ cells/microtiter well in 24 well plates or at $10^5$ or $5 \times 10^4$ cells/microtiter well when grown in protein-free medium. After 24 h the cells were rinsed twice with medium and fresh medium containing sense or antisense oligomers was added (0.25 ml medium/microtiter well). Cell counts were determined in duplicate samples at each time point in each of the experiments. Viability of the cells was analyzed by trypan blue exclusion.

$5 \times 10^3$ WM983-B cells were resuspended in a final concentration of 0.3% soft agar medium containing 5% serum and 25 μM of oligomer. The cells were plated into 35 mm Petri dishes and soft agar colonies were counted after 14 days of incubation.

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