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Induction of Different Morphologic Features of Malignant Melanoma and Pigmented Lesions After Transformation of Murine Melanocytes with bFGF-cDNA and H-*ras*, *myc*, *neu*, and E1a Oncogenes

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Malignant melanomas show a remarkable degree of heterogeneity because of different morphologic features, biologic behavior, and prognosis. In this communication, the authors attempted to correlate morphologic heterogeneity of melanomas with transformation by different activated oncogenes; they studied the histologic features of melanocytic lesions induced by murine melanocytes transformed by basic fibroblast growth factor (b-FGF-cDNA) or H-ras, neu, myc, and E1a oncogenes, and the lesions were compared with those observed in human pathology. Tumors formed after grafting onto syngenic mice or subcutaneous injections in nude mice were studied. In syngenic mice, benign melanocytic lesions reminiscent of intradermal nevus were observed with melanocytes transformed with b-FGF-cDNA, and myc and E1a oncogenes. Benign lesions were also formed by neu-transformed melanocytes when they were grafted concomitantly with keratinocytes, whereas malignant tumors were formed by the same cells when grafted alone or together with fibroblasts. In contrast, H-ras melanocytes always formed malignant tumors. In nude mice, b-FGF–transformed melanocytes induced benign lesions, whereas transformed melanocytes by the other oncogenes formed malignant tumors with distinctive and homogeneous morphologic features that depended on the transforming oncogene. Melanomas with either epithelioid cell, spindle cell, small round cell, and anaplastic cell growth patterns could be distinguished after transformation with H-ras, neu, E1a, and myc oncogenes, respectively. These various histologic types are analogous to those that may be observed in human melanomas, even within the same tumor. These studies suggest a possible molecular mechanism for tumor heterogeneity in which distinct oncogenes or oncogenelike activities can be activated in different tumors or discrete parts of the same tumor. (Am J Pathol 1991, 138:349–358)

The study of cellular oncogenes provides an important tool for clarifying the various genetic events involved in carcinogenesis.[1,2] Tumor formation may require escape from the inhibitory effects of a normal cellular environment[3] and the control by the immune system.[4]

Malignant melanomas provide a good example of multistep tumor development.[5–7] Histologically malignant melanomas are characterized by a high degree of heterogeneity, with a broad spectrum of morphologic features that may simulate any other human neoplasia.[8] The study of the molecular basis for this heterogeneity is completely unknown and could be useful in understanding the biology and evolution of these tumors as well as to establish better prognostic criteria. Prognostic factors of malignant melanomas used so far include tumor thickness, mitotic rate,[9] and the study of the growth fraction of malignant melanoma determined by immunostaining with the monoclonal antibody Ki 67.[10] Cell type has been

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shown to be an independent variable for patients with melanomas less than 2.00 mm in thickness.[11]

In a previous report,[12] we tried to cause melanoma formation by transforming normal murine melanocytes with specific genes such as b-FGF-cDNA and H-*ras*, *neu*, *myc*, and E1a oncogenes. The resultant transformants showed different *in vivo* behavior depending on the immune system (Syngenic [SM] *versus* nude mice [NM]) and the surrounding cellular environment.

In the present communication, we studied the possible role of selective oncogene activation in the induction of tumor heterogeneity as evaluated by morphologic parameters. We performed a detailed histologic analysis of the lesions induced by the transformed melanocytes in mice. All examined tumors displayed distinctive and homogeneous histologic patterns, which depended on the type of oncogene activated, including epithelioid cell, spindle cell, small round cell, and anaplastic growth patterns. Oncogene-induced melanomas appear histologically similar to human melanomas; thus tumor heterogeneity may be due to distinct oncogenelike activities in different tumors or discrete parts of the same tumor.

Materials and Methods

In Vitro Transformation

L-BIOBR melanocytes[13] were transformed with retroviral vectors carrying the viral Harvey *ras* and avian Mc29 *gag-myc* oncogenes,[14] the rat *neu* oncogene,[15] and the E1a region of adenovirus 5.[16] A retrovirus carrying a full-length copy of bovine b-FGF-cDNA was also used. *In vitro* characterization of these various transformants has been described.[12]

Grafting and Injection in Nude Mice

The number of cells used for injection of each type were 2×10^5 melanocytes, 2×10^6 keratinocytes, and 8×10^6 dermal fibroblasts. Cells were grafted onto the back of syngeneic mice (female, 6 to 8 weeks old) as previously described.[3,17,18] The same amount of cells were used for subcutaneously injection in nude mice (male, 6 to 8 weeks old).[12] Mice were killed 21 to 28 days later.

Light Microscopy

Tumors and lesions obtained were quickly fixed in 10% buffered formalin and embedded in paraffin. Five- to six-micron–thick sections were stained with hematoxylin and eosin (H&E). At least four tumors or lesions of each type of transformed melanocytes, both in SM and NM, were studied. Mice were also examined for distant tumor spread. Metastatic lesions were not detected in any cases, presumably because of the short time (4 weeks) chosen for termination of the experiment.

Immunopathologic Study

Formalin-fixed, paraffin-embedded tissue sections of tumors in each group were stained with S-100 protein antibodies (diluted 1:100; Dakopatts, Santa Barbara, CA) by the avidin-biotin complex (ABC) technique.

Histologic Evaluation

Tumors were evaluated according to their cytologic and architectural features. Cytologically, nuclear atypia, nucleoli, cytoplasmic features, number of mitoses, and necrosis were evaluated. According to the histologic growth pattern, melanomas were classified as: 1) predominant spindle cell; 2) epithelioid cell (round cells with abundant cytoplasm, often disposed in nests or 'theques'); 3) undifferentiated (small- to medium-sized cells with a diffuse pattern of growth and loss of nesting pattern); and 4) anaplastic (large bizarre cells, with big nucleoli and marked cytologic atypia). Features considered as indicative of higher differentiation were the formation of nests (theques), melanin pigment production by the tumor cells, and the degree of expression of S-100 protein.

Results

Summary of Lesions Induced in Syngenic Mice and Nude Mice

In syngenic mice, melanocytes transformed with *ras* or *neu* oncogenes gave rise to malignant neoplasms. With cells transformed by the other oncogenes (E1a, *myc*) or bFGF cDNA, benign lesions resembling intradermal nevus were observed.

In nude mice, a prominent black lesion corresponding to an intradermal nevuslike growth was observed after the injection of the b-FGF L-BIOBR melanocytes; malignant tumors were detected with the other oncogenes (Table 1).

When melanocytes transformed with the various oncogenes were injected concomitantly with keratinocytes or dermofibroblasts, only those transformed with *neu* showed different histologic features and biologic behavior (see Table 1).

Table 1. *Lesions Induced by Oncogene-transformed Melanocytes in Nude and Syngenic Mice*

Cells	Syngenic mice	Nude mice
LBIO · BRb FGF	Intradermal nevuslike	Intradermal nevuslike
LBIO · BR *myc*	Intradermal nevus or scattered pigmented cells	Anaplastic melanoma with sarcomatoid appearance
LBIO · BR E1a	Intradermal nevus or scattered pigmented cells	Small round cell tumor with lymphoblastoid appearance
LBIO · BR *neu* (±)Fibroblasts	Spindle cell melanoma	Spindle and epitheliod cell melanoma
LBIO · BR Neu Ker.	Scattered pigmented cells or small spindle cell lesion with regression features	Spindle and epitheliod cell melanoma
LBIO · BR H-*ras*	Epitheliod cell melanoma with nesting formation; Occasional spindle cell areas	Epitheliod cell melanoma

L-BIOBR b-FGF Melanocytes

In all cases, only a black spot at the injection was observed. No tumors were identified. Histologically nests or scattered pigmented cells were observed in the dermis, displaying an epithelioid or dendritic morphology. No atypia and very few lymphocytes were identified. In nude mice, similar lesions were present (Figure 1A). Well-differentiated melanocytes were also seen in the dermis or reconstituted epidermis after grafting of normal, untransformed L-BIOBR cells. In this case, however, cells were sparse and present in much lower numbers.

L-BIOBR-myc and L-BIOBR-E1a-Melanocytes

In syngeneic mice, cells transformed by either *myc* or E1a oncogenes produced similar lesions. Only scattered cells or cell clusters with the features of intradermal nevus were observed at the site of grafting (Figure 1B). The number of pigmented cells was variable. Sparse lymphoid cells were present, and no atypia, mitoses, or necrosis was identified (Table 2).

In nude mice, the L-BIOBR-*myc* melanocytes produced very aggresive tumors, with abundant areas of

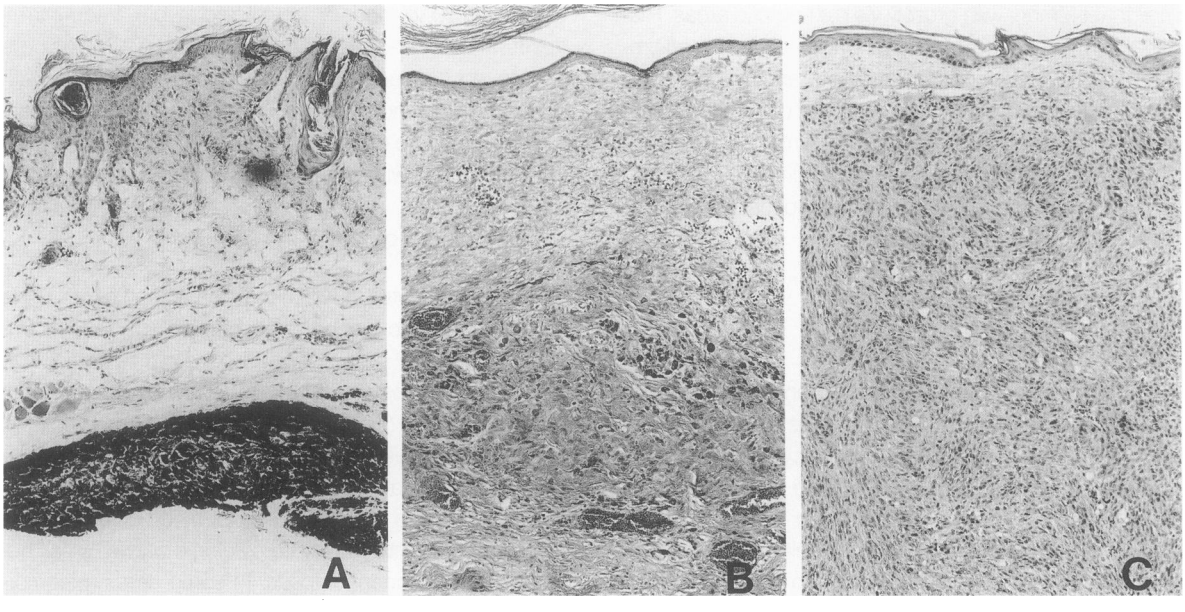

Figure 1. *Lesions observed after grafting of b-FGF-(A), E1a− (B) and* Neu− *(C) transformed melanocytes together with keratinocytes onto syngenic mice.* **A**: *b-FGF-cDNA transformed melanocytes. Clusters of deeply pigmented melanocytic cells in deep dermis (H&E, ×40).* **B**: *E1a-transformed melanocytes. Scattered melanin-laden melanocytes are seen in reticular dermis (H&E, ×40).* **C**: Neu-*transformed melanocytes grafted concomitantly with keratinocytes. Bland and spindle cell population is seen replacing the dermis containing lymphocytes (H&E, ×40).*

Table 2. *Morphologic Parameters of the Lesions Induced in Syngenic (SM) and Nude Mice (NM)*

Cells	Cit.Atyp	Mit	Necr	Pigm	Lymp.Inf.	S100
LBIO · BR FGF						
SM	−	−	−	+ + +	− +	+ + +
NM	−	−	−	+ + +	−	+ +
LBIO · BR *myc*						
SM	−	−	−	+	−	+ +
NM	+ + +	+ + +	+ + +	− +	−	− +
LBIO · BR E1a						
SM	−	−	−	+ +	− +	+
NM	+ +	+ +	+ +	−	−	−
LBIO · BR Neu						
SM	+	+	−	− +	+	+
NM	+	+ +	+	−	−	− +
LBIO · BR Neu + Ker.						
SM	−	+	−	− +	+ +	+ +
NM	+	+ +	+ +	− +	−	−
LBIO · BR H-*ras*						
SM	+ +	+ +	+	− +	− +	+ +
NM	+ + +	+ + +	+	−	−	− +

Mit = mitoses (+: occasionals; + +: less than 5 mitoses/10 HPF (high power field); + + +: more 5m/10HPF)
Necr = necrosis (+: scarse; + +: less than 1/3 tumor size; + + + more than 1/3 tumor size)
Pigm = Pigmented cells (− +: only in some cases, scattered cells; +: occasional cells; + +: less 10% cells; + + +: more 10%)
Lymp.Inf = Lymphoid inflamatory (− +: only in some cases, few cells; +: sparse; + +: less than 10% HPF; + + + more 10% HPF)
S-100 = Protein S-100 (− +: only in some cases, scattered cells; +: occasional; + +: less than 10% cells; + + + more 10% cells)

necrosis. These tumors achieved the largest size of all the tumors studied. Histologically, the neoplasms were composed of very undifferentiated and anaplastic cells, with large and prominent nucleoli, and acidophylic cytoplasm. Numerous mitoses, extensive areas of necrosis, and sparse, occasional pigmented cells were observed (Figure 4A).

In nude mice, L-BIOBR-E1a melanocytes grew as malignant tumors with grossly visible necrotic areas. Histologically they were composed of small and medium-sized cells with hyperchromatic nuclei and multiple small nucleoli. The tumors showed a diffuse pattern of growth with a distinctive monomorphic and lymphoblastoid appearance. They showed numerous mitoses and extensive areas of necrosis. No pigmented cells were observed (Figure 4B).

L-BIOBR-neu-Melanocytes

In syngeneic mice, the melanocytes transformed with the *neu*-oncogene–induced benign lesions or small tumors when they were grafted with keratinocytes (Figure 1C), and malignant tumors when they were grafted alone or with fibroblasts. The tumors observed when they were grafted alone displayed a spindle cell growth pattern, with the cells disposed in fascicles with occasional scattered epithelioid cells. Mild cytologic atypia, few mitoses, sparse lymphocytes, and occasional pigmented cells were present (Figure 2A,B). The lesions found when these transformants were grafted concomitantly with keratinocytes displayed a bland cytologic appearance and a striking number of lymphocytes. Conversely, the tumors showed more aggressive histologic features when they were grafted with fibroblasts.

In nude mice, *neu*-transformed melanocytes produced malignant melanomas in all cases with and without keratinocytes. Histologically, a spindle cell growth pattern with scattered epithelioid cells was observed that showed mild to moderate cytologic atypia, frequent mitoses, and sparse areas of necrosis (Figure 2C). The cells displayed a predominantly spindle morphology with small nucleoli and focal foci of anaplasia. No significant differences were observed when the L-BIOBR-*neu* were injected mixed with keratinocytes or fibroblasts.

L-BIOBR-RAS-Melanocytes

These melanocytes induced malignant neoplasms with a similar histologic appearance both in syngeneic and nude mice. The cells showed epithelioid features, with abundant eosinophilic cytoplasm and well-defined cytoplasmic borders (Figure 3A). Sometimes pigmented cells could be observed in the center of the lesions. Nesting formation and a pseudoalveolar pattern were distinguished in some areas (Figure 3B). The nuclei were hyperchromatic, with moderate cytologic atypia, small nucleoli, and abundant mitoses. In some areas, pleomorphic and elongated cells could be observed. Necrosis was present in some areas both in syngenic and nude mice, whereas inflammatory cells were sparse or absent. The tumors invaded adjacent structures.

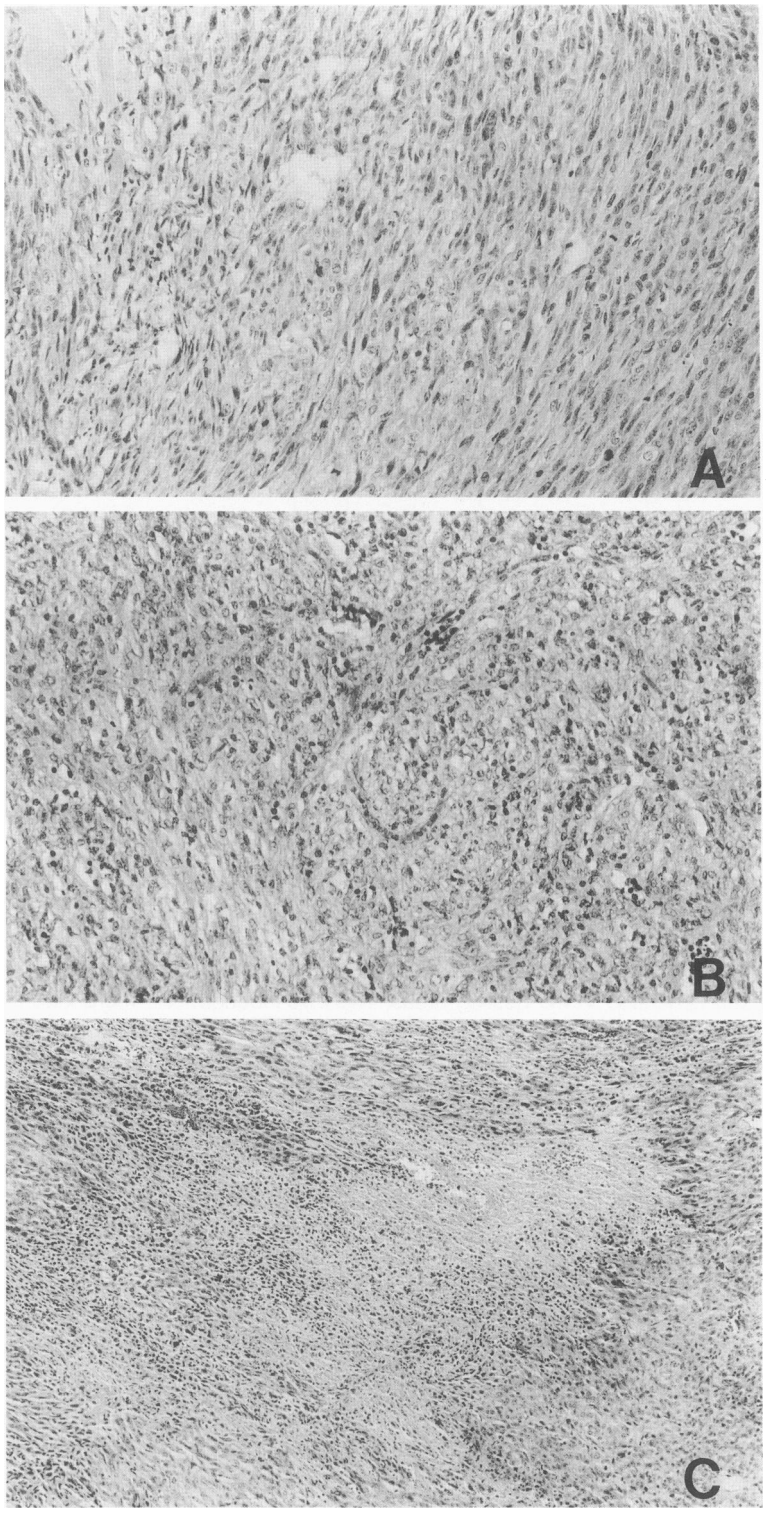

Figure 2. *Tumors formed by* Neu-*transformed melanocytes, in the absence of keratinocytes, in syngenic mice (A,B) and nude mice (C).* **A**: Neu-*transformed melanocytes. Compact proliferation of elongated spindle-shaped cells with mild nuclear atipia and brisk mitotic activity (H&E, ×200).* **B**: Neu-*transformed melanocytes plus keratinocytes. Mix cell population with bland cytologic features displaying prominent limphocytic infiltration (H&E, ×200).* **C**: *Neu-transformed melanocytes in nude mice. Spindle cell proliferation with extensive areas of necrosis (H&E, ×100).*

Expression of S-100 Protein

Most of the benign lesions observed in syngeneic mice contained a high number of S-100 protein-positive cells (Table 2). The reaction was observed both in pigmented and unpigmented cells. In malignant tumors, most of the S-100-positive cells had an epithelioid morphology, and only scattered spindle cells reacted with the antibody. According to the pattern of expression, tumors induced by H-*ras* oncogene showed a larger number of positive

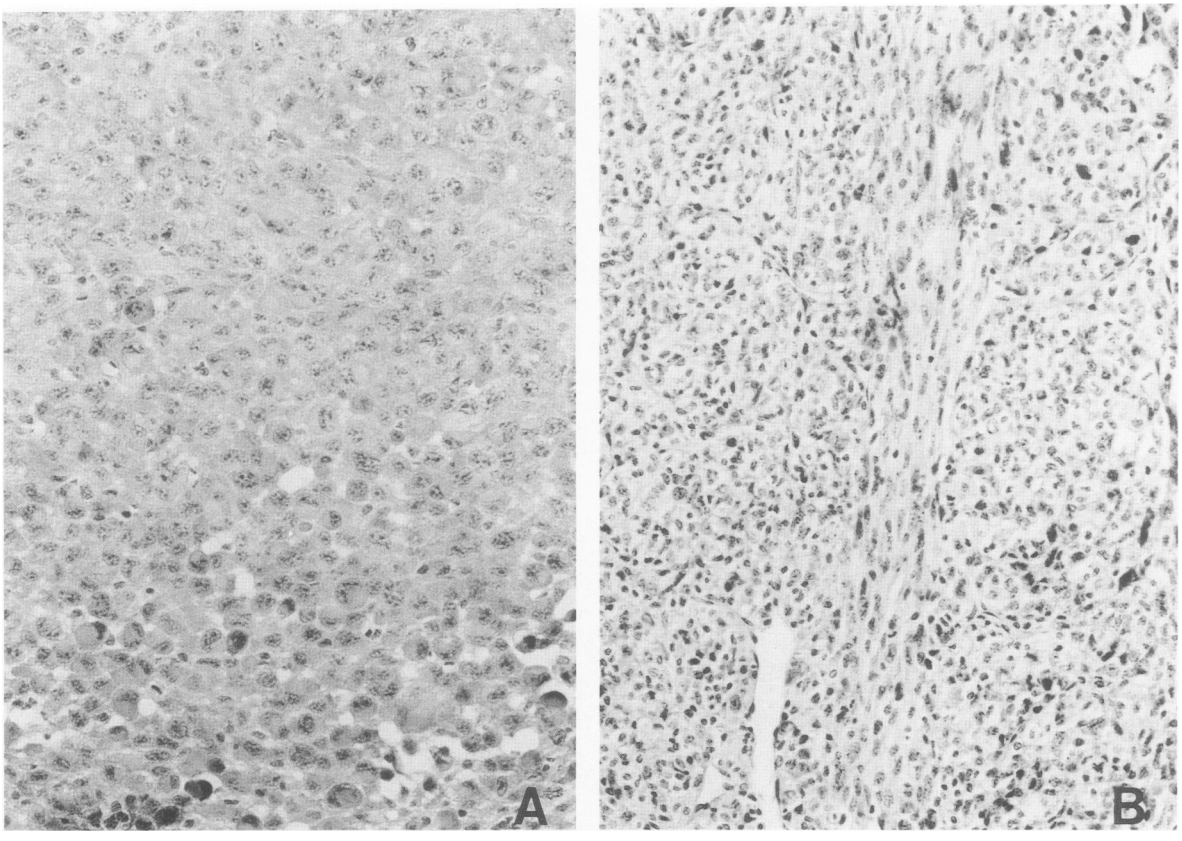

Figure 3. *Tumors formed by* ras*-transformed melanocytes in syngenic mice.* **A:** *H-*ras*–transformed melanocytes. Uniform population of large epithelioid cells containing abundant and eosinophilic cytoplasm (H&E, ×200).* **B:** *H-*ras *transformed melanocytes. Focal areas of nesting pattern are present reminiscent of well-differentiated melanocytes tumors in humans (H&E, ×200).*

cells than those induced by *neu* oncogene. The positivity was enhanced in the nesting areas. The *myc*-induced melanomas showed very few positive cells. Melanomas induced by E1a were uniformly negative for S-100 protein.

Discussion

In this paper we describe the histologic features of lesions induced in syngenic and nude mice by melanocytes transformed with different oncogenes. L-BIOBR melanocytes were transformed with retroviral vectors carrying the avian MC29 *gag-myc* oncogene, the Harvey *ras*, the rat *neu* oncogene, the adenovirus E1a-13s region, and a complete cDNA copy of bovine bFGF.[12] These genes transform normal cells by presumably quite different mechanisms.[1,19–23] Cells transformed with these oncogenes induced benign or malignant lesions, depending on the activated gene and the immune system of the animals (nude mice versus syngenic mice) (Table 1). Histologically distinctive features could be related to activation of the specific oncogenes. A similar underlying mechanism could be related to the various morphologic growth patterns of human melanomas and explain the tumor heterogeneity usually found in human tumors. In fact, the distinctive growth patterns that we observed with H-*ras*-, *neu*-, E1a-, and *myc*-melanomas appear remarkably similar to those found in human pathology and classified as nodular melanomas or melanomas with morphologic features of fibrosarcomas, round cell neoplasms, and anaplastic sarcomas, respectively (see Rosai,[8] Figures 4 to 11).[24] With this model, we suggest that, even in human melanomas, activation of specific oncogenes is responsible for the different morphologic features usually observed in human tumors. Study of human melanomas should be the next step to correlate the morphologic features, oncogene activation, and clinical prognosis.

Most oncogenes can stimulate as well as suppress expression of many cellular genes, and such abnormal gene expression may contribute to the neoplastic phenotype.[1,25,26] Although experiments with different transforming oncogenes in melanocytic and other cell lines have been carried out, very few morphologic illustrations of the tumors induced have been reported.[27,28] In the present system, because the various oncogenes

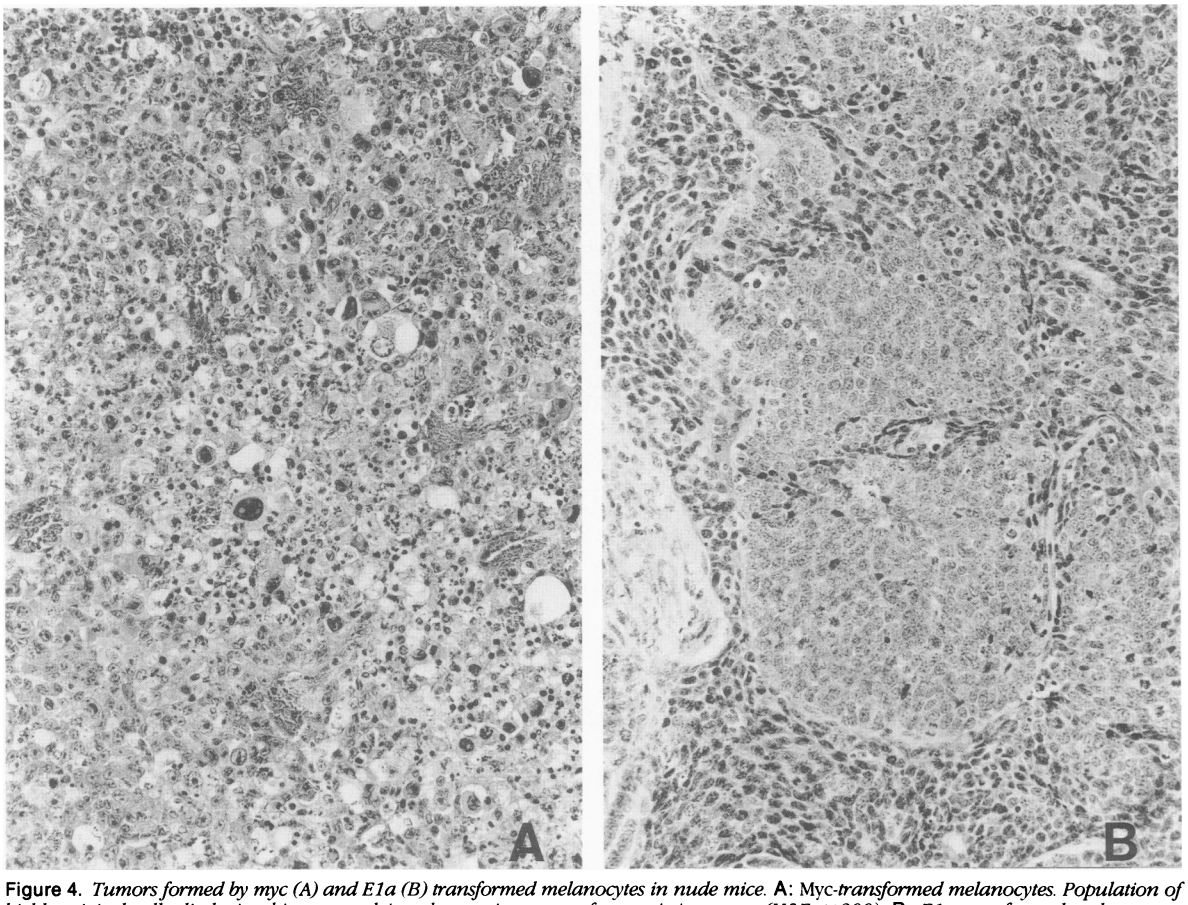

Figure 4. *Tumors formed by myc (A) and E1a (B) transformed melanocytes in nude mice.* **A:** Myc-*transformed melanocytes. Population of highly atipical cells displaying bizarre nuclei and extensive areas of necrosis is present (H&E, ×200).* **B:** *E1a-transformed melanocytes. Monotonous proliferation of undifferenciated, small, round cells containing multiple small nuclei is seen, resembling "small blue cell tumors" in humans (H&E, ×200).*

were introduced into the same melanocytic cell line, we may assume that the consistent morphologic changes that were observed are a specific result of the activity of the various transforming oncogenes. Thus, our results indicate that intrinsic genetic differences among cells are an important determinant of tumor heterogeneity. A similar conclusion is also suggested by the fact that even in human pathology tumor heterogeneity is frequently found within a single tumor[8] and genetic heterogeneity is a well-documented fact.[6,20,24,29,30]

Microenvironmental factors as well as the general conditions of the host, however, are likely to play a second, key determinant role in tumor heterogeneity. In previous works, the cellular environment and the site of the metastasis were found to be involved in the tumor heterogeneity.[31–33] In malignant cell populations, the heterogeneity could be explained by specific deficits in their cell surface receptors[25] and the differences could be mediated by local factors controlling proliferation or differentiation.[34]

In this communication, we have shown that the immune system can also play an important role in control of tumor development and it can exert different effects, which are specifically dependent on which oncogene transformed the target cell. Modulation of the immune response against *neu*-transformed melanocytes by surrounding keratinocytes also provides support to the notion that heterotopic cell interactions can have significant tumor-suppressing effects.[3]

Dotto et al[18] showed that a specific and more differentiated cell line (p117) obtained through chemically induced mouse papillomas produced either differentiated or undifferentiated tumors, depending on the oncogene with which they were transformed. This model showed that malignant progression of benignly transformed keratinocytes can be substantially altered by the action of different oncogenes, paralleling specific steps that frequently occur *in vivo*. In the present study, using normal melanocytes, we could reproduce different growth patterns observed in human melanoma. In other systems, the expression of v-*myb* and v-*myc* are able to regulate the phenotype of myelocytic cells with a mature, intermediate, or an immature phenotype depending on the oncogene(s) activated.[25] Buckley[26] proposes that such an

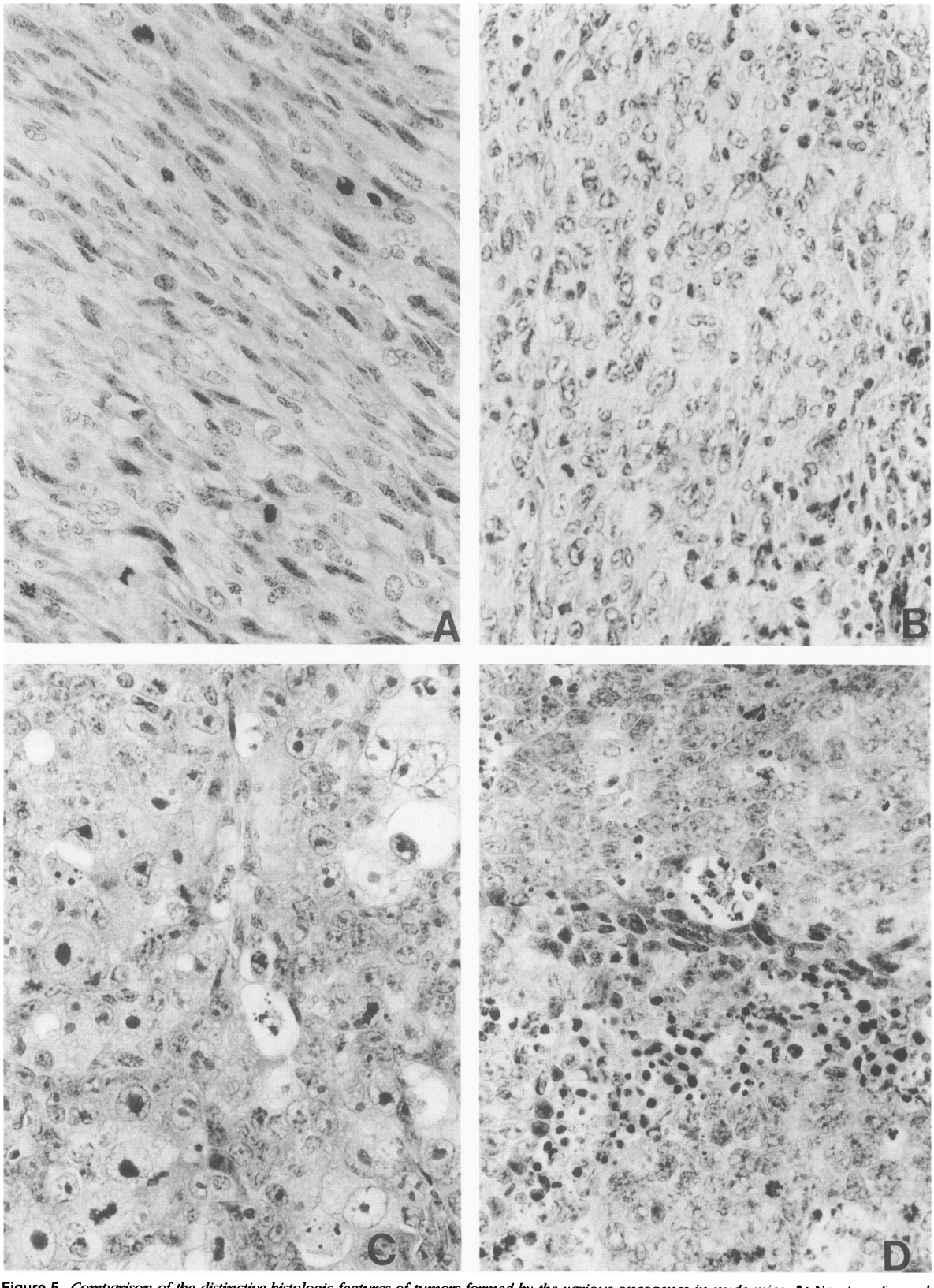

Figure 5. *Comparison of the distinctive histologic features of tumors formed by the various oncogenes in nude mice.* **A:** Neu-*transformed melanocytes. Highly atipical spindle cell proliferation containing numerous abnormal mitotic figures resembling growth pattern of fibrosarcoma (H&E, ×400).* **B:** *H-ras transformed melanocytes. Population of predominant epithelioid cells containing round nuclei with abundant cytoplasm (H&E ×400).* **C:** Myc-*transformed melanocytes. Proliferation of anaplastic large cells with round vesicular nuclei and prominent nucleoli (H&E, ×400).* **D:** *E1a-transformed melanocytes. Population of uniform round cells with blastic appearance showing multiples small nucleoli and scant cytoplasm (H&E, ×400).*

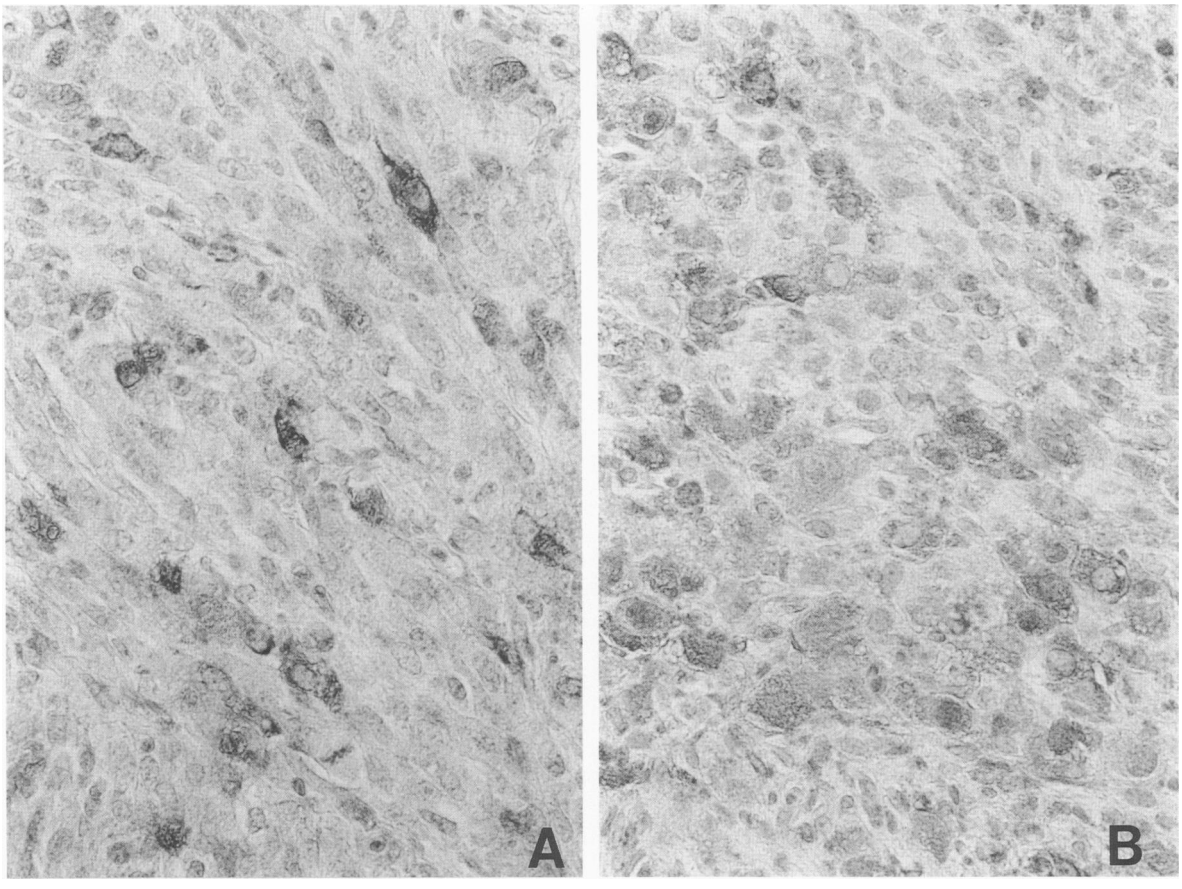

Figure 6. *Staining pattern of S100 protein.* **A:** *S-100 protein stains occasional cells in spindle cell areas of* neu-*transformed melanocytes.* **B:** *S-100-protein stains higher number of cells in epitheliod areas of* ras-*transformed melanocytes.*

effect is not achieved by blocking differentiation totally but rather by distorting the normal pattern of expression of individual phenotypic features, presumably by anomalous gene activation and suppression. Genetic damage remains undetected in the great majority of human tumors,[1] and tumor progression constitutes a cascade of complex events. The cooperation between different oncogenes and transcriptional factors and the study of other tissue regulatory factors may help explain the frequent variability observed in the pathology of human neoplasms.[24] In summary, we showed that melanocytes transformed with various oncogenes may induce malignant tumors with distinctive histologic features that parallel the different patterns of growth of human melanomas. With this model, tumors induced experimentally with different oncogenes may be correlated with similar human tumors to study their biologic behavior.

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