Localization of Basic Fibroblast Growth Factor mRNA in Melanocytic Lesions by In Situ Hybridization

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Basic fibroblast growth factor (bFGF) is a mitogen for normal human melanocytes and keratinocytes in culture. Experiments in vitro suggest that keratinocytes supply bFGF to melanocytes through a paracrine mechanism and that the aberrant expression of bFGF in melanomas confers growth independence from bFGF-producing cells. To determine whether bFGF is expressed in vivo, we examined a series of benign and malignant melanocytic lesions in situ using bFGF riboprobes on tissue sections, and correlated bFGF expression with histologic phenotype. Seventeen melanocytic neoplasms were studied, including four common acquired nevi, four dysplastic nevi, four primary malignant melanomas, and five metastatic melanomas. Nevic cells in benign intradermal nevi showed low signal intensity (1+), whereas compound and dysplastic nevi showed 2+ to 3+ expression in the junctional nevic cell population and 1+ expression in the dermal nevic cell population. Melanocytes in primary melanomas had intermediate (2+) and those in metastatic melanomas had low (1+) levels of bFGF gene transcripts. Fibroblasts expressed high levels (3+) and epidermal and adnexal keratinocytes moderate (2+) levels of bFGF in all cases studied. Basic FGF expression in endothelial cells, known to produce and respond to this growth factor in vitro, was lower than that in the fibroblast and keratinocyte cell population and, in 10 of 17 cases, no bFGF mRNA was detectable. This study shows that bFGF is expressed in nevomelanocytes in vivo in all melanocytic lesions studied and thus cannot be used as a marker for transformation. The presence of bFGF gene transcripts in the various dermal cell types and in keratinocytes suggests that it may act as an autocrine and paracrine growth factor in regulating cellular proliferation in the skin. *J Invest Dermatol 96:318–322, 1991*

The factors that regulate the growth and malignant transformation of melanocytes in vivo are unknown. Recently, basic fibroblast growth factor (bFGF), known to stimulate the growth of mesodermally and neuroectodermally derived cells (for review see [1]), has been shown to be a natural mitogen for human melanocytes cultured in vitro [2]. Normal human melanocytes cultured in vitro, unlike other cell types that respond to bFGF, do not produce this factor [2]. However, other skin cells, such as proliferating keratinocytes and dermal fibroblasts, produce bFGF and in co-culture with melanocytes maintain melanocyte viability [3]. The production of a melanocyte mitogen by cells that are in intimate contact with melanocytes in vivo suggests that keratinocyte-derived bFGF may act as a paracrine growth factor in the regulation of the growth of these cells in vivo.

The majority of metastatic melanomas and some primary melanomas, particularly those in the vertical growth phase, are independent of melanocyte growth factors because of self-stimulation through aberrant production of bFGF [4]. Metastatic melanoma cells in culture produce mRNA species that hybridize with bFGF cDNA and are similar in size to bFGF mRNA in other cell types [4]. bFGF in melanomas confers growth advantage to these cells because internalized neutralizing anti-bFGF–antibodies and antisense, but not sense, oligodeoxynucleotides targeted against three different sites of human bFGF mRNA, inhibit growth [4,5].

Although the expression of bFGF in cultured skin cells is well studied, its expression in vivo is largely unexplored. Melanoma is an ideal model in which to study the stage-related expression of tumor-associated growth factors because melanocytic malignancy evolves in a step-wise progression from dysplasia to melanoma in situ, invasive melanoma, and finally metastatic melanoma, with each step identifiable by defined histologic criteria. This investigation reports on the relative amounts and distribution of bFGF mRNA in intact melanocytic lesions using in situ hybridization with tritium-labeled riboporobes. The expression of bFGF was correlated to specific histologic stages of melanocytic tumor progression.

MATERIALS AND METHODS

Tissue Preparation Skin biopsies of 17 melanocytic lesions were fixed immediately after excision in 10% buffered formaldehyde, pH 7.4, for 6 to 12 h, embedded in paraffin, and sectioned at 5 μ. Each section was mounted on 3-aminoptopyltriethoxysilane (TES) coated slides and stored at room temperature. Routine tissue sections were prepared with hematoxylin and eosin stains. Human neonatal fibroblasts, known to contain abundant mRNA for bFGF [6], were used as a positive control. Cells were cultured in RPMI media (Hazelton Biol., KS, USA) with 10% fetal calf serum, scraped off the culture dish with a rubber policeman, and sedimented by centrifuga-

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Abbreviations:
bFGF: basic fibroblast growth factor
TES: 3-aminoptopyltriethoxysilane
cDNA: complimentary deoxynucleic acid
RNA: ribonucleic acid
dpm/mg: disintegrations per milligram
SSC: sodium chloride sodium citrate

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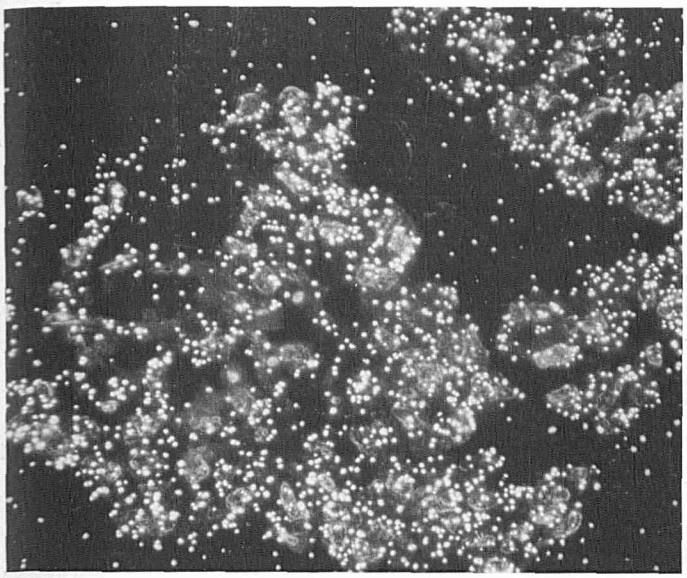

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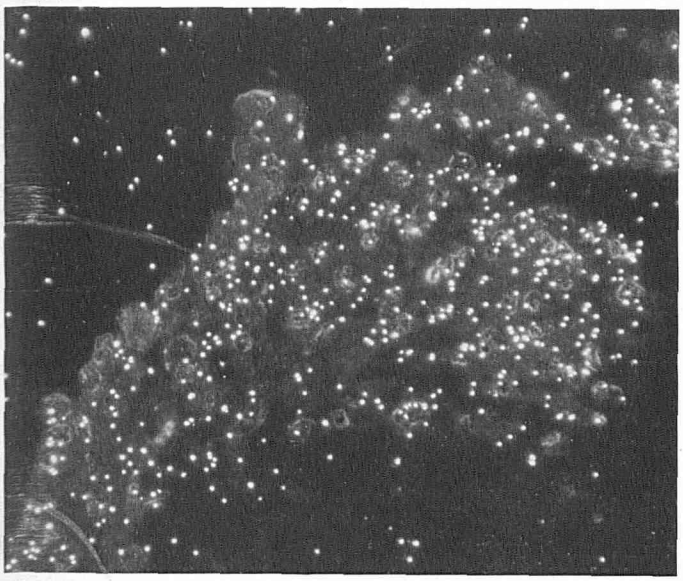

B

Figure 1. Cultured fibroblasts hybridization with bFGF antisense probe shows 2+ signal above the background level *(A)* as compared with the same cells hybridized with the sense probe *(B)*. (Magnification ×400.)

tion at 10,000 rpm for 5 min. The pellet was suspended in a solution of 1% agar plus 10% formalin and processed as described below.

The cases in this study include four common acquired nevi, four dysplastic nevi, four primary malignant melanomas, and five melanomas metastatic to lymph nodes. Dysplastic nevi were sporadic and judged clinically as such by the patient's dermatologist. The histologic parameters of dysplasia used fulfilled all the criteria established by Clark et al [7].

Preparation of RNA Probes The 1.4-kb bovine cDNA fragment for bFGF was obtained from Drs. J.A. Abraham and J.C. Fiddes, California Biotechnology, Inc., Mountain View, CA [8]. The unmodified bFGF cDNA was cloned into the Eco-R1 site of the RNA transcription vector Gemini 3Z (Promega Biotec, Madison, WI). Its orientation relative to the SP6 and T7 transcriptional promoter sites was determined by restriction enzyme digestion with Fsp and Hae II (New England Biolabs,). Tritium-labeled asymmetric RNA transcripts were synthesized using commerically available SP6 and T7 polymerase to a specific activity of 1.2×10^8 dpm/mg DNA.

In Situ Hybridization of bFGF Riboprobes In situ hybridization was performed as previously described [9,10] with the following modifications. Briefly, tissue sections were dewaxed by heating to 65°C with subsequent xylene washes. After hydrating through a graded series of alcohol baths, slides were placed in a 1 mg/ml proteinase K solution at 37°C for 30 min and then acetylated in acetic anhydride-triethanolamine, pH 8.0. The slides were then washed in 2 × SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) and set to air dry. Hybridization was carried out under moderate stringency at hybrid midmelting temperature (Tm, 25°C) overnight (approximately 16 h). After hybridization, the slides were passed through three changes of chloroform and four changes of 4 × SSC and were then treated with RNase A (20 mg/ml, Sigma, Deisenhofen, FRG) and RNase T1 (1 mg/ml, Boehringer, Mannheim, FRG) for 30 min at 37°C, washed in RNase A buffer (5 M NaCl, 1 M Tris-HCl, 1 mM EDTA) for 30 min at 37°C and then incubated in 2 × SSC for 30 min at room temperature. The sections were then subjected to high stringency, post-hybridization wash in 0.1 × SSC at 56°C followed by a wash in 0.1 × SSC at room temperature for 30 minutes and finally dehydrated in graded ethanols up to 95% containing 300 mM ammonium acetate. Autoradiography was performed with NTB2 nuclear track emulsion (Eastman Kodak, Rochester, NY) diluted 1 : 1 with 600 mM ammonium acetate. After 21 d at 4°C, the slides were developed in D19, fixed in Kodak fixer (Eastman Kodak, Rochester, NY), and lightly stained with hematoxylin and eosin.

To confirm the specificity of the hybridization reaction, bFGF sense RNA probes that were complementary in nucleotide sequence to the test (antisense) probes, and synthesized to the same specific activity, were hybridized to identical tissue sections from each case. Sections were evaluated blind with respect to diagnosis first independently by two pathologists (GS and MS) and then by consensus over a double-headed scope using dark field microscopy. The essential principle of darkfield optics is the formation of a hollow cone of light whose apex occurs in the plain of the specimen. When the

Table I. Localization and Histology Results of In Situ Hybridization of bFGF Riboprobes in 17 Melanocyte Neoplasms

Histology	In Situ Hybridization	
	Keratinocytes	Nevomelanocytes
Common acquired nevi		
1. Dermal nevus	+	+
2. Dermal nevus	+	+
3. Compound nevus	+	+
4. Compound nevus	++	++
Dysplastic nevi		
1. Dysplastic compound nevus	++	++
2. Dysplastic compound nevus	+++	+++
3. Dysplastic junctional nevus	+	+
4. Dysplastic junctional nevus	++	++
Primary malignant melanoma		
1. Compound dysplastic nevus with transition to malignant melanoma level II; depth, 0.5 mm	+++	++
2. Level II; depth, 0.9 mm	+	+
3. Level IV; depth, 1.15 mm	++	++
4. nLevel V; depth, 5 mm	++	++
Metastatic Malignant Melanoma to Lymph Nodes		
1.	N/A[a]	+
2.	N/A	+/++
3.	N/A	+
4.	N/A	0
5.	N/A	++

[a] N/A, not applicable.

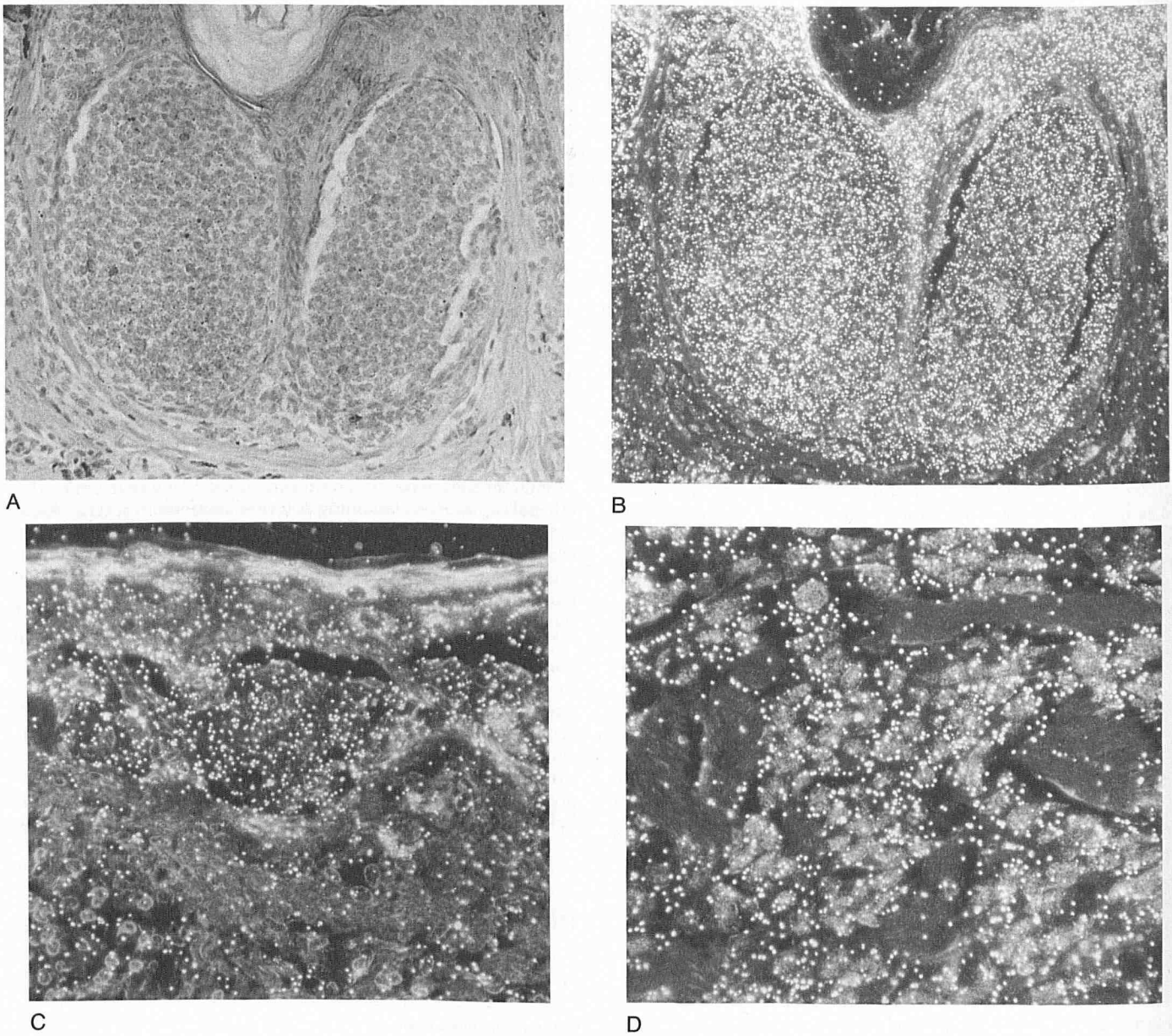

Figure 2. A common acquired nevus hybridized with the bFGF antisense probe seen under bright field *(A)* and dark field *(B)* illumination. Well-defined nests of nevic cells are present at the dermal-epidermal junction. Dark field microscopy *(B)* reveals moderate signal (2+) in the keratinocytes and the nevic cells. A dysplastic nevus *(C)* shows 1–2+ signal in the junctional dysplastic nevic cells and 1+ signal in the epidermal keratinocytes. Infiltrating malignant melanoma cells from a vertical growth phase melanoma *(D)* shows 2+ signal in the infiltrating malignant cells. (Magnification for *A, B,* and *C,* ×200; for *D,* ×400.)

light is focused on the specimen, the silver grains will scatter light into the objective and appear as bright white dots. Elements on the slide that do not scatter light, such as the tissue, will appear as a dark background.

The relative signal intensities were converted to a rough numerical grading system. 1+ is approximately 5 grains/cell over background and is considered low. 2+ is 5–10 grains/cell over background and is moderate and 3+ is more than 10 grains/cell over background and is high.

RESULTS

Positive and Negative Controls The specificity of hybridization in tissues was confirmed by comparing the levels of hybridization of the sense and antisense RNA probes in each case. In addition, cultured human fibroblasts hybridized with antisense probes were used as positive controls. Significant hybridization signal in cultured fibroblasts was observed with the antisense probes as compared with background levels with the sense probe (Fig 1).

In Situ Localization of bFGF mRNA in Nevomelanocytic Lesions Results of the localization of bFGF mRNA gene transcripts in 17 melanocytic lesions is summarized in Table I and illustrated in Fig 2.

In the four cases of common acquired nevi, the expression of basic FGF mRNA by nevic cells was low (1+) in three cases, with one case displaying high signal intensity (3+) in the junctional nevic cell population (Fig 2*A*,*B*). Signal intensity was generally higher in dysplastic nevi than in common nevi (2+ to 3+, Fig 2*C*), but one case showed only 1+ signal. In three cases of primary malignant melanoma, the malignant melanocytes displayed intermediate signal intensity (2+, Fig 2*D*) and in one case low signal intensity (1+). In a case of malignant melanoma contiguous with a dysplastic

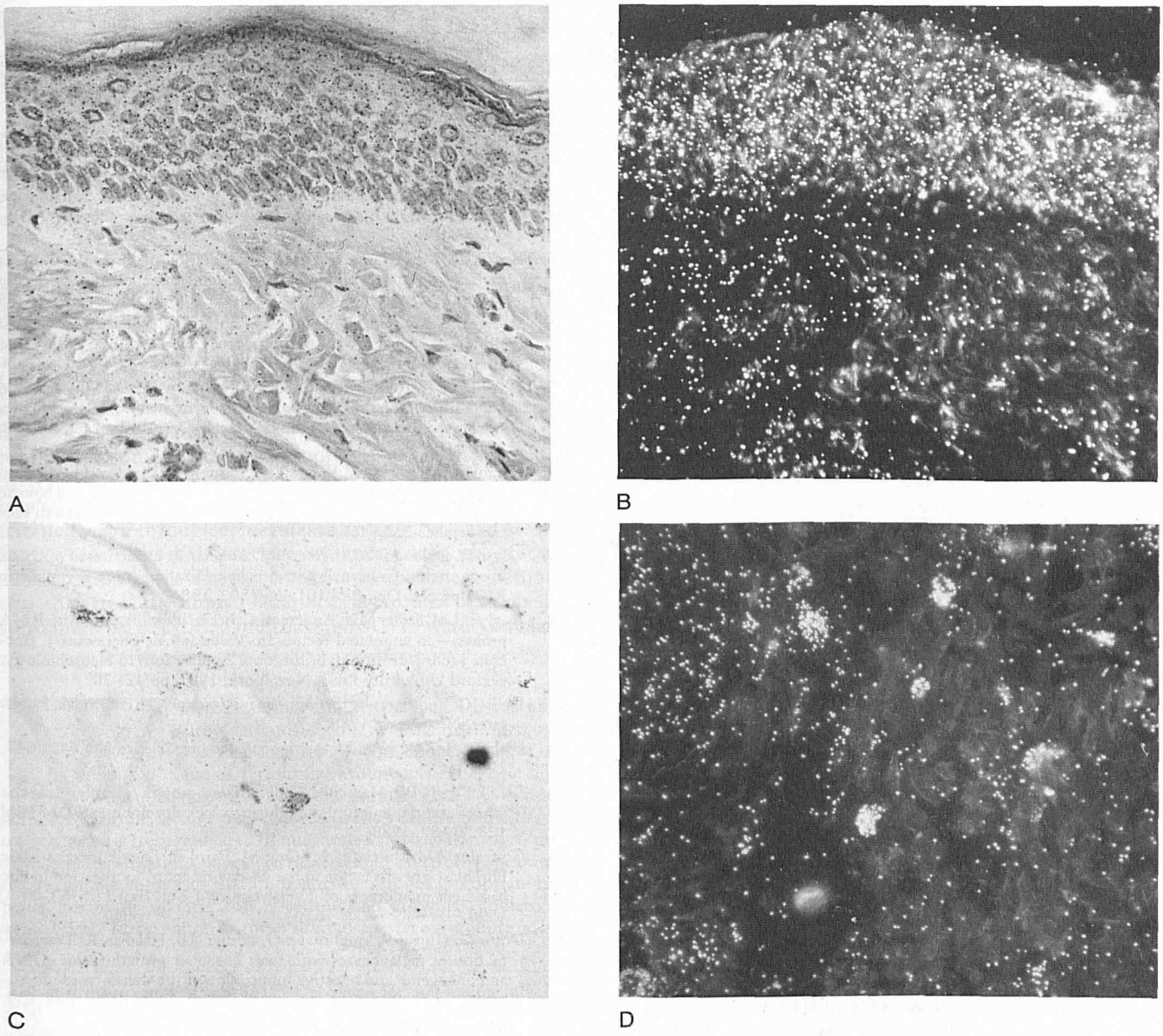

Figure 3. Normal skin hybridized with the bFGF probe shows 2+ signal in epidermal keratinocytes as seen under bright field *(A)* and dark field *(B)* illumination. Dermal fibroblasts under bright field *(C)* and dark field *(D)* illumination show 3+ signal. (Magnification ×200.)

nevus, dysplastic nevic cells expressed similar levels of bFGF gene transcripts (2+) as the invasive malignant melanocytes. Metastatic malignant melanoma cells showed intermediate to low signal intensity and in one case no signal was detectable above background levels (data not shown).

In Situ Localization of bFGF mRNA in Normal Skin Epidermal and adnexal keratinocytes expressed in bFGF mRNA in all cases at intermediate levels (2+, Fig 3*A*,*B*). Fibroblasts expressed abundant bFGF mRNA (Fig 3*C*,*D*) but endothelial cells showed variable expression (data not shown). In the five cases of metastatic melanoma, expression could not be detected in endothelial cells lining blood vessels or in endothelial cells from six of the 12 cutaneous melanocytic lesions.

DISCUSSION

In situ hybridization with riboprobes for bFGF mRNA shows that nevomelanocytes from both benign and malignant melanocytic lesions express bFGF gene transcripts and that bFGF expression cannot be used as a marker for transformation. In sections of normal skin, bFGF mRNA was detected in keratinocytes, fibroblasts, and endothelial cells. The method did not allow us to determine whether normal melanocytes in vivo produced bFGF because of the difficulty in differentiating individual intraepithelial melanocytes from keratinocytes in the basal layer without the use of immunocytochemical or electron microscopic techniques. The data on keratinocytes, fibroblasts, and endothelial cells are in accordance with preceding studies showing bFGF gene transcripts in proliferating human keratinocytes, dermal fibroblasts, and endothelial cells in culture [3,4,12]. Although there are reports describing the immunolocalization of bFGF in various tissues [13,14], this is the first report of the distribution of bFGF mRNA gene transcripts in melanocytic lesions in vivo.

That expression of bFGF alone is insufficient to confer a malignant phenotype is in agreement with results obtained by introducing a bFGF cDNA clone into normal melanocytes experimentally [15]. The introduction of bFGF cDNA into primary cultures of murine melanocytes via a retroviral expression vector led to low expression of bFGF that was insufficient to confer autonomous growth from external factors. In contrast, introduction of bFGF

cDNA clones into a murine melanocyte cell line, L-B10BR, that had lost growth dependency on cAMP stimulator, led to high expression of bFGF, autonomous growth in culture, and loss of all differentiated functions, including pigmentation. However, even the factor-independent, bFGF-transformed melanocytes did not grow as tumors in syngeneic or nude mice but instead regained their differentiated functions, including the ability to produce melanin, and appeared in all respects like normal melanocytes.

The significance of bFGF production by nevic cells in vivo is uncertain but experimental evidence suggests that expression of low levels of bFGF may confer a growth advantage to these cells. Nevic cells have a longer life span in culture than do normal melanocytes, once growth factors are removed from the medium and remain viable for a longer period of time [16]. Although nevic cells cultured from common and dysplastic nevi generally display complete dependence on melanocyte growth factors, melanocytes derived from two dysplastic nevi from a 28-year-old patient, who had a history of cutaneous melanoma and had the dysplastic nevus syndrome, were able to proliferate in the presence of only cAMP stimulators in the absence of bFGF [16]. These data suggest that the low levels of expression of bFGF in nevic melanocytes confer a growth advantage and increased viability under conditions of deprivation that kill normal melanocytes.

Studies by other investigators on the transforming capacity of bFGF are in agreement with our observations. These studies suggest that bFGF confers growth advantage and a transformed phenotype to cells in culture when expressed at moderate levels but only becomes a transforming growth factor in vivo when modified to be secreted by the insertion of a signal peptide [17] or when made to be expressed at extremely high levels (up to 0.1% of total cellular protein) [18].

The interrelationship of keratinocytes and melanocytes in vitro is well recognized. Melanocytes cluster around keratinocytes and melanocytes co-cultured with keratinocytes or cultured with keratinocyte cell extracts proliferate at a faster rate than when cultured alone. Recent work using the skin equivalent model suggests that the proliferative rate or state of differentiation of the keratinocytes may regulate the position of melanocytes at the basal layer [19]. The molecular mechanisms of these interactions are unknown, but because bFGF is a melanocyte mitogen it has been suggested that bFGF may be one of the factors that mediates these interactions [3]. The demonstration of bFGF mRNA gene transcripts within keratinocytes in vivo would support a theory of paracrine regulation of melanocytes by neighboring keratinocytes through local production of bFGF.

This study clearly identifies bFGF mRNA within several cell types in the skin. In addition to confirming work done on the distribution of bFGF in cells in skin cells in culture, we have established that benign, dysplastic, and malignant melanocytes produce bFGF mRNA in vivo and that bFGF expression alone does not confer a malignant phenotype. Because much is still unknown about the mechanism by which bFGF is released, where it is stored and how it acts on its target tissue, it remains necessary to establish the physiologic function of bFGF in the skin in vivo.

Note: Since submission of this manuscript a survey of the distribution of bFGF in normal, inflammatory, and tumorous tissues by immunocytochemistry was done by Schulze-Osthoff et al [20]. They found that in normal skin bFGF was expressed by basal keratinocytes and hair follicle epithelium, sweat gland epithelium, and sebaceous glands. Histiocytes and endothelial cells rarely expressed bFGF.

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