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Differences in Basic Fibroblast Growth Factor RNA and Protein Levels in Human Primary Melanocytes and Metastatic Melanoma Cells[1]

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ABSTRACT

Cultivation of human melanocytes requires several growth factors for cell proliferation. For example, basic fibroblast growth factor (bFGF) is an essential growth agent for melanocyte proliferation *in vitro* and has been proposed to be an autocrine growth factor in human melanoma cells. Studies using either anti-bFGF antibodies or antisense oligonucleotides partially inhibited the proliferation of human melanoma cells. However, one group was unable to detect bFGF RNA transcripts in human melanoma cells using a human complementary DNA probe. These contradictory results prompted us to investigate the bFGF gene expression in human primary melanocytes and metastatic melanoma cells using Southern, Northern, and Western blot analyses. No gross rearrangements in the bFGF gene were detected in the genomic DNA. Although high levels of bFGF RNA transcripts were detected in melanocytes, no bFGF protein was detected using Western blot analysis. In contrast, melanoma cells expressed much lower levels of bFGF RNA transcripts, and cells from three of four cell strains synthesized the multiple isoforms of bFGF protein. In one of the melanoma cell strains, no bFGF protein was detected using Western blot analysis. Although three of four melanoma cell strains expressed bFGF protein, this molecule does not appear to function as an autocrine growth factor, and expression of the bFGF protein was not a consistent alteration in all melanoma cell strains.

INTRODUCTION

The altered expression of growth factors is a frequent occurrence that accompanies transformation of mammalian cells. bFGF[3] has been shown *in vitro* to be a potent mitogen for many cell types of mesodermal and neuroectodermal origin (1). Cloning of the bFGF gene has allowed its characterization at the RNA and DNA level (2, 3). Expression of the bFGF gene has been measured in various cells, and four RNA transcripts have been detected. Two major RNA transcripts (7.0 and 3.7 kilobases) are observed as well as several low-abundance RNA transcripts (1.0–1.8 kilobases) in several cell lines (4, 5). Interestingly, the smallest RNA transcript (1 kilobase) has been shown to be an antisense RNA transcript to the bFGF gene and may function in its regulation (6, 7).

Antibodies to the bFGF protein have also been generated and the bFGF protein has been shown to have a basic isoelectric point (>9.0) and to have a high affinity for heparin and glycosaminoglycans. Heparin-affinity chromatography and bFGF antibodies have been used to investigate the translational control of bFGF. Four bFGF polypeptides (18, 21, 22.5, and 24 kDa) have been detected in the human cell line, SK-HEP-1. Utilization of non-AUG codons produced the three larger bFGF isoforms (8, 9).

bFGF is a mitogen for human primary neonatal melanocytes *in vitro* (10, 11). Halaban *et al.* (10, 11) were able to detect a low expression level of the bFGF RNA transcripts in two melanoma cell lines but were unable to detect the expression of the bFGF RNA transcripts in neonatal melanocytes using Northern blot analysis with a bovine bFGF cDNA probe.

However, Chenevix-Trench *et al.* (12) were unable to detect bFGF RNA transcripts in human primary and metastatic melanoma cell lines using Northern blot analysis with a human bFGF cDNA probe. bFGF RNA transcripts have been shown to be expressed at various stages of human melanocyte progression and were detected in tissue from dermal nevi, primary melanomas, and metastatic melanomas using *in situ* hybridization analysis (13). Interestingly, the expression level of bFGF RNA transcripts detected decreased with increasing progression toward malignant tissue (nevi, +2 to +3; primary, +2; metastatic melanoma, +1).

The effect of bFGF-neutralizing antibodies on human metastatic melanoma cell growth has also been investigated (14). A decrease in melanoma proliferation was observed following incubation of cells with antibodies to bFGF protein. Inhibition of cell proliferation by antisense oligonucleotides to the bFGF gene has also been studied (15). Using antisense oligodeoxynucleotides targeted against the bFGF gene, Becker *et al.* (15) observed a slight inhibition of the cell proliferation of melanoma cells as well as inhibition of anchorage-independent growth.

An investigation of the bFGF gene at the DNA, RNA, and protein levels would be required to determine the role of bFGF protein in human melanocyte and melanoma cell proliferation. In our study, the expression of the bFGF gene was investigated in human primary neonatal melanocytes and in metastatic melanoma cell strains using a human cDNA probe. The expression level of the four bFGF RNA transcripts in melanocytes was high in nonproliferative and proliferative conditions, but the bFGF protein was undetectable using Western blot analysis. However, in comparison, bFGF RNA transcripts and proteins were expressed at very low levels in metastatic melanoma cells.

MATERIALS AND METHODS

Culture of Melanocytes. The method used is a combination of the procedures developed by others (16, 17) and have been described in detail elsewhere (18, 19). Human foreskin samples were collected from newborn infants; melanocytes were isolated and transferred to a T-75 flask. Primary newborn melanocytes were cultivated in MCDB 153 medium (Irvine Scientific, Irvine, CA) with 2% fetal calf serum, 100 μM 3-isobutyl-1-methyl-xanthine, 52 μg/ml bovine pituitary extract (Clonetics, San Diego, CA), 2.0 mM calcium chloride, 5 μg/ml insulin (Clonetics), 10 ng/ml TPA, 1% glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) as described by Halaban *et al.* (14), modified by Jambrosic *et al.* (20), and designated melanocyte complete

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[3] The abbreviations used are: bFGF, basic fibroblast growth factor; cDNA, complementary DNA; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; SDS, sodium dodecyl sulfate; SSC, standard sodium citrate (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate); TE, 10 mM Tris, 1 mM EDTA, pH 8.0.

medium. Human metastatic melanoma cell strains were cultured in F-10 with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg/ml) (21–23) and was designated melanoma complete medium. The passage number for the various cell strains used in these experiments was <8. Viable cell counts were determined by trypan blue exclusion.

Experimental Conditions. Primary melanocytes and melanoma cells were cultivated in melanocyte complete medium and melanoma medium, respectively, until 70–80% confluent. Melanocytes were washed and incubated with prewarmed complete medium or medium without various growth agents. Melanoma cells were incubated with prewarmed medium with or without serum. There was no change in cell morphology when the human melanocytes were cultivated in medium without serum and TPA or in the melanomas cultivated in medium without serum for 24 h. Following incubation for 24 h, the cells were isolated, and total RNA was recovered.

Isolation of DNA and Southern Blot Analysis. Genomic DNA was isolated from the cells using the 4 M guanidine isothiocyanate-0.1% Sarkosyl solution procedure described by Chirgwin *et al.* (24). The DNA was recovered from the solution just above the 5.7 M CsCl cushion and extracted with phenol-chloroform-isoamyl alcohol (25:24:1), chloroform-isoamyl alcohol (24:1), and ethanol precipitated. The DNA was spooled out and resuspended in TE buffer. The genomic DNA was digested with various restriction enzymes as described by the manufacturer, ethanol precipitated, and resuspended in TE buffer with 100 mM NaCl. The Southern blot was prepared as described by Maniatis *et al.* (25). Digested DNA (10 μg) was electrophoresed on a 1.0% agarose gel, transferred by capillary action to a nylon filter (Magna NT; MSI, Westborough, MA), and prehybridized for 2–4 h at 65°C. The probe was labeled by the method of Feinsberg and Vogelstein (26) using a random priming kit (Promega, Madison, WI). Fresh hybridization solution containing 2–4 × 10^6 cpm/ml of radiolabeled probe was added to the filter as described by the manufacturer. Hybridization was done overnight at 65°C and the filter was washed (50–60°C with 0.1% SDS and 0.1× SSC). The filter was exposed to Kodak X-OMAT film from 2–20 h at −80°C. The filters were stripped of probe by using two washes of 0.1% SDS and 0.1× SSC at 80–90°C.

Isolation of RNA and Northern Blot Analysis. The procedure was a modification of that described by Chirgwin *et al.* (24). Two to six T-175 flasks were used per growth condition. The cells were pelleted and lysed using a 4 M guanidine isothiocyanate-0.1% Sarkosyl solution. The sheared homogenate was layered on top of a 5.7 M CsCl cushion and centrifuged at 55,000 rpm for 3 h in a TLS-55 rotor. The RNA pellet was resuspended in TE buffer with 0.1% SDS, extracted, ethanol precipitated, and resuspended in sterile water. The Northern blot was prepared as described by Fourney *et al.* (27). Total RNA (10 μg/sample) was electrophoresed on a denaturing formaldehyde agarose gel, transferred by capillary action to a nylon filter (Magna NT; MSI), and prehybridized for 2–4 h at 42°C. Fresh hybridization solution containing 1 × 10^6 cpm/ml of a randomly primed, radiolabeled probe was added to the filter as described by manufacturer. Hybridization was done overnight at 42°C, followed by two to three stringent washouts (40–50°C with 0.1% SDS and 0.1× SSC). The filter was exposed to Kodak X-OMAT film from 2–20 h at −80°C. The filters were stripped of probe by using two washes of 0.1% SDS and 0.1× SSC at 80°C. For quantitation of individual RNA transcripts, films were exposed for times which produced band intensity that was linear with respect to time. Films were scanned with a densitometer (Hoefer Scientific Instruments, San Francisco, CA), and RNA transcript abundance was determined from the area of the peak corresponding to each RNA transcript. Values for bFGF gene expression were normalized using an 18S rRNA probe. RNA standards were used to determine RNA sizes (0.24- to 9.5-kilobase RNA ladder; BRL, Gaithersburg, MD).

Western Blot Analysis. Cell extracts were prepared for Western blot analysis according to the following procedures (28, 31). One ml of ice cold lysis buffer (1% Nonidet P-40, 0.4% deoxycholate, 50 mM Tris, 62 mM EDTA, 150 mM NaCl, 0.01 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml Aprotinin, 0.5 mg/ml leupeptin, and 0.5 mg/ml pepstatin, pH 7.5) was added to each T-75 flask which was 70% confluent. Cell lysates were clarified by microfuge centrifugation (16,000 × *g*) for 10 min at 4°C. Supernatants were removed and incubated with prewashed heparin-Sepharose for 2 h at 4°C. Heparin-Sepharose and bound protein were pelleted, resuspended and washed twice with lysis buffer, three times with rinse buffer (20 mM Tris, 0.5 M NaCl, 5 mM EDTA, 2 mM ethyleneglycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and protease inhibitors, pH 7.5), and three times with buffer containing 1.0 M NaCl. Bound protein was eluted directly into SDS-sample buffer for 12% polyacrylamide gel electrophoresis as described by Laemmli (29). Proteins were transferred to nitrocellulose by Western blotting in a buffer containing 25 mM 3-[(1,1-dimethyl-2-hydroxyethyl)amino]-2-hydroxypropanesulfonic acid, pH 9.5, in 20% methanol. Filters were blocked in a buffer containing 5% powdered milk, incubated with guinea pig anti-18-kDa bFGF antisera diluted 1:500, and then incubated with ^{125}I-protein A.

Probes and Antibodies. The human cDNA bFGF probe has been described by Abraham *et al.* (2). The bFGF data were obtained with a 0.8-kilobase *Eco*RI restriction fragment from the plasmid, pHFL1–7, which includes the region from exon 2 to the end of the gene. The 18S data was obtained with a 5.6-kilobase *Eco*RI restriction fragment from the plasmid, pB (30). The guinea pig anti-bFGF antibody was previously described (28, 31).

RESULTS

Southern Blot Analysis. Genomic DNA was prepared from human primary neonatal melanocytes and metastatic melanoma cell strains, digested with various restriction enzymes (*Eco*RI, *Hind*III, *Bam*HI, and *Pvu*II), and analyzed by Southern blotting to detect rearrangements in the bFGF gene. Figs. 1 and 2 are representative Southern blot analyses using the human bFGF cDNA as a probe. Digestion of DNA with *Pvu*II detected three bands of 14.5, 5.8, and a faint band of 2.5 kilobases (Fig. 1). Southern blot analyses of *Bam*HI-digested DNA hybridized

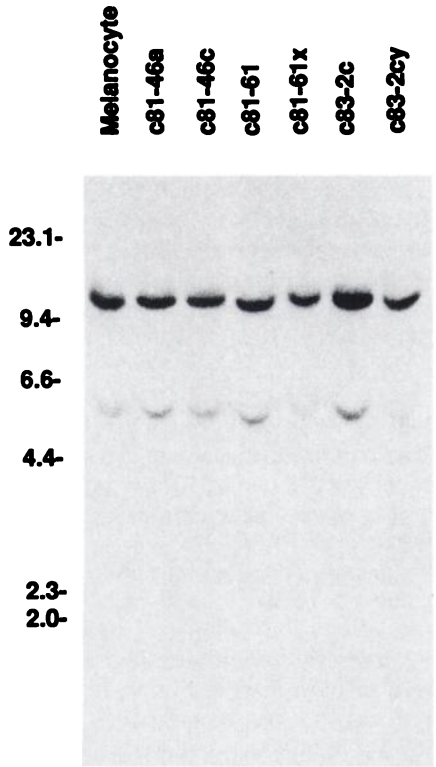

Fig. 1. Southern blot analysis of bFGF gene in human primary neonatal melanocytes and metastatic melanoma cell strains. Genomic DNA digested with the restriction endonuclease, *Pvu*II, was hybridized with ^{32}P-labeled restriction fragments of bFGF cDNA. *Left lane,* positions of migration by λ-DNA digested with the restriction endonuclease *Hind*III with indicated kilobases.

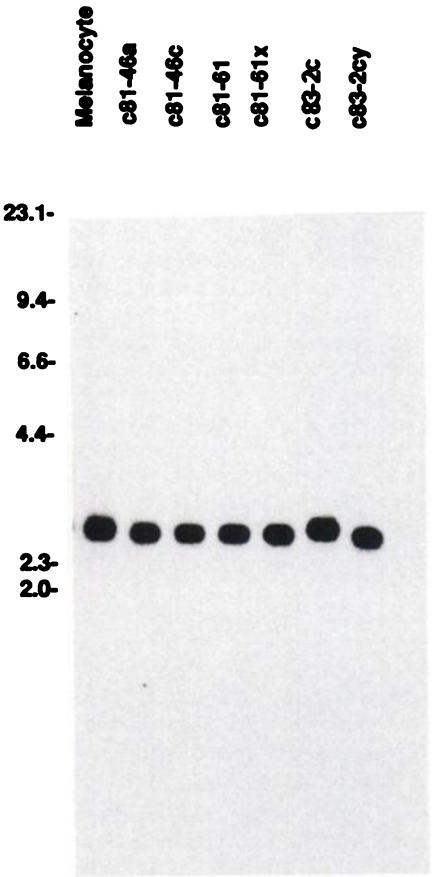

Fig. 2. Southern blot analysis of bFGF gene in human primary neonatal melanocytes and metastatic melanoma cell strains. Genomic DNA digested with the restriction endonuclease, *Bam*HI, was hybridized with ^{32}P-labeled restriction fragments of bFGF cDNA. *Left lane*, positions of migration by λ-DNA digested with the restriction endonuclease *Hind*III with indicated kilobases.

with the human bFGF probe identified a single fragment of 2.7 kilobases and a faint band of 9.0 kilobases (Fig. 2). Genomic DNA digested with the restriction enzyme *Eco*RI (6.4 and 2.1 kilobases) or *Hind*III (8.0, 4.0, and 1.0 kilobases) did not allow detection of any gross rearrangements in the melanocytes and melanoma cells (data not shown). No gross rearrangements in the bFGF gene were detected with the four restriction enzymes used. Our inability to detect any gross rearrangements in the bFGF gene was similar to the results obtained by Theillet *et al.* (32) and Adelaide *et al.* (33) using different restriction enzyme digestions.

Northern Blot Analysis. Total RNA was isolated and screened for the expression of the bFGF gene in human primary melanocytes and metastatic melanoma cell strains. The densitometric scans of the four bFGF RNA transcript expression levels in melanocytes and melanoma cell strains are summarized in Table 1.

Northern blot analysis detected four bFGF RNA transcripts (7.0, 3.7, 2.1, and 1.2 kilobases) in melanocytes (Fig. 3). Melanocytes cultivated in plain medium expressed a low level of two of the four transcript bFGF gene (7.0 and 3.7 kilobases). When cultivated in more complete growth medium, melanocytes expressed higher levels of the two smaller RNA transcripts (2.1 and 1.2 kilobases) compared to melanocytes cultivated in plain medium. The other two RNA transcripts (7.0 and 3.7 kilobases) decreased in expression level as the melanocytes were cultivated in more complete medium (plain medium with calcium to complete medium without TPA and serum). The two RNA transcripts (7.0 and 3.7 kilobases) were detected at the highest RNA transcript expression level in melanocytes which were cultivated in complete medium.

Expression of the bFGF RNA transcripts was different in the metastatic melanoma cell strains compared to the melanocytes. Low basal expression levels of the bFGF gene was detected in metastatic melanoma cells. Two RNA transcripts (7.0 and 3.7 kilobases) were detected in five of six melanoma cell strains. An increase in the expression level of the bFGF RNA transcripts (7.0, 3.7, 2.1, and 1.2 kilobases RNA transcripts) was detected in melanoma cell strains (c81–46c, c81–61, and c81–61x) when the cells were cultivated in medium with serum compared to medium without serum. A repression in the expression of the bFGF RNA transcripts was detected in melanoma cell strains c81–46a and c83–2cy when cultivated in medium with serum compared to without serum.

Melanoma cell strain c83–2c was unique, since these cells expressed only the 1.2-kilobase bFGF RNA transcript. The other three bFGF RNA transcripts detected in melanocytes were undetectable. A slight increase in the 1.2-kilobase RNA transcript was observed when the cells were cultivated in medium with serum compared to medium without serum.

Western Blot Analysis. Expression of the bFGF protein isoforms was determined using Western blot analysis. The 18-, 21-, 22.5-, and 24-kDa bFGF protein isoforms were detected in the primary neonatal fibroblasts, but these protein isoforms were undetectable in melanocytes (Fig. 4 and data not shown).

Expression of the bFGF protein was different in the melanoma cell strains (Figs. 4 and 5). Three of the bFGF protein isoforms with molecular masses of 18, 21, and 22.5 kDa were detected in three of the four melanoma cell strains (c81–46a, c81–46c, and c81–61, respectively). Very low levels of bFGF

Table 1 *Densitometric quantification of bFGF RNA transcripts in human primary neonatal melanocytes and metastatic melanoma cell strains*

Primary melanocytes were cultivated in various growth conditions. Metastatic melanoma cell strains were cultivated in medium without (−) or with (+) serum. Values are standardized to bFGF RNA transcript expression levels in primary melanocytes cultivated in plain medium with calcium ions for 24 h.

	bFGF RNA transcripts (kilobases)			
	1.2	2.1	3.7	7.0
Melanocytes				
P[a]	N.D.	N.D.	0.1	8.5
PC	100	100	100	100
PCI	1.0	133	95	85
CTS	1.2	166	84.7	67.6
C	1.6	200.9	106.4	105.4
Melanomas				
c81–46a				
−	N.D.	N.D.	7.7	7.9
+	N.D.	N.D.	6.2	3.5
c81–46c				
−	N.D.	N.D.	10.4	6.1
+	0.2	0.1	15.9	12.1
c81–61				
−	N.D.	N.D.	N.D.	3.1
+	0.2	0.1	10.8	13.4
c83–2c				
−	0.5	N.D.	N.D.	N.D.
+	0.6	N.D.	N.D.	N.D.
c83–2cy				
−	N.D.	N.D.	8.6	9.6
+	N.D.	N.D.	2.2	2.0
c81–61x				
−	N.D.	N.D.	5.1	3.5
+	N.D.	N.D.	15.5	13.1

[a] P, MCDB 153 medium only; PC, plain with calcium chloride; PCI, PC with insulin; CTS, complete medium without TPA and serum; C, complete medium; N.D., not detected.

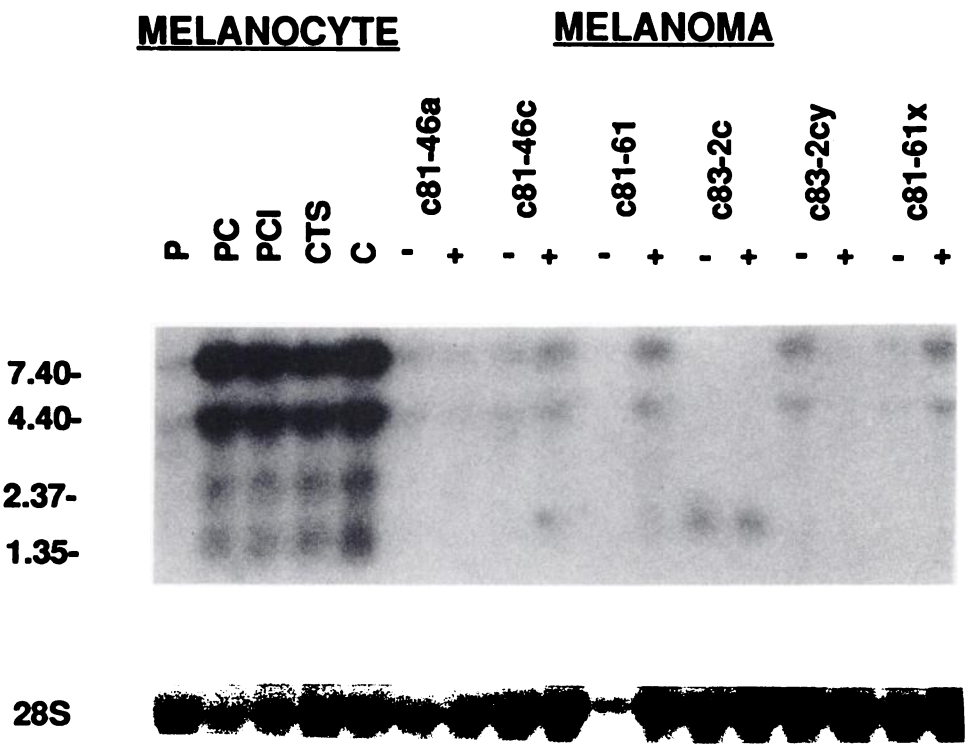

Fig. 3. Expression of bFGF RNA transcripts in human primary neonatal melanocytes and metastatic melanoma cell strains. Cells were cultivated and treated for 24 h in various conditions. Total RNA (10 μg) was hybridized with ^{32}P-labeled restriction fragments of the human bFGF cDNA probe. Melanocytes were cultivated in the following growth medium conditions: P, MCDB 153 medium only; PC, plain with calcium chloride; PCI, PC with insulin; CTS, complete medium without TPA and serum; and C, complete medium. Melanoma cells were cultivated in medium without (−) or with (+) serum. *Left lane*, positions of migration by RNA standards.

protein were detected in the melanoma cells. However, expression of the bFGF protein was undetectable in the melanoma cell strain c83–2c when the cells were cultivated in medium without or with serum.

DISCUSSION

The function of the bFGF gene using Southern, Northern, and Western blot analysis was investigated in human primary neonatal melanocytes and metastatic melanoma cell strains. Human neonatal melanocytes expressed all four of the bFGF RNA transcripts (7.0, 3.7, 2.1, and 1.2 kilobases) cultivated in all growth conditions, except when the melanocytes were grown in basal medium. Optimal expression of the bFGF RNA transcripts was observed when TPA and serum were included in the melanocyte medium. These growth agents may be inducing the expression of the bFGF RNA transcripts through the postulated AP-1 and SP-1 DNA-binding sites in the bFGF promoter (31). However, when Western blot analysis was used, heparin-Sepharose-binding bFGF protein was not detected in melanocytes cultivated under any of the growth conditions.

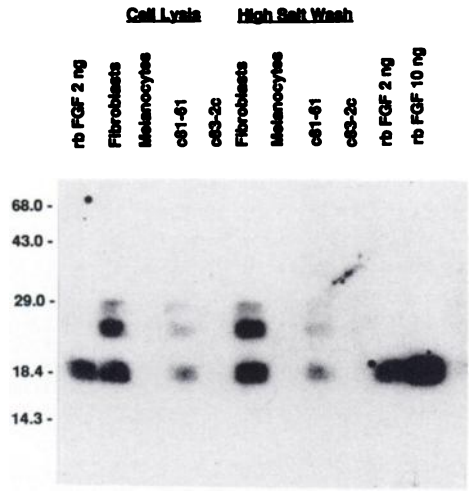

Fig. 4. Amount of bFGF protein produced in human primary melanocytes, primary fibroblasts, and metastatic melanoma cells. Heparin-Sepharose-binding protein was isolated from cell lysates and eluted directly into SDS-sample buffer. Protein was electrophoresed on a 12% polyacrylamide gel and transferred to a nitrocellulose filter. Filters were incubated with a guinea pig anti-18-kDa bFGF antisera and then with ^{125}I-protein A. *Left lane*, positions of migration by protein standards.

The expression of bFGF RNA transcripts in human metastatic melanoma cell strains demonstrated four patterns. In general, only two of the four RNA transcripts (7.0 and 3.7 kilobases) were detected in the melanoma cell strains. In the first case (c81–46c, c81–61, and c81–61x), bFGF RNA transcripts were induced when the cells were cultivated in medium with serum compared to medium without serum. In contrast, in melanoma cells c81–46a and c83–2cy, bFGF RNA transcripts were repressed when the cells were cultivated in medium with serum compared to medium without serum. In the third case (c83–2c), the only bFGF RNA transcript detected was the smaller 1.2-kilobase RNA transcript, and it was present when the cells were cultivated in medium with or without serum.

The expression of the bFGF gene at the protein level was detected in the melanoma cell strains. Low levels of protein were detected in all of the melanoma cell strains but was undetectable in the melanoma cell strain c83–2c. Melanoma cell strains c81–46a, c81–46c, and c81–61 expressed the 18-, 21-, and 22.5-kDa bFGF protein isoforms, respectively, but the expression level was low (approximately 1 pg/10^5 cells). Expression of the bFGF protein isoforms in melanoma cell strain c83–2c was undetectable using Western blot analysis. This result correlates with the RNA expression level, since only the smaller 1.4-kilobase RNA transcript was detected. The 1.4-kilobase bFGF RNA transcript may be an antisense bFGF RNA transcript in human melanocytes and melanoma cells, which would be consistent with previous data of Volk *et al.* (7) and Kimelman and Kirschner (6) in *Xenopus*.

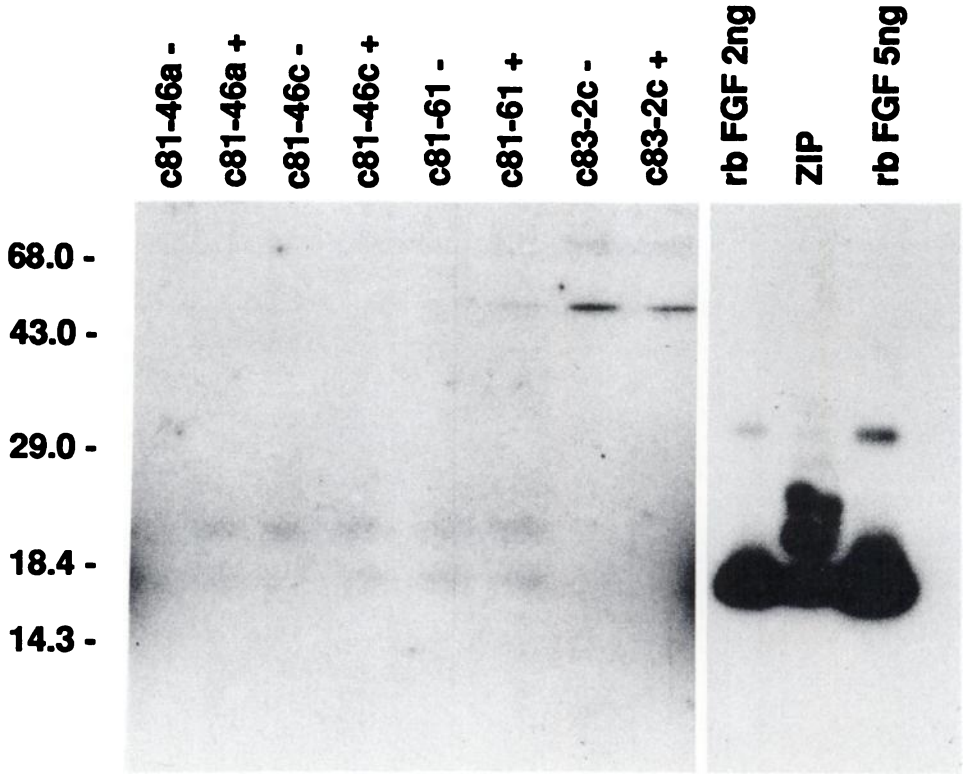

Fig. 5. Amount of bFGF protein produced in human metastatic melanoma cell strains. Melanoma cells were cultivated in medium without (−) or with (+) serum. Heparin Sepharose-binding protein was isolated from cell lysates and eluted directly into SDS-sample buffer. Protein was electrophoresed on a 12% polyacrylamide gel and transferred to a nitrocellulose filter. Filters were incubated with a guinea pig anti-18-kDa bFGF antisera and then with ^{125}I-protein A. *Left lane*, positions of migration by protein standards.

Expression of the bFGF RNA transcripts did not result in expression of the bFGF proteins in human melanocytes. Melanocytes expressed high levels of the bFGF RNA transcripts, but protein levels were undetectable by Western blot. This suggests a tight translational regulation of the bFGF RNA in melanocytes. However, melanoma cells expressed low levels of the bFGF RNA transcripts and proteins, suggesting a less tightly regulated control in the malignant cells.

In several studies, the bFGF protein has been proposed to be an autocrine growth factor for human melanomas (10, 11, 34). However, bFGF RNA transcripts were detected at the highest levels in premalignant lesions, lower in primary melanomas, and lowest in metastatic melanoma using *in situ* hybridization (13). We were able to detect bFGF RNA transcripts in melanocytes using a human bFGF cDNA probe, while Halaban *et al.* (10, 11) were unable to detect bFGF RNA transcripts using a bovine bFGF cDNA probe. The only major difference in the melanocyte complete growth medium used by Halaban *et al.* and our group is the presence of insulin in our medium and the amount of serum (10% calf serum for Halaban *et al.* and 2% fetal calf serum for ours). However, there was little difference in the expression levels of the two major bFGF RNA transcripts (3.7 and 7.0 kilobases) when the melanocytes were cultivated in the various growth mediums. Using the same bovine bFGF cDNA probe, we were also unable to obtain clean Northern blots[4] and were also barely able to detect the two major bFGF RNA transcripts. These results suggest that the human bFGF cDNA probe used in our studies was better at detecting bFGF RNA transcripts in human cells than its bovine counterpart; this may explain why Halaban *et al.* were unable to detect any expression of the bFGF RNA transcripts in melanocytes using Northern blot analysis.

Our studies indicate that melanoma cells expressed very low levels of bFGF RNA transcripts and proteins. Although transfection of the bFGF gene in a recombinant retroviral vector allowed murine melanocytes to become independent of the bFGF growth requirement, transformation of the murine melanocytes did not result (34). While three of four cell strains expressed bFGF RNA transcripts and protein isoforms, the cell strain c83–2c only expressed the 1.2-kilobase RNA transcript and did not synthesize any detectable levels of bFGF protein as measured by Western blot analysis. These data suggest that, although exogenous bFGF protein may be required for the proliferation of human primary melanocytes and was expressed in three of four melanoma cell strains, expression of the bFGF protein does not appear to be a consistent alteration in melanocyte transformation.

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