Progression of Squamous Carcinoma Cells to Spindle Carcinomas of Mouse Skin Is Associated with an Imbalance of H-ras Alleles on Chromosome 71

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Abstract

Analysis of benign and malignant mouse skin tumors had previously shown that amplification of a mutant H-ras allele or loss of the normal allele was generally seen only in high grade or spindle cell tumors. The normal:mutant ras gene dosage has been studied directly by polymerase chain reaction amplification of DNA derived from paraffin sections of carcinomas of defined histological types. Some tumors had virtually no signal corresponding to the normal allele and these were invariably spindle cell carcinomas. In four cases where both squamous and spindle cell components could be identified within the same tumor the spindle cell component had a higher mutant:normal gene ratio. Additional experiments on cell lines derived from squamous or spindle cell tumors have demonstrated a good correlation between the ratio of normal:mutant ras and the degree of invasiveness of the cells in in vitro assays.

Introduction

The biological and biochemical features of tumor initiation and progression have been studied extensively using the mouse skin carcinogenesis model system (1). The initiating event, at least for a proportion of tumors and depending upon the nature of the initiating carcinogen, appears to involve mutation of the cellular Harvey-ras (H-ras) gene (for review, see Ref. 2). In mouse skin the same gene appears to be involved in tumor progression, since many carcinomas appear to exhibit amplification of the mutant ras allele and/or loss of the corresponding normal allele (3-7). Inspection of skin tumors induced by the standard DMBA+/TPA carcinogenesis protocol (4) showed that there was a strong correlation between loss of normal ras alleles and the development of the anaplastic phenotype. The frequency of induction of anaplastic tumors can be increased by treatment of developing skin tumors with benzoyl peroxide, an agent which induces DNA damage (8). We have used the polymerase chain reaction to show that spindle cell carcinomas exhibit the same ras mutation as adjacent squamous carcinoma cells but differ in that they have an altered ratio of normal to mutant ras alleles. These genetic changes are accompanied by the acquisition of more aggressive growth properties in vitro and in vivo.

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4 The abbreviations used are: DMBA, 7,12-dimethylbenz(a)anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate.
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Materials and Methods

Tumor Induction. Tumor induction was carried out as described previously (4) or using either a three stage carcinogenesis protocol, consisting of initiation with 10 nmol DMBA, 20-week promotion with a weekly dose of 2 µg TPA, followed by 10 weeks of weekly applications of 20 mg benzoyl peroxide, or a complete carcinogenesis protocol consisting of 50 weekly applications of 20 nmol DMBA (8). All tumors were fixed with 10% buffered formaldehyde, embedded in paraffin, and stained with hematoxylin-eosin. Normal skin structures were dissected from the tumor tissue using a stereomicroscope. Squamous and spindle cell components were divided and normal skin structures as well as inflammatory infiltrates and extensive stromal components were dissected from the tumor. The dissected components were reembedded in paraffin and new sections were studied to assess the separation of the two tumor components.

Extraction and Amplification of DNA from Formalin-fixed Tissues. DNA was extracted from tumors which had been fixed in formalin and embedded in paraffin essentially according to the method of Dubeau et al. (9). One µg of DNA from each sample was used in a polymerase chain reaction to amplify exon 2 of the mouse H-ras gene as described previously (10). Each filter containing amplified DNA was probed successively with end-labeled oligonucleotides corresponding to the normal (CAA) or mutant (CTA) sequences at codon 61 (10). The intensity of the dots was quantitated by densitometric analysis. In order to take account of differences in the specific activity of the oligonucleotide probes, the exposure times were adjusted to reflect the known ratios of normal to mutant ras alleles in PDV and PDVC57 cell lines (approximately 2:1 and 1:2, respectively) (4, 11).

Cell Lines. Cells were maintained under an atmosphere of 5% CO2 in antibiotic-free minimal essential medium supplemented with 10% fetal calf serum. Cell lines carB, carC, and SN161 were derived from the primary spindle cell carcinomas described in the text. These and other cell lines used in this study have been described in detail elsewhere (4, 11).

Tumorigenicity, Spontaneous Metastasis, and Experimental Lung Colonization Assays. Viable cells (10⁶) were injected s.c. in the backs of 8-10 week-old nude mice of BALB/c background. Eight to 10 weeks following the observation of tumors, mice were sacrificed and tumor and lung tissues were examined histologically. Experimental lung colonization was examined by injecting 5×10⁶ viable cells in 0.05 ml 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid-buffered saline into the tail vein. After 8 weeks, or before the mice became sick, animals were sacrificed and metastatic foci in the lungs were examined histologically.

Chemo invasion and Chemotaxis Assay. These assays were performed by a modification of previous methods (12, 13).

Type IV Collagenolytic Assay. Type IV collagenase activity in cell supernatants was assayed as described previously (14).

Results

Cell Lines Which Have Lost the Normal ras Allele Are Derived from Anaplastic Carcinomas. It has been shown previously that a decrease in the normal:mutant ras gene ratio is associated with a more aggressive tumorigenic phenotype (11). This is demonstrated by comparison of cell lines PDV and...
IMBALANCE OF H-ras ALLELES ON CHROMOSOME 7

PDVC57, which have normal:mutant H-ras gene ratios of 2:1 and 1:2, respectively (4, 11). Both of these lines exhibit the characteristic DMBA-associated mutation at codon 61 of the H-ras gene and express normal and mutant ras p21 in accordance with the gene dosage (11). Injection of PDV cells s.c. into nude mice gives rise to tumors only in about 30–50% of injection sites, whereas PDVC57 is more strongly tumorigenic, with tumors diagnosed as squamous carcinomas arising at all injection sites (Table 1).

The cell lines SN161, carB, and carC were all developed from carcinomas induced by initiation with DMBA and promotion with TPA (3, 4) and all of these lines showed complete loss of the normal ras allele in Southern blots (Ref. 4 and data not shown). Each line also gave rise to highly anaplastic spindle cell carcinoma or fibrosarcoma-like tumors after injection into nude mice (Fig. 1). Inspection of the histology of the original tumors showed that carB (Fig. 1) and carC (data not shown) were high grade spindle carcinomas before explanting, indicat-

Fig. 1. Histological appearance of original spindle cell tumors and nude mouse tumors produced by s.c. inoculations of cell lines. (A) Original tumor carB; (B) growth at the s.c. injection site in nude mice; (C) original SN161 tumor. The corresponding nude mouse tumor was similar to that shown in B; (D) carcinoma produced in a Sencar mouse by a multistage carcinogenesis protocol with both a spindle cell component (right) and a squamous cell component (left); (E) higher magnification of the spindle cell component shows numerous fusiform and giant atypical cells; (F) squamous cell carcinoma component is a well differentiated tumor with little stroma. A–C, × 110; D, × 30; E, F, × 90.
ing that the undifferentiated phenotype was not a consequence of prolonged growth in culture. The primary SN161 tumor showed mainly a spindle cell phenotype but exhibited some areas of moderate epithelioid differentiation (Fig. 1C). These epithelioid cells did not, however, grow in vitro, inasmuch as the SN161 nude mouse tumor was composed exclusively of undifferentiated cells (data not shown).

Spindle Cell Carcinomas Are Derived from Squamous Carcinomas in Vivo. The above observations on the histology of tumors which had lost the normal ras gene suggested that the spindle cell tumors might arise by loss of the ras allele or of other genes close to the H-ras locus on mouse chromosome 7 from preexisting squamous tumors. The use of the polymerase chain reaction enabled us to address this question more directly, by analyzing the normal:mutant ras gene ratio in paraffin sections from tumors of defined histological types. DNA extracted from paraffin sections was amplified using H-ras-specific amplimers and the normal:mutant ras allele ratio was determined by probing with labeled mutation-specific oligonucleotides.

Five tumors were analyzed that contained both a spindle and a squamous component (Fig. 1D). The spindle component was usually homogeneous with fusiform cells arranged in fascicles or whorls. Mitotic figures were moderately abundant (Fig. 1E). The squamous component of these tumors exhibited variable differentiation that included well differentiated as well as moderately differentiated squamous cell carcinoma areas. The squamous carcinoma cells were clearly different from the spindle cells, i.e., large epithelial polyhedral cells with variable atypia and keratinization patterns (Fig. 1F). The five independent spindle cell tumors studied had the same histopathology as the spindle cell component of the mixed spindle/squamous tumors.

The controls for this experiment are shown in Fig. 2A. The signal intensities obtained using DNA from PDV, PDVC57, or carC cells probed with oligonucleotides corresponding to the normal (CAA) or mutant (CTA) sequences at codon 61 are in approximate proportion to the relative gene dosages as shown by Southern blots (4).

All of the primary tumors analyzed showed the presence of the codon 61 → T mutation, including 2 tumors induced by repeated treatment with benzo(a)pyrene, a carcinogen not previously associated with the induction of this particular mutation (Fig. 2B). The ratios of the normal and mutant ras alleles were verified by densitometric analysis (data not shown), using the intensities observed for the control samples shown in Fig. 2A as standards.

All of the pure spindle cell carcinomas showed very low levels and in some cases virtually complete absence of the normal H-ras allele (Fig. 2B). More importantly, in four cases where it was possible to analyze separately the sections derived from differentiated squamous or spindle cell components, there was a decrease in the normal:mutant ratio on progression from the squamous to the spindle cell component. The extent of the decrease varied between individual tumors. For tumor 3, the normal ras allele was almost completely lost, whereas in tumors 2, 4 and 5 the relative intensity of the normal allele was reduced such that the ratio was altered in favor of the mutant. In the fifth tumor showing both components (tumor 1), there was an apparent slight increase in the normal to mutant allele ratio, but histological examination of tumor sections indicated that both components were contaminated by cells of the other type or by abscess tissue, thus complicating the analysis. However, the other tumors all showed a relative decrease in the amount of normal H-ras allele in the spindle component. We conclude that the spindle carcinomas do in fact arise by progression from well-differentiated squamous tumors, in an event generally associated with altered normal:mutant ras gene dosage.

![Fig. 2. (A) Ratios of normal (CAA) to mutant (CTA) H-ras alleles in control cell lines. The approximate ratios as determined by karyotype analysis (not shown) and Southern blotting (4) are shown to the left. (B) Ratios of normal to mutant H-ras alleles in primary tumors. Squamous (Sq) or Spindle (Sp) sections of the same tumor are grouped together. Tumors from animals 1–4 were induced by the three stage protocol (8) (see "Materials and Methods"). Others were induced by repeated treatment with DMBA (animal 5), benzo(a)pyrene (animals 6 and 7), or two stage initiation/promotion (animals 8, 9, and 10).](cancerres.aacrjournals.org)
Spindle Cell Carcinomas Show Elevated Invasive and Chemotactic Properties. A series of the cell lines described above was tested in an assay for tumorigenicity, as well as for the invasive and chemotactic properties associated with highly malignant carcinomas. The results are shown in Tables 1 and 2. It can be seen that all of the cell lines which showed loss of the normal ras allele were highly tumorigenic in nude mice. SN161, carB, carC, and PDVC57 were clearly the most malignant murine skin tumor cell lines in terms of their tumor incidence, latency for tumor appearance, and lung colonization ability (Table 1). These four skin cell lines were considerably more invasive and chemotactic than PDV, C5N, and C50 and displayed higher collagenolytic activity at all time points assayed. Moreover, histological examination revealed that they all yielded tumors with a poorly differentiated phenotype, i.e., spindle cell tumors. These results demonstrate strong agreement among the in vitro and in vivo parameters of malignant potential assessed in these studies.

Discussion

We have demonstrated here that loss of the differentiated phenotype in mouse skin tumors takes place in a specific progression event from squamous to spindle cell carcinomas. The origin of spindle cell carcinomas has been the subject of debate for some considerable time, with a number of investigators believing that they constitute a separate tumor class of fibroblastic origin (for review, see Ref. 15). Our results, however, show that in mouse skin tumors containing both differentiated and spindle cell parts, both components carry the same H-ras mutation, suggesting that they do in fact have the same clonal origin.

The most significant difference detected between squamous and spindle carcinomas is the reduction in the normal:mutant H-ras gene dosage. Although well differentiated squamous tumors have a higher proportion of normal stromal cells which are impossible to remove completely, we do not think that the presence of stromal cells can explain the observations made, for several reasons: (a) the difference in the ratios of normal and mutant alleles for some tumors in as high as 10-20-fold on progression from squamous to spindle cells [e.g., the tumor isolated from mouse 3 (Fig. 2B)]; this difference is far too high to be accounted for by stromal cells; (b) analysis of cell lines from squamous or spindle tumors, which are free of stromal cell contamination, has shown that most squamous lines retain the normal ras allele, while in the spindle variants it is frequently lost (Ref. 4 and data not shown). It is, however, not possible as yet to determine whether the crucial feature of this progression event is the loss of the normal ras allele, increased absolute expression of the mutant allele (16), or a combination of both (for a further discussion of this point, see Ref. 17).

Preliminary attempts to reintroduce a normal H-ras gene into spindle cell carcinomas have not yet provided evidence for a reduction in tumorigenicity, although some morphological changes were observed.

An additional complicating factor is that the distal part of mouse chromosome 7 harbors a cluster of genes which are frequently amplified in undifferentiated human squamous tumors (18). These genes, which include the int-2, hst, bel-1, and D11S287 loci, are localized on chromosome 11q13 and have been implicated in the development of epithelial malignancies (18). We therefore have a complicated situation where distal mouse chromosome 7 contains genes which are either deleted (11p15) (19, 20) or amplified (11q13) (18) in human carcinomas. Detailed studies will therefore be required to determine the relative importance of the different candidate loci in determining the anaplastic phenotype. The morphological and behavioral alterations seen in spindle carcinoma cells are themselves striking. The cells become more refractile, invasive, and chemotactic and show aberrant expression of the simple keratins 8 and 18.· One of our primary goals is therefore to identify the specific mechanisms involved in loss of differentiation control in these cells.

References

11. Quintanilla, M., Haddow, S., Jonas, D., Jaffe, D., Bowden, G. T., and Balmain, A. Comparison of ras activation during epidermal carcinogenesis

3 S. Haddow, R. Crombie, and A. Balmain, unpublished results.
4 M. Diaz-Guerra, C. Bauluz, S. Haddow, J. L. Jorcano, A. Cano, A. Balmain, and M. Quintanilla, submitted for publication.


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